Title: Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes

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Abstract

Background: Pan-bacterial 16S rRNA microbiome surveys performed with massively parallel DNA sequencing technologies have transformed community microbiological studies. Current 16S profiling methods, however, fail to provide sufficient taxonomic resolution and accuracy to adequately perform species-level associative studies for specific conditions. This is due to the amplification and sequencing of only short 16S rRNA gene regions, typically providing for only family- or genus-level taxonomy. Moreover, sequencing errors often inflate the number of taxa present. Pacific Biosciences' (PacBio's) long-read technology in particular suffers from high error rates per base. Herein we present a microbiome analysis pipeline that takes advantage of PacBio circular consensus sequencing (CCS) technology to sequence and error correct full-length bacterial 16S rRNA genes, which provides high-fidelity species-level microbiome data

Results: Analysis of a mock community with 20 bacterial species demonstrated 100% specificity and sensitivity. Examination of a 250-plus species mock community demonstrated correct species-level classification of >90% of taxa and relative abundances were accurately captured. The vast majority of the remaining taxa were demonstrated to be multiply, incorrectly, or incompletely classified making true resolution difficult. Using this methodology we examined the microgeographic variation present among the microbiomes of six sinonasal sites, by both swab and biopsy, from the anterior nasal cavity to the sphenoid sinus from 12 subjects undergoing trans-sphenoidal hypophysectomy. We found greater variation among subjects than among sites within a subject, although significant within-individual differences were also

observed. *Propiniobacterium acnes* (recently renamed *Cutibacterium acnes* [1]) was the predominant species throughout, but was found at distinct relative abundances by site.

Conclusions: Our microbial composition analysis pipeline for single-molecule real-time 16S rRNA gene sequencing (MCSMRT, https://github.com/jpearl01/mcsmrt) overcomes deficits of standard marker gene based microbiome analyses by using CCS of entire 16S rRNA genes to provide increased taxonomic and phylogenetic resolution. Extensions of this approach to other marker genes could help refine taxonomic assignments of microbial species and improve reference databases, as well as strengthen the specificity of associations between microbial communities and dysbiotic states.

Keywords: microbiome; 16S rRNA; paranasal sinuses; sinonasal; database; long-read DNA sequencing, circular consensus sequencing

Background

The advent of culture- and cloning-free methods to analyze bacterial phylogenetic marker genes by deep sequencing ushered in a new era of microbial community analysis, dramatically reducing the labor and cost of profiling the identities and abundances of microbes from different environments, independent of their ability to be cultivated [2-5]. The small subunit ribosomal RNA gene (16S rRNA) is shared by all bacteria and has been sequenced in thousands of distinct named species. Because of this, polymerase chain reactions (PCR) using primers that target conserved regions can amplify variable segments of the 16S rRNA gene from across the bacterial domain in a relatively unbiased fashion for amplicon-based deep sequencing [6, 7]. 16S sequence databases can then be used to classify a given sequence read's taxonomic source. Combined with increasingly powerful ecological methods for analyzing microbial community dynamics and inferring community-level metabolic networks, profiling the taxonomic composition of bacterial communities by 16S rRNA gene sequencing has become a standard part of microbiome analysis [8-11].

Unfortunately, the use of the 16S rRNA gene as a taxonomic marker has, in part, been constrained by the short read length of the most commonly used sequencing platform for microbial community profiling (the Illumina MiSeq), which only allows interrogation of up to 3 of 9 variable regions in the 16S rRNA gene (called V1-V9), often targeting only V3-V5, V1-V3, or V4 alone [10, 12-16]. This constraint limits the taxonomic resolution to which short reads can be classified, typically only to the family- or genus-level, and furthermore taxonomic resolution varies for different groups of bacteria when using different portions of the 16S rRNA gene [10]. Low-resolution classification in turn limits

not only the accuracy and precision of ecological inferences and metabolic reconstructions, but also the ability to identify appropriate bacterial strains to use in follow-up experimental and translational studies. Metagenomic shotgun sequencing has been shown to often provide higher taxonomic resolution [17, 18], but these approaches continue to be prohibitively expensive in many cases (particularly when in the presence of excess host DNA), and consensus remains in flux regarding the best pipelines for shotgun metagenomics-based community analysis [17] .

An alternative is to use "3rd generation" long-read sequencing technology to obtain full-length 16S rRNA gene sequences (V1-V9). This increases taxonomic and phylogenetic resolution by increasing the number of informative sites sequenced, while continuing to use a well-studied pan-bacterial marker gene. Initial applications of Pacific Biosciences (PacBio) single-molecule real-time (SMRT) sequencing were hampered by the technology's high intrinsic error rate [19-21], but improvements to the chemistry have since allowed for the generation of high-quality "circular consensus sequence" (CCS) reads, in which individual 16S rRNA genes are sequenced many times using circularized library templates combined with highly processive polymerases that provide for single-molecule, consensus-sequence error correction [22]. Recent studies evaluating full-length 16S sequencing by PacBio have found that, with appropriate processing and filtering, CCS reads of full-length 16S rRNA genes can be generated that are of sufficiently high quality to offer higher taxonomic resolution than partial 16S rRNA sequences [23-26].

The composition of the human sinonasal microbiome and how it changes in health and disease remains poorly understood, largely due to differences in methodology among

studies resulting in large variations in reported bacterial profiles [27-32]. Culture-based approaches capture <15% of resident bacterial taxa when compared to nucleic acid-based techniques, since fast-growing bacteria like *Staphylococcus sp.* tend to predominate in culture specimens, and recovery of anaerobes and slow-growing bacteria is limited [29, 33, 34]. Comparing across recent surveys of the sinonasal bacterial community reveals broadly similar results, but few specific assertions can be made; agreement between studies and results have been limited by an inability to distinguish bacteria at the species level [35-39] but as discussed above does not give a complete reflection of the microbial community. Thus, despite the vastly superior ability of molecular techniques to identify bacterial phylotypes, species-specific identification of bacteria remains superior in culture-based techniques [40]. For this reason, improved specificity of molecular detection techniques is necessary for not only a more complete understanding of the human sinonasal microbiome and other microbial communities, but also to be able to use this approach for decision making in the clinical context. Lastly, identifying the microbial taxa at play in different diseases with higher specificity will enable more directed experimental follow-up studies.

To take advantage of newer PacBio sequencing chemistry, improve upon data processing methods, and apply full-length 16S rRNA gene sequencing to a clinically relevant context, we describe a new pipeline (MCSMRT, “Microbiome Classification by Single Molecule Real-time Sequencing”). We show using two mock communities (one with 280 bacterial species) that full-length 16S CCS reads offer unprecedented accuracy and precision. We then explore bacterial diversity in the human nose and

paranasal sinuses using results from MCSMRT, investigating not only bacterial diversity among subjects but also diversity within subjects at distinct sub-anatomical sites.

Results

Microbial community profiling by full-length 16S rRNA deep sequencing and CCS error correction

The taxonomic resolution of microbial community profiling via 16S rRNA gene sequencing was increased by using Pacific Biosciences (PacBio) RSII to generate full-length 16S gene sequences from mock and human sinonasal microbial communities. We combined a circular sequencing template approach with the long DNA polymerase read-lengths provided by the PacBio sequencing technology. This provided for multiple sequencing passes of each molecule, enabling the generation of circular consensus sequence (CCS) reads of exceptionally high quality [20, 22]. To analyze these data, we developed a new bioinformatics pipeline, MCSMRT, building upon the uparse pipeline [41], which (a) processes and filters PacBio CCS reads generated from multiplexed samples, (b) *de novo* clusters high-quality full-length 16S sequences into "operational taxonomic units" (OTUs), (c) taxonomically classifies each OTU and assigns confidence values at each taxonomic level, and (d) quantifies the abundance of each OTU based on the full CCS read dataset (**Figure 1**). This processed data is suitable for downstream microbiome analyses using standard tools [42, 43]. Full details are in the **Methods** and **Additional File 1**, and the MCSMRT software documentation which is freely available (https://github.com/jpearl01/mcsmrt).

Figure 1
Overview of the MCSMRT pipeline represented as a flowchart.

The MCSMRT method for analysis of 16S rRNA reads from the PacBio is carried out in two steps: In the pre-clustering step, CCS reads are generated during demultiplexing, labeled by sample, pooled together, and then filtered based on several criteria (length distribution, terminal matches to the primer sequences, and not aligning to a host or background genome sequence). Before the clustering step, CCS reads are filtered based on cumulative expected error (EE<1). The clustering pipeline sorts unique sequences based on their abundance, then clusters CCS reads into OTUs using uclust, filtering out chimeric reads during clustering, and then by using uchime after clustering. An OTU count table is created by mapping the filtered results from the end of the pre-clustering pipeline, and each OTU is taxonomically classified based on a representative "centroid" sequence.

Below, we demonstrate the robustness and high taxonomic resolution of our experimental and bioinformatics approach using results from two distinct mock microbial communities: one from the Biodefense and Emerging Infections Research Resource (BEI) and the other from the Joint Genome Institute (JGI) used as part of their "Critical Assessment of Metagenome Interpretation" (CAMI) project. We then applied full-length 16S rRNA gene sequencing to ask how the healthy human sinonasal microbiome varies among individuals and among sub-anatomical sites within individuals (**Table 1**; expected mock community compositions in **S1 Table, Additional File 2** and **S2 Table, Additional File 2**).

Table 1
Community Characteristics

	BEI-EC	**JGI-CAMI**	**HSNM-MS**
Source	Biodefense and Emerging Infections Research Resource Repository (BEI)	Joint Genome Institute (JGI)	Philadelphia Veterans Affairs Medical Center
Type	Mock Community	Mock Community	Wild Community

Details	BEI Even C (Catalog Number)	CAMI competition	Human Sinonasal Microbiome
Number of bacterial species	20	282 (308 strains)	Unknown
Number of other species	2 (archaeal and fungal)	2 (archaeal)	Unknown
Species distribution	Reportedly even	Widely varying	Unknown
Other	Pooling DNA based on 16S qPCR	Pooling DNA based on genomic DNA mass	12 subjects, 6 sites, swab and biopsy

Source and composition characteristics of BEI-EC and JGI-CAMI (mock communities), and HSNM-MS (Human sinonasal community).

CCS and filtering for full-length 16S rRNA reads

From each sample across the three types of communities, PCR was used to amplify full-length 16S rRNA genes (~1.5 kilobases [kb]) from total DNA purifications using primers that targeted conserved regions at both ends of the gene that also contained terminal asymmetric barcodes to allow for pooling and subsequent demultiplexing of multiple samples into the same SMRTcell (**S3 Table, Additional File 2**). For the simpler BEI mock community, we tested PCR parameters, varying the polymerase (GoTaq vs. AccuPrime), cycle number (22 vs. 35), and the presence of excess off-target DNA (*i.e.* 10-fold excess of genomic DNA from U937 lymphoblast lung cell line).

We used the PacBio RSII (P6-C4 chemistry) to collect 3,446,849 polymerase reads in total across the 3 communities (**Table 2**). We typically obtained ~50K-60K polymerase reads per SMRTcell, in which 3-4 barcoded 16S amplimer libraries were pooled together and subsequently demultiplexed. More than half of polymerase reads were

typically >20 kb, such that the average polymerase read included ~12 complete sequencing passes around each molecule (average 1422 basepair [bp] inserts). Those polymerase reads with >4 passes were used to generate error-corrected CCS reads, whose quality was dramatically improved (mean cumulative expected errors, EE, of 4.9 per kb) compared to polymerase reads to (EE / kb = 139.4). CCS reads with 1-4 passes had considerably lower quality (mean EE / kb = 193; 98.9% of these reads had EE > 10), and these were not considered further. The requirement for at least 5 passes resulted in a 20 - 30% reduction in overall yield compared to total polymerase reads but massively increased confidence in base calling (**Table 2**).

Table 2
Bulk sequencing and filtering stats for all three communities

Source[1]	**BEI-EC[2]**	**JGI-CAMI[3]**	**HSMC-MS[4]**
Type	**mock**	**mock**	**human**
Total Samples	8	1	122
Total # SMRTcells	4	1	85
Total Pol Reads	396,625	53,164	2,997,060
N_{50} Pol Read length in kb	23523	21220	18837
Avg Read Length in Pol Reads	12997	11232	11940
Avg Phred-quality in Pol Reads	9.89	9.5	9.35
Avg EE of Pol Reads[5]	1868.82	1727.44	1653.9
EE per kb of Pol Reads	143.79	153.8	138.52
Avg CCS passes	14.04	13.06	11.48
Avg Phred-quality in CCS Reads	40.46	40.84	39.26
Avg CCS length	1454	1481	1417
Avg EE for all CCS Reads[5]	5.36	3.15	7.16
EE per kb of CCS Reads	3.69	2.13	5.05
Total CCS Yield (>4 passes)	163,689	19,576	787,302
Size filtered (0.5-2kb)	131,413	16,061	498,007

Host filtered	163,670	19,574	704,935
Primer matched	131,856	16,156	498,820
Percent passed primary filters	80.4	81.8	63.1

Sequencing and pre-clustering filter statistics of BEI-EC and JGI-CAMI (mock communities), and HSNM-MS (Human sinonasal community).
[1]In addition to mixed species communities, 7 independent negative reagent controls and 4 positive controls (2 x DNA from pure cultures of *Escherichia coli* and *Agrobacterium tumefaciens*) were run.
[2]Three conditions: Polymerase (GoTaq vs. AccuPrime) x PCR Cycles (35 vs. 22) x Excess DNA (no human DNA vs. 10-fold excess).
[3]Four independent libraries.
[4]Twelve subjects with healthy sinuses sampled at 6 sinonasal anatomic locations, both swab and biopsy. Twenty samples (mostly sites E and F) were not collected or not run.
[5]EE is cumulative expected error across the full read.

A series of additional filters were applied to CCS reads to eliminate off-target sequences: (a) a size filter, (b) a filter against background (host) sequences, and (c) a primer matching filter. Collectively, these filters eliminated ~20-40% of CCS reads (**Figure 1A**, **Table 2**, **S1 Figure, Additional File 3**, **S4 Table, Additional File 2**).

Size filter: CCS reads were removed if they were outside the thresholds of full-length 16S sequences (those between 0.5-2 kb were retained). This 2-3% of all CCS reads were mostly dimeric 16S sequences ~3 kb long, most likely created during the ligation step of library preparation (**S2 Figure, Additional File 3**).

Host filter: CCS reads were removed if they aligned to a background genome (in this case, the human GRCh37, or hg19, reference). Notably, only 19 of ~160K reads from the BEI mock community samples mapped to the human genome, despite half of these samples including a 10-fold excess human DNA (extracted from U937 human

lymphoblast lung cell line to minimize contamination from the human microbiome). This indicates no appreciable off-target priming or contaminating fragments from U937 cell line DNA added at a 10:1 excess (by mass). However, samples with added human DNA had marginally lower sequencing yields (~25%), possibly indicating a weak inhibitory effect by excess off-target DNA (**S3A Figure, Additional File 3**, effect on CCS yield, Tukey's HSD $p=0.048$ for 10-excess human DNA, but $p>0.2$ for polymerase or cycle number).

By contrast, a much larger proportion of CCS reads from the human sinonasal communities mapped to the human genome (9.9%), suggesting off-target amplification of human DNA when in vast excess over bacterial DNA (alternatively, the U937 cell line DNA used for the BEI experiment may have lacked some or all off-target priming sites present in the human reference). Supporting this interpretation, biopsy samples had substantially higher total DNA yields after extraction than swabs (though no obvious differences in PCR yield, **S4 Figure, Additional File 3**), and reads derived from human were significantly more abundant in biopsy samples (**S3BC Figure, Additional File 3,** comparing biopsies and swabs, Tukey's HSD $p<<0.01$ for total CCS yield or % human-contaminants, but $p>0.8$ when for patient or site). The 105,801 reads in the sinonasal dataset that mapped to the human genome aligned to 9,716 distinct genomic positions, but they were highly enriched at only a few (67.9% of mapped positions had only a single mapped read, but 58.2% of reads started at only 16 positions and had >1,000-fold coverage, **S5 Figure, Additional File 3**). These data indicate an off-target priming effect at high excess concentrations of human DNA with "hotspots" for off-target priming, along with a proportion of library molecules carrying apparently random human

genomic DNA fragments. To confirm that reads mapped to the human genome were not improperly aligning true bacterial 16S genes, utax classification of all human-mapping reads showed that all had extremely low confidence assignments to the bacterial domain (<0.1), indicating a probable host origin.

Primer matching: CCS reads were required to have the forward and reverse primer sequences each found once and oriented correctly at the ends of the sequence, and this removed 12-18% of reads (**Table 2 and Tables S4 and S7**, **Additional File 2, S6 Figure, Additional File 3, Additional File 1**). Primer matching also served to determine the orientation of the 16S gene in each CCS read, so reads were reverse complemented when the reverse primer came first. Finally, primers were trimmed from reads. In principle, this loses several taxonomically informative sites, since the primers contained four degenerate bases; however, in practice, the primer sequence seen in a given read was random with respect to the taxonomic source of that 16S gene. This is most easily illustrated from control sequencing of 16S rRNA genes amplified from clonal cultures of *Escherichia coli* K12 MG1655 and *Agrobacterium tumefaciens* NTL1 (**S7 Figure and S8 Figure, Additional File 3,**).

Clustering CCS reads into OTU

Profiling the bacterial composition of a microbiome often begins by clustering sequences with high sequence identity into OTU, with a standard cutoff of 97% [44, 45], though sometimes other cut-offs are used [46-48]. To cluster reads and determine centroid OTU sequences, we used the UCLUST algorithm [49](**Clustering Pipeline,**

Additional File 1), which incorporates a filter against chimeric 16S sequences by excluding apparent hybrids between distinct OTU as they accumulate in the dataset (here called CHIM1). A second chimera filter (CHIM2, using uchime) [50] then removes centroid OTU that appear to be hybrids of distinct 16S sequences in a curated Ribosomal Database Project (RDP) database [51] (**Clustering Pipeline, Additional File 1**). The abundance of each OTU in each sample was then determined by counting the number of filtered CCS reads that aligned to each centroid (**Figure 1B**). Though OTU clustering may collapse or separate distinctly named species into the same or different OTU, it systematically defines taxa in a uniform way that does not depend on taxonomic nomenclature [52, 53].

***Sequencing error increases observed OTU counts*:** Erroneous base calls in CCS reads risk artificially inflating the number of OTU, since reads with sequencing errors in similar 16S rRNA genes can be spuriously separated into distinct OTU, especially if their actual divergence is near the 3% divergence cut-off and/or they are short. Thus, a final pre-clustering filter was applied based on cumulative expected error (EE, or the sum of error probabilities across all positions in a read as determined from Phred-scaled base quality scores). This measure has previously been shown to discriminate against error prone sequences better than the average quality score [54].

Analysis of the BEI mock community: Using the BEI mock community to examine the relationship between CCS passes and EE, we found, as expected, that reads with more CCS passes had a lower median EE (**Figure 2**, linear model of log(EE) vs. CCS passes gives $p<<0.001$ and $R^2=0.22$). 98.9% of reads with less than five CCS passes

had EE>10 (across ~1.4 kb total length) and were not considered further (**Figure S9, Additional File 3**).

Figure 2 - Distribution of reads at different CCS passes and cumulative expected error values (EE). Only reads with CCS passes > 4 are considered.

2A. Violin plot of EE as a function of CCS passes
2B. Histogram for EE distribution

To empirically determine an appropriate EE cut-off for clustering CCS reads into OTU, we compared the expected number of OTU in the BEI mock community to that obtained by clustering OTU at different EE cut-offs. The expected number of OTU was 19, since 2 of the 20 species' 16S rRNA genes differed by at most 23 nucleotides (*Staphylococcus aureus* and *Staphylococcus epidermidis* have only 1.4% divergence, less than the OTU clustering cut-off of 3% divergence, **S1 Table, Additional File 2**). As expected, increasing the stringency of the EE filter reduced the total number of CCS reads available for OTU clustering, as well as the total number of OTU detected (**Figure 3A**). Using EE≤1 (one or fewer expected errors per read) retained less than half (40.1%) of filtered CCS reads, but these clustered into exactly the 19 OTU expected. Decreased stringency (higher EE cut-offs) increased the total OTU detected, dramatically for cut-offs of EE ≤8 and above; using no expected error threshold (EE ≤128), 3,453 OTU were detected, more than 100-fold greater than the true number. In summary, using a high stringency expected error cut-off for OTU clustering reduced the

number of reads available for clustering but provided exact total OTU counts for the BEI mock community.

Figure 3 - Clustering of post-filtered CCS reads into OTU.

3A. Count of total, unique, CHIM1 and centroid OTU reads at different maximum EE thresholds.
3B. Count of total OTU detected using full-length or truncated reads at different maximum EE thresholds

Sequence length and OTU clustering: To examine how OTU clustering would be affected by using partial instead of full-length 16S rRNA gene sequences for the BEI mock community, we performed *in silico* primer matching and trimming on the full-length CCS reads for three short-read primer pairs commonly used for microbial community profiling, namely primers targeting the V1-V3, V3-V5, or V4 hypervariable regions of the 16S rRNA gene, which are amenable to analysis using Illumina short-read sequencing (**S3 Table, Additional File 2**). Using these *in silico* short-read data produced dramatically higher OTU counts than predicted, even when using substantially more stringent EE cut-offs (**Figure 3B**). Thus, for example, whereas full-length V1-V9 reads clustered into the expected 19 OTU at EE ≤1, reads truncated to include only the V3-V5 hypervariable regions (average length = 536 nucleotides) clustered into 116 OTU. The other truncated sequences (V1-V3 and V4) had even higher elevated total OTU counts. Even when the shorter length of partial sequences was compensated for by using an 8-fold higher stringency cut-off (EE ≤ 0.125), spurious OTU were still detected, e.g. 58 OTU were detected with the V3-V5 truncated reads, substantially higher than expected. Though these comparisons relied on *in silico* truncation of full-length reads from the

same PacBio dataset, inflated OTU counts have also been seen in published V3-V5 sequences from the BEI mock community collected using both 454 pyrosequencing and Illumina MiSeq short-read technology [55-57]. This suggests that inflated OTU counts when using partial 16S sequences is independent of our specific PCR conditions or the particular error profile of PacBio CCS reads (which we demonstrated to be very low).

The above results underline the value of using full-length 16S rRNA to minimize the effect of sequencing errors on OTU cluster counts. They also indicate that methods that profile taxonomic composition using partial 16S rRNA genes may be prone to overestimating bacterial diversity. For all subsequent analyses, we used only CCS reads with EE ≤1 for OTU clustering, and then mapped all reads passing all pre-clustering filters onto these centroid OTU's to obtain abundance data (**Pre-clustering Pipeline, Clustering Pipeline, Additional File 1)**.

Taxonomic classification of centroid OTU

Bacterial taxonomic nomenclature has traditionally been based on physiological and other microbiological traits (e.g. virulence) rather than 16S rRNA gene sequences, so the accuracy and precision with which a centroid OTU can be taxonomically defined is dictated by: a combination of organism-specific criteria used for naming species; the quality and completeness of the database used; and the distribution of informative variable sites within the 16S rRNA among named taxa [10]. Commonly used databases for classifying 16S rRNA gene sequences, namely RDP and Silva [51, 58, 59], do not provide species-level taxonomic identifiers [60]. Another problem with these databases

is the absence of representative sequences from genera present in our mock communities (for example the genus *Clostridium* was not found in RDP). Though 285,289 sequences in the popular Greengenes database do have species labels, only 631 of these are unique species. Both Greengenes (N=1,262,986) and Silva (N=1,922,213) have vastly more taxonomically classified sequences than RDP (N=8,978), in part because they computationally assign taxonomies to sequences from environmental microbiome surveys [58, 61]. While these databases are appropriate in many cases, we needed a database of full-length 16S sequences with species-level taxonomic information.

To classify OTU centroids based on bacterial taxonomy to the species level and also provide confidence values at each taxonomic level, we trained a utax classifier on a custom-built database of full-length 16S rRNA gene sequences downloaded from NCBI (**16s rRNA Microbial Database, Additional File 1**). Most full-length 16S sequences available at NCBI (N=17,764) could be associated with a taxonomic ID (txid) using the gid accession number, allowing us to extract, parse, and configure sequences in the database to create a utax-compatible Linnaean hierarchy (**16s rRNA Microbial Database, Additional File 1**). We compared the accuracy of this full-length classifier to one trained on partial 16S rRNA genes derived from the full-length sequences (from V3 to V5) by classifying the database sequences themselves (N=17,391, since V3-V5 primer matching failed for 373 full-length entries). In this context, incorrect classification could arise in particular due to distinct named species with highly similar sequences. The full-length classifier gave an incorrect label only 1.0% of the time (N=173 mistakes), compared to 13.2% of the time using the truncated classifier (N=2295 mistakes).

Indeed, when the two classifiers disagreed, the full-length call was much more frequently correct (2x2 contingency table: 15,081 both correct, 2,137 only full-length correct, 15 only truncated correct, and 158 neither correct; Fisher's exact test p-value << 0.001). Furthermore, species-level confidence values were higher 81.3% of the time using the full-length classifier (mean 81.7%, median 92.7%) compared to the truncated classifier (mean 71.1%, median 82.7%). These results show the value of using full-length compared to partial 16S gene sequences for accurate taxonomic assignment.

Only a single taxonomic assignment was made for each OTU centroid by the utax classifier. This assignment is associated with confidence values at each taxonomic level, though low values could arise for several reasons aside from the quality of the sequence data. In particular, sequences labeled as a distinct named species could have other equally good matches, or nearly so. In order to determine what species might be assigned to a particular centroid OTU, we clustered the NCBI database sequences (17,776 in total, 99.1% unique) at the same threshold level (97% identity), thereby grouping species belonging to the same "database OTU" (dbOTU). Since uclust relies on abundant unique sequences to initiate centroids and also drops putative chimeric sequences during clustering, we instead applied hierarchical clustering (average linkage, using pairwise percent identity values from all-by-all blast, and separating dbOTU clusters at a 3% difference level). This method is unaffected by the order of the sequences and included all database entries.

Hierarchical clustering of NCBI sequences resulted in 6,065 dbOTU, of which 66.9% consisted of a single species (93.2% encompassed a single genus), whereas 14.6% contained species split over more than one dbOTU (**Additional File 4**). Some dbOTU

consisted of many species. For example, the top three most species-rich dbOTU collectively contained 453 distinctly named *Streptomyces* species indicating that 16S rRNA clustering at 3% divergence poorly discriminates among named species in this genus [62]. These results reflect the variability with which different bacterial taxa are named compared to how they group based on divergence in their 16S rRNA [47, 48] (**S10 Figure, Additional File 3**). Collectively, clustering the full-length 16S rRNA gene sequences from NCBI indicated that centroid OTU assignments will result in high-confidence species-level classification for most full-length 16S rRNAs; imprecision due to distinct species belonging to the same dbOTU will affect about a third of 16S rRNA sequences in the database, but these can be flagged by low confidence values from the utax classifier and by cross-referencing to dbOTU clusters to identify other possible "nearly best hits".

Nearly all reads collected from reagent controls failed filtering and classification steps. In advance of the studies described above, we pre-screened multiple PCR reagents and DNA polymerases to identify those that produced no observable amplification when using reagent controls. In addition, seven negative control reagent samples were sequenced in parallel with mock community and sinonasal samples. After demultiplexing, these controls produced a total of only 54 reads. Of these, only 3 reads passed the filtering criteria described above. Taxonomic classification on all 54 reads returned only 5 reads with >10% bacterial domain-level confidence values (all 5 gave 100% confidence). These 5 reads had genus-level classification as *Finegoldia* (95.3% confidence), *Propionibacterium* (16.6%), *Streptococcus* (22.7%) and two as

Staphylococcus (0.8%). 4 had 0% species-level confidence, while *Finegoldia magna* had 94% species-level confidence. These results demonstrate that our laboratory and bioinformatics methods produce extremely low levels of contamination from off-target bacterial nucleic acids generated from our reagents.

BEI mock community composition

OTU classification: The 19 distinguishable OTU in the BEI mock community were readily identified and accurately classified by MCSMRT (**Table 3**). All but 3 of the centroid OTU—including 3 distinct *Streptococcus* species—were correctly classified to the species level. The 3 discrepancies were, however, reflected by low confidence values assigned by utax, as well as by clustering of discrepant taxa into the same dbOTU.

Table 3
Taxonomic classification of BEI

DB Full Length			**Centroids v3-v5**			**Clustered from v3-v5 truncated reads**		
Genus_Species	**Genus Conf**	**Species Conf**	**Genus_Species**	**Genus Conf**	**Species Conf**	**Genus_Species**	**Genus Conf**	**Species Conf**
A. baumannii	0.9796	0.9392	*A. baumannii*	0.8784	0.4225	*A. baumannii*	0.8784	0.4225
A. odontolyticus	0.9897	0.9392	*A. odontolyticus*	0.9728	0.6189	*A. odontolyticus*	0.9728	0.6189
B. anthracis	0.9876	0.494	*B. cereus*	0.963	0.4225	*B. cereus*	0.963	0.3114
B. vulgatus	0.993	0.9438	*B. vulgatus*	0.9847	0.897	*B. vulgatus*	0.9847	0.897
C. beijerinckii	0.989	0.7194	*C. roseum*	0.9669	0.4225	*C. roseum*	0.965	0.3114
D. radiodurans	0.9935	0.9438	*D. radiodurans*	1	0.897	*D. radiodurans*	0.9994	0.897
E. faecalis	0.9862	0.9392	*E. faecalis*	0.8784	0.8265	*E. faecalis*	0.8784	0.8265
S. flexneri	0.6878	0.494	*S. flexneri*	0.6661	0.4225	*S. flexneri*	0.6072	0.3114
H. pylori	0.9918	0.9346	*H. pylori*	0.9787	0.9022	*H. pylori*	0.9787	0.9022

L. gasseri	0.9928	0.7194	*L. gasseri*	0.9728	0.4225	*L. gasseri*	0.9728	0.4225
L. monocytogenes	0.989	0.7194	*L. ivanovii*	0.961	0.4225	*L. ivanovii*	0.961	0.3114
N. meningitidis	0.973	0.9346	*N. meningitidis*	0.9533	0.6189	*N. meningitidis*	0.9533	0.6189
P. acnes	0.973	0.9575	*P. acnes*	0.9669	0.9099	*P. acnes*	0.965	0.9099
P. aeruginosa	0.973	0.9346	*P. aeruginosa*	0.9329	0.6189	*P. aeruginosa*	0.9329	0.6189
R. sphaeroides	0.973	0.5993	*R. sphaeroides*	0.9228	0.4225	*R. sphaeroides*	0.9228	0.3114
NA	NA	NA	*NA*	NA	NA	*S. aureus*	0.1509	0.0089
S. epidermidis	0.9869	0.7194	*S. epidermidis*	0.6661	0.4225	*S. epidermidis*	0.6661	0.3114
S. agalactiae	0.9897	0.9483	*S. agalactiae*	0.9728	0.8996	*S. agalactiae*	0.9728	0.8996
S. mutans	0.9918	0.9529	*S. mutans*	0.9768	0.8996	*S. mutans*	0.9768	0.8996
S. pneumoniae	0.9904	0.494	*S. pneumoniae*	0.9768	0.4225	*S. pneumoniae*	0.9768	0.4225

Classification results from the BEI mock community. The performance of full-length 16S centroid OTU assignments compared to those truncated to V3-V5. The last two columns indicate the top 20 most abundant OTU from truncating to V3-V5 prior to clustering.

All 19 centroid OTU were correctly classified to the family level, with one genus-level discrepancy: classification of *Escherichia coli* as *Shigella flexneri*. This incorrect assignment is not a surprise; indeed, the matching dbOTU contained 54 sequences that were assigned to *Escherichia*, *Shigella*, *Citrobacter*, and *Salmonella*, all genera previously demonstrated to have low levels of divergence among their 16S rRNA genes[63, 64]. Two species-level assignments were incorrect: (a) *Bacillus cereus* was classified as *Bacillus anthracis*; these two differ by only 2 nucleotides in their 16S genes and share the same dbOTU with 8 other *Bacillus* species; and (b) *S. aureus* and *S. epidermidis*—whose 16S genes differ by 23 nucleotides—were collapsed into a single OTU, with the centroid called *S. epidermidis* (the matched dbOTU consisted of 39

additional *Staphylococcus* sp., though see below). Species-level confidence values were 0.49 and 0.71 respectively. By contrast, only one correctly classified OTU, *Streptococcus pneumoniae*, had a species-level confidence value <0.50 and this species shared the same dbOTU with 13 additional *Streptococcus* species.

Truncation of CCS reads prior to clustering considerably worsened classification; in addition to increasing the number of total OTU, CCS reads truncated to their V3-V5 region prior to clustering resulted in seven misclassified OTU among the top 20 most abundant OTU (**Table 3**). To isolate the effects of truncation on classification alone, rather than both clustering and classification, we also truncated the 19 full-length centroid OTU to their V3-V5 region and classified these using a utax classifier built from a database of sequences also truncated to V3-V5. This showed reduced species-level confidence values but also more miscalled taxa, further underlining the value of full-length 16S sequence (**Table 3**). These results show that use of full-length 16S rRNA gene sequences provides substantially improved taxonomic identification of centroid OTU compared to truncated 16S rRNA sequences, to the extent that bacterial nomenclature allows.

Relative abundance: The abundance of each OTU was estimated by assigning all filtered CCS reads to a centroid OTU using usearch global with a maximum of 3% divergence for a hit to be counted (with no EE threshold). The 20 bacterial species in the BEI mock community were expected to have equimolar abundances of their 16S rRNA genes, and for most species, we detected a roughly even mock community composition for most species (**S11A Figure, Additional File 3**). Several were outliers: (a) the two *Staphylococcus* species were binned together as *S. epidermidis* (as

described above); (b) *Bacteroides vulgatus* and *Helicobacter pylori* were overrepresented, especially at high PCR cycle number; and (c) five species were found at lower than expected abundances across PCR conditions.

We next evaluated the impact of (a) chimeric sequences on relative abundance measurements and (b) “true” substitution errors. First, all CCS reads were run through uclust with no filters other than requiring >4 CCS passes to identify likely CHIM1 chimeras, and then all CCS reads were aligned to the 16S reference sequences from the BEI community to determine their likely source. This again found relatively even abundances for each taxon with the exception of those mentioned above. Increased cycle number also increased the variance among taxa in their relative abundances, but the inclusion of chimeric reads had little effect (**S11B Figure, Additional File 3**). Second, the number of base mismatches (excluding gap characters) was calculated between each non-chimeric read and its most similar reference sequence, estimating the number of “true” substitution errors made during sequencing (though intragenomic variation in 16S rRNA gene sequences [65] contributes to putative substitution errors). This analysis indicates that the AccuPrime polymerase made fewer errors than GoTaq polymerase but that errors made by either polymerase were insufficient to inflate OTU numbers when using full-length sequence (**S12 Figure, Additional File 3**).

Independent analyses of the same BEI mock community by Illumina MiSeq for V3-V5 have previously found the same taxa elevated or depleted, suggesting that these taxa actually are at unequal concentrations in this mock community [66, 67]. The primers we used have perfect identity with all BEI bacterial strains’ reference 16S rRNA gene

sequences and are distinct from the Illumina-based analyses, so the compositional biases seen are not likely to be due to primer choice or PCR conditions.

Species discrimination below the OTU level: While the two clinically defined *Staphylococcus* species in the BEI mock community belonged to the same dbOTU and were not distinguished during clustering as separate OUT, the presence of distinct clades could still be determined. This was clarified by multiple sequence alignment of all *Staphylococcus* sequences in the NCBI database and a subsample of 10 thousand filtered CCS reads that had been assigned to the BEI *Staphylococcus* OTU. Phylogenetic trees and ordination using NMDS (non-metric multidimensional scaling) of all pairwise distances shows that *Staphylococcus* 16S rRNA genes in the BEI mock community were roughly equally divided between *S. epidermidis* and *S. aureus* and clearly separated from other staphylococci, as expected (**Figure 4A**). Rare reads that appear as hybrids between the two “true” species likely represent chimeric sequences; the algorithm that removes CHIM1 chimera would not identify these, since it requires distinct OTU representing each organism in order to identify chimeric sequence between those OTU. By contrast, clustering sequences truncated to the V3-V5 region failed to distinguish between the two Staphylococcal species in the database and the BEI mock community (**Figure 4B,** NMDS stress values of 0.067 and 0.139 for NMDS of full-length and truncation, respectively; the lower the stress value, the more reliable the ordination, and as a rule of thumb stress values <0.1 are generally considered ‘fair’). These results show that distinguishing among closely related organisms—even when sequence differences are insufficient to separate these into distinct OTU—is strongly facilitated by use of full-length 16S rRNA gene sequences.

Figure 4 - NMDS of *Staphylococcus* reads from the BEI mock community.
4A. Full Length
4B. V3-V5
Ordination of pairwise distances among all BEI mock community reads assigned to the *Staphylococcus* OTU and all full-length *Staphylococcus* 16S rRNA gene sequences in the NCBI database. Pink dots are individual reads (down-sampled to 10,000 total) from the BEI mock community. Blue dots are all full-length 16S rRNA genes in the NCBI database classified as *Staphylococcus*. The large group on the left represent the *S. aureus* reads, and those on the right are *S. epidermidis* reads.

CAMI mock community composition

OTU classification: Because a curated set of full-length 16S rRNA gene sequences was not available for the 280 unique bacterial species present in the CAMI mock community, we first cross-referenced the denoted bacterial composition (**S2 Table, Additional File 2**) with the full-length 16S rRNA gene sequences in the NCBI database, finding one or more full-length sequences for all but 3 species, for which a taxonomy was available but not a corresponding 16S rRNA gene sequence (*Mameliella alba*, *Fusobacterium naviforme* and *Promicromonospora flava*). In addition, 11 species names used by CAMI and NCBI were synonyms, due to revisions in species names (particularly members of the *Clostridiales* family, **S5 Table, Additional File 2**). By cross-referencing the denoted species with the NCBI dbOTU, we found the 280 CAMI species would cluster into 253 OTU at the 3% divergence level, each associated with a distinct dbOTU (except the three missing species). For example, 5 species from the genus *Prauserella* (*aidingensis, alba, flava, halophila, salsuginis*) existed in the CAMI community, and while there was an instance of each of those species in the NCBI database, none of these NCBI 16s sequences differed from each other by greater than

3%. Therefore, the expectation was a single OTU associated with the genus *Prauserella*, which was indeed our result. Most of these expected clusters had three or fewer named species in their dbOTU (84.4% of CAMI clusters), but they collectively comprised 586 distinct species calls in the NCBI database. This clustering allowed us to cross-reference the centroid OTUs identified by PacBio sequencing to members of the CAMI community and identify ambiguities in the extant taxonomic classification (**S5 Table, Additional File 2**).

Full-length 16S rRNA gene sequencing by PacBio had exceptionally high specificity and sensitivity for identifying the bacterial constituents within the complex CAMI mock community (**Figure 5**). The ~16K filtered CCS reads (final yield from one PacBio SMRTcell) clustered into 227 OTU (using 6,878 reads at EE ≤1) with 216 unique species names. Of these, 192 centroid assignments perfectly matched up with an expected cluster in the CAMI community, thus giving 89% exact species-level matches with the centroid OTU. Nineteen more centroids could be connected to CAMI-defined clusters, either via a dbOTU (13 centroids), equivalent to 95% species accuracy, or a correct genus-level assignment (6 centroids). Illustrating the value of full-length compared to partial 16S sequences, **S13 Figure, Additional File 3** shows NMDS ordinations and phylogenetic trees for a subsample of reads classified as *Lachnospiraceae*, along with all associated NCBI database entries for that family, showing clear discrimination among family members.

Figure 5 - CAMI community composition
Observed versus expected relative abundances, based on matching centroid OTU assignments with expected species composition.

Of the 5 remaining OTU detected by PacBio that did not belong to a CAMI cluster, 2 could be accounted for with family-level matches to CAMI clusters (the Rhodobacteraceae *Mameliella alba*, absent from the NCBI database, was classified as *Paracoccus versutus*, and *Promicromonospora flava* was identified as *Isoptericola variabilis*). This left only 3 “false positive” OTU, which accounted for a total of 7 CCS reads. Two of these misidentifications were species belonging to families represented in the CAMI community—the Rhizobiaceaen *Agrobacterium larrymoorei* (1 CCS read) and the Lachnospiraceaen *Moryella indoligenes* (5 CCS reads)—and one was not represented (the Moraxellaeceaen *Acinetobacter septicus* had a single CCS read). There were 40 expected CAMI clusters that were not identified among the centroid OTU (*i.e.* “false negatives”). This was, at least in part, due to under-sampling: The relative abundance of 16S rRNA genes for most CAMI species was expected to be very low (some well below our limit of detection), and all missing CAMI members had expected abundances of <1% (**Figure 5**). Thus, with a single SMRTcell, we detected 84.2% of taxa present with 95% species-perfect identification, and nearly every single OTU identified by full-length 16S rRNA gene sequencing could be attributed to a member of the CAMI community. By contrast, truncating CCS reads (or their centroid OTUs) to the V3-V5 region was less accurate and showed lower species-level confidence values, as seen above for the database sequences themselves (see above and **S6 Table, Additional File 2**).

Relative abundances in the CAMI community: The expected relative abundance of each species in the CAMI mock community was accurately reflected by the number of reads assigned to each CAMI centroid OTU by usearch (**Figure 5**, **S5 Table, Additional File 2**). The expected relative concentration of each species' 16S rRNA genes in the CAMI community was calculated using: (a) the genome size estimated by JGI from *de novo* assembly of shotgun sequence collected from each CAMI accession [68] (**S5 Table, Additional File 2**), and (b) an estimate of 16S rRNA gene copy number using rrnDB [69]. For CAMI species missing from the rrnDB, the lowest Linnaean rank with members of the database was determined, and the average 16S rRNA copy number of all species under that rank was used (**S5 Table, Additional File 2**). Remarkably, we observed a strong linear fit between observed and expected abundances (**Figure 5**, $R^2 = 0.63$), showing that we not only accurately identified the species present by centroid OTU, but also accurately quantified their relative abundances, despite the low expected relative abundance of most species' 16S rRNA genes.

The composition of the human sinonasal bacterial microbiome

Rhinosinusitis effects 16% of the US population [70] and accounts for 1 in 5 antibiotic prescriptions to adults in the US in the outpatient setting, making it the number 1 diagnosis for outpatient antibiotic use in our country [71]. Thus, a more complete understanding of the resident microbial community of the upper respiratory track is paramount to improved therapeutic interventions and reduction of inappropriate

antibiotic prescriptions. Thus, we applied our bacterial microbiome profiling method to the human sinonasal cavity. We obtained samples from 12 subjects undergoing pituitary gland adenoma removal, utilizing the sinonasal cavity as a surgical corridor for access to the gland. None of the total 12 patients examined had objective or subjective findings of infectious or inflammatory disorders of their sinonasal complex. In creating a surgical corridor for access to the skull base, 6 distinct anatomical locations within the sinonasal cavity were sampled by both swab and biopsy (**Figure 6, Table 4, S7 Table, Additional File 2)**.

Figure 6 - Schematic diagrams in the sagittal and coronal planes of the human sinonasal cavity. Surgical access to the sella turcica (pituitary) is shown by the shaded arrow. Sites of sampling for microbiome analysis: deep nasal vestibule swab, deep to the vibrissae past the squamous mucosal epithelial junction (A), head of inferior turbinate swab (B), middle meatus swab (C), uncinate process biopsy (D), maxillary sinus swab (E) and biopsy (F), ethmoid sinus swab (G) and biopsy (H), superior meatus swab (I) and biopsy (J), and sphenoid sinus swab (K) and biopsy (L). Figure adapted from "Atlas of Endoscopic Sinus and Skull Base Surgery," ed. Palmer, J.N., Chiu, A.G., Adappa N.D. Elsevier, Philadelphia (2013).

Table 4 Codes used for Swab/Biopsy sites

Swabs		**Biopsies**	
Code	**Site**	**Code**	**Site**
A	Nasal Vestibule	B	Head of Inferior Turbinate Tissue
C	Middle Meatus	D	Uncinate Process Tissue
E	Maxillary Sinus	F	Maxillary Sinus Tissue
G	Ethmoid Culture (Deep to Ethmoid Bulla)	H	Ethmoid Tissue (Deep to Ethmoid Bulla)
I	Superior Meatus	J	Sphenoethmoidal Recess Tissue
K	Sphenoid	L	Sphenoid Tissue

To determine the bacterial constituents of the human sinonasal microbiome and the extent to which it varies among healthy individuals and among distinct sinonasal sites, we sequenced full-length 16S amplicons by PacBio from paired swabs and biopsies at the 6 anatomical sites from the 12 subjects (**S7 Table, Additional File 2**, 122 specimens total across 12 individuals). No filtered CCS reads were generated from 23 samples, primarily from the maxillary sinus (both swabs and biopsies), suggesting little colonization of this site by bacteria (**Figure 6**), and significantly fewer reads were collected from biopsy samples than from swab samples, potentially indicating lower overall bacterial load compared with the mucosal surface (**S3 Figure, Additional File 3**). Filtering, clustering at an EE ≤1, taxonomic assignment, and counts per OTU per sample were conducted as above (counting all filtered CCS reads against all non-chimera centroid OTU sequences via usearch). Complete information about counts per OTU per sample, as well as the taxonomic assignments of each centroid OTU are in **S8 Table, S9 Table** and **S10 Table, Additional File 2**.

The overall diversity of the sinonasal microbiomes collected here were relatively low. Across all specimens, clustering resulted in a total of 300 OTU (plus 6 centroids that were removed before classification by the uchime filter), and the corresponding centroid OTU sequences were classified into 271 named species comprising 150 genera. Although 300 OTU were detected overall, the top 20 OTU accounted for 96.7% of reads (**Figure 7**), and only 61 OTU had >50 read counts summed across all >460K primer-match CCS reads. As previously seen, the dominant taxa in the sinonasal microbiome were *Staphylococcus* (OTU_2; see below) and *Propionibacterium acnes* (OTU_1), which together comprised 65.2% of all read counts [35]. Three of the top 20 OTU (and 7

in total) were classified as *Anaerococcus octavius*, which suggests either intragenomic variation among rRNA operon copies or the existence of novel *Anaerococcus* species (**S14 Figure, Additional File 3**). Notably, while the centroid assignment for the most abundant OTU (OTU_2) was *S. epidermidis,* NMDS ordination clearly showed that members of this OTU consisted of 4 distinct clusters, mostly corresponding to *S. epidermidis* and *S. aureus* but also many sequences clearly clustering with *S. lugdenensis* and *S. simiae*, along with more distantly related staphylococci at low abundance (**Figure 8A**). By contrast, truncating reads to V3-V5 failed to show these relationships (**Figure 8B**).

We next investigated the relationship between species-level confidence values for each centroid and how many species are shared in the same dbOTU (**S15 Figure, Additional File 3**). This analysis identified several OTU whose centroid assignment belonged to a dbOTU with only 1 or 2 species. These may represent other novel or poorly described species, or alternatively some may represent problems with the taxonomy assignments in the NCBI database.

Figure 7 – Composition of the sinonasal community. Multiple dots indicate that more than one OTU was classified as the same species.
7A. Overall relative abundance of the top 20 most abundant species.
7B. Number of species observed in 10 or more samples.

Figure 8 - NMDS of *Staphylococcus* reads from the sinonasal community.
8A. Full length
8B. V3-V5
Ordination of pairwise distances among all sinonasal reads assigned to the OTU belonging to *Staphylococcus* along with full-length *Staphylococcus* 16s rRNA gene sequences from the NCBI database. Stress of full length NMDS 0.101, stress of v3v5 is 0.139

Variation in sinonasal microbial communities among subjects and anatomical sites

Considerable variation in microbial composition was seen among sinonasal specimens, ranging from 3 to 56 OTU per sample. OTU richness was lower among individuals, ranging from 50 to 108 per subject. The “core” sinonasal microbiome consisted of 11 OTU that were present across all 12 subjects, whereas most taxa were found in only a few individuals (**Figure 7B**). For subsequent analyses of microbial diversity, absolute counts were normalized to relative abundances after first removing low yield samples and rare taxa, though results were qualitatively similar even with no filtering. We set a minimum sample size of 500 read counts, reducing the number of samples from 122 to 108 (the number of reads collected per sample was highly variable, ranging from 0 to 17,548, mean 3,842 ± 3,229). We also set a minimum OTU size of 50 read counts across the whole set of samples, reducing the number of taxa to only 59 OTU across the dataset. Though rare taxa may play important roles in the sinonasal microbiome, as has been shown in other environments [6], in the absence of dense longitudinal sampling, we could not tell whether these were resident to the sinonasal passages, transients, or contaminants.

Overall, the taxonomic profiles across samples were distinctly more similar within-subjects than within-site, as illustrated by hierarchical clustering and NMDS ordination of samples (**Figure 9, Figure 10A**). This suggests that though the bacterial composition varies at distinct sub-anatomical sites, differences in microbial composition among individuals is much higher.

Figure 9 –
Heatmap of human sinonasal microbiome from 12 subjects. Columns are subjects; rows are species. OTU counts were summed by species-level centroid classification, samples with <500 reads were excluded, then species with <0.2% relative abundance in all samples were dropped. Remaining OTU counts were converted to relative abundances and then log-transformed after adding a pseudocount (1 / # of reads in sample) before hierarchical clustering, showing strong clustering by subject (horizontal colored strip, with different colors indicating the sample's subject)

Figure 10 –
10A. NMDS ordination of log-transformed Euclidean distance matrix of relative OTU abundances in human sinonasal specimens. Some clustering is observed by patient (color), little to no clustering by site (size) or type (shape). Stress value=0.0052.
10B. Box-plot of the variation in diversity among sites. The x-axis has all the sites used in the sino-nasal community sequencing and the y-axis represents the diversity. Coloring is based on the sample type, swab or biopsy.
10C. OTU richness and Shannon's effective number of OTU. Box-plot of number of OTUs observed in each patient. The colors are based sample type, swab or biopsy.
10D. Box-plot of Shannon's effective number of species observed in each patient. The colors are based sample type, swab or biopsy.

Because many OTU were only found in a subset of specimens, we next examined differences in the overall diversity of the samples with respect to subject, anatomical site, and whether obtained by swab or biopsy. Instead of using OTU richness (*i.e.* the total number of OTUs in each sample), we calculated Shannon's diversity index (which accounts for the relative abundance of distinct OTUs). Analysis-of-variance (ANOVA) of Shannon's diversity found that, by far, the most important factor accounting for variation in Shannon's diversity was the subject the sample had come from (**Figure 10B,** $p < 2e-16$). Sample type (swab versus biopsy) showed no significant effect ($p=0.116$). Furthermore, anatomical site was a significant contributor to the variance though no obvious trends were seen; variation among subjects was much higher (**Figure 10C**, $p=0.0082$). Swab/biopsy pairs from the same site and subject were extremely similar

(p=0.96), indicating no major shift in bacterial composition between the mucosal layer and the tissue immediately beneath, though the latter likely had fewer bacteria overall. These findings were robust to changing the filters used, to using genus or species level classification, and to reformulating the ANOVA model with different factor orders and interaction terms. Thus, these results suggest underlying community structure in the sinonasal cavity, though much of this effect is hidden by the much larger differences in overall microbial composition among subjects.

Finally, to examine whether bacteria might partition differently within the sinonasal cavity, we performed ANOVA on transformed relative abundance measurements for each OTU. Only a single OTU showed a significant effect by anatomical site (*Propiniobacterium acnes*, p=0.009 after Benjamini-Hochsberg FDR correction), and none showed variation by swab versus biopsy. Interestingly, *P. acnes* was least abundant relative to other bacteria in the nasal vestibule (site AB) the largest and most aerated part of the sinonasal cavity, whereas its abundance often made up a major component of the bacterial signature at other less accessible sites (**Figure 10D**).

Discussion

We report a novel high specificity pan-bacterial molecular diagnostic pipeline for profiling the bacterial composition of microbiome samples, applying amplification and sequencing of full-length 16S rRNA genes with the Pacific Biosciences (PacBio) platform. We exploit circular consensus sequencing (CCS), in which we obtain >10 passes on average of each single molecule sequenced, resulting in CCS reads with

exceptionally high average Phred-scaled base quality scores of >37. This single molecule correction system is not possible on other modern DNA sequencers (https://microbiomejournal.biomedcentral.com/articles/10.1186/2049-2618-1-10; https://academic.oup.com/nar/article/38/15/e159/2409757). Notably, our MCSMRT software is modular and can easily provide inputs to well-established and commonly used downstream microbiome analysis pipelines (namely QIIME and Mothur) at several points before or after OTU clustering, taxonomic assignment, or abundance calculations. Two main innovations of MCSMRT extend and improve upon previous applications of PacBio sequencing to bacterial composition profiling using the 16S rRNA gene as a taxonomic marker [19-25]:

(1) We identify and implement a series of stringent filters that eliminate many sources of sequencing artifacts, and in particular we show that using only the highest fidelity consensus reads for the OTU clustering step (those with a cumulative expected error, EE, of ≤ 1) effectively eliminates over-calling the number of OTU, which has been a pervasive problem in methods using shorter partial 16S sequences [52].

(2) To assign taxonomy and confidence values to centroid OTU sequences, we needed to create a custom-built database constructed from all available full-length 16S rRNA sequences at NCBI, since many commonly used 16S rRNA gene databases lack species-level classifications or lack full-length 16S genes for many taxa. This custom database allowed us to use a uniform Linneaen hierarchy based on NCBI's txid and build a classifier that defined full-length 16S gene sequences to the species level along with associated confidence values. Our analysis of this utax trained classifier showed improved accuracy and higher confidence when using full-length 16S rRNA sequences

compared to partial sequences, which are typical when using short-read Illumina MiSeq 16S survey methods that normally capture only up to ~500 nt using paired-end sequencing (*e.g.* http://aem.asm.org/content/79/17/5112.short).

We tested our experimental and bioinformatics pipeline on two distinct "mock communities", showing that use of high-quality CCS reads from full-length 16S rRNA genes has exceptional precision and accuracy at identifying and quantifying the bacteria in complex mixtures. We correctly classified all bacterial species in the relatively simple BEI mock community, and showed the ability to differentiate between *Staphylococcus aureus and S. epidermidis*, which have 99% identity between their 16S rRNA genes thereby belonging to the same OTU. More impressively, we correctly identified most species in the more complex CAMI community, making only 3 false identifications represented by only 5 CCS reads. Remarkably, we also accurately measured the relative abundance of most species in this complex CAMI community, indicating that our pipeline not only has high taxonomic specificity but high accuracy for quantitative measures of species abundance in complex microbial communities. Underlining the accuracy and precision of our experimental and bioinformatics procedures, 99.81% of filtered reads from monoclonal positive control samples were correctly classified to the species level, and 7 independent negative controls yielded almost no reads that passed our filters.

Following validation of our pipeline using complex mock communities, we applied it to the human sinonasal microbiome, finding that the community has a relatively low complexity (with 61 OTU at a frequency of >0.1% across samples); *Staphylococcus* species and *Propionibacterium acnes* dominated across subjects and anatomical sites

[35]. Nevertheless, although microbial composition varied much more substantially among subjects than among anatomical sites in the same subject, we nevertheless observed trends in the overall diversity of different sites, with the easily accessible swabs just deep to the nasal vestibule overall reflecting the majority of the healthy sinonasal cavity with the least diverse and least dominated by *P. acnes*. Importantly, we find that swab and biopsy sampling at the same site in the same subject have highly correlated microbial composition, indicating that invasive biopsy sampling is not needed. The large differences among the sinonasal microbiomes among healthy subjects will be of interest in future studies that examine links between sinonasal disease states (particularly chronic rhinosinusitis), bacterial composition, and the innate immune response [72-79].

Conclusion

Beyond improving the accuracy of species-level classification by capturing all nine variable regions of the bacterial 16S rRNA gene, we anticipate two additional applications for marker-based long amplicon sequencing for microbiome analysis: (1) higher power to discriminate below the OTU or species levels using oligotyping and related approaches [80, 81], and (2) expanding the scope of marker-based taxonomic and phylogenetic classification scheme through, for example, joint single-molecule sequencing of eukaryotic ITS and 18S rRNA genes from fungi to enrich and extend marker-based databases [82].

Methods

Ethics statement: Patients were recruited from the Division of Rhinology of the Department of Otorhinolaryngology - Head and Neck Surgery at the University of Pennsylvania with full approval of the Institutional Review Board (Protocol 800614). Informed consent was obtained during the pre-operative clinic visit or in the pre-operative waiting room. Selection criteria for recruitment were patients undergoing sinonasal surgery for non-rhinologic disease entities, *e.g*. pituitary pathology or other cranial base pathologies.

Sinonasal sample collection: Sinonasal samples were obtained from patients undergoing sinonasal surgery for non-inflammatory and non-infectious indications (predominately pituitary tumors or other skull base neoplastic process) who had not received antibiotics in the preceding 8 weeks. The Institutional Review Board at The University of Pennsylvania School of Medicine provided full study approval and informed consent was obtained pre-operatively from all patients. Sinonasal specimens were collected as both swabs (S) (BD ESwab collection and transport system) and Tissue (T) (placed in MP lysing matrix tubes). Multiple locations in the sinonasal cavity were sampled including the nasal vestibule (S), inferior turbinate head (T), uncinate process (T), middle meatus (S), maxillary sinus (S)(T), ethmoid sinus (S)(T), superior meatus (S), superior turbinate (T), and sphenoid sinus (S)(T) for a maximum of 12 specimens per patient.

DNA extractions: Total DNA was isolated from all samples (swabs and biopsies) using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturers recommendations with slight modifications. Biopsy material was incubated overnight at 56°C with 570 µl ATL lysis buffer with 30 µl Proteinase K in a Lysing Matrix E tube (MP Biomedicals

LLC), homogenized by SPEX 1600 MiniG (Fisher Sci.) for 10min. at 1500 Hz, and centrifuged 1min x 13000 rpm. Swab tubes were treated similarly but initially vortexed for 1min. and spun for 10 seconds and incubated for only 5 min. at 56 °C prior to homogenization. DNA was eluted with 200 µl of the Elution Buffer. DNA quality, and quantity were analyzed by agarose gel electrophoresis and Nanodrop 2000 spectrophotometry.

Control DNA samples: The BEI mock community was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project (www.beiresources.org): We used genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S RNA Gene Sequencing, HM-782D. The complex CAMI mock community was obtained from the JGI, which had been constructed for the CAMI (Critical Assessment of Metagenomic Interpretation) Hosts Community Challenge for Assessing Metagenomes. Human DNA was isolated from the U937 lymphoblast lung cell line as an off-target control template.

Full-length 16S rDNA PCR reactions: Amplifications were performed using 1µl total DNA as template, universal 16S primers F27 and R1492 with four sets of asymmetric barcodes at 0.25 µM (Table S3) [83, 84], and GoTaq Hot Start Master Mix (Promega) or AccuPrime Taq High Fidelity Polymerase with 1µl of 10mM dNTP Mix (Fisher Sci.) in 50µl final volume. Cycling conditions were: 94°C, 3 min; then 22 or 35 cycles of 94°C 30 sec, 54°C 30 sec, 72°C 2 min; following by 5 min final elongation at 72°C. PCR products were cleaned with AxyPrep™ MagPCR (Corning Life Sciences) according to the manufacturer's protocol and eluted in 40µl of water. Cleaned PCR products were quantified using both using Quant-iT™ dsDNA Assay Kit, high sensitivity (Invitrogen) on

BioTek™ FLx800™ Microplate Fluorescence Reader, and AccuClear Ultra High Sesitivity sDNA Quantitation Kit (Biotium). Based on the results, amplicons were normalized to the same concentration prior to pooling amplicons with distinct barcodes into multiplexed sets of 2-4 samples per pool.

Pacific Biosciences circular consensus sequencing: Library construction used Pacific Biosciences (PacBio) SMRTbell™ Template Prep Kit V1 on normalized pooled PCR products, and sequencing was performed using the PacBio RS II platform using protocol "Procedure & Checklist - 2 kb Template Preparation and Sequencing" (part number 001-143-835-06). DNA Polymerase Binding Kit P6 V2 was used for sequencing primer annealing and polymerase binding. SMRTbell libraries were loaded on SMRTcells V3 at final concentration 0.0125 nM using the MagBead kit. DNA Sequencing Reagent V4 was used for sequencing on the PacBio RS II instrument, which included MagBead loading and stage start. Movie times were 3 hours for all SMRTcells. PacBio sequencing runs were set up using RS Remote PacBio software and monitored using RS Dashboard software. Sequencing performance and basic statistics were collected using SMRT® Analysis Server v2.3.0.

Pre-Clustering Pipeline: The MCSMRT pipeline accepts CCS data from the PacBio RSII sequencer, and is divided into pre-clustering and clustering steps (**Figure 1, Pre-clustering Pipeline, Additional File 1**). Sequences were generated using the reads of insert (ROI) protocol within Pacific Biosciences SMRT® Analysis Server, reads which had 4 or fewer CCS passes were removed. To further filter low quality or off target sequences, reads failing 3 filters were removed: (a) CCS reads outside the range of 500 to 2000 bp, (b) those that aligned to the hg19 human genome with bwa v0.7.10-r789,

and (c) those that did not match both primer sequences with usearch v8.1.1861 [15, 49]. Primer sequences were then trimmed, and reads were oriented 5' to 3' with respect to 16S rRNA transcription. Venn diagrams defining read filtration subsets were created using Venny [85].

OTU clustering, taxonomic classification of centroids, and OTU abundances: OTUs were generated using the uparse [41] algorithm in the usearch software, using parameters tuned for full-length sequence. In short, reads were de-replicated (and the number, or size, of identical sequences tracked in the header), then sorted by abundance. OTUs were iteratively created at a threshold of 3% divergence from any other existing OTU centroid sequence (*i.e*. reads within 97% similarity to an existing OTU centroid became a member of an existing cluster; otherwise a new OTU was formed with that sequence).

To obtain a database capable of providing a species-level classification of the full-length sequences, all sequences annotated as full-length 16S rRNA genes were downloaded from NCBI in Oct. 2015, and taxonomies were inferred from each read's 16S gid identifier via the associated txid. This newly formatted database contained species-level taxonomic information for OTU classification (**16s rRNA Microbial Database, Additional File 1**). Representative OTU sequences were assigned a taxonomy using a utax classifier built from this database.

Chimeric sequences were removed during the clustering process based on previously seen OTU centroid sequences (CHIM1 filtering), followed by removal of chimeric

centroid OTU using uchime to filter the final OTU sequences using the RDP ‘gold’ sequences [50].

OTU abundance was determined using usearch for filtered reads prior to the expected error threshold, reported as CCS read counts assigned to each centroid OTU. 16S rRNA copy number for the BEI community was estimated from provided quality control data, and OTU abundance by 16S rRNA copy number was calculated in R (Table S1, Figure 8). OTU abundances for the CAMI and sinonasal datasets were calculated using the same rrndb database to the closest matching taxonomic level, however differences between the relative abundance of OTU before copy number correction, and after copy number correction were not significantly different and therefore not used further (**Figure S16, Additional File 3**).

Sub-OTU ordination and phylogenetic methods: A 3% divergence cutoff for OTU clustering is commonly used in comparing various partial 16S fragments [6, 10, 86, 87]. To further examine how individual reads were related to one another, mafft [88] alignments of individual genus/species sequences (including sequences from both OTU and matching NCBI database) were created. Pairwise distance matrices from the alignments were created using ape v4.1 [89] and seqinr v 3.3-6 [90]. 2D non-metric multidimensional scaling (NMDS) ordinations and neighbor-joining trees used vegan v2.4-0 and ggtree v1.8.1 respectively. Data were visualized using ggplot2 v2.2.1 [91-93]. Additionally, maximum likelihood trees (FastTree v2.1.8) were calculated and visualized with ggtree [94].

Ecological analyses of the healthy sinonasal microbiome: Measures of ecological diversity (number of species observed, Shannon's diversity index) were calculated for each sample using vegan before and after filtering to eliminate samples with <500 CCS reads, and OTU with <50 CCS reads across all samples. Count tables were transformed to relative abundances prior to calculating dissimilarity and distance matrices by either Bray-Curtis or Euclidean distance metrics. NMDS ordinations of samples were generated with vegan, and heatmaps created using the gplots package for R.

List of abbreviations

BEI - Biological and Emerging Infections Resources Program
CCS – Circular consensus sequence
CAMI – Critical Assessment of Metagenome Interpretation
EE – Expected Error
JGI – Joint Genome Institute
MCSMRT – Microbiome Classifier using Single Molecule Real-time Sequencing
NCBI – National Center for Biotechnology Information
NIAID - National Institute of Allergy and Infectious Diseases
NMDS – Non-metric Multidimensional Scaling
nt - nucleotide
OTU – Operational Taxonomic Unit
Pacbio – Pacific Biosciences
ROI – Reads of Insert
RDP - Ribosomal Database Project
rrnDB - Ribosomal RNA Operon Copy Number Database

Declarations

- Ethics approval and consent to participate

  The Institutional Review Board at The University of Pennsylvania School of Medicine provided full study approval and informed consent was obtained pre-operatively from all patients.

- Consent for publication

  Not applicable.

- Availability of data and material
  - The dataset(s) supporting the conclusions of this article is(are) available in the NCBI SRA repository, [unique persistent identifier and hyperlink to dataset(s) in http:// format].
  - Software
    - Project name: MCSMRT
    - Project home page: http://github.com/jpearl01/mcsmrt
    - Archived version: DOI or unique identifier of archived software or code in repository (e.g. enodo)
    - Operating system(s): linux 64 bit
    - Programming language: ruby
    - Other requirements: usearch v.8.1.1861
    - License: e.g. GNU GPL, FreeBSD etc.
    - Any restrictions to use by non-academics: none
  - For databases, this section should state the web/ftp address at which the database is available and any restrictions to its use by non-academics.

- Competing interests

  The authors declare that they have no competing interests.

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- Authors' contributions
  - Conceived and designed study: JPE, NAC, GDE, JCM
  - Carried out experiments: All
  - Carried out analyses: All
  - Coded software: JPE, AB, RLE

- Provided materials and reagents: NDA, JNP, NAC, GDE
- Wrote the manuscript: JPE, JCM, AB, GDE, NAC, JK, SB, RLE, JH
- Read and approved the final manuscript: All authors

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Figure 1: Pipeline for microbiome classification by single-molecule real-time sequencing

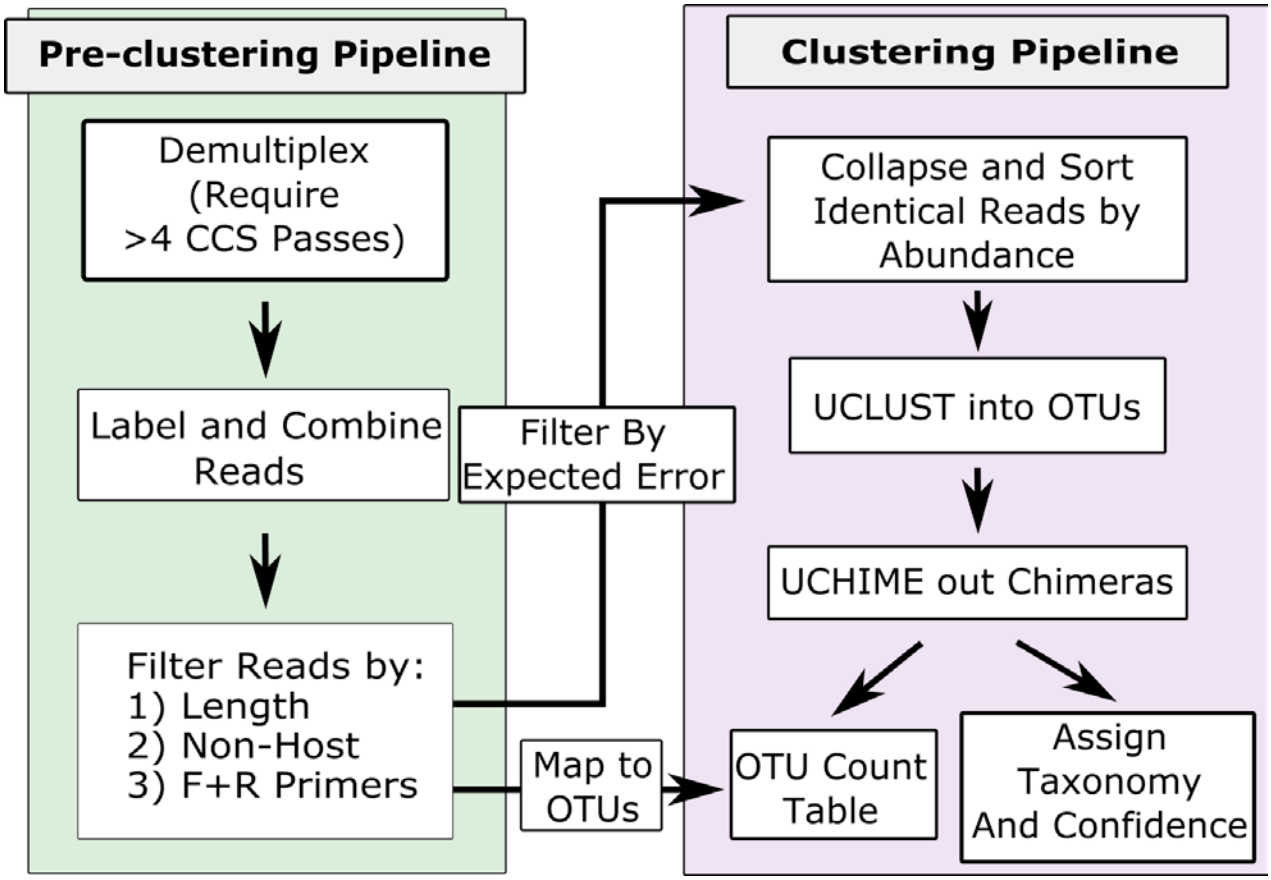


Figure 2: EE vs CCS in BEI mock community

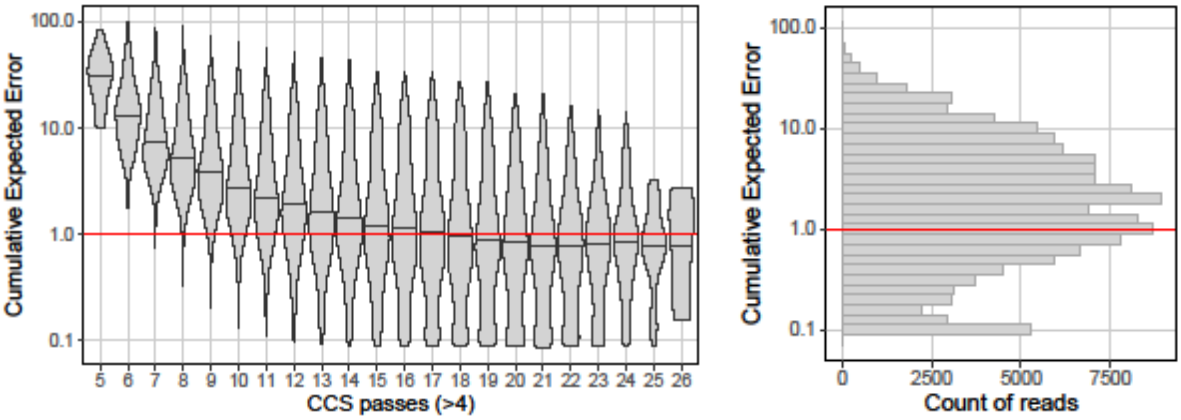


Figure 3: Read and OTU yields X EE

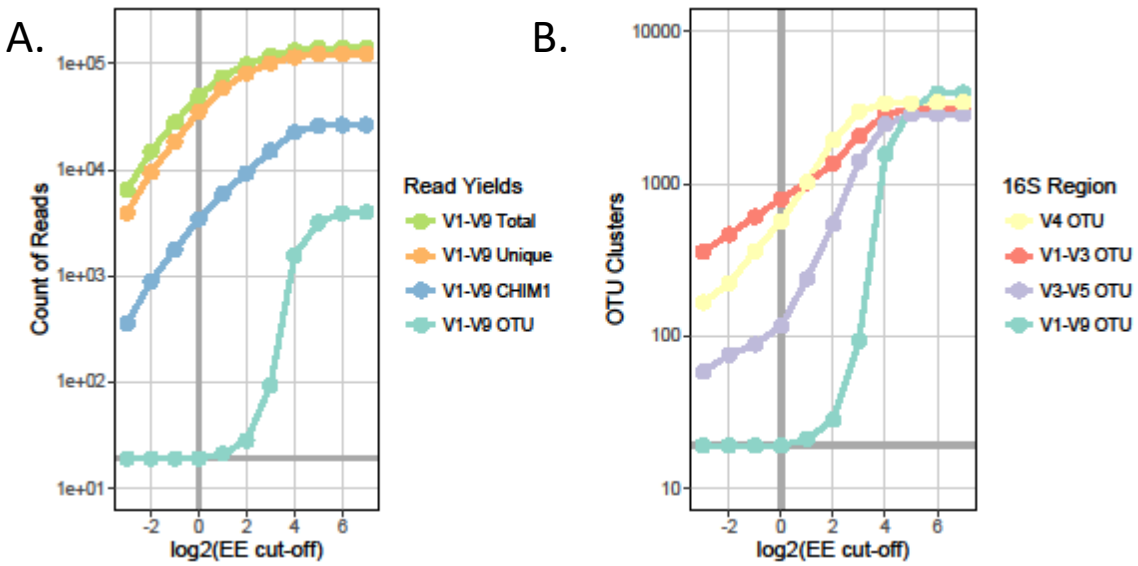
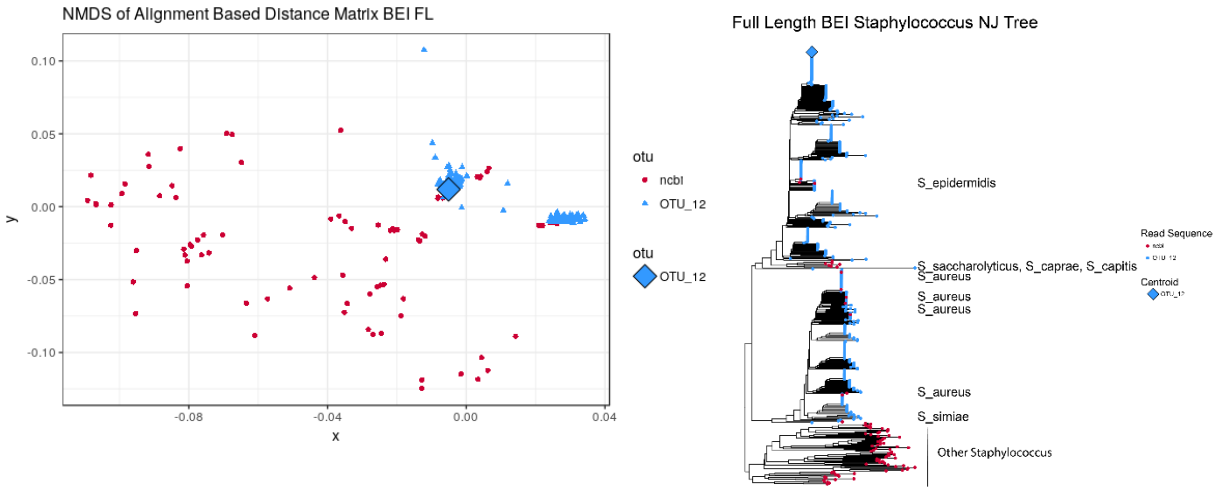


Figure 4: BEI NMDS and NJ trees for Staphylococcus spp.

A.



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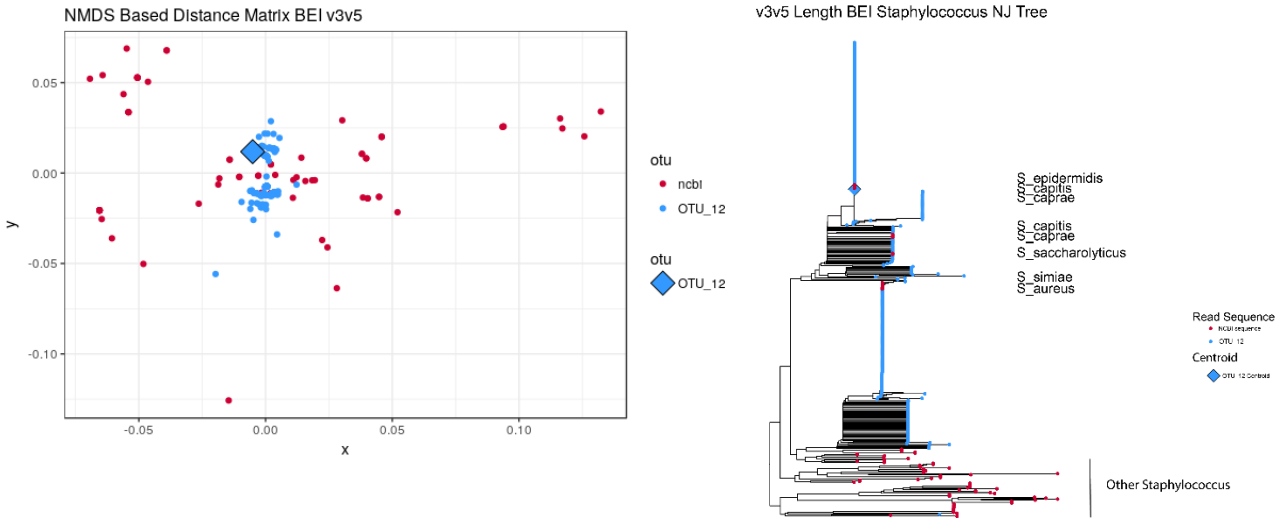


Figure 5: CAMI community composition

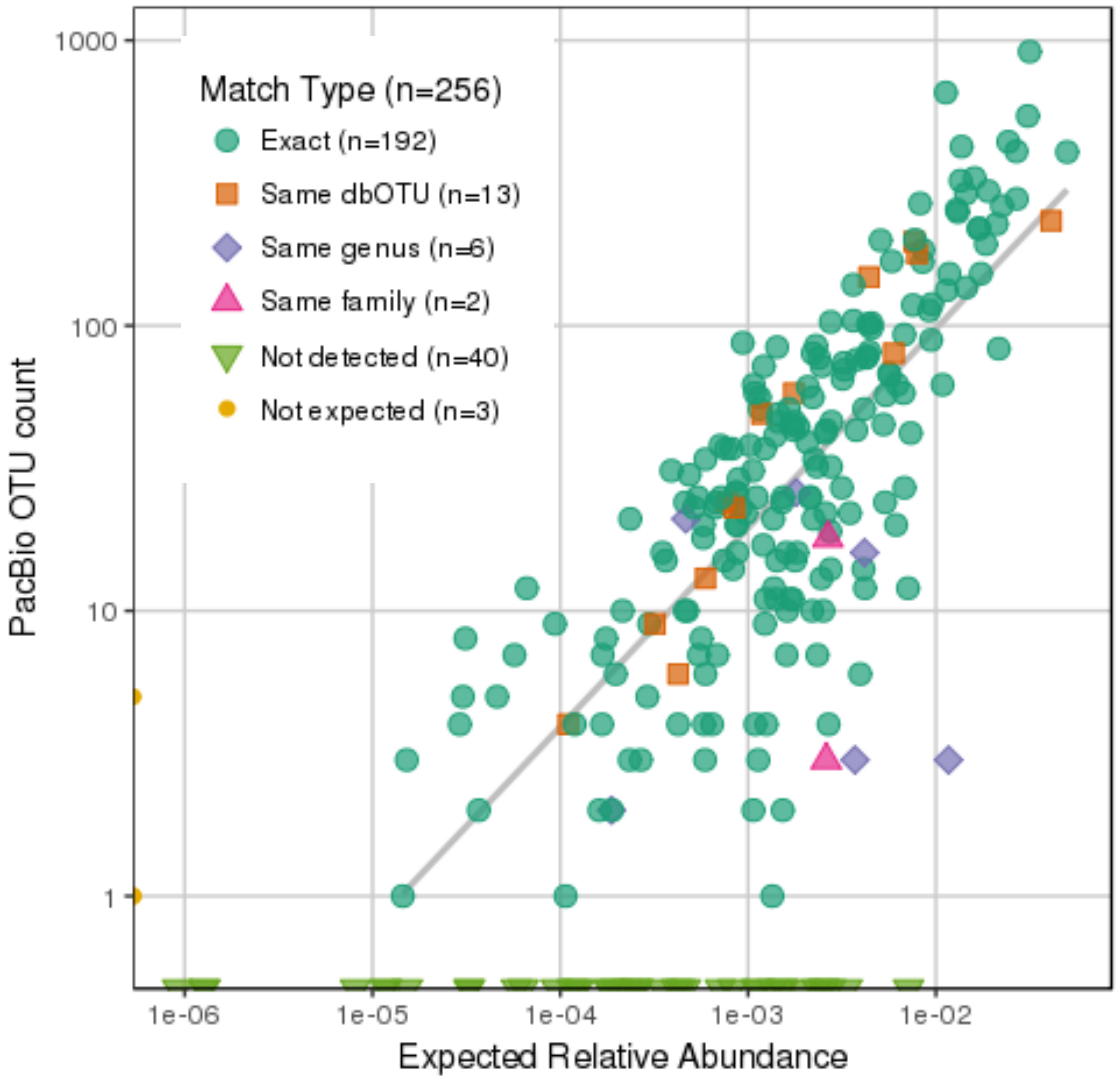


Figure 6

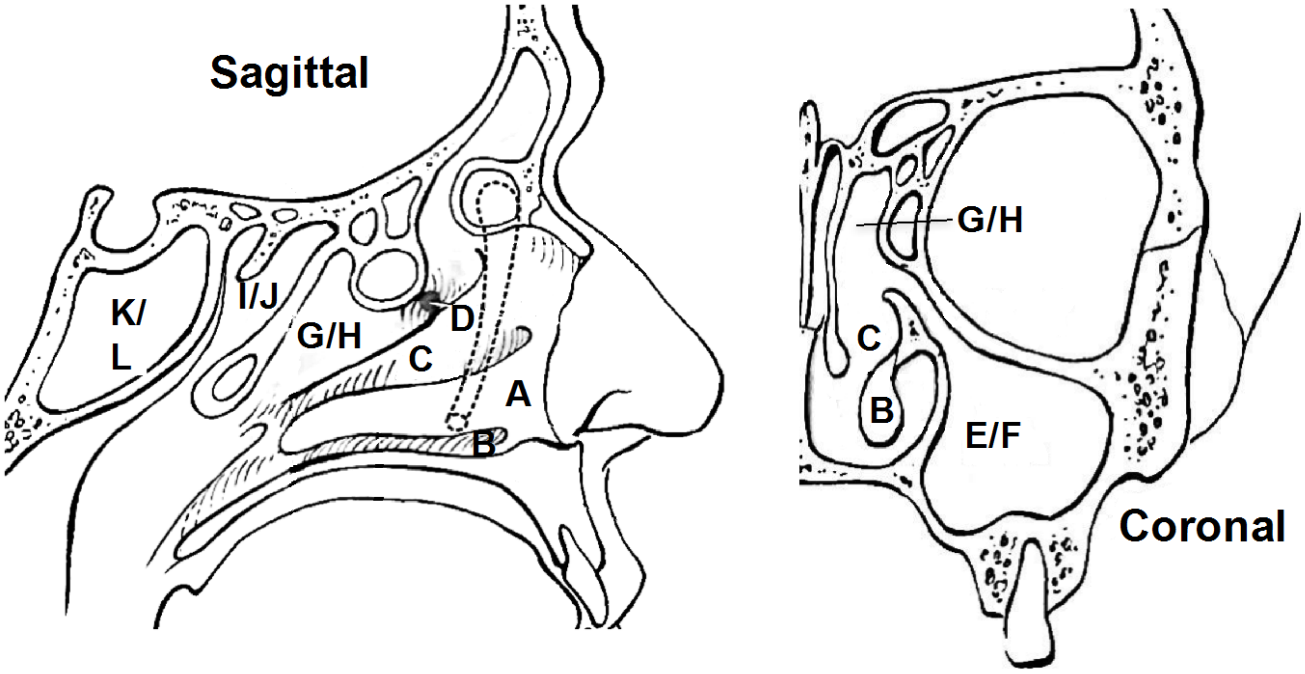


Figure 7: Frequently observed species

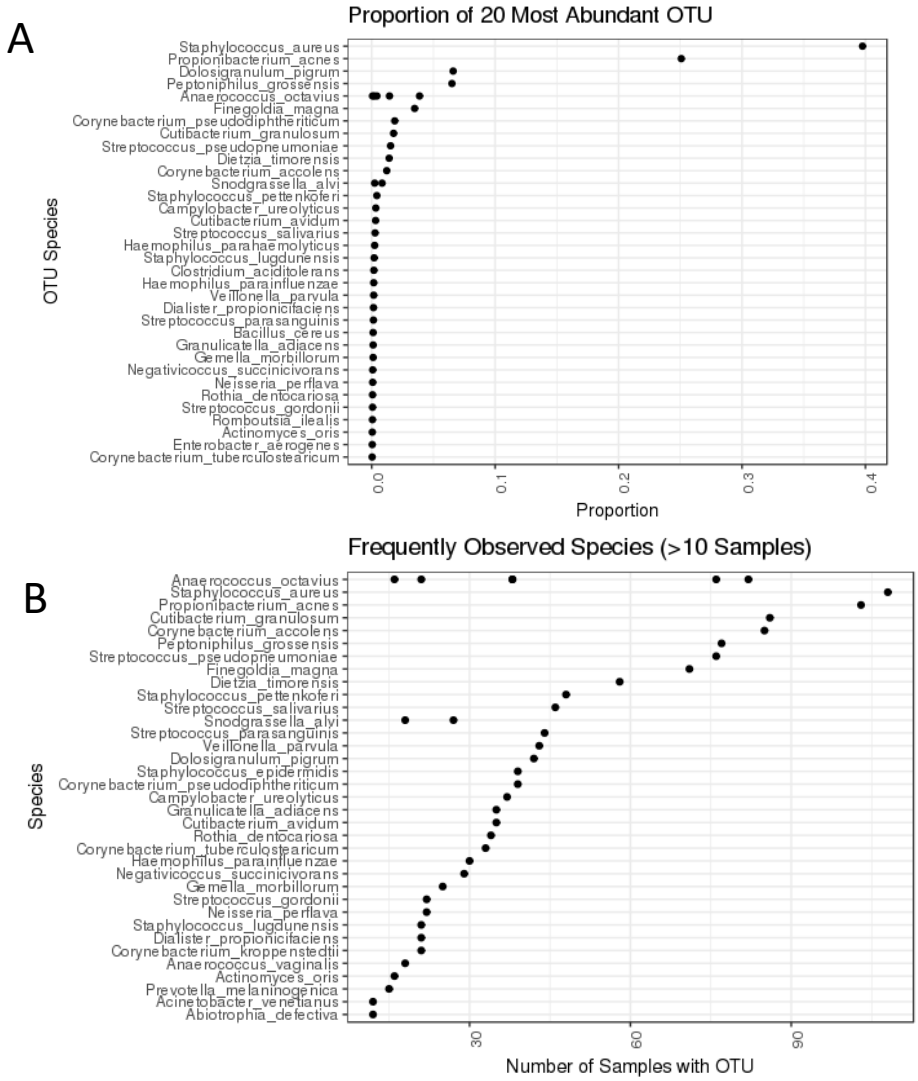
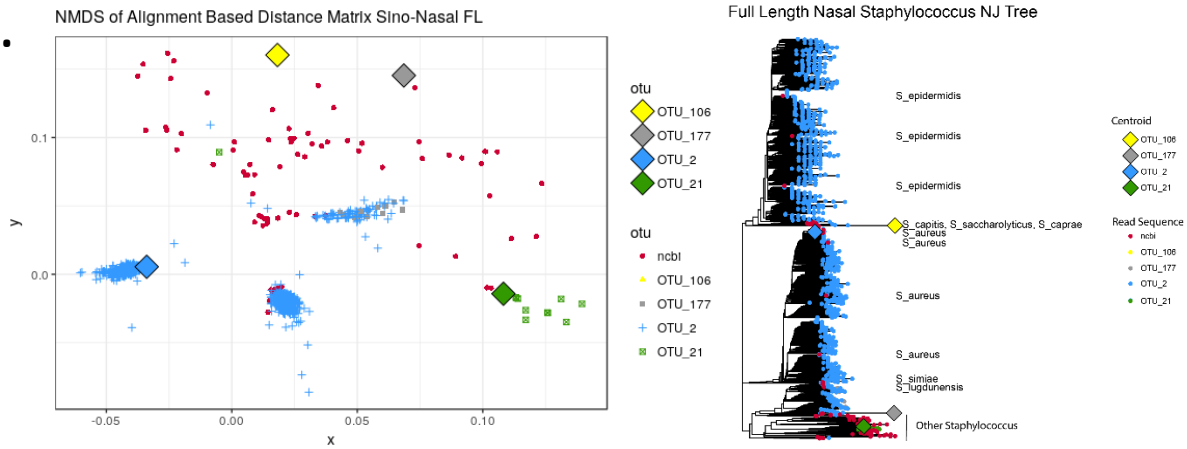


Figure 8: Sinonasal NMDS and NJ trees for Staphylococcus spp.

A.



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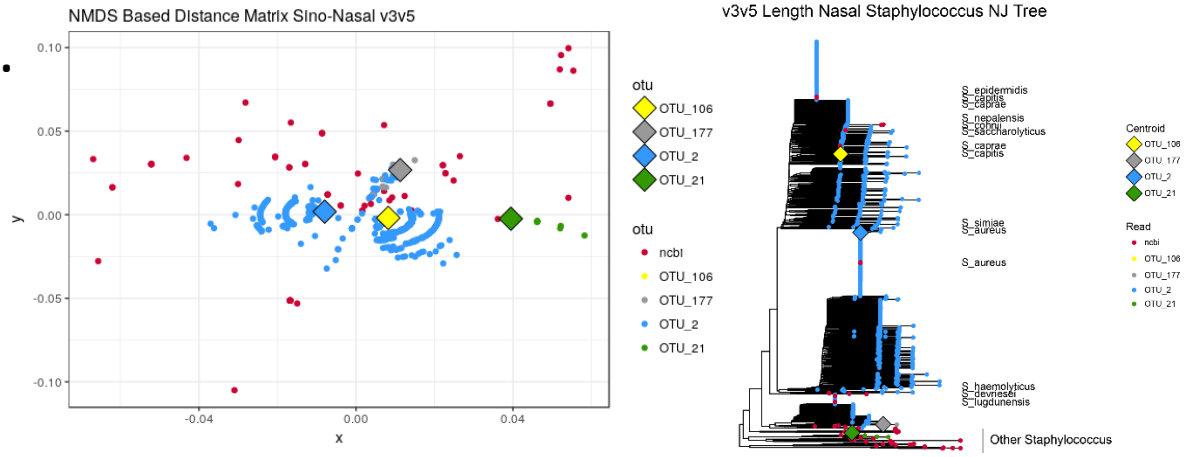


Figure 9: Heatmap of human sinonasal microbiome from 12 subjects.

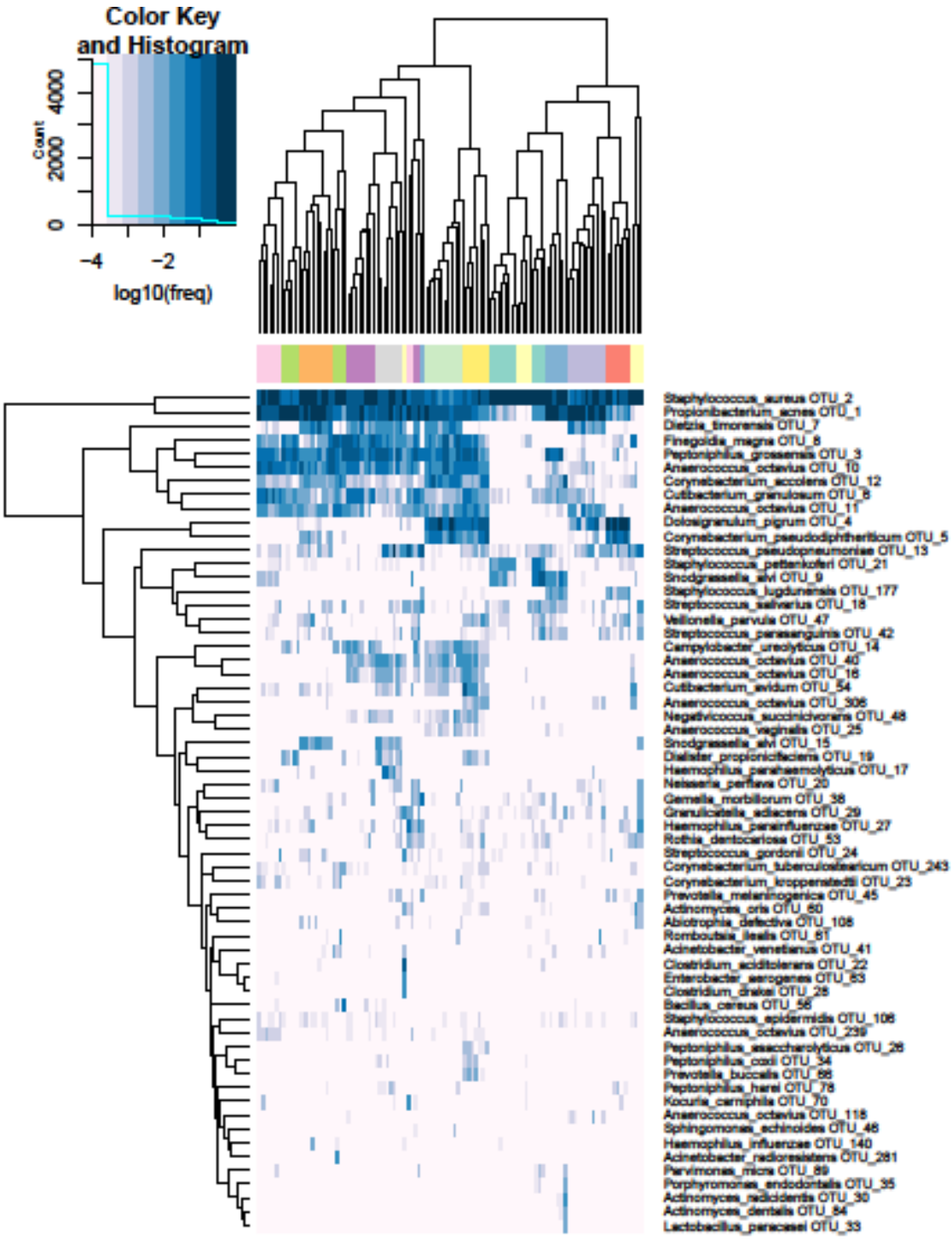
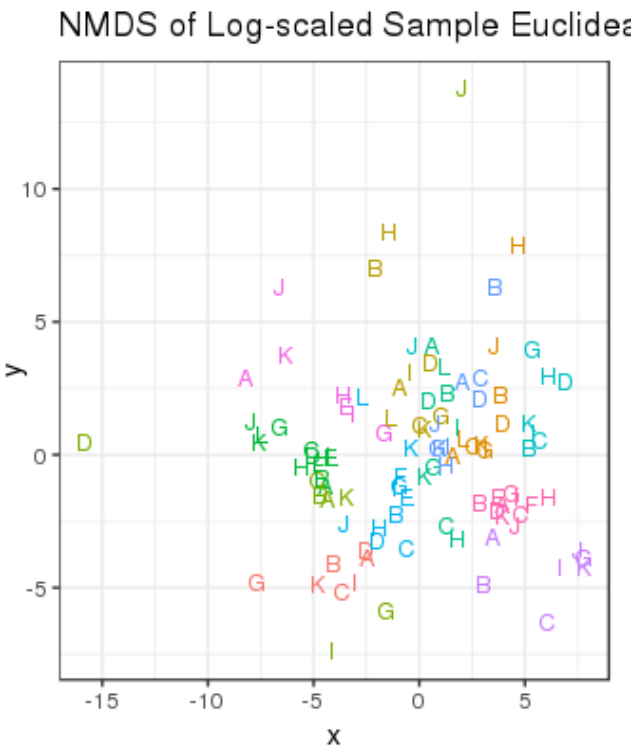


Figure 10:

A



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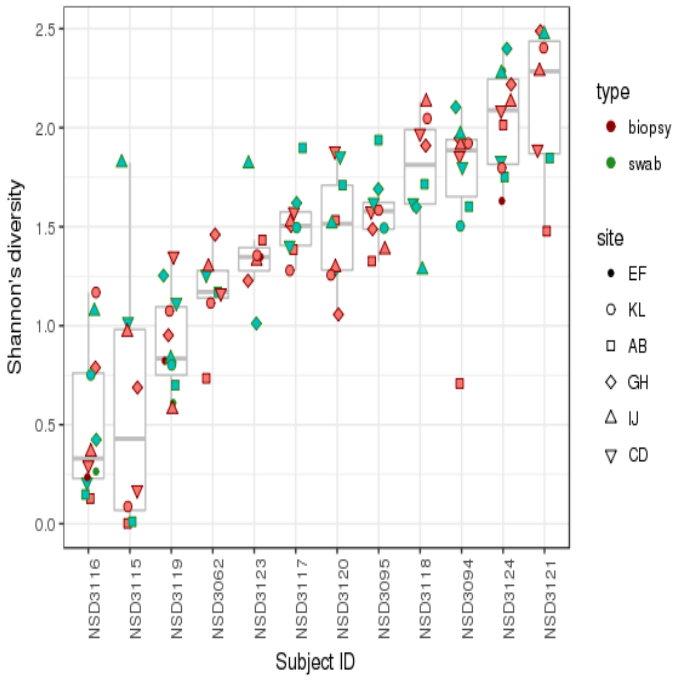
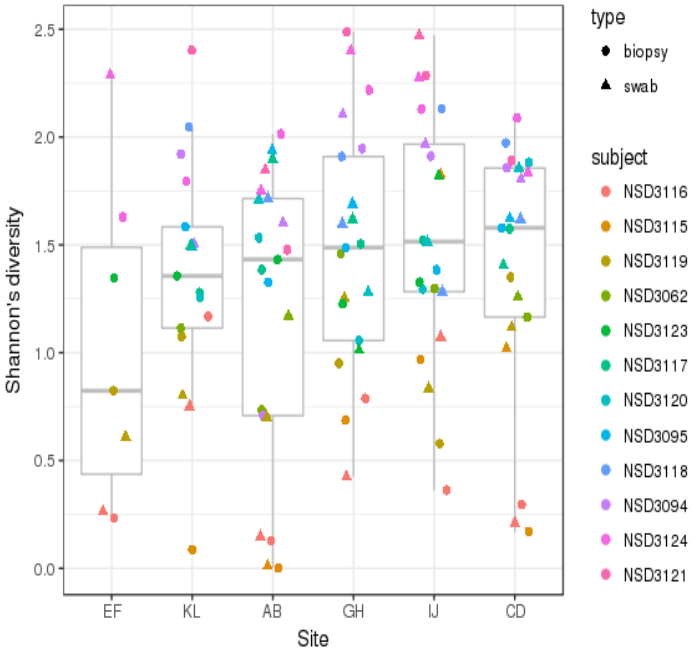
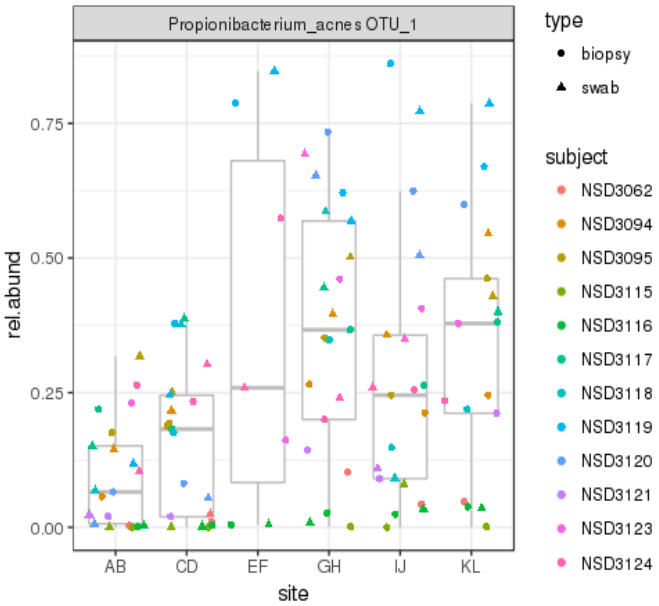


C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