Microbiome Analysis with QIIME2: A Hands-On Tutorial

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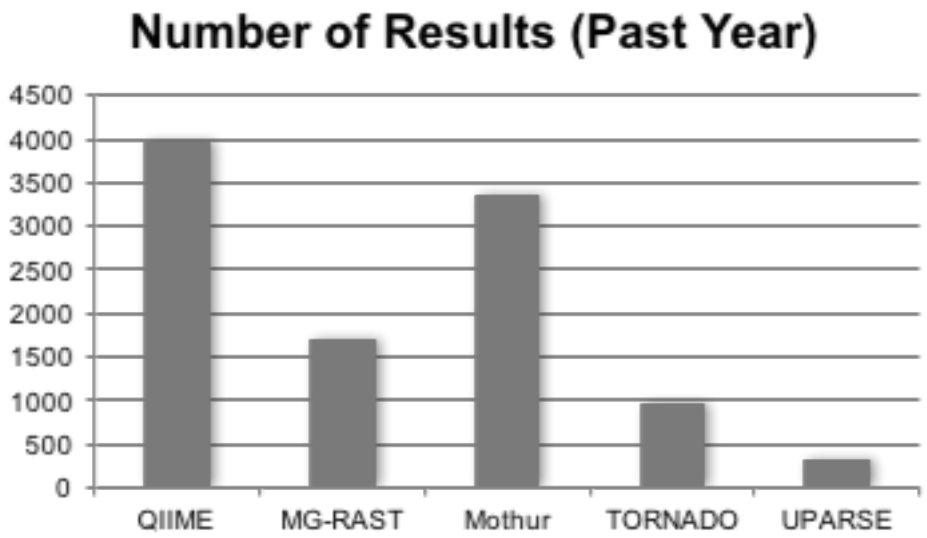
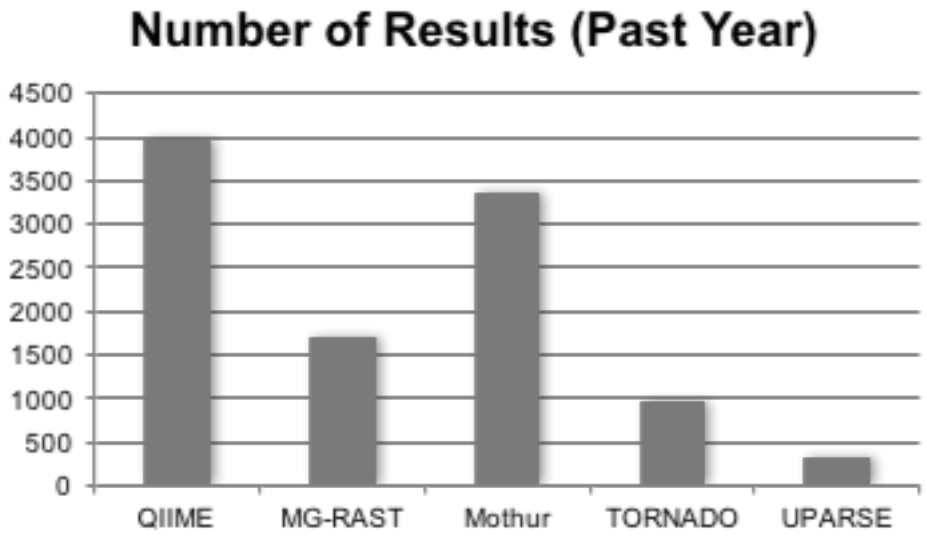
Software Selection

- Google “16S analysis <program name>”; main contenders are
- Mothur
  - Name: not an acronym (play on DOTUR, SONS)
  - Philosophy: single piece of re-implemented software
  - Top pro: easy to install
  - Top con: re-implementations could be buggy
  - Language: C++
  - Model: open-source
  - License: GPL
  - Published: 2009
  - Developed: at Umichigan



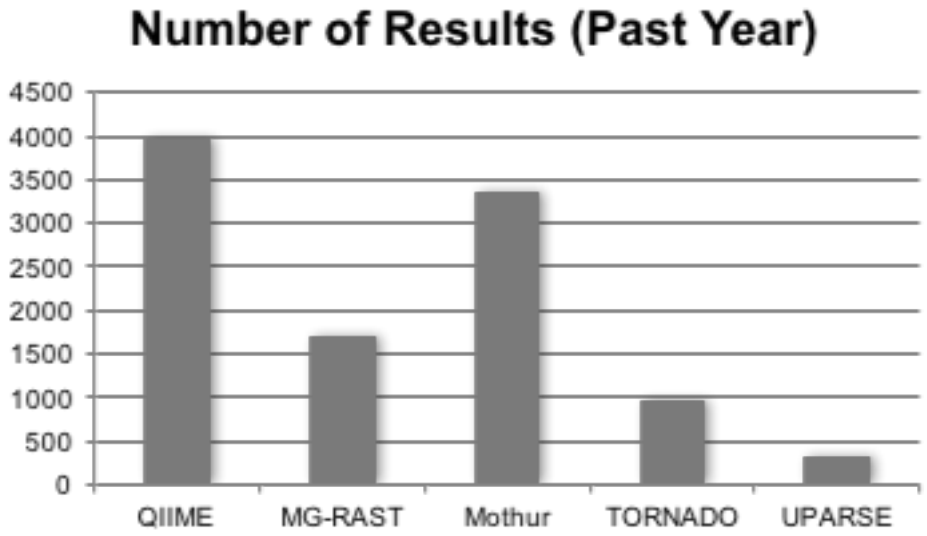
Software Selection

- Google “16S analysis <program name>”; main contenders are
- QIIME
  - Name: Quantitative Insights Into Microbial Ecology
  - Philosophy: wrapper of best-in-class software
  - Top pro: extremely flexible
  - Top con: QIIME 2 not yet feature-complete
  - Language: python (wrapper)
  - Model: open-source
  - License: mixed
  - Published: 2010
  - Developed: At UCSD, NAU



Software Selection

- Google “16S analysis <program name>”
  - Main contenders are Mothur and QIIME
  - Both widely used
  - Both pride themselves on quality of support
- Will discuss only QIIME in this tutorial
- QIIME 1 vs QIIME 2
  - QIIME 1 is no longer supported (since end of 2017)
  - This tutorial uses QIIME 2 **only**
- **I’m not a QIIME 2 developer**
  - I’m not taking credit for this tool, just demonstrating it!
  - Today’s practicum is an expansion of the QIIME2 “Moving Pictures” tutorial



Prologue: Tuning

- Show of hands, please:
  - How many have analyzed 16S data before?
  - How many know what the “command line” is?
  - How many are comfortable with unix shell commands?

Approach:

- Practicum on 16S analysis with QIIME 2
  - Alternating lecture and tutorial on command-line software
- Suggest you pair up with a partner
  - Two eyes are better than one for finding mistakes and patterns
- Use the provided post-it notes to signal your status
  - None—command not yet completed
  - Green—command completed, no problems
  - Red—having problems!
- Red post-its will be visited by my lovely assistant ☺

Get Ready To Practice!

- **“Why are you making me type?!”**
  - QIIME 2 has a GUI—but still under development
  - QIIME 2 command-line interface is easy to install and ready to run
  - Typing is better than copy/pasting commands because in your real analyses, you will need to type in the appropriate commands for your data
    - Need to make realistic typing mistakes now so you know how to correct them later!

Tips to Help

- When typing a file name or directory path, you can use **tab completion**
  - Start typing file/directory path, then hit tab—if only one file/directory matches what you already typed, shell fills that in
    - Very helpful for correctly entering long file names
    - If >1 matches, shell fills in as much as it can
- Press **up arrow** to get back previous commands you typed
- If you type a command, press enter, and "nothing happens", **don't just run it again**
  - Many unix commands produce no visible output to shell—just get back command prompt
  - That doesn't mean they do nothing, so running them *again* can screw up results
  - **Do not store commands in a word processing program** (or PowerPoint, etc)
    - E.g., MS Word changes hyphens to "m dash"—which command line can't understand
  - Shell commands are **case-sensitive**

Getting Data

- Data acquisition method is project-specific
  - Public data can often be pulled down from internet with `wget` or `curl` commands
  - Sequencing data from a core usually available by ftp
    - Can use browser, Cyberduck, Filezilla, etc
  - If all else fails, use a flash drive ☺

Getting Data (cont.)

- For today's tutorial, we will use public data from the QIIME2 website

```
mkdir qiime2-moving-pictures-tutorial

cd qiime2-moving-pictures-tutorial

wget -O "sample-metadata.tsv" \
"https://data.qiime2.org/2018.4/tutorials/moving-pictures/sample_metadata.tsv"

mkdir emp-single-end-sequences

wget -O "emp-single-end-sequences/barcodes.fastq.gz" \
"https://data.qiime2.org/2018.4/tutorials/moving-pictures/emp-single-end-
sequences/barcodes.fastq.gz"

wget -O "emp-single-end-sequences/sequences.fastq.gz" \
"https://data.qiime2.org/2018.4/tutorials/moving-pictures/emp-single-end-
sequences/sequences.fastq.gz"

wget -O "gg-13-8-99-515-806-nb-classifier.qza" \
"https://data.qiime2.org/2018.4/common/gg-13-8-99-515-806-nb-classifier.qza"
```

Making a Mapping File

#SampleID	LinkerPrimerSequence	BarcodeSequence	ReportedAntibioticUsage	DaysSinceExperimentStart	SampleType
L1S140	GTGCCAGCMGCCGCGGTAA	ATGGCAGCTCTA	Yes	0	gut
L2S155	GTGCCAGCMGCCGCGGTAA	ACGATGCGACCA	No	84	left palm

- "Mapping file" contains metadata for study
  - Must contain info needed to process sequences and test YOUR hypotheses
- QIIME 1 required certain columns in certain order, but QIIME 2 is more flexible
  - Tab-separated text file with column labels in first line + at least one data line
    - Column label values must be unique (i.e. no duplicate values)
  - First column is the "identifier" column (sample ID)
    - All values in the first column must be unique (i.e. no duplicate values)
  - See https://docs.qiime2.org/2017.6/tutorials/metadata/
- The easiest way to make a mapping file is with a spreadsheet
  - But **Excel is not your friend!**
    - Routinely corrupts gene symbols, anything interpreted as a dates, etc, & isn't reversible

Practicum: Viewing A Mapping File

- Open Terminal
  - For below, remember to try tab completion!
  - Ensure you are in the tutorial directory:
    - `qiime2-moving-pictures-tutorial`

```
source activate qiime2-2018.4
```

```
ls
```

```
nano sample-metadata.tsv
```

- Stretch the window so you can look at the contents; then, to close, type

```
Ctrl + x
```

- Mapping file errors can lead to QIIME 2 errors—or worse, garbage results!
  - Keemei (pronounced 'key may') tool checks for errors in **Google Sheets**
    - **Chrome only,** and must have Google account to use; see http://keemei.qiime.org/

Mapping File View

```
 GNU nano 2.0.6                                File: sample-metadata.tsv

#SampleID       BarcodeSequence LinkerPrimerSequence    BodySite        Year    Month   Day     Subject ReportedAntibioticUsage DaysSinceExperimentStart        Description
L1S8    AGCTGACTAGTC    GTGCCAGCMGCCGCGGTAA     gut     2008    10      28      subject-1       Yes     0       subject-1.gut.2008-10-28
L1S57   ACACACTATGGC    GTGCCAGCMGCCGCGGTAA     gut     2009    1       20      subject-1       No      84      subject-1.gut.2009-1-20
L1S76   ACTACGTGTGGT    GTGCCAGCMGCCGCGGTAA     gut     2009    2       17      subject-1       No      112     subject-1.gut.2009-2-17
L1S105  AGTGCGATGCGT    GTGCCAGCMGCCGCGGTAA     gut     2009    3       17      subject-1       No      140     subject-1.gut.2009-3-17
L2S155  ACGATGCGACCA    GTGCCAGCMGCCGCGGTAA     left palm       2009    1       20      subject-1       No      84      subject-1.left-palm.2009-1-20
L2S175  AGCTATCCACGA    GTGCCAGCMGCCGCGGTAA     left palm       2009    2       17      subject-1       No      112     subject-1.left-palm.2009-2-17
L2S204  ATGCAGCTCAGT    GTGCCAGCMGCCGCGGTAA     left palm       2009    3       17      subject-1       No      140     subject-1.left-palm.2009-3-17
L2S222  CACGTGACATGT    GTGCCAGCMGCCGCGGTAA     left palm       2009    4       14      subject-1       No      168     subject-1.left-palm.2009-4-14
L3S242  ACAGTTGCGCGA    GTGCCAGCMGCCGCGGTAA     right palm      2008    10      28      subject-1       Yes     0       subject-1.right-palm.2008-10-28
L3S294  CACGACAGGCTA    GTGCCAGCMGCCGCGGTAA     right palm      2009    1       20      subject-1       No      84      subject-1.right-palm.2009-1-20
L3S313  AGTGTCACGGTG    GTGCCAGCMGCCGCGGTAA     right palm      2009    2       17      subject-1       No      112     subject-1.right-palm.2009-2-17
L3S341  CAAGTGAGAGAG    GTGCCAGCMGCCGCGGTAA     right palm      2009    3       17      subject-1       No      140     subject-1.right-palm.2009-3-17
L3S360  CATCGTATCAAC    GTGCCAGCMGCCGCGGTAA     right palm      2009    4       14      subject-1       No      168     subject-1.right-palm.2009-4-14
L5S104  CAGTGTCAGGAC    GTGCCAGCMGCCGCGGTAA     tongue  2008    10      28      subject-1       Yes     0       subject-1.tongue.2008-10-28
L5S155  ATCTTAGACTGC    GTGCCAGCMGCCGCGGTAA     tongue  2009    1       20      subject-1       No      84      subject-1.tongue.2009-1-20
L5S174  CAGACATTGCGT    GTGCCAGCMGCCGCGGTAA     tongue  2009    2       17      subject-1       No      112     subject-1.tongue.2009-2-17
L5S203  CGATGCACCAGA    GTGCCAGCMGCCGCGGTAA     tongue  2009    3       17      subject-1       No      140     subject-1.tongue.2009-3-17
L5S222  CTAGAGACTCTT    GTGCCAGCMGCCGCGGTAA     tongue  2009    4       14      subject-1       No      168     subject-1.tongue.2009-4-14
L1S140  ATGGCAGCTCTA    GTGCCAGCMGCCGCGGTAA     gut     2008    10      28      subject-2       Yes     0       subject-2.gut.2008-10-28
L1S208  CTGAGATACGCG    GTGCCAGCMGCCGCGGTAA     gut     2009    1       20      subject-2       No      84      subject-2.gut.2009-1-20
L1S257  CCGACTGAGATG    GTGCCAGCMGCCGCGGTAA     gut     2009    3       17      subject-2       No      140     subject-2.gut.2009-3-17

^G Get Help     ^O WriteOut     ^R Read File    ^Y Prev Page    ^K Cut Text     ^C Cur Pos
^X Exit         ^J Justify      ^W Where Is     ^V Next Page    ^U UnCut Text   ^T To Spell
```

Common Issues in Marker Gene Studies

- Neglecting metadata
  - Analysis can not test for effects of, or discard bias from, categories you didn't record!
- Picking novel 16S primers—not all created equal
  - Earth Microbiome Project recommends 515f-806r primers, error-correcting barcodes
- Not taking precautions to support amplicon sequencing
  - Some Illumina machines require high PhiX, low cluster density
- Selecting an inappropriate reference database
  - E.g., Greengenes (16S) reference database when sequencing ITS
- Expecting species-level taxonomy calls
  - Most sequence variants only specify to family or genus level
- Using inappropriate statistical tests
  - Taxa abundance requires a compositionality-aware test like ANCOM
  - Differences in β diversity distances across groups requires test like PERMANOVA, not ANOVA

Importing Data

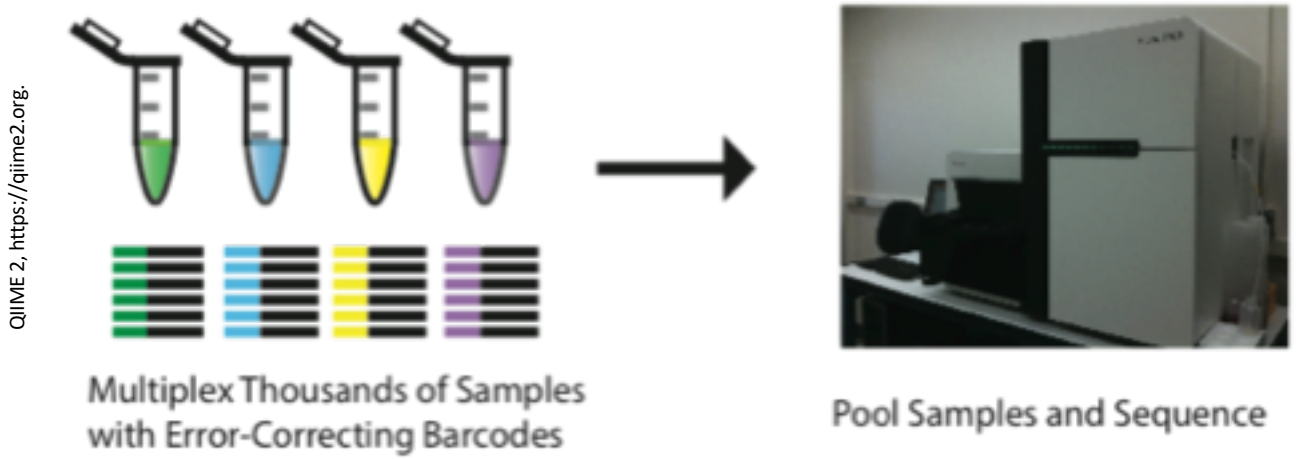
- After sequence data is on your machine, must be imported to a QIIME 2 “artifact”
  - Artifact = data + metadata
  - QIIME 2 artifacts have extension `.qza`
  - Different kinds of input data (e.g., single-end vs paired-end) and different formats of input data (e.g., sequences & barcodes in same or different file) need different imports
    - See “Importing data” tutorial at https://docs.qiime2.org/

Practicum: Importing Data

```
qiime tools import \
  --type EMPSingleEndSequences \
  --input-path emp-single-end-sequences \
  --output-path emp-single-end-sequences.qza
```

- Backslash is line continuation
  - Could leave out and just type whole command as one run-on line ☺
- Note structure of arguments to `qiime` command
  - Plugin name then method name then arguments
    - Order matters

Demultiplexing



- Must assign resulting sequences to samples to analyze
- **You may not need to do this!**
  - If sequencing done by a core, results may be demultiplexed before returned to you

Practicum: Demultiplexing

```
qiime demux emp-single \
 --i-seqs emp-single-end-sequences.qza \
 --m-barcodes-file sample-metadata.tsv \
 --m-barcodes-column BarcodeSequence \
 --o-per-sample-sequences demux.qza
```

- Arguments have a naming convention
  - Inputs (--i-<whatever>), metadata (--m-<whatever>), parameter (--p-<whatever), output (--o-<whatever>)
  - Order *doesn't* matter

Practicum: Demultiplexing (cont.)

- Presumably you'd like to know how your demultiplexing worked
- But the artifact doesn't show you that info, so create a *visualization*

```
qiime demux summarize \
  --i-data demux.qza \
  --o-visualization demux.qzv
```

  - Note that visualizations have the extension `.qzv` instead of `.qza`

- Now view the visualization, locally

```
qiime tools view demux.qzv
```

- When done examining, in Terminal, type **JUST** `q`
  - Don't need to hit Enter afterwards
  - Beware: quitting visualization doesn't close web page (but page becomes unreliable)

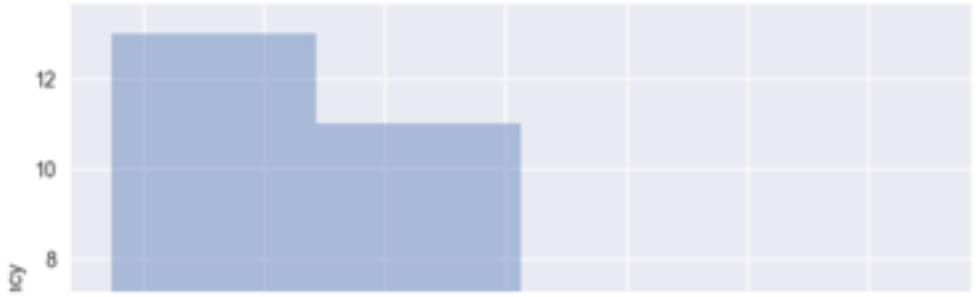
Demultiplexing Summary View

qiime2

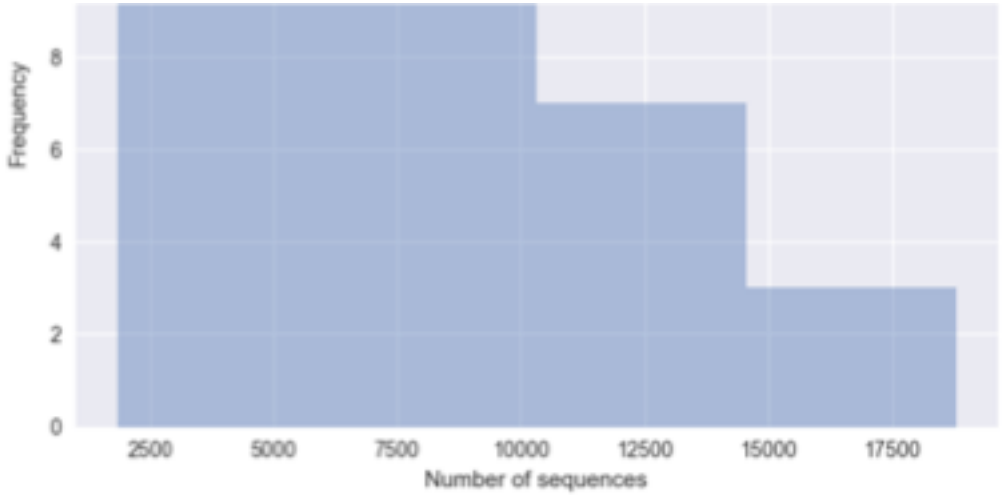
Overview | Interactive Quality Plot

Demultiplexed sequence counts summary

Minimum:	1853
Median:	8645.0
Mean:	7761.11764706
Maximum:	18787
Total:	263878



Demultiplexing Summary View (cont.)

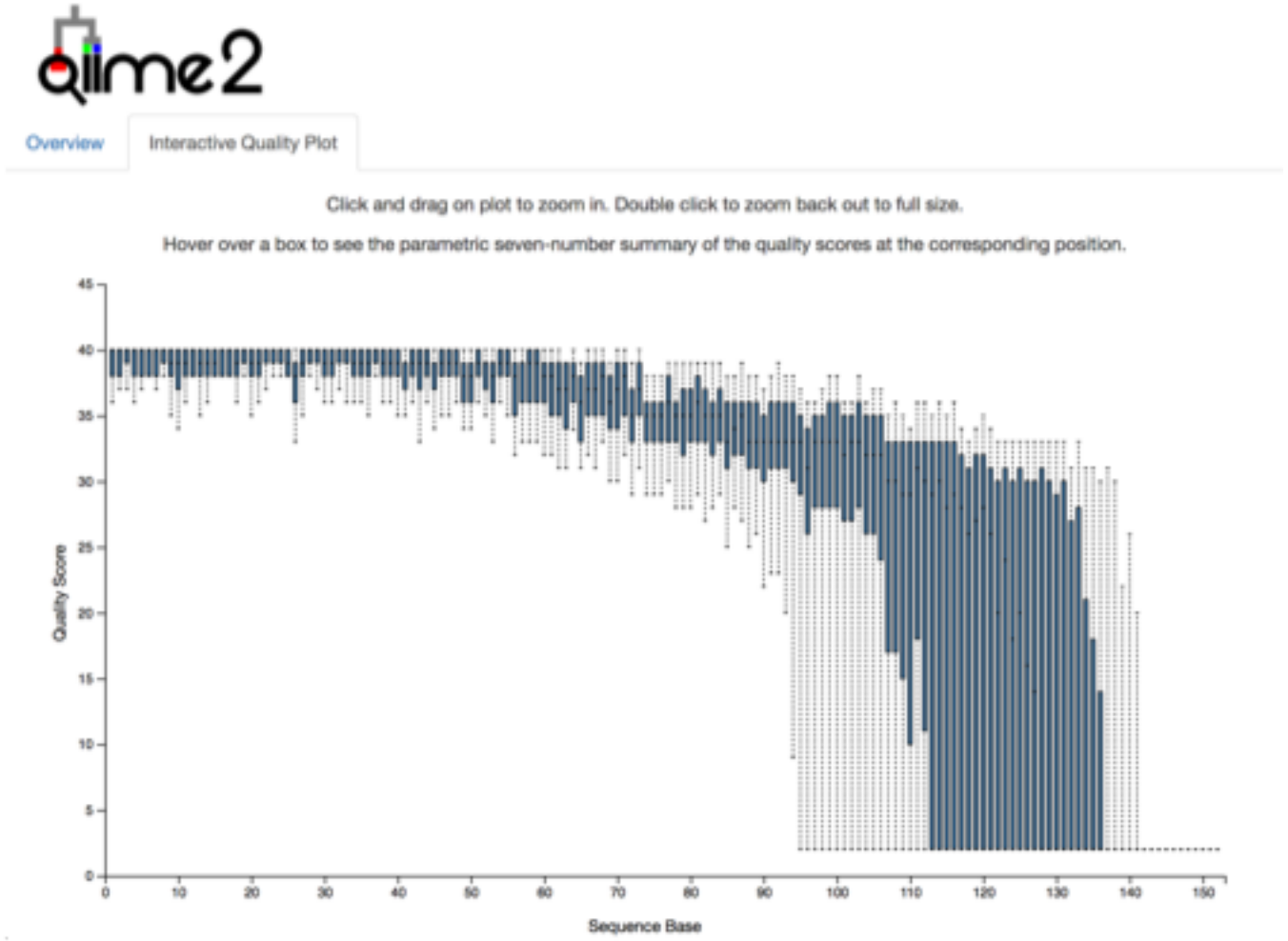


Download as PDF

Per-sample sequence counts

Sample name	Sequence count
L4S137	18787
L4S63	17167
L4S112	16265
L1S8	12386

Demultiplexing Summary View (cont.)



Practicum: Peeking At An Artifact

- What happens if you type

```
qiime tools view demux.qza
```

Practicum: Peeking At An Artifact (cont.)

- What happens if you type

```
qiime tools view demux.qza
```

- You get

```
Usage: qiime tools view [OPTIONS] VISUALIZATION_PATH

Error: Invalid value: demux-filtered.qza is not a QIIME 2
Visualization. Only QIIME 2 Visualizations can be viewed
```

- Instead, run

```
qiime tools view demux.qza
```

Practicum: Peeking At An Artifact (cont.)

- What happens if you type

```
qiime tools view demux.qza
```

- You get

```
Usage: qiime tools view [OPTIONS] VISUALIZATION_PATH

Error: Invalid value: demux-filtered.qza is not a QIIME 2
Visualization. Only QIIME 2 Visualizations can be viewed
```

- Instead, run

```
qiime tools view demux.qza
```

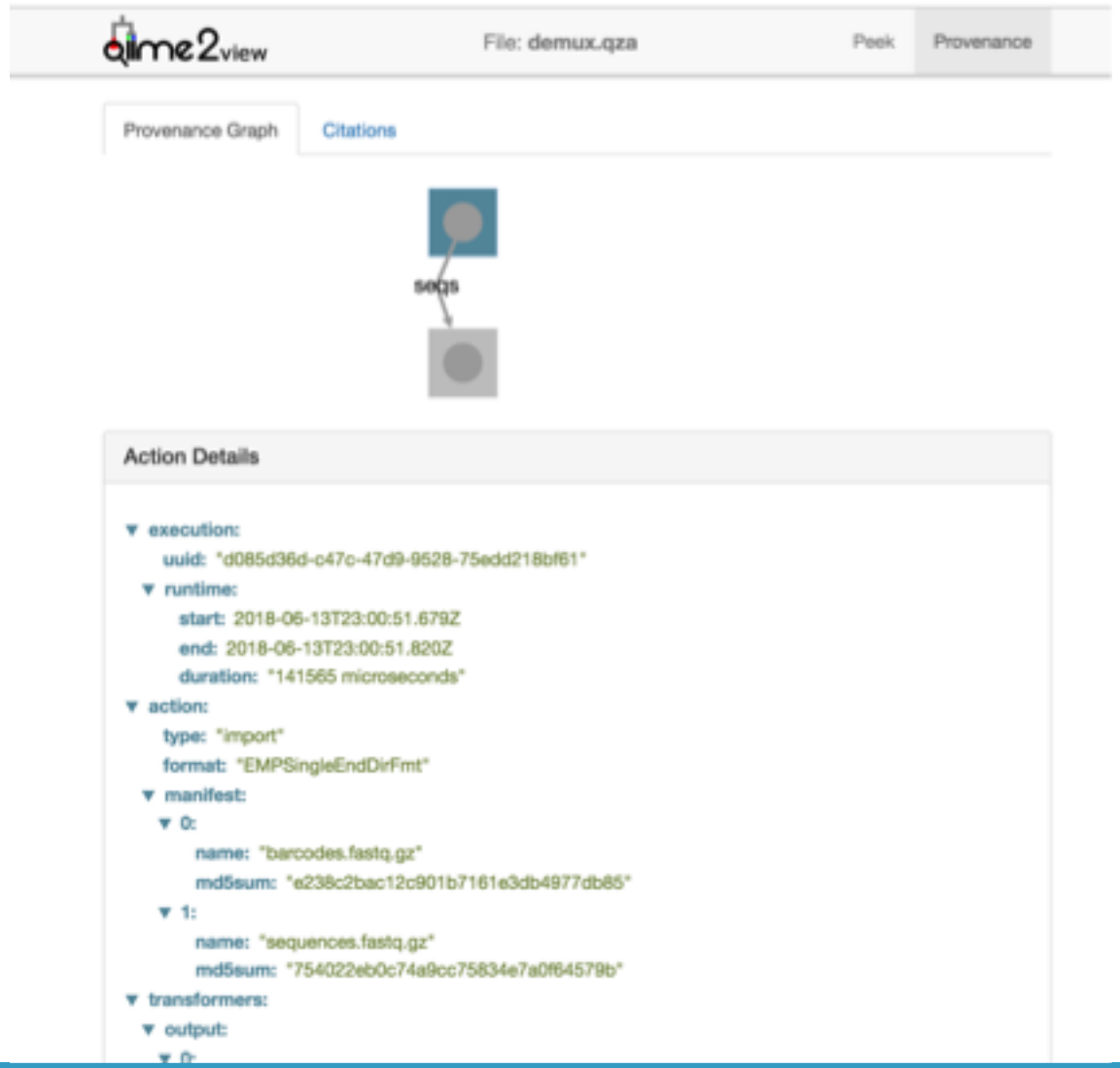
- See something like

```
UUID:          cce55836-0f04-42de-8476-83224254b419
Type:          SampleData[SequencesWithQuality]
Data format: SingleLanePerSampleSingleEndFastqDirFmt
```

Aside: Viewing Artifact Provenance

- Provenance tracking is **absolutely critical** to reproducible analyses
  - Almost no tool actually tracks it **for** you—really a fantastic new QIIME 2 feature
- Provenance can be viewed through the QIIME2 View website
  - Open Chrome and go to https://view.qiime2.org
  - Drag and drop file `demux.qza`
  - Click on “Provenance” tab

Aside: Viewing Artifact Provenance (cont.)



- Click on square to see action details
- Click on circle+arrow to see file passed between actions
- Note that citations are also provided!

Quality Control

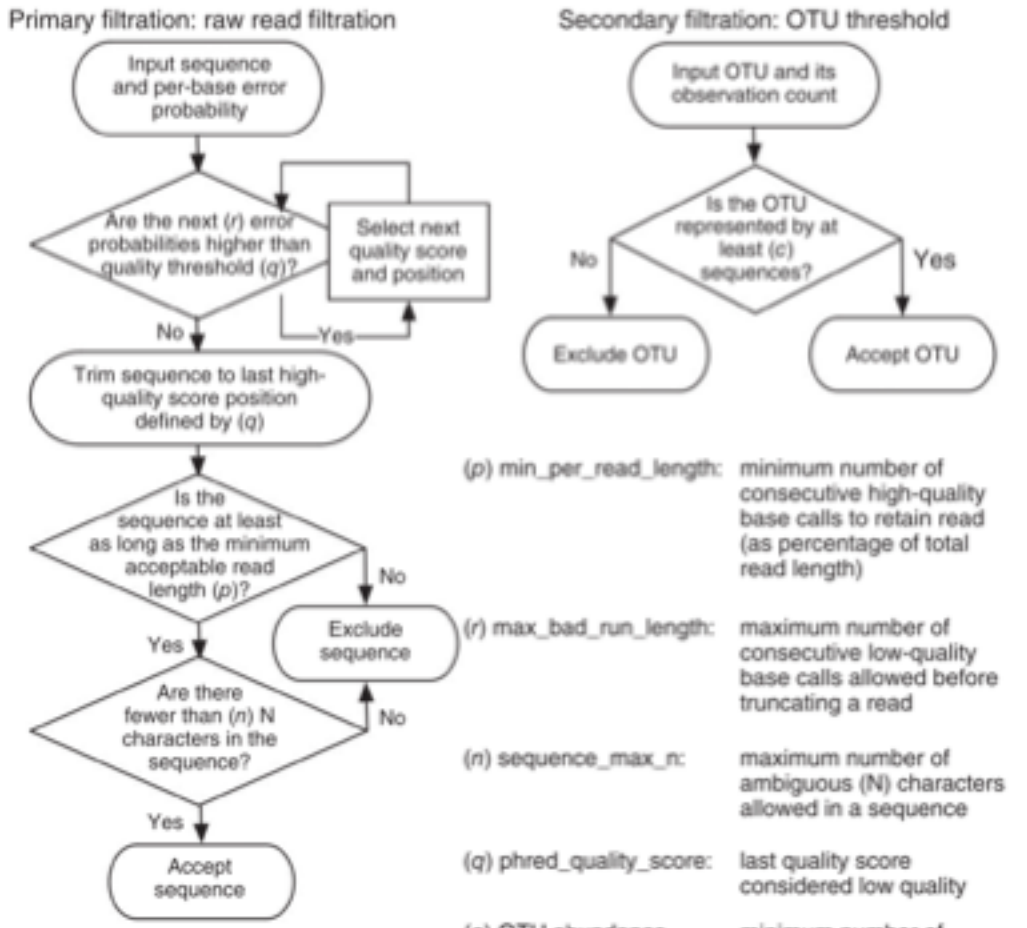


Figure 1 | Quality-filtration process flow in QIIME v1.5.0.

Bokulich, N. et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods, 10*(1), 57–59.

QIIME defaults:

- r = 3
- q = 3
- p = 0.75
- n = 0
- c = 0.005% or 2

Practicum: Quality Control

```
qiime quality-filter q-score \
 --i-demux demux.qza \
 --o-filtered-sequences demux-filtered.qza \
 --o-filter-stats demux-filter-stats.qza
```

Practicum: Quality Control (cont.)

```
qiime quality-filter q-score \
 --i-demux demux.qza \
 --o-filtered-sequences demux-filtered.qza \
 --o-filter-stats demux-filter-stats.qza

qiime metadata tabulate \
  --m-input-file demux-filter-stats.qza \
  --o-visualization demux-filter-stats.qzv
```

Quality Control Summary View

Download metadata TSV file

This file won't necessarily reflect dynamic sorting or filtering options based on the interactive table below.

Search:

sample-id #q2:types	total-input-reads numeric	total-retained-reads numeric	reads-truncated numeric	reads-too-short-after-truncation numeric	reads-exceeding-maximum-ambiguous-bases numeric
L1S105	11340	9232	10782	2066	42
L1S140	9736	8584	9457	1113	39
L1S208	11335	10148	10667	1161	26
L1S257	8216	7302	7672	876	38
L1S281	8904	7763	8343	1117	24
L1S57	11750	10000	11000	1716	34
L1S76	10100	8984	9678	1092	24
L1S8	12386	8433	12035	3916	37
L2S155	9261	5066	8932	4167	28
L2S175	10691	5574	10216	5092	25

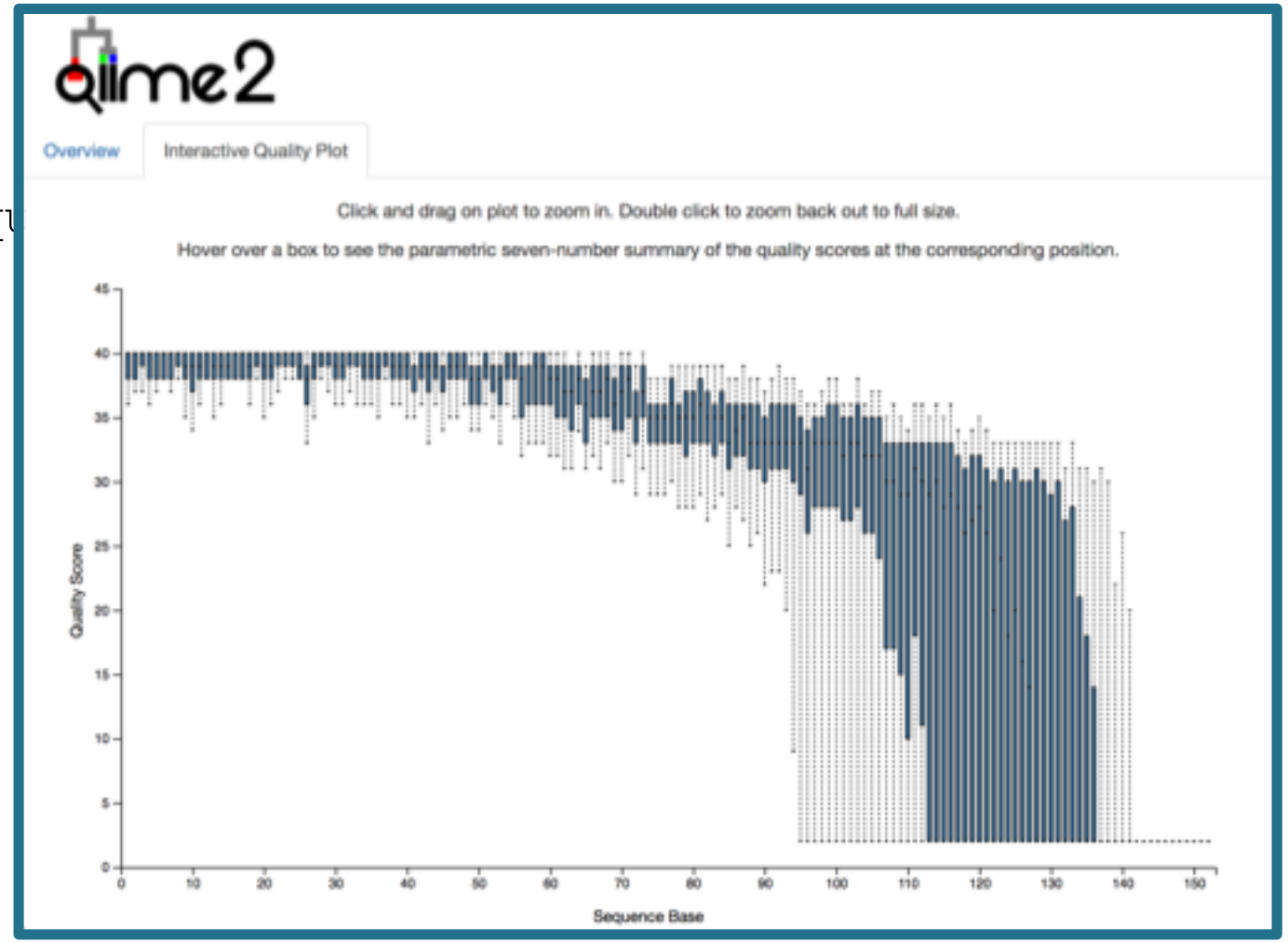
Practicum: Feature Table Creation

```
qiime deblur denoise-16S \
  --i-demultiplexed-seqs demux-filtered.qza \
  --p-trim-length 120 \
  --o-representative-sequences rep-seqs.qza \
  --o-table table.qza \
  --p-sample-stats \
  --o-stats deblur-stats.qza
```

- This can take up to 10 minutes to run, so while we wait …
  - Where do you guess the number 120 came from?

Practicum: Feature Table Creation

```
qiime deblur denoise-16S
  --i-demultiplexed-seqs
  --p-trim-length 120 \
  --o-representative-sequ
  --o-table table.qza \
  --p-sample-stats \
  --o-stats deblur-stats
```

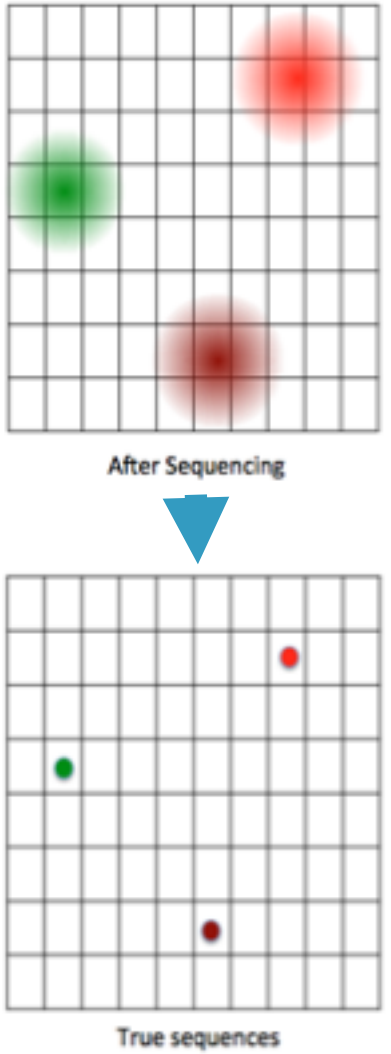


Feature Table Creation—The Past

- Last year: OTU (Operational Taxonomic Unit)
  - "an operational definition of a species used when only DNA sequence data is available"
  - Sequences at/above a given similarity threshold considered part of the same OTU
    - 97% is the usual "species-level" threshold
      - Similarity determined using alignment (time-consuming)
    - Purpose is to minimize impact of sequencing errors
      - But also masks fine (sub-OTU) variation in real biological sequences
  - Results very difficult to compare across studies if done *de novo*
    - "Closed reference", "open reference" methods increase comparability require reference database
- Output is a "feature table":
  - Rows are samples
  - Columns are OTUs (arbitrary identifiers if **de novo**, from reference database if closed reference)
  - Values are frequency of reads from that OTU in that sample

Feature Table Creation—The Present

- This year: sOTU (sub-OTU) methods
  - Use error modeling to *in silco* correct sequencing mistakes
    - Sounds impossible but is actually quite accurate, with right error model
      - Error model is specific to the sequencing type (e.g., 454, Illumina Hi/MiSeq)
  - Result: only sequences likely to have been input to the sequencer
  - Options include (NOT a complete list):
    - DADA2 (2016)
    - Deblur (2017)
- Output is STILL a feature table:
  - Rows are samples
  - Columns are SEQUENCES
  - Values are frequency of reads from that SEQUENCE in that sample



QIIME 2, https://qiime2.org.

Practicum: Feature Table Creation (cont.)

```
qiime deblur visualize-stats \
  --i-deblur-stats deblur-stats.qza \
  --o-visualization deblur-stats.qzv
```

Deblur Statistics View

Per-sample Deblur stats

Click on a Column header to sort the table.

Mouse over a Column header to get a description.

	sample-id	reads-raw	fraction-artifact-with-minsize	fraction-artifact	fraction-missed-reference	unique-reads-derep	reads-derep	unique-reads-deblur	reads-deblur	unique-reads-hit-artifact	reads-hit-artifact	unique-reads-chimeric	reads-chimeric	unique-reads-hit-reference	reads-hit-reference	unique-reads-missed-reference	reads-missed-reference
0	L3S360	1341	0.518270	0.0	0.011785	118	646	111	600	0	0	1	6	73	512	3	7
1	L2S222	4459	0.498094	0.0	0.019086	327	2238	287	1999	0	0	4	8	147	1603	3	38
2	L2S309	1904	0.457458	0.0	0.003185	124	1033	99	942	0	0	0	0	76	895	1	3
3	L3S341	1293	0.438515	0.0	0.000000	95	726	86	675	0	0	0	0	78	653	0	0
4	L2S204	4349	0.429064	0.0	0.012851	236	2483	152	2102	0	0	1	1	106	1968	2	27
5	L2S357	3100	0.419032	0.0	0.000000	149	1801	87	1554	0	0	0	0	75	1533	0	0
6	L3S294	1523	0.401182	0.0	0.002398	82	912	65	836	0	0	1	2	52	800	1	2
7	L3S313	1340	0.391045	0.0	0.000000	85	816	69	747	0	0	1	1	66	741	0	0
8	L2S240	7110	0.390717	0.0	0.000000	253	4332	78	3578	0	0	9	17	59	3535	0	0
9	L2S155	5066	0.388077	0.0	0.006227	260	3100	178	2730	0	0	0	0	119	2579	3	17
10	L2S175	5574	0.371726	0.0	0.001308	281	3502	177	3059	0	0	1	1	132	2954	1	4

Practicum: Feature Table Creation (cont.)

```
qiime feature-table summarize \
  --i-table table.qza \
  --o-visualization table.qzv \
  --m-sample-metadata-file sample-metadata.tsv
```

Feature Table Summary View

qiime2

Overview | Interactive Sample Detail | Feature Detail

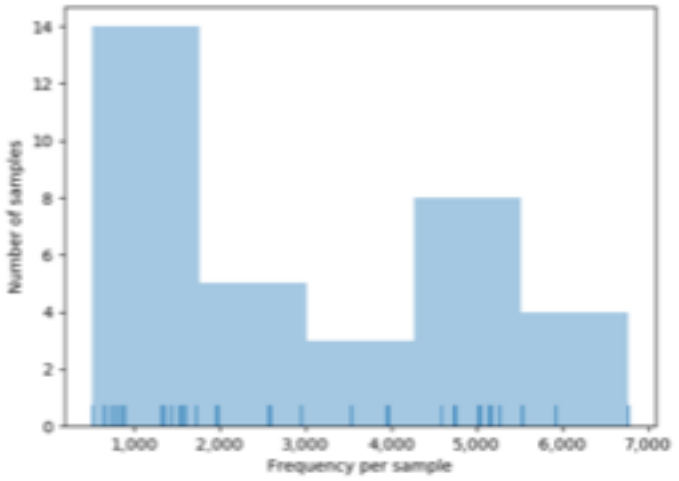
Table summary

Metric	Sample
Number of samples	34
Number of features	485
Total frequency	102,545

Frequency per sample

	Frequency
Minimum frequency	512.0
1st quartile	1,367.5
Median frequency	2,581.0
3rd quartile	4,952.0
Maximum frequency	6,770.0
Mean frequency	3,016.029411764706

Frequency per sample detail (csv | html)



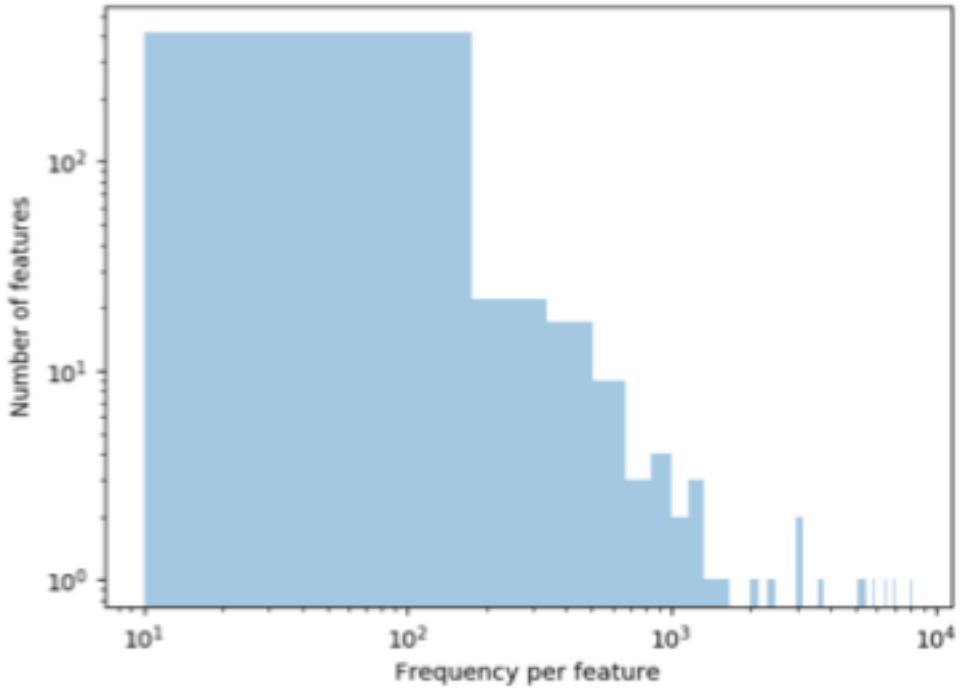
Download as PDF

Feature Table Summary View (cont.)

Frequency per feature

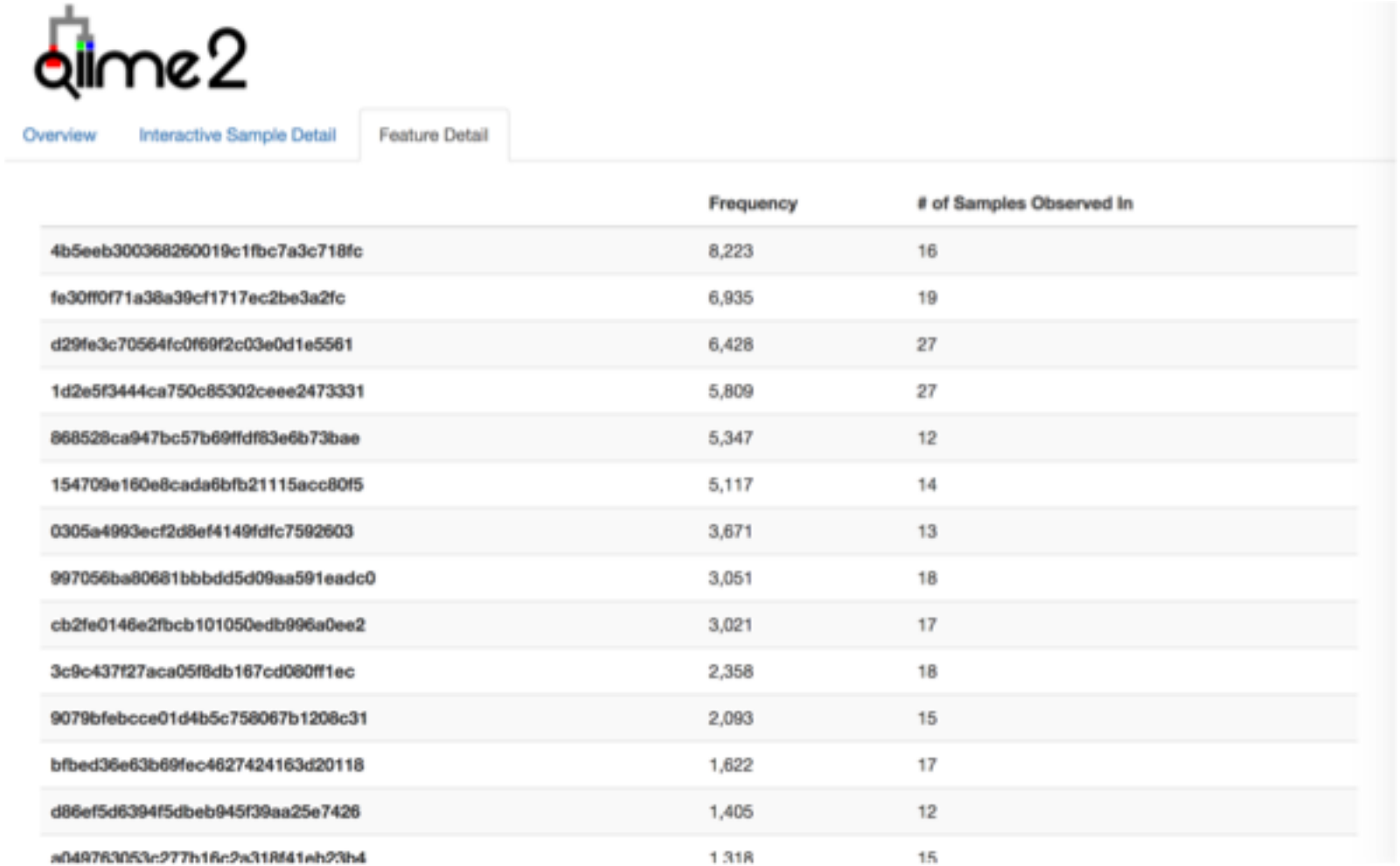
	Frequency
Minimum frequency	10.0
1st quartile	16.0
Median frequency	33.0
3rd quartile	88.0
Maximum frequency	8,223.0
Mean frequency	211.43298969072166

Frequency per feature detail (csv | html)



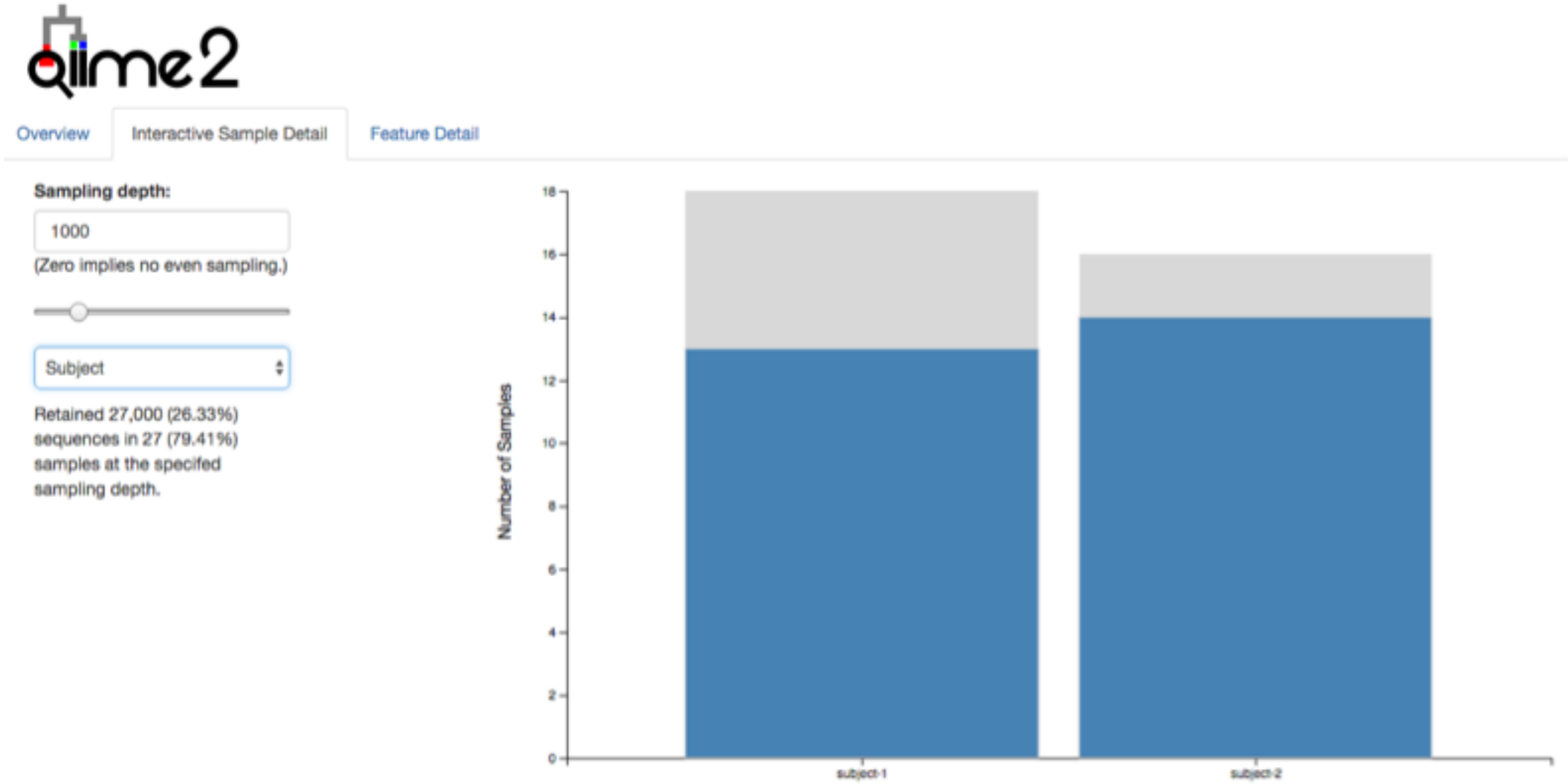
Download as PDF

Feature Table Summary View (cont.)



	Frequency	# of Samples Observed In
4b5eeb3003682600l9c1fbc7a3c718fc	8,223	16
fe30ff0f71a38a39cf1717ec2be3a2fc	6,935	19
d29fe3c70564fc0f69f2c03e0d1e5561	6,428	27
1d2e5f3444ca750c85302ceee2473331	5,809	27
868528ca947bc57b69ffdf83e6b73bae	5,347	12
154709e160e8cada6bfb21115acc80f5	5,117	14
0305a4993ecf2d8ef4149fdfc7592603	3,671	13
997056ba80681bbbdd5d09aa591eadc0	3,051	18
cb2fe0146e2fbcb101050edb996a0ee2	3,021	17
3c9c437f27aca05f8db167cd060ff1ec	2,358	18
9079bfebcce01d4b5c758067b1208c31	2,093	15
bfbed36e63b69fec4627424163d20118	1,622	17
d86ef5d6394f5dbeb945f39aa25e7426	1,405	12
a04976305[illegible]	1,318	15

Feature Table Summary View (cont.)



Feature Table Summary View (cont.)

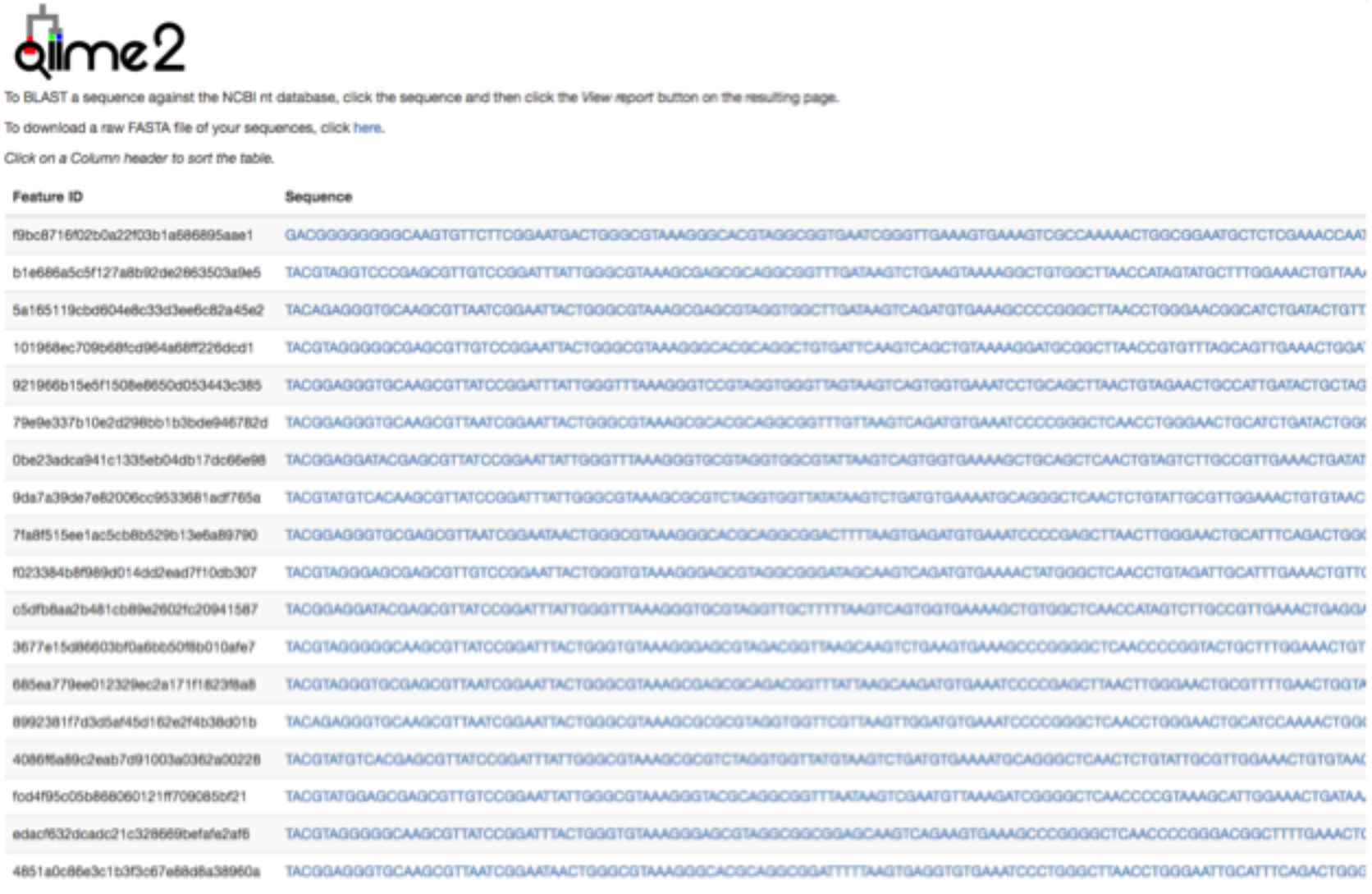
Sample ID	Sequence Count
L4S137	6,770
L4S63	5,912
L1S57	5,525
L4S112	5,523
L6S93	5,261
L6S20	5,170
L1S208	5,136
L1S76	5,037
...	
L5S155	1,347
L5S240	1,329
L2S309	895
L3S378	849
L3S294	800
L3S313	741
L3S242	660
L3S341	653
L3S360	512

Practicum: Feature Table Creation (cont.)

```
qiime feature-table summarize \
  --i-table table.qza \
  --o-visualization table.qzv \
  --m-sample-metadata-file sample-metadata.tsv

qiime feature-table tabulate-seqs \
  --i-data rep-seqs.qza \
  --o-visualization rep-seqs.qzv
```

Feature Table Tabulation View



To BLAST a sequence against the NCBI nt database, click the sequence and then click the *View report* button on the resulting page.

To download a raw FASTA file of your sequences, click here.

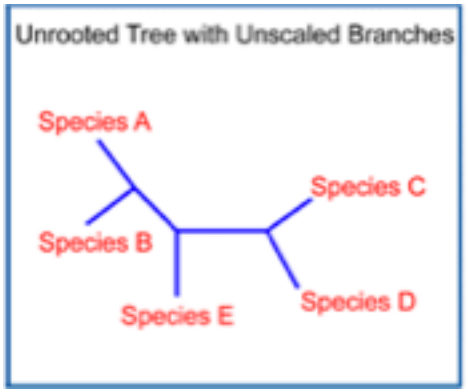
Click on a Column header to sort the table.

Feature ID	Sequence
f9bc8716f02b0a22f03b1a686895aae1	GACGGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGTGAATCGGGTTGAAAGTGAAAGTCGCCAAAAACTGGCGGAATGCTCTCGAAACCAAT
b1e686a5c5f127a8b92de2863503a9e5	TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTAAAAGGCTGTGGCTTAACCATAGTATGCTTTGGAAACTGTTAA
5a165119cbd604e8c33d3ee6c82a45e2	TACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGATAAGTCAGATGTGAAAGCCCCGGGCTTAACCTGGGAACGGCATCTGATACTGTT
101968ec709b68fcd964a68ff226dcd1	TACGTAGGGGGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGGGCACGCAGGCTGTGATTCAAGTCAGCTGTAAAAGGATGCGGCTTAACCGTGTTTAGCAGTTGAAACTGGA
921966b15e5f1508e8650d053443c385	TACGGAGGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGTGGGTTAGTAAGTCAGTGGTGAAATCCTGCAGCTTAACTGTAGAACTGCCATTGATACTGCTAG
79e9e337b10e2d298bb1b3bde946782d	TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGC
0be23adca941c1335eb04db17dc66e98	TACGGAGGATACGAGCGTTATCCGGAATTATTGGGTTTAAAGGGTGCGTAGGTGGCGTATTAAGTCAGTGGTGAAAAGCTGCAGCTCAACTGTAGTCTTGCCGTTGAAACTGATAT
9da7a39de7e82006cc9533681adf765a	TACGTATGTCACAAGCGTTATCCGGATTTATTGGGCGTAAAGCGCGTCTAGGTGGTTATATAAGTCTGATGTGAAAATGCAGGGCTCAACTCTGTATTGCGTTGGAAACTGTGTAAC
7fa8f515ee1ac5cb8b529b13e6a89790	TACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCACGCAGGCGGACTTTTAAGTGAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTCAGACTGGC
f023384b8f989d014dd2ead7f10db307	TACGTAGGGAGCGAGCGTTGTCCGGAATTACTGGGTGTAAAGGGAGCGTAGGCGGGATAGCAAGTCAGATGTGAAAACTATGGGCTCAACCTGTAGATTGCATTTGAAACTGTTC
c5dfb8aa2b481cb89e2602fc20941587	TACGGAGGATACGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGTTGCTTTTTAAGTCAGTGGTGAAAAGCTGTGGCTCAACCATAGTCTTGCCGTTGAAACTGAGGA
3677e15d86603bf0a6bb50f8b010afe7	TACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGTTAAGCAAGTCTGAAGTGAAAGCCCGGGGCTCAACCCCGGTACTGCTTTGGAAACTGT
685ea779ee012329ec2a171f1823f8a8	TACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGCAGACGGTTTATTAAGCAAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCGTTTTGAACTGGTA
8992381f7d3d5af45d162e2f4b38d01b	TACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGC
4086f6a89c2eab7d91003a0362a00228	TACGTATGTCACGAGCGTTATCCGGATTTATTGGGCGTAAAGCGCGTCTAGGTGGTTATGTAAGTCTGATGTGAAAATGCAGGGCTCAACTCTGTATTGCGTTGGAAACTGTGTAAC
fcd4f95c05b868060121ff709085bf21	TACGTATGGAGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGGGTACGCAGGCGGTTTAATAAGTCGAATGTTAAAGATCGGGGCTCAACCCCGTAAAGCATTGGAAACTGATAA
edacf632dcadc21c328669befafe2af6	TACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGGCGGCGGAGCAAGTCAGAAGTGAAAGCCCGGGGCTCAACCCCGGGACGGCTTTTGAAACTC
4851a0c86e3c1b3f3c67e88d8a38960a	TACGGAGGGTGCAAGCGTTAATCGGAATAACTGGGCGTAAAGGGCACGCAGGCGGATTTTTAAGTGAGGTGTGAAATCCCTGGGCTTAACCTGGGAATTGCATTTCAGACTGGC

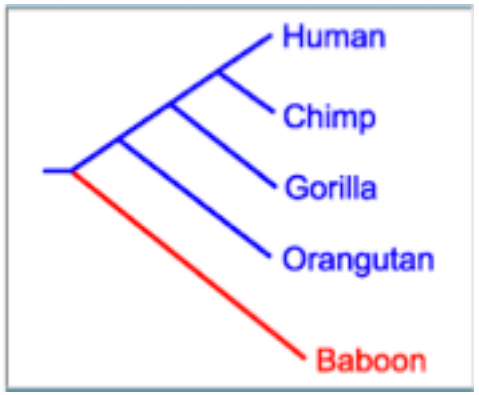
Phylogenetic Tree Creation

- Evolution is the core concept of biology
  - There's only so much you can learn from microbes while ignoring evolution!
- Evolution-aware analyses of a dataset need a phylogenetic tree of its sequences
  - *De novo*: infer tree using only sequences from dataset
  - Reference-based: insert sequences from dataset into an existing phylogenetic tree
    - Not all existing phylogenies are created equal—have strengths and weaknesses based on intended purpose when developed
- Phylogenetically based analyses in QIIME 2 need a **rooted** tree

Unrooted:



Rooted:



Geer, R.C., Messersmith, D.J, Alpi, K., Bhagwat, M., Chattopadhyay, A., Gaedeke, N., Lyon, J., Minie, M.E., Morris, R.C., Ohles, J.A., Osterbur, D.L. & Tennant, M.R.

2002. NCBI Advanced Workshop for Bioinformatics Information Specialists. [Online] http://www.ncbi.nlm.nih.gov/Class/NAWBIS/.

Practicum: Phylogenetic Tree Creation

```
qiime alignment mafft \
 --i-sequences rep-seqs.qza \
 --o-alignment aligned-rep-seqs.qza
```

- Note: here we are doing *de novo* phylogenetic tree creation
  - Not necessarily the BEST approach, but an easy one to show you ☺

Practicum: Phylogenetic Tree Creation (cont.)

```
qiime alignment mafft \
 --i-sequences rep-seqs.qza \
 --o-alignment aligned-rep-seqs.qza

qiime alignment mask \
  --i-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza
```

Practicum: Phylogenetic Tree Creation (cont.)

```
qiime alignment mafft \
 --i-sequences rep-seqs.qza \
 --o-alignment aligned-rep-seqs.qza

qiime alignment mask \
  --i-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza

qiime phylogeny fasttree \
  --i-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza
```

Practicum: Phylogenetic Tree Creation (cont.)

```
qiime alignment mafft \
 --i-sequences rep-seqs.qza \
 --o-alignment aligned-rep-seqs.qza

qiime alignment mask \
  --i-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza

qiime phylogeny fasttree \
  --i-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza

qiime phylogeny midpoint-root \
  --i-tree unrooted-tree.qza \
  --o-rooted-tree rooted-tree.qza
```

- No visualizations provided for these artifacts

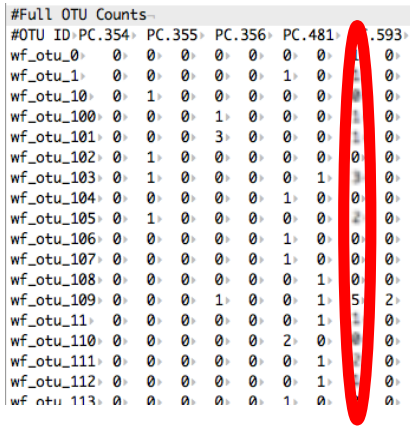
Core Metrics

- So how do you actually compare microbial communities?
  - Can't just eyeball the (gigantic, sparse) feature tables and look for differences
  - Instead, calculate metrics that compress a lot of info into a single number
  - Then do statistical tests on metrics to look for significant differences
    - **BE CAREFUL**—microbiome data is sparse, compositional, etc, so requires unusual tests
    - QIIME 2 uses appropriate tests; if doing your own, **MUST** check the literature first
- These metrics are lossy!
  - No metric exposes all the information in the full feature table
    - If it did, it would BE the feature table
  - Different metrics capture different aspects of the communities
- **Thus …**
  - **Don't ask, "Which metric should I use?" UNTIL you know what you're looking for!**

Core Metrics (cont.)

- QIIME 2 calculates a smorgasbord of metrics for you with one command
- Alpha diversity
  - Shannon's diversity index (a quantitative measure of community richness)
  - Observed OTUs (a qualitative measure of community richness)
  - Faith's Phylogenetic Diversity (a qualitiative measure of community richness that incorporates phylogenetic relationships between the features)
  - Evenness (or Pielou's Evenness; a measure of community evenness)
- Beta diversity
  - Jaccard distance (a qualitative measure of community dissimilarity)
  - Bray-Curtis distance (a quantitative measure of community dissimilarity)
  - unweighted UniFrac distance (a qualitative measure of community dissimilarity that incorporates phylogenetic relationships between the features)
  - weighted UniFrac distance (a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features)

Normalization for Core Metrics



#Full OTU Counts

#OTU ID	PC.354	PC.355	PC.356	PC.481	.593
wf_otu_0	0	0	0	0	0
wf_otu_1	0	0	0	1	0
wf_otu_10	0	1	0	0	0
wf_otu_100	0	0	0	1	0
wf_otu_101	0	0	0	3	0
wf_otu_102	0	1	0	0	0
wf_otu_103	0	1	0	0	0
wf_otu_104	0	0	0	0	0
wf_otu_105	0	1	0	0	0
wf_otu_106	0	0	0	0	0
wf_otu_107	0	0	0	1	0
wf_otu_108	0	0	0	0	0
wf_otu_109	0	0	1	0	2
wf_otu_11	0	0	0	0	0
wf_otu_110	0	0	0	2	0
wf_otu_111	0	0	0	0	0
wf_otu_112	0	0	0	0	0
wf_otu_113	0	0	0	1	0

- Calculated metric values depend on sampling depth
- Ex: circled column has more non-zero counts than others
  - Is its community really more diverse—or do we just SEE more?
  - Samples with more sequences (greater sampling depth) show more diversity
- Normalization is necessary for valid comparisons of abundance/diversity
  - "**But how**?!"
    - Longstanding approach: rarefaction (reduce all samples to uniform sampling depth)
    - Recent publication caused concern
      - *Waste not, want not: why rarefying microbiome data is inadmissible*. McMurdie PJ, Holmes S. PLoS Comput Biol. 2014;10(4).
    - Further work demonstrated concern is excessive
      - *Normalization and microbial differential abundance strategies depend upon data characteristics*. Weiss S, et al. Microbiome. 2017 Mar 3;5(1):27. (Note: I'm an author, so not objective)

Rarefaction

- What is rarefaction?
  - randomly subsampling the same number of sequences from each sample
  - NB: samples without that number of sequences are discarded
- Concerns:
  - Too low: ignore a lot of samples' information
  - Too high: ignore a lot of samples
  - *Still* a good choice for normalization (Weiss S, et al. Microbiome. 2017):
    - "Rarefying more clearly clusters samples according to biological origin than other normalization techniques do for ordination metrics based on presence or absence"
    - "Alternate normalization measures are potentially vulnerable to artifacts due to library size"
- Researcher must choose sampling depth—but how?

Sampling Depth Selection

- Don’t sweat it too much
  - “Low” depths (10-1000 sequences per sample) capture all but very subtle variations

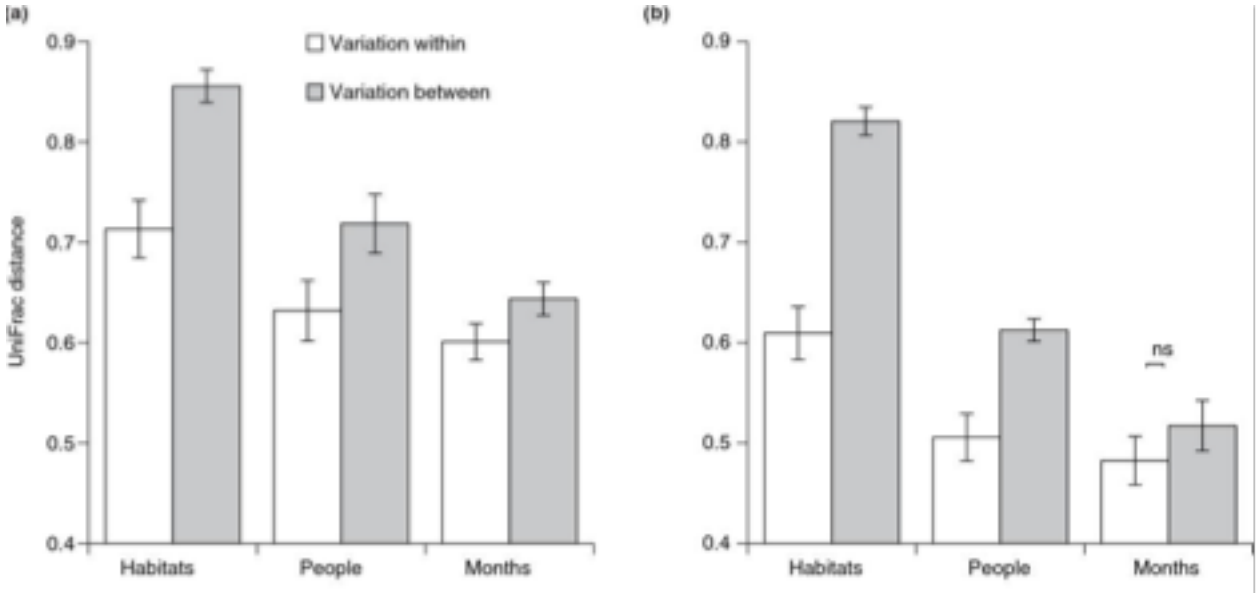


Fig. 2, Kuczynski, J. et al., "Direct sequencing of the human microbiome readily reveals community differences", Genome Biology, 2010

  - Retaining samples is usually more important than retaining sequences
    - May care not just how many samples are left out but WHICH samples are left out

Practicum: Core Metrics

```
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny rooted-tree.qza \
  --i-table table.qza \
  --p-sampling-depth ??? \
  --m-metadata-file sample-metadata.tsv
  --output-dir metrics
```

- Which sampling depth should we use?
  - How can we decide?

Exercise: Core Metrics

```
qiime diversity core-metrics-phyl
  --i-phylogeny rooted-tree.qza \
  --i-table table.qza \
  --p-sampling-depth ??? \
  --m-metadata-file sample-metada
  --output-dir metrics
```

- Which sampling depth should we use?
  - How can we decide?

    ```
    qiime tools view table.qzv
    ```

  - Work with your partner to choose a sampling depth, then answer:
    - Why did you choose this value?
    - How many samples will be excluded from your analysis based on this choice?
    - How many total sequences will you be analyzing in the core metrics command?

Answers: Core Metrics

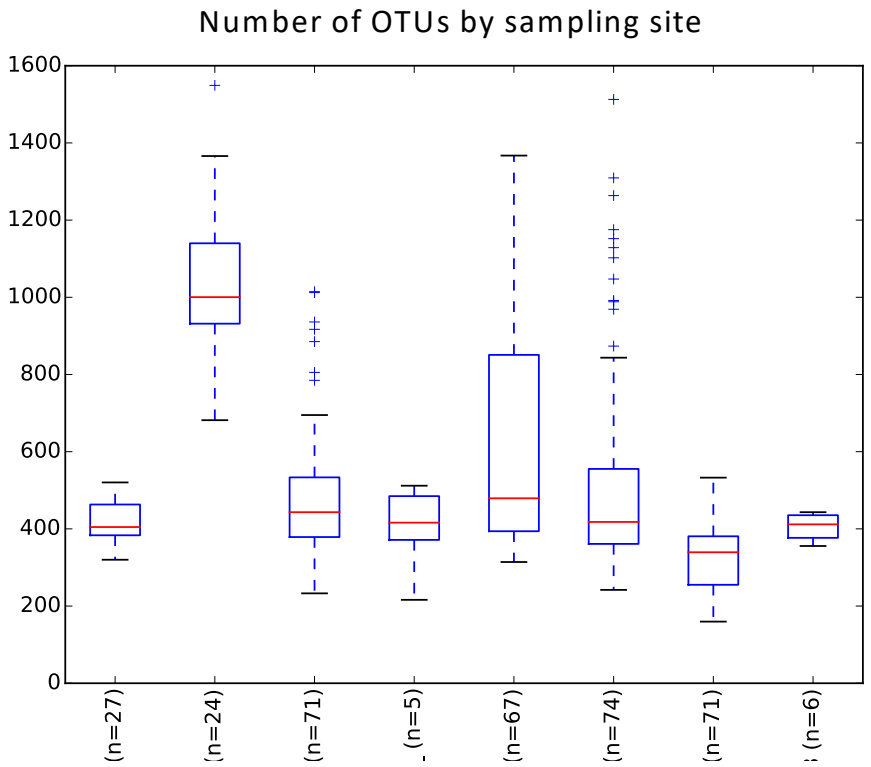
```
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny rooted-tree.qza \
  --i-table table.qza \
  --p-sampling-depth 800 \
  --m-metadata-file sample-metadata.tsv
  --output-dir metrics
```

- My answers:
  - Why did you choose this value?
    - Anything higher excludes >= half of right palm samples
  - How many samples will be excluded from your analysis based on this choice?
    - 4, all from right palm of subject 1
  - How many total sequences will you be analyzing in the core metrics command?
    - 24,000 (23.40%)
- Note: there is no single visualization for core metrics

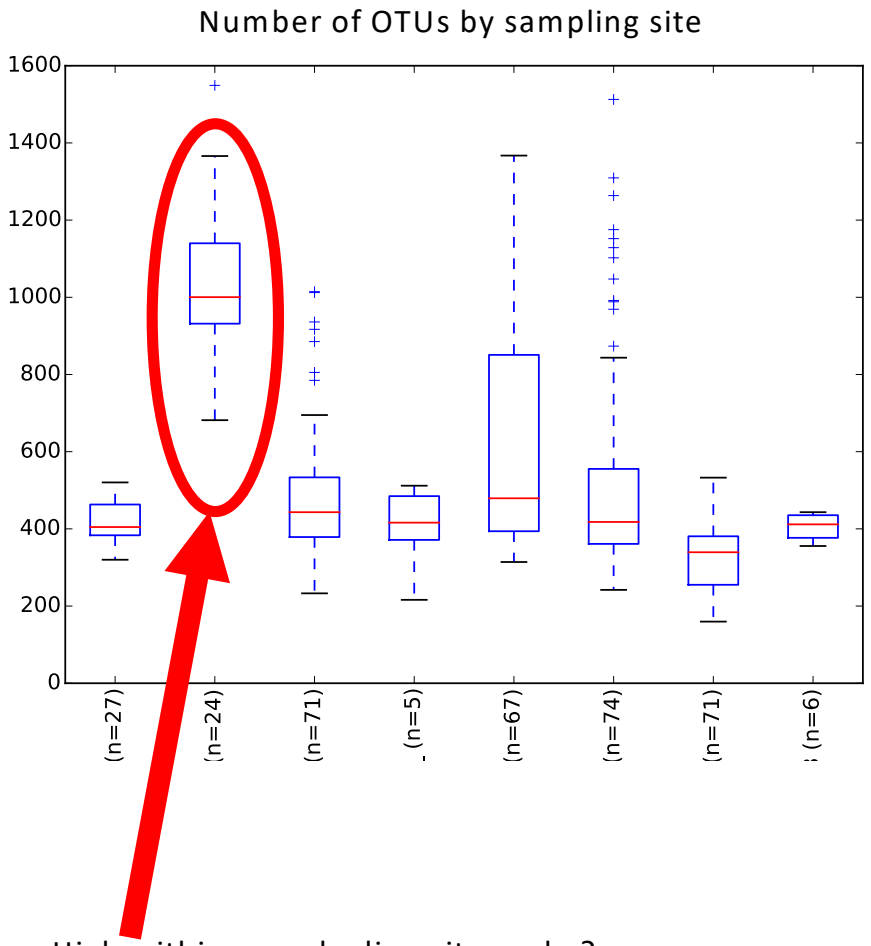
Alpha Diversity

- "Within-sample" diversity
  - Many different metrics exist
    - Taxonomy-based (e.g., number of observed OTUs)
      - Assume everything is equally dissimilar
      - More likely to see differences based on close relatives
    - Phylogeny-based (e.g., phylogenetic diversity over whole tree)
      - Treat less related items as more dissimilar
      - Better at scaling the observed differences
  - The "correct" metric(s) are those relevant to your hypothesis
    - Please do HAVE a hypothesis!
- Testing approach:
  - Examine alpha diversity metric by metadata values
  - Test whether differences in metric distribution is different between groups (if metadata is categorical) or correlated with metadata (if metadata is continuous)

Alpha Diversity



Alpha Diversity



High within-sample diversity—why?

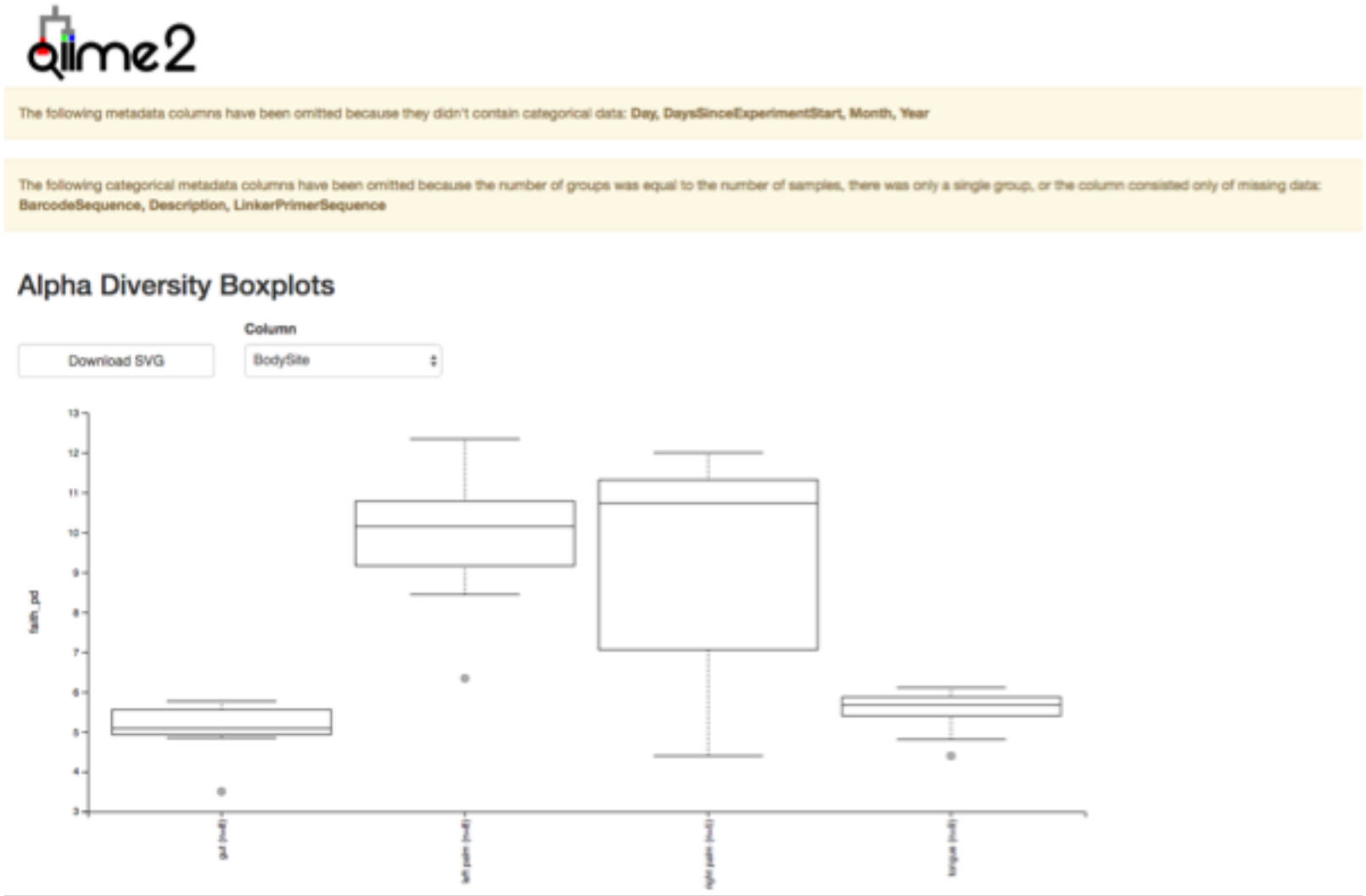
Practicum: Alpha Diversity Group Significance

```
qiime diversity alpha-group-significance \
  --i-alpha-diversity metrics/faith_pd_vector.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/faith-pd-group-significance.qzv
```

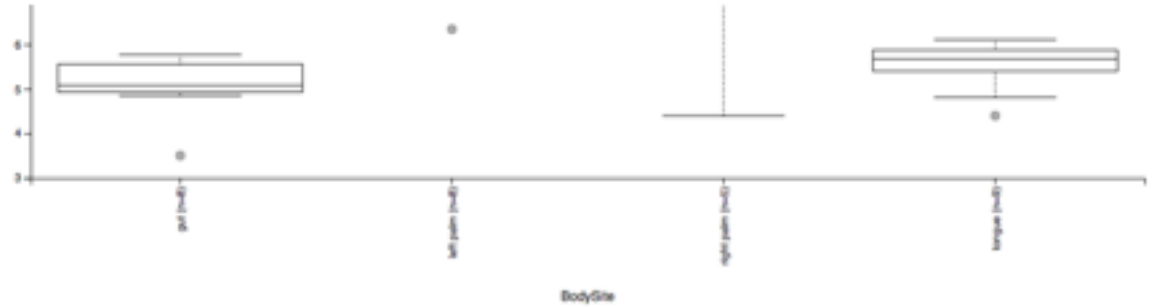
- Note: only showing you the group significance visualization of ONE alpha diversity metric
  - Remember that 3 others are calculated by `core-metrics-phylogenetic` alone
  - *The one I am showing is not "the correct one"—pick the one that fits your hypothesis*
- To check the group significance of a different metric, just input a different vector file
  - To find them:

```
cd metrics/
ls *_vector.qza
```

Alpha Diversity Group Significance View



Alpha Diversity Group Significance View



Kruskal-Wallis (all groups)

	Result
H	16.60616487455198
p-value	0.0008515500394924999

Kruskal-Wallis (pairwise)

Download CSV

		H	p-value	q-value
Group 1	Group 2			
gut (n=8)	left palm (n=8)	11.294118	0.000778	0.002333
	right palm (n=5)	3.621429	0.057040	0.107791
	tongue (n=9)	1.564815	0.210962	0.253154
left palm (n=8)	right palm (n=5)	0.000000	1.000000	1.000000
	tongue (n=9)	12.000000	0.000532	0.002333
right palm (n=5)	tongue (n=9)	3.240000	0.071861	0.107791

Exercise: Alpha Diversity Group Significance

```
qiime diversity alpha-group-significance \
  --i-alpha-diversity metrics/faith_pd_vector.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/faith-pd-group-significance.qzv
```

- Work with your partner to answer these questions:
  - Is BodySite value associated with significant differences in phylogenetic diversity?
  - Which two sites have the most significant difference in phylogenetic diversity distributions?
    - Note different between p-value and q-value
  - Is Subject value associated with significant differences in phylogenetic diversity?

Answers: Alpha Diversity Group Significance

```
qiime diversity alpha-group-significance \
  --i-alpha-diversity metrics/faith_pd_vector.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/faith-pd-group-significance.qzv
```

- My answers:
  - Is BodySite value associated with significant differences in phylogenetic diversity?
    - Yes, with p < 1 E-3
  - Which two sites have the most significantly difference in phylogenetic diversity distributions?
    - Left palm is (equally) most significantly different from gut and tongue
      - Consider: any idea why perhaps left palm but not right?
  - Is Subject value associated with significant differences in phylogenetic diversity?
    - No

Practicum: Alpha Diversity Correlation

```
qiime diversity alpha-correlation \
  --i-alpha-diversity metrics/evenness_vector.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/evenness-alpha-correlation.qzv
```

- Same caveat as before:
  - Only showing the correlation visualization of ONE alpha diversity metric
    - Not necessarily “the correct one”!

Alpha Diversity Correlation View

Beta Diversity

- "Between-sample" diversity
  - Has similar categories, caveats as α diversity
- A popular phylogenetic option is 'UniFrac':
  - Measures how different two samples' component sequences are

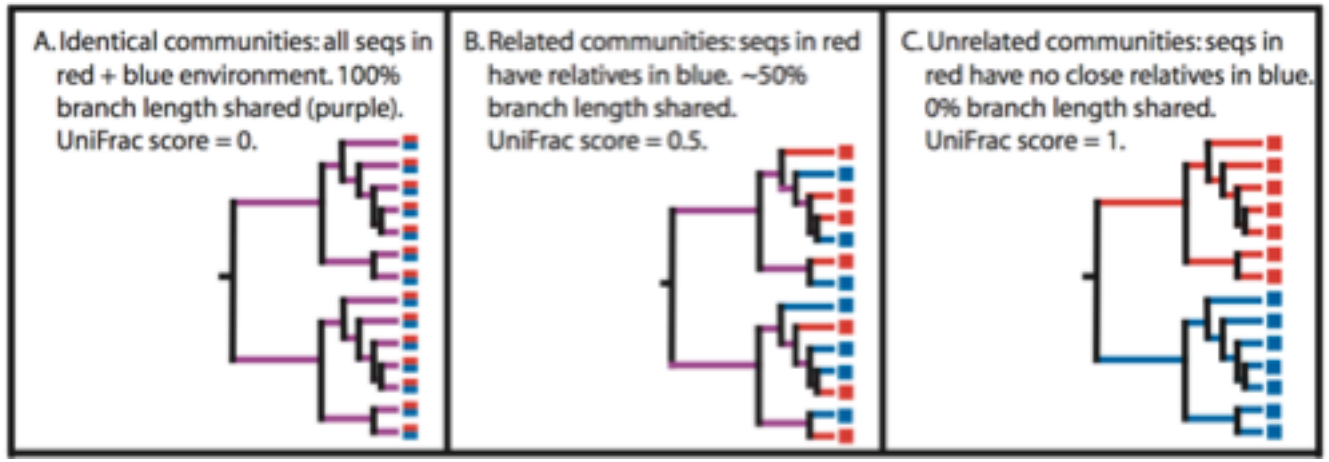


Illustration courtesy of Dr. Rob Knight

  - Weighted UniFrac: takes abundance each sequence into account

Beta Diversity Ordination

- **Ordination**: multivariate techniques that arrange samples along axes on the basis of composition
- **Principal Coordinates Analysis**: a way to map non-Euclidean distances into a Euclidean space to enable further investigation
  - Abbreviated as PCoA, not to be confused with PCA (Principal Component Analysis)
  - Starting point is distance matrix
    - NOT the full set of independent variables for each sample
  - n pairwise distances are projected into n-1 dimensions
  - PCA performed to reduce the dimensionality back down

	A	B	C
A	0	.3	.7
B	.3	0	.6
C	.7	.6	0

Distance Matrix

- PCoA axes can't be decomposed into independent variable contributions
  - But results can be compared to metadata to identify patterns

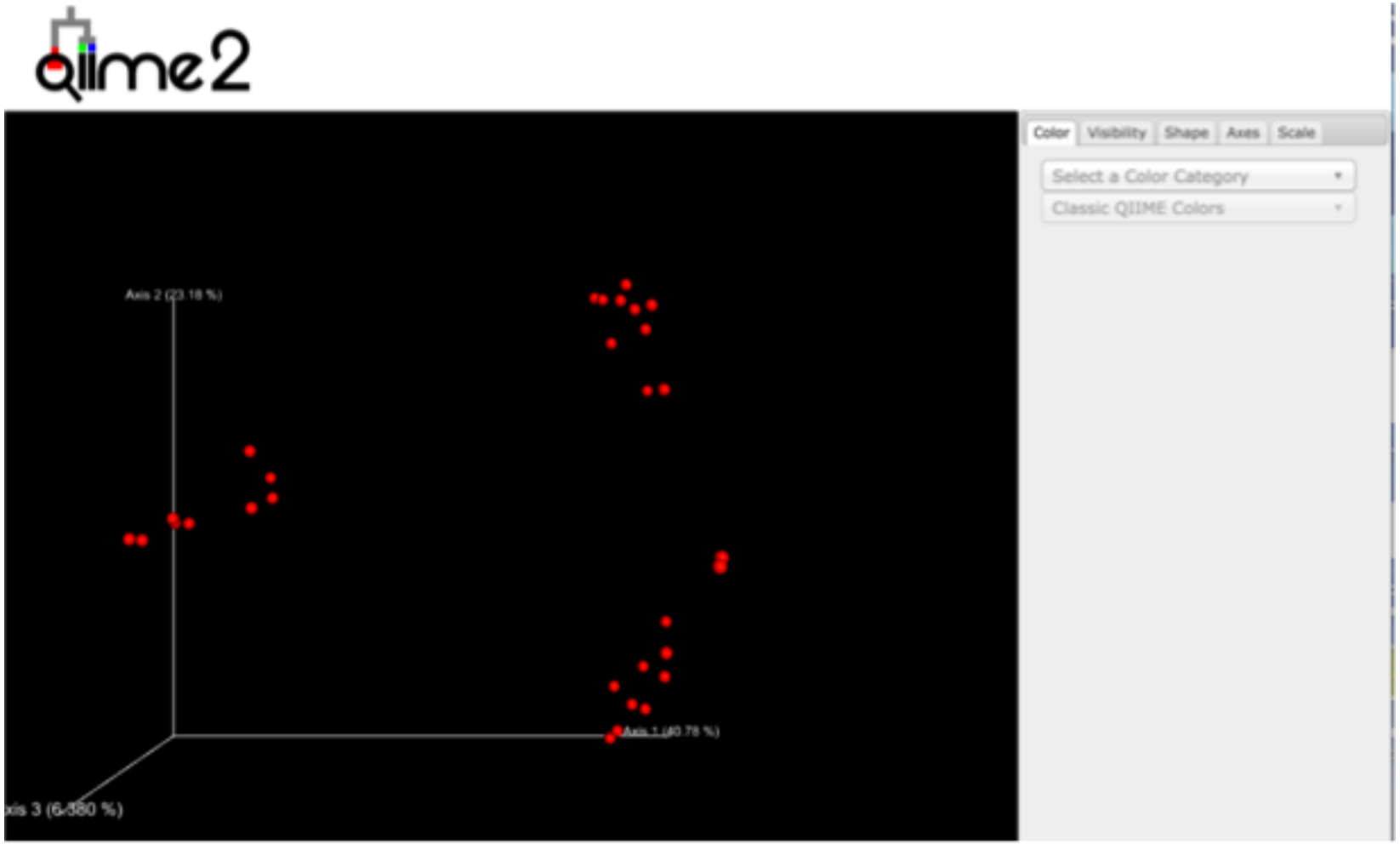
Practicum: Beta Diversity Ordination

```
qiime emperor plot \
  --i-pcoa metrics/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/unweighted-unifrac-emperor.qzv
```

- Same caveat as before:
  - Only showing the PCoA visualization of ONE beta diversity metric
    - Not necessarily “the correct one”!
  - Remember that 3 others are calculated by `core-metrics-phylogenetic` alone
- To check the group significance of a different metric, just input a different vector file
  - To find them:

```
cd metrics/
ls *_pcoa_results.qza
```

Beta Diversity Ordination View



Exercise: Beta Diversity Ordination

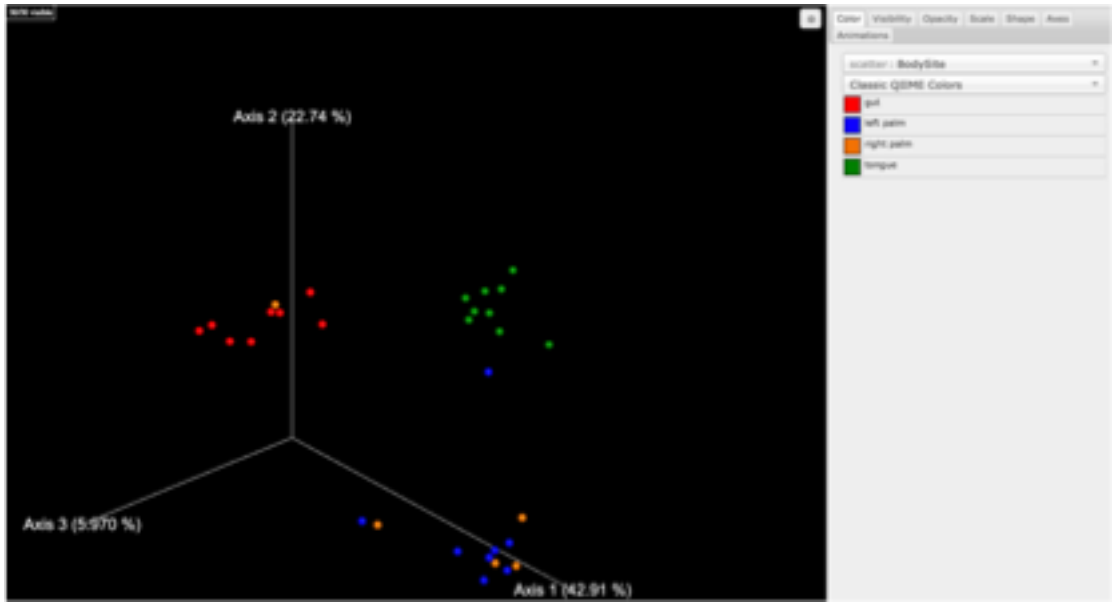
```
qiime emperor plot \
  --i-pcoa metrics/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/unweighted-unifrac-emperor.qzv
```

- Work with your partner to answer the following question:
  - Can you find a metadata category that appears associated with the observed clusters?
    - Hint: Experiment with coloring points by different metadata

Answers: Beta Diversity Ordination

```
qiime emperor plot \
  --i-pcoa metrics/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization metrics/unweighted-unifrac-emperor.qzv
```

- My answer:
  - Can you find a metadata category that appears associated with the observed clusters?
    - Yep: BodySite



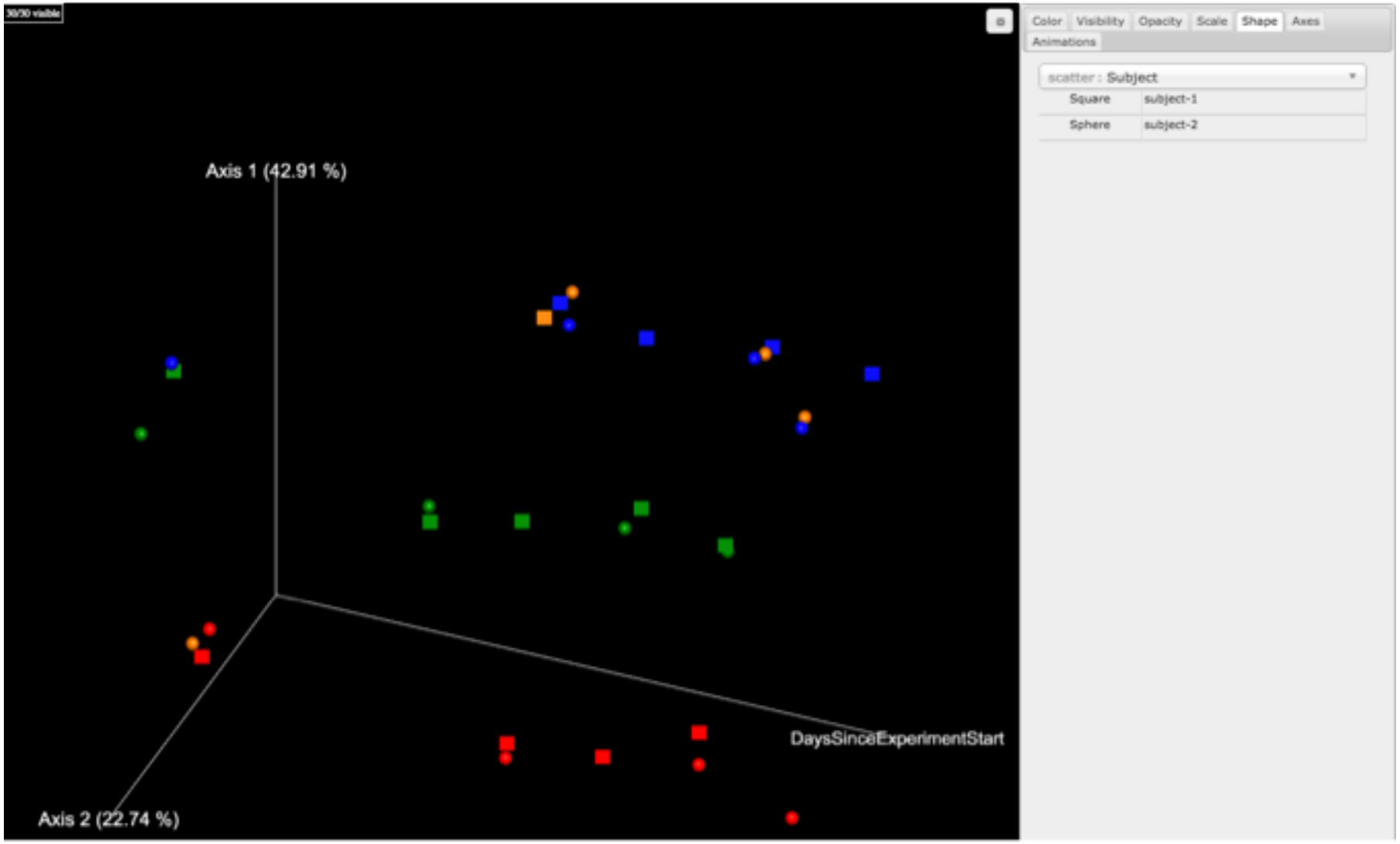
Practicum: Beta Diversity Ordination (cont.)

- But wait, this is time-series data!

```
qiime emperor plot \
  --i-pcoa metrics/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --p-custom-axes DaysSinceExperimentStart \
  --o-visualization metrics/unweighted-unifrac-emperor-bydayssince.qzv
```

- Standard caveats apply

Beta Diversity Ordination View (cont.)



Practicum: Beta Diversity Group Significance

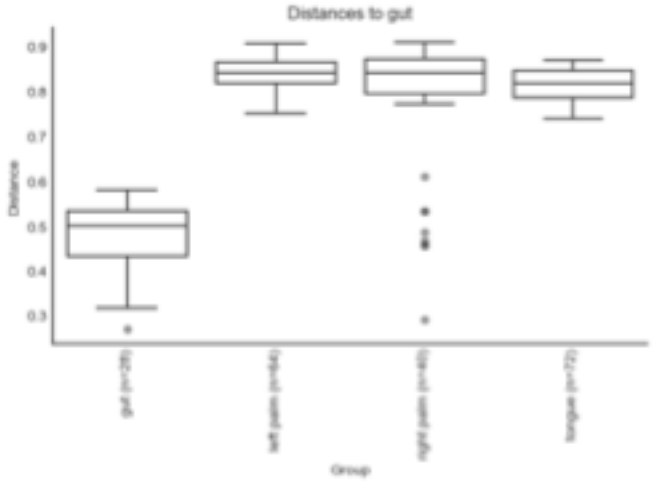
```
qiime diversity beta-group-significance \
  --i-distance-matrix metrics/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-column BodySite \
  --p-pairwise  \
  --o-visualization metrics/unweighted-unifrac-bodysite-significance.qzv
```

- Standard caveats apply

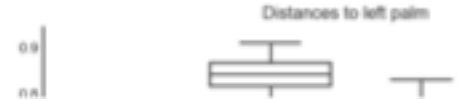
Beta Diversity Group Significance View

qiime2

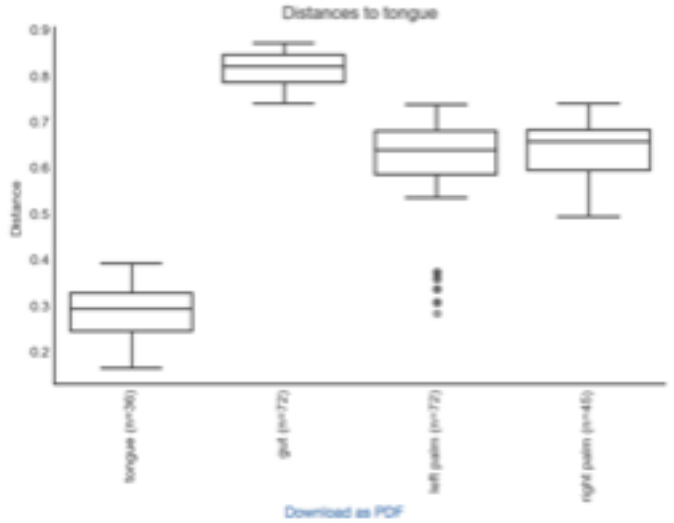
PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	30
number of groups	4
test statistic	11.3089
p-value	0.001
number of permutations	999



Download as PDF



Beta Diversity Group Significance View



Pairwise permanova results

Download CSV

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
gut	left palm	16	999	16.532145	0.002	0.0024
	right palm	13	999	7.442433	0.001	0.0020
	tongue	17	999	28.727307	0.001	0.0020
left palm	right palm	13	999	0.495504	0.974	0.9740
	tongue	17	999	12.465506	0.001	0.0020
right palm	tongue	14	999	6.999375	0.002	0.0024

Exercise: Beta Diversity Group Significance

```
qiime diversity beta-group-significance \
  --i-distance-matrix metrics/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-category BodySite \
  --p-pairwise  \
  --o-visualization metrics/unweighted-unifrac-bodysite-significance.qzv
```

- Work with your partner to answer these questions:
  - Does the group significance analysis bear out your intuition from the ordination?
    - If so, are the differences statistically significant?
    - Are there specific pairs of BodySite values that are significantly different from each other?
  - How about Subject?
    - Hint: you will need to run a new command!

Answers: Beta Diversity Group Significance

```
qiime diversity beta-group-significance \
  --i-distance-matrix metrics/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-category BodySite \
  --p-pairwise  \
  --o-visualization metrics/unweighted-unifrac-bodysite-significance.qzv
```

- My answers:
  - Does the group significance analysis bear out your intuition from the ordination?
    - Yes
    - If so, are the differences statistically significant?
      - Yes, with p <= 0.001 (bonus: why do I say "**less than** or equal to"?)
    - Are there specific pairs of BodySite values that are significantly different from each other?
      - Yes, all of the pairs except left palm/right palm

Taxonomic Assignment

- Sequence features or OTUs have limited utility
  - At some point, you'll want to link your findings to published work
  - That requires identifying the taxonomy of each sequence feature
- Steps:
  - Pick reference database
    - I hear you cry, "Which one should I use?"
  - Train a classifier algorithm to assign taxonomies to sequences
    - Use the reference database as the training set
  - Run the classifier algorithm on your sequence features

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Common Issues in Marker Gene Studies

- Neglecting metadata
  - Analysis can not test for effects of, or discard bias from, categories you didn't record!
- Picking novel 16S primers—not all created equal
  - Earth Microbiome Project recommends 515f-806r primers, error-correcting barcodes
- Not taking precautions to support amplicon sequencing
  - Some Illumina machines require high PhiX, low cluster density
- Selecting an inappropriate reference database
  - E.g., Greengenes (16S) reference database when sequencing ITS
- Expecting species-level taxonomy calls
  - Most O sequence variants only specify to family or genus level
- Using inappropriate statistical tests
  - Taxa abundance requires a compositionality-aware test like ANCOM
  - Differences in β diversity distances across groups requires test like PERMANOVA, not ANOVA

Marker Gene Reference Databases

- NOT a complete list:
  - Greengenes: 16S
  - Silva: 16S/18S
  - RDP: 16S/18S/28S
  - UNITE: ITS
- Another not complete list at eukref.org/databases (not just eukaryotic)
- At the very least, choose a database that includes your marker gene!
  - Beyond that, formal guidance is hard to find
  - But off the record you might get some informal guidance ☺

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Taxonomy: Expectation Vs Reality

	Ideal Result	Real Result
Kingdom	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria
Class	Gammaproteobacteria	Gammaproteobacteria
Order	Enterobacteriales	Enterobacteriales
Family	Enterobacteriaceae	Enterobacteriaceae
Genus	*Eschericia*	---
Species	*coli*	OTU 2445338
Strain	O157:H7	--

Practicum: Taxonomic Assignment

```
qiime feature-classifier classify-sklearn \
  --i-classifier gg-13-8-99-515-806-nb-classifier.qza \
  --i-reads rep-seqs.qza \
  --o-classification taxonomy.qza
```

- Note: the classifier has already been trained for you
  - trained on the Greengenes 13_8 99% OTUs
  - sequences trimmed to only include 250 bases from the region of the 16S that was sequenced in this analysis
    - the V4 region, bound by the 515F/806R primer pair
- Other pre-trained classifiers available in Data Resources page on docs.qiime2.org

Practicum: Taxonomic Assignment

```
qiime feature-classifier classify-sklearn \
  --i-classifier gg-13-8-99-515-806-nb-classifier.qza \
  --i-reads rep-seqs.qza \
  --o-classification taxonomy.qza

qiime metadata tabulate \
  --m-input-file taxonomy.qza \
  --o-visualization taxonomy.qzv
```

Taxonomic Assignment Tabulation View

Download metadata TSV file

This file won't necessarily reflect dynamic sorting or filtering options based on the interactive table below.

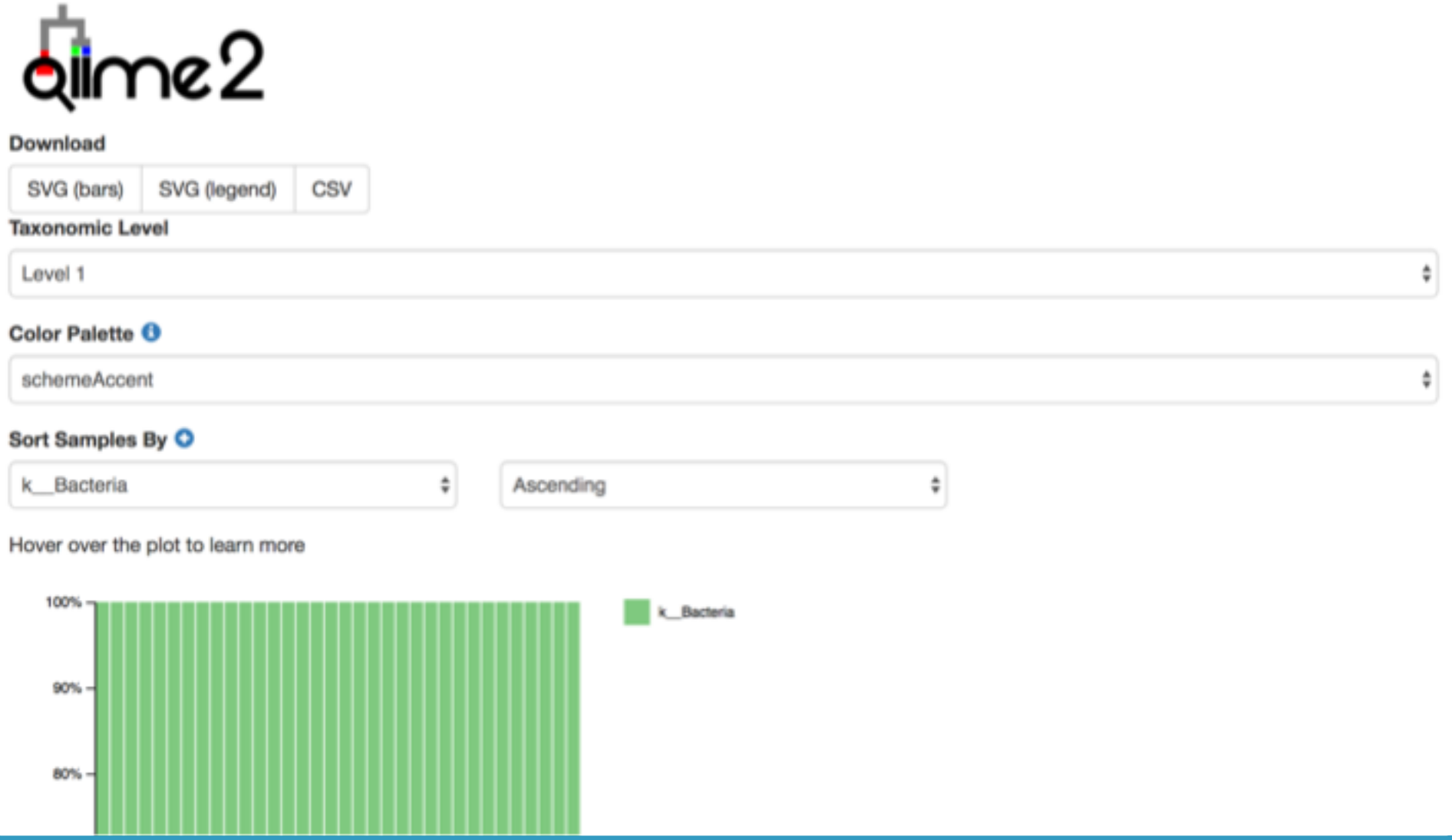
Search:

Feature ID #q2:types	Taxon categorical	Confidence categorical
0160e14a78b18b903618f11bc732746e	k__Bacteria; p__Verrucomicrobia; c__Verrucomicrobiae; o__Verrucomicrobiales; f__Verrucomicrobiaceae; g__Akkermansia; s__muciniphila	0.9999999999838138
01b99cb344ed2530f7d80897ffe257a9	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae	0.9934747834222519
01ce91fd8dbecf637eb5e67cdab5c5aa	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__[Mogibacteriaceae]; g__; s__	0.9992651687229891
01e0b7ac306895be84179f2715af269b	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Lachnospira; s__	0.9999998747932687
02ef9a59d6da8b642271166d3ffd1b52	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__	0.9782760999752006
0305a4993ecf2d8ef4149fdfc7592603	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__uniformis	0.9967281012905461

Practicum: Taxonomic Assignment

```
qiime taxa barplot \
  --i-table table.qza \
  --i-taxonomy taxonomy.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization taxa-bar-plots.qzv
```

Taxonomic Assignment Bar Plot View



Exercise: Taxonomic Assignment

```
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  --i-table table.qza \
  --i-taxonomy taxonomy.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization taxa-bar-plots.qzv
```
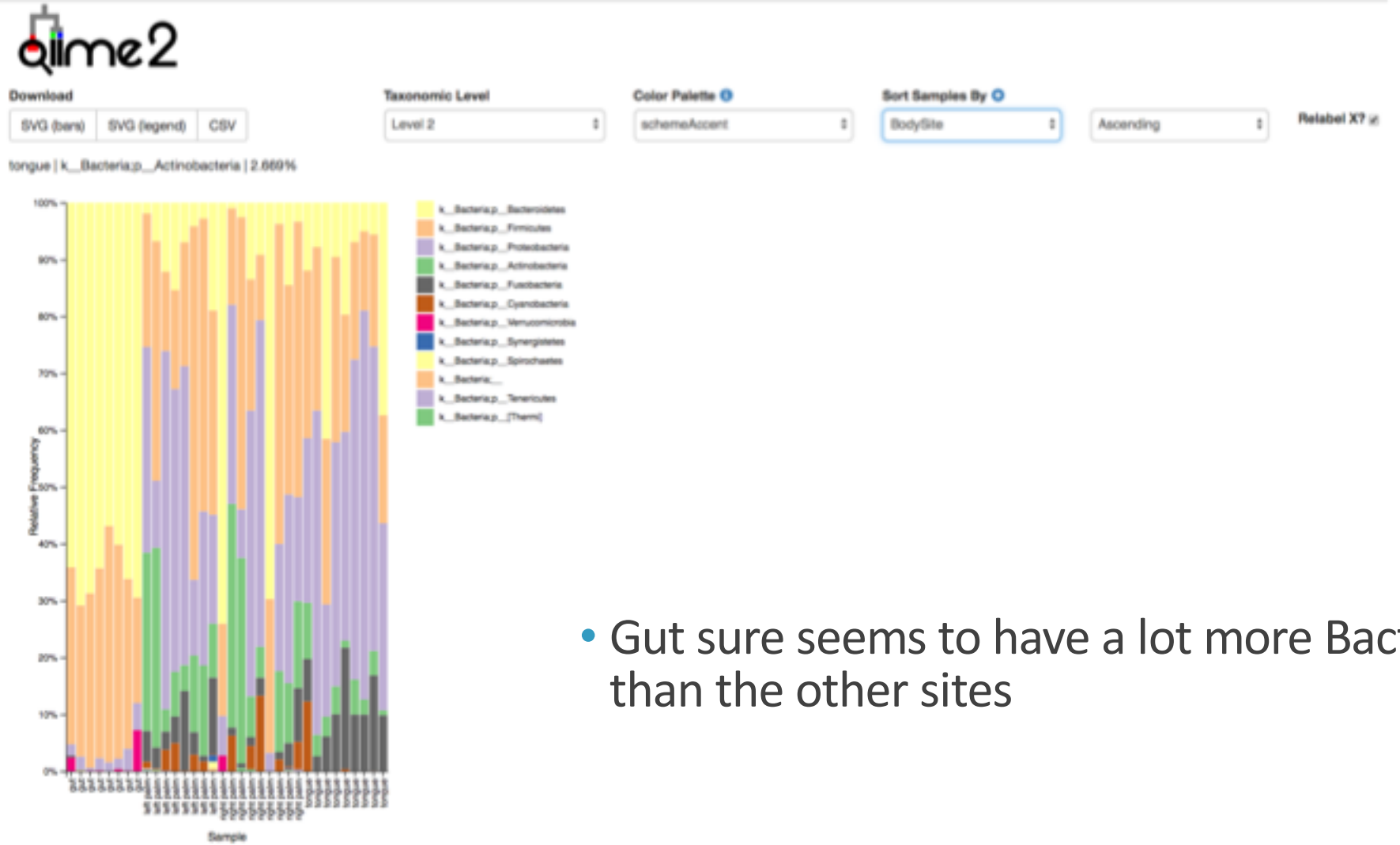

- “Level 1” = kingdom, “Level 2” = phylum, etc
- Work with your partner to:
  - Visualize the taxa at level 2
  - Sort the samples by BodySite
  - Do you see anything suggestive?

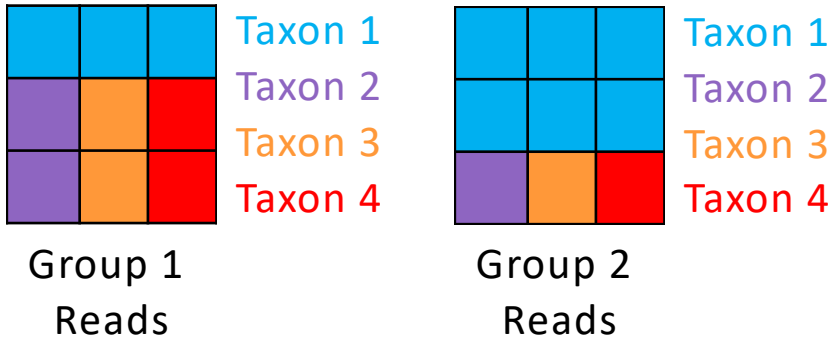
Answers: Taxonomic Assignment



- Gut sure seems to have a lot more Bacteroidetes than the other sites

Differential Abundance Analysis

- Why go to the trouble of assigning taxonomies?
  - Probably you want to know whether any particular taxa are differentially abundant
    - In different individuals, environments, time points, etc
- How to test for differential abundance?
  - Microbiome datasets are “compositional” (fixed sum)



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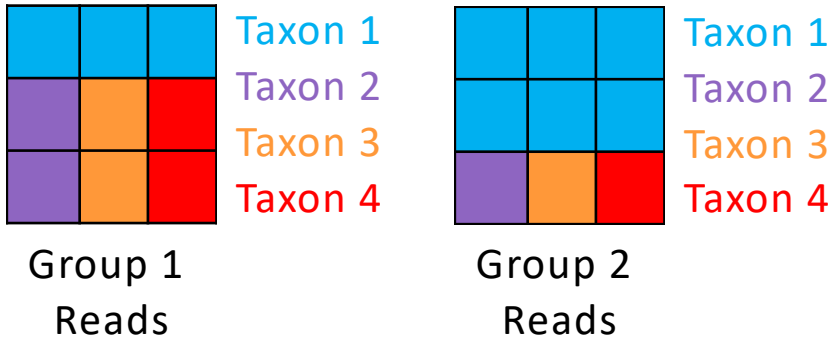
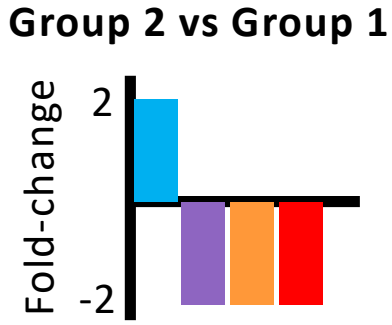




  - **Watch out**: “traditional” statistical methods perform badly for this sort of data
    - e.g., t-test, ANOVA
    - False discovery rate can be as high as 90%!

Differential Abundance Analysis (cont.)

- What to use instead?
  - ANCOM (Analysis of Composition of Microbiomes)
    - Identifies taxa that are present in different abundances across sample groups
      - Compares log ratio of the abundance of each taxon to abundance of all remaining taxa one at a time
    - Assumes that <25% of the features are changing between groups—not a given!
  - ilr (Isometric Log Ratio transforms—a.k.a. balance trees or gneiss)
    - Identifies microbial subcommunities that present in different abundances across sample groups
  - Neither require rarefication of inputs
  - Both recommend filtering out taxa that don't contain much info, such as
    - Features that have few reads (i.e. less than 10 reads across all samples).
    - Features that are rarely observed (i.e. present in less than 5 samples in a study).
    - Features that have very low variance (i.e. less than 10e-4)
      - This is left as an exercise for the reader ☺ Check out `feature-table filter-samples`

Practicum: ANCOM Analysis

- We already saw in that different body sites look very different
- Given that, probably many features (sequences) are changing in abundance across body sites
  - **This violates ANCOM's statistical assumption!**
- Therefore, limit ANCOM analysis to samples from a single body site:

```
qiime feature-table filter-samples \
  --i-table table.qza \
  --m-metadata-file sample-metadata.tsv \
  --p-where "BodySite='gut'" \
  --o-filtered-table gut-table.qza
```

Practicum: ANCOM Analysis (cont.)

- Can run ANCOM on individual “features” (sequences) but isn’t very informative
- Often more useful to collapse features to a chosen taxonomic level before ANCOM
  - Here I chose level 6, e.g., genus

```
qiime taxa collapse \
  --i-table gut-table.qza \
  --i-taxonomy taxonomy.qza \
  --p-level 6 \
  --o-collapsed-table gut-table-level6.qza
```

Practicum: ANCOM Analysis (cont.)

- Internally, ANCOM takes logs—and log of zero is undefined
  - Common approach: add one count to every value (pseudocount)
  - Not limited to ANCOM—used for any log-based method

```
qiime composition add-pseudocount \
  --i-table gut-table-level6.qza \
  --o-composition-table comp-gut-table-level6.qza
```

Practicum: ANCOM Analysis (cont.)

```
qiime composition ancom \
  --i-table comp-gut-table-level6.qza \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-column Subject \
  --o-visualization ancom-subject-level6.qzv
```

ANCOM View

ANCOM statistical results

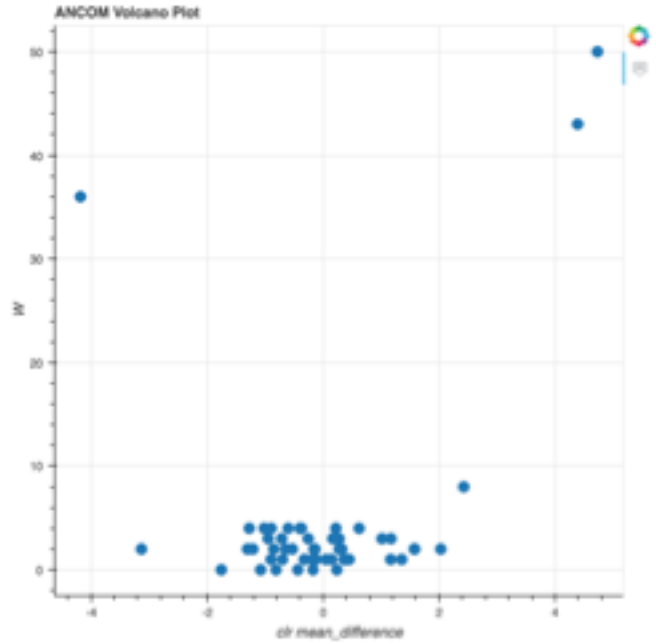
Download complete table as CSV

	W
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	50

Percentile abundances of features by group

Download complete table as CSV

Percentile	0.0	25.0	50.0	75.0	100.0	0.0	25.0	50.0	75.0	100.0
Group	subject-1	subject-1	subject-1	subject-1	subject-1	subject-2	subject-2	subject-2	subject-2	subject-2
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	1.0	1.0	1.0	1.0	1.0	76.0	100.75	112.0	158.0	287.0

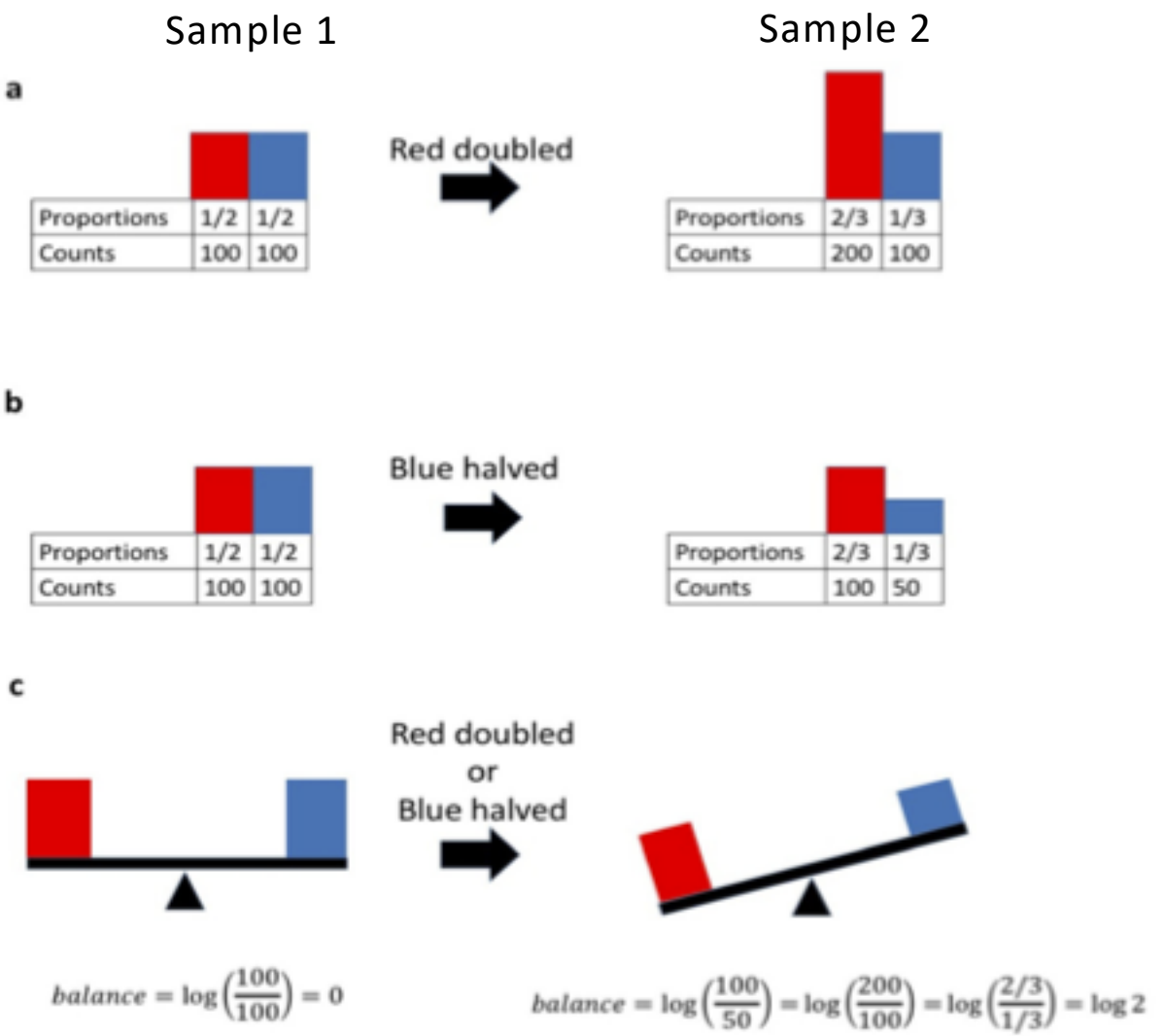


Details: ANCOM View

- W-statistic
  - # of features that a single feature is tested to be significantly different against
  - ANCOM internally decides what W value indicates significance, returns only significant results
    - You aren't going to find a p-value here, no matter how hard you look ☺
- Percentile abundance table:
  - A table of features and their percentile abundances in each group
  - Rows are features or taxa
  - Columns are percentile within a group
  - Values are abundance of reads for given percentile for that group
    - e.g.: The lowest-in-this-taxon 25% of samples in "group" subject-2 had 100.75 or fewer sequences assigned to this taxon

ilr and Balance Trees

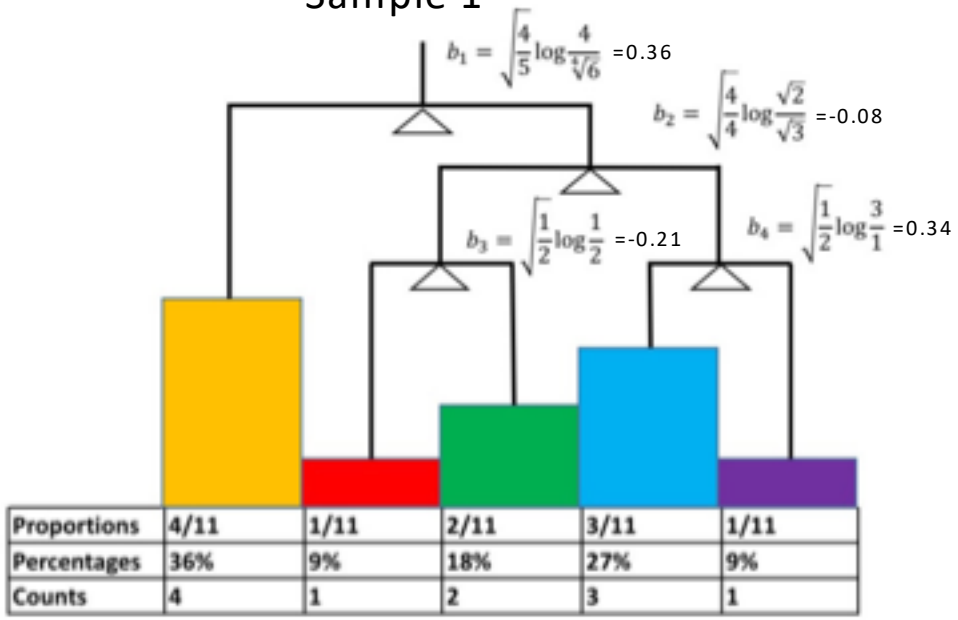
- Microbiome sequence data give *proportions* of taxa abundance
  - Because of compositionality
- "[B]ased on proportions alone, it is impossible to determine whether the growth or decline of any individual species has truly occurred"
- Balance trees instead ask an answerable question:
  - Has the balance of sub-communities changed?



Quote and figure from Morton et al, mSystems 2017

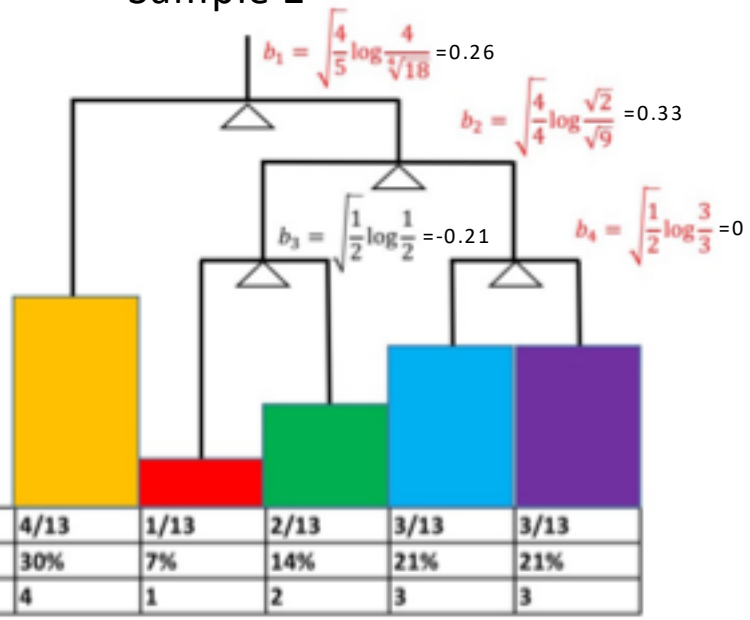
ilr and Balance Trees (cont.)

Sample 1



Proportions	4/11	1/11	2/11	3/11	1/11
Percentages	36%	9%	18%	27%	9%
Counts	4	1	2	3	1

Sample 2



Proportions	4/13	1/13	2/13	3/13	3/13
Percentages	30%	7%	14%	21%	21%
Counts	4	1	2	3	3

Fig modified from Morton et al, mSystems 2017

- The subcommunities of interest are defined by a tree of all the species
  - Each internal node in the tree is a “balance” between the subcommunities in its left and right children
- For each sample, at each balance, calculate the isometric log ratio transform (ilr)
  - Gives a measure of relative abundance of subcommunities on each side of the balance

ilr and Balance Trees (cont.)

- After ilr, result is a matrix
  - ilr values by sample by balance
- Simple case:
  - Only one balance is of interest
  - Can just do Student's t-test
- More realistic case:
  - All balances are of interest
    - Want to characterize whole community
  - Do multivariate response linear regression
    - Fit a linear regression model for each balance based on all samples for that balance
      - e.g., $Y_{b1} = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \ldots. \beta_i X_i + \varepsilon_{b1}$
    - Coefficients significantly ≠ 0 = categories associated w/difference in subcommunities of balance

	b1	b2	b3	b4
Sample 1	0.36	-0.08	-0.21	0.34
Sample 2	0.26	0.33	-0.21	0.00

		b1	b2	b3	b4
Group 1	Sample 1	0.36	-0.08	-0.21	0.34
	Sample 3	0.33	-0.11	-0.20	0.29
	Sample 5	0.37	-0.03	-0.18	0.35
Group 2	Sample 2	0.26	0.33	-0.21	0.00
	Sample 4	0.28	0.30	-0.22	0.01
	Sample 6	0.25	0.34	-0.20	0.02

Practicum: gneiss Analysis

- Balance trees were first developed in geology
  - “gneiss” (say: nice) is a kind of rock
- Like ANCOM, gneiss uses logs, so requires a pseudocount

```
qiime gneiss add-pseudocount \
  --i-table table.qza \
  --p-pseudocount 1 \
  --o-composition-table composition.qza
```

Practicum: gneiss Analysis (cont.)

- The tree used defines which balances are assessed
  - i.e., which subcommunities of species are compared
  - How to choose it?
- Use an externally defined tree (e.g., phylogeny)
- Build a tree based on a numeric metric related to your hypothesis
  - e.g., from your metadata, such as pH
  - Using `gradient clustering` gneiss command
- Build a tree with unsupervised clustering across all your data
  - Group together organisms based on how often they co-occur with each other

```
qiime gneiss correlation-clustering \
  --i-table composition.qza \
  --o-clustering hierarchy.qza
```

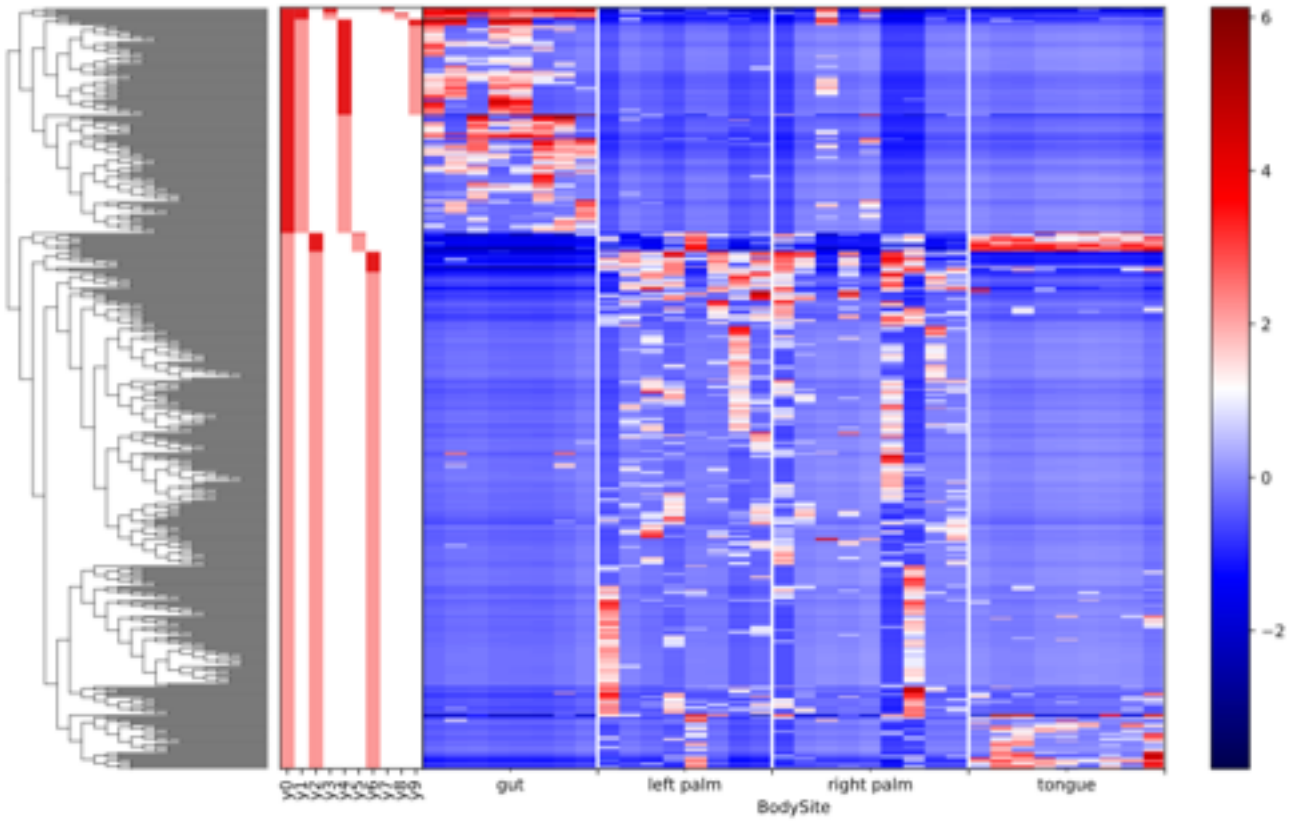
Practicum: gneiss Analysis (cont.)

- What does the tree actually look like?

```
qiime gneiss dendrogram-heatmap \
  --i-table composition.qza \
  --i-tree hierarchy.qza \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-column BodySite \
  --p-color-map seismic \
  --o-visualization tree_heatmap_by_bodysite.qzv
```

gneiss Tree View

Dendrogram heatmap



Numerator Denominator

Practicum: gneiss Analysis (cont.)

- Calculate the ilr transforms

```
qiime gneiss ilr-transform \
  --i-table composition.qza \
  --i-tree hierarchy.qza \
  --o-balances balances.qza
```

Practicum: gneiss Analysis (cont.)

- Fit the linear regression model
  - Requires deciding on your formula, based on your metadata

```
qiime gneiss ols-regression \
  --p-formula "Subject+BodySite+DaysSinceExperimentStart" \
  --i-table balances.qza \
  --i-tree hierarchy.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization regression_summary.qzv
```

- As this is a time-course, could instead use `lme-regression` grouped by Subject

gneiss Regression Summary View

Simplicial Linear Regression Summary

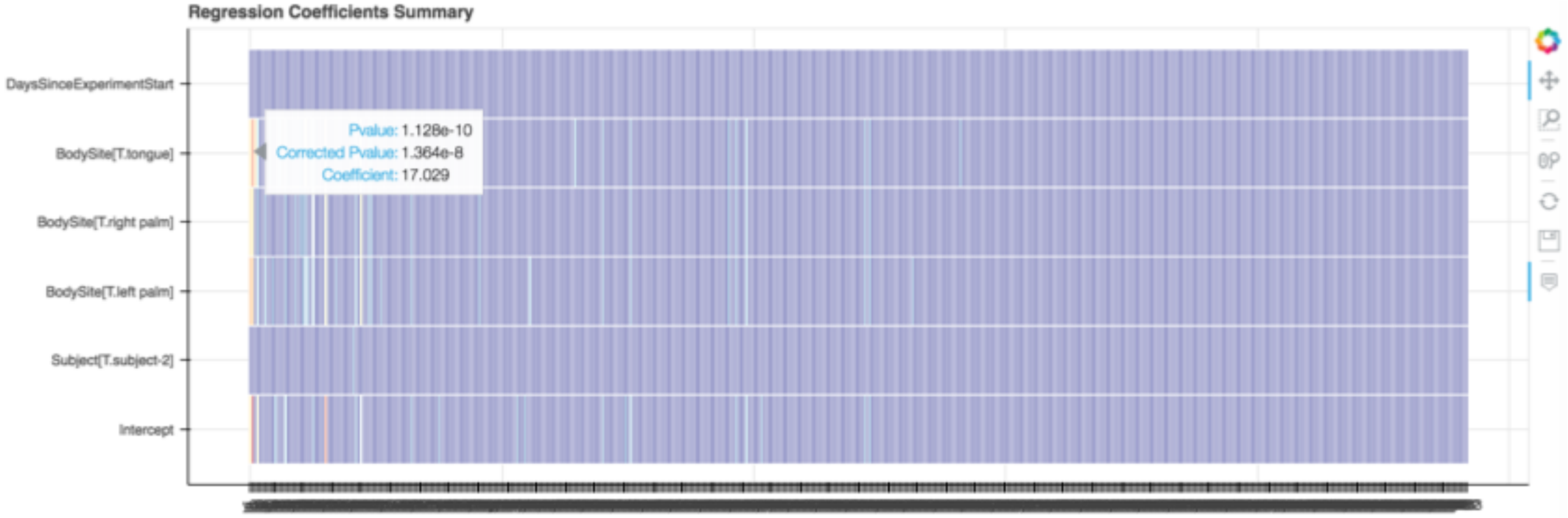
No. Observations 34.0000
Model: OLS
Rsquared: 0.4975

	mse	Rsquared	R2diff
Intercept	18.2693	0.3309	0.1665
Subject[T.subject-2]	15.2408	0.4418	0.0556
BodySite[T.left palm]	19.2142	0.2963	0.2011
BodySite[T.right palm]	18.5499	0.3207	0.1768
BodySite[T.tongue]	19.8765	0.2721	0.2254
DaysSinceExperimentStart	14.5560	0.4669	0.0305

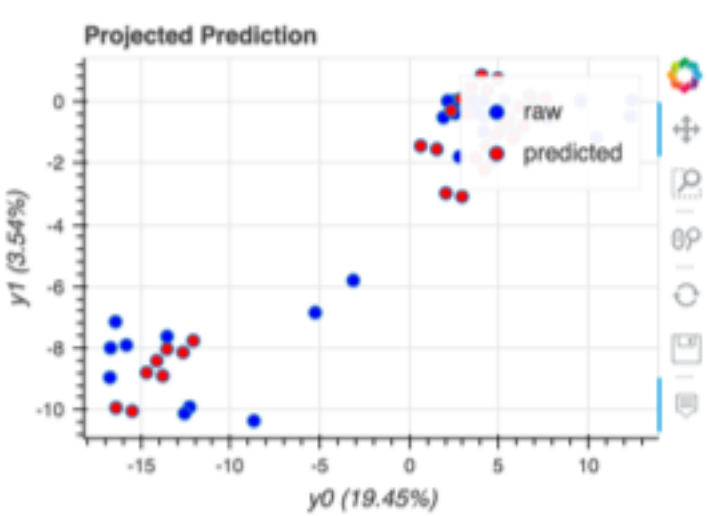
	model_mse	Rsquared	pred_mse
fold_0	11.0272	0.4668	1.8216
fold_1	10.9444	0.4592	2.1930
fold_2	10.8385	0.4979	2.1128
fold_3	10.6139	0.5143	2.5960
fold_4	10.8593	0.5234	2.4676
fold_5	10.6387	0.5243	2.2926
fold_6	11.3915	0.5102	1.4510
fold_7	9.6503	0.5400	3.6218
fold_8	10.6161	0.5135	2.3444
fold_9	12.0860	0.4713	0.7668

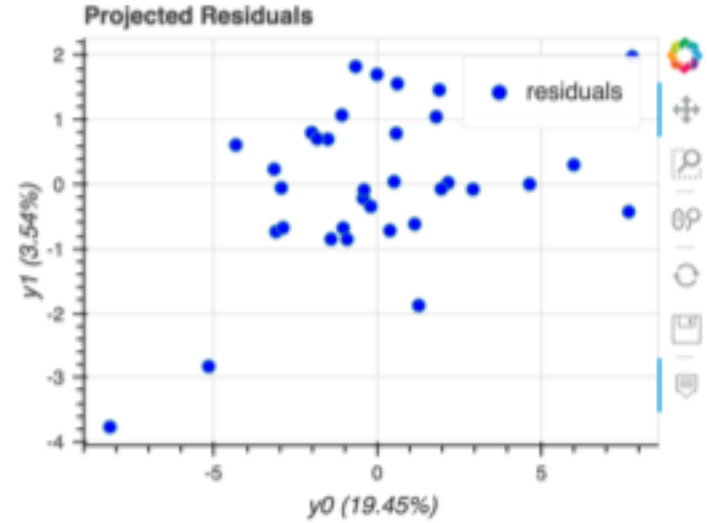
Coefficients Download as CSV

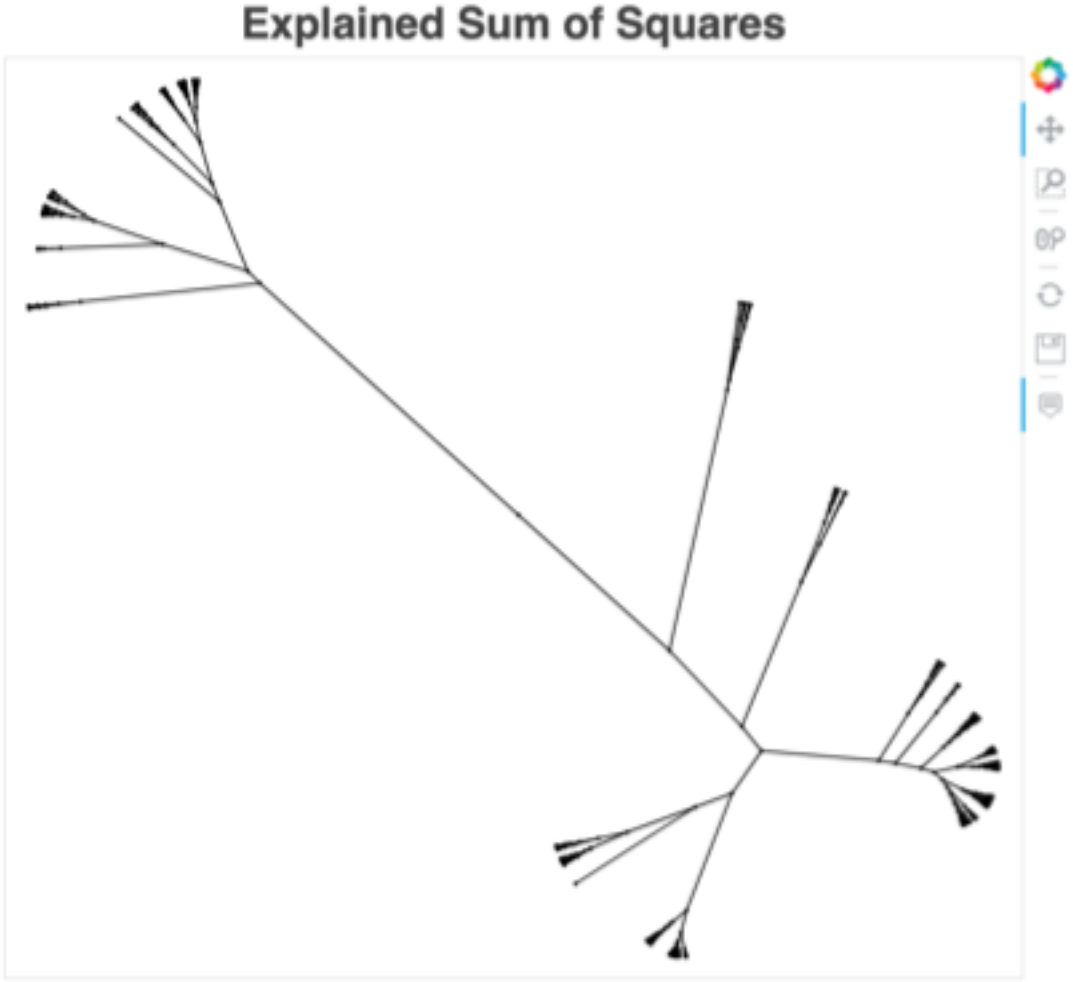
gneiss Regression Summary View (cont.)



gneiss Regression Summary View (cont.)





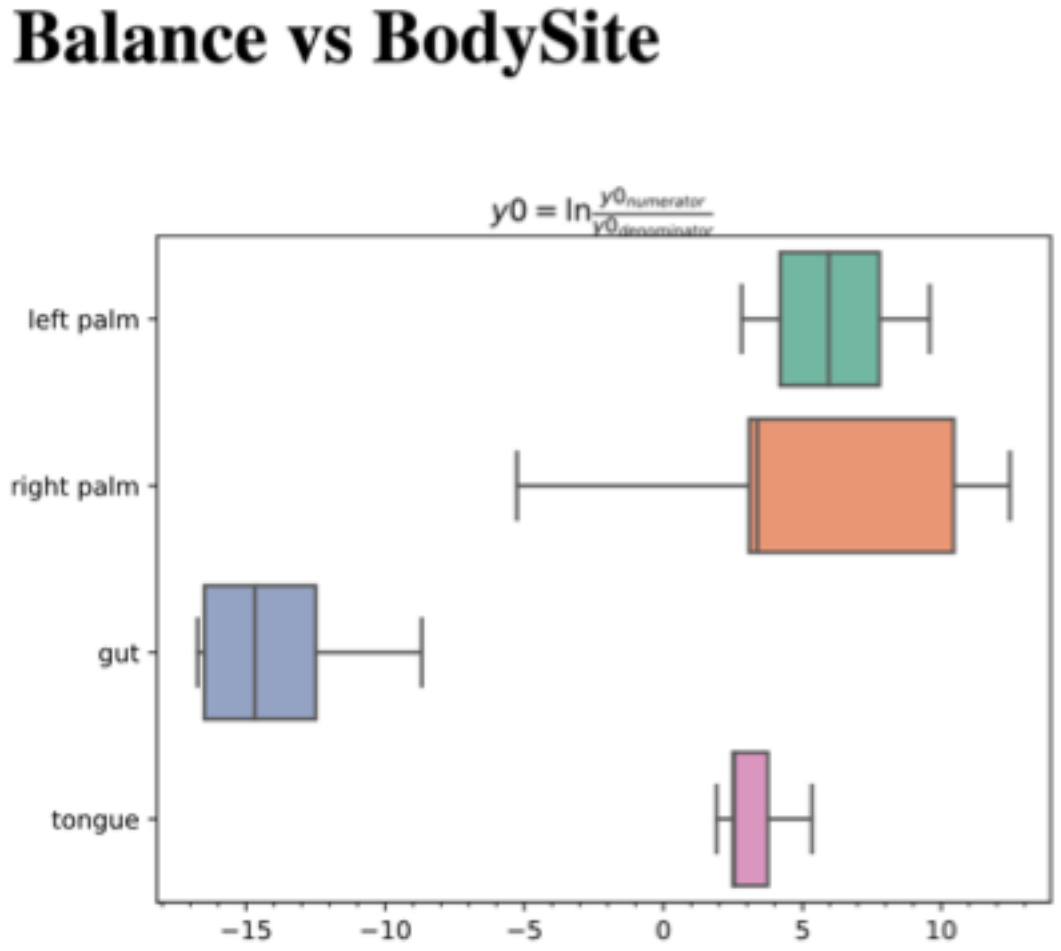


Practicum: gneiss Analysis (cont.)

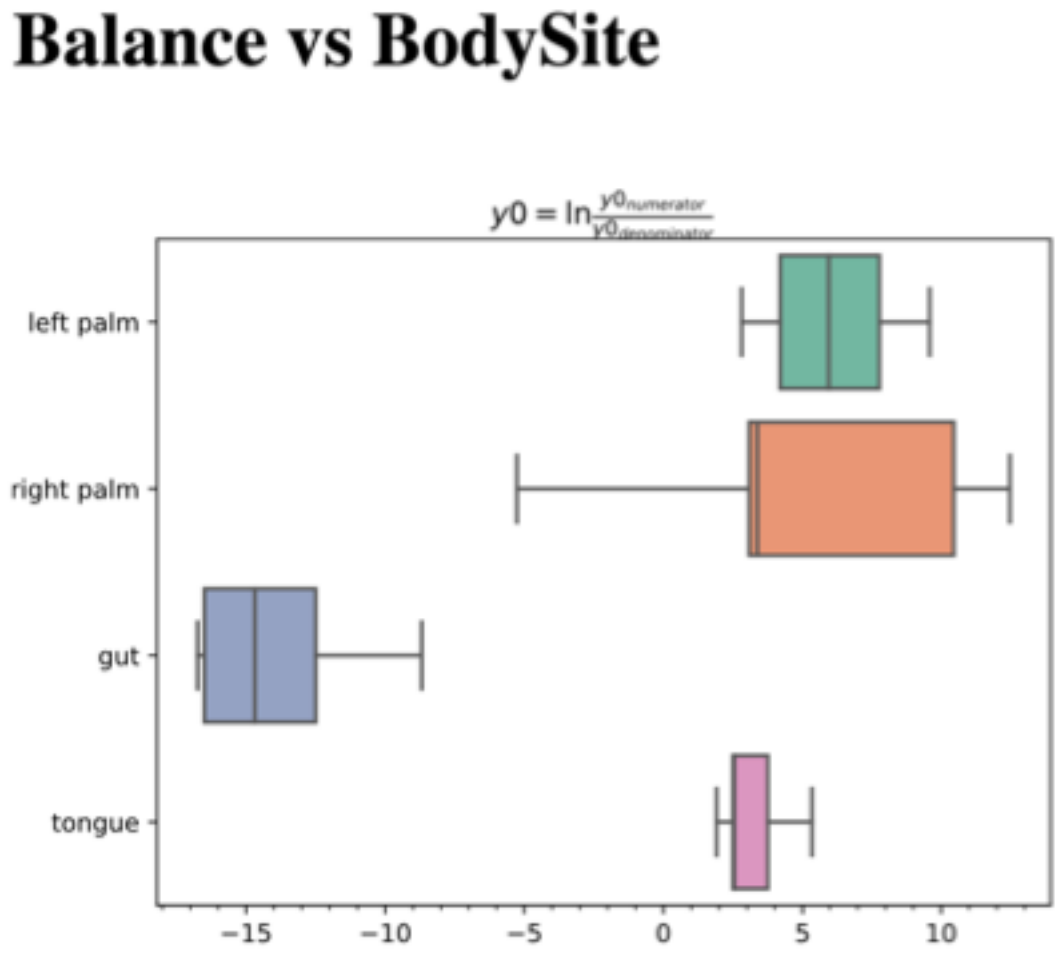
- Well, great, but we seem to have strayed a long way from actual taxa!

```
qiime gneiss balance-taxonomy \
  --i-table composition.qza \
  --i-tree hierarchy.qza \
  --i-taxonomy taxonomy.qza \
  --p-taxa-level 2 \
  --p-balance-name 'y0' \
  --m-metadata-file sample-metadata.tsv \
  --m-metadata-column BodySite \
  --o-visualization y0_taxa_summary.qzv
```

gneiss Balance Taxa Summary View



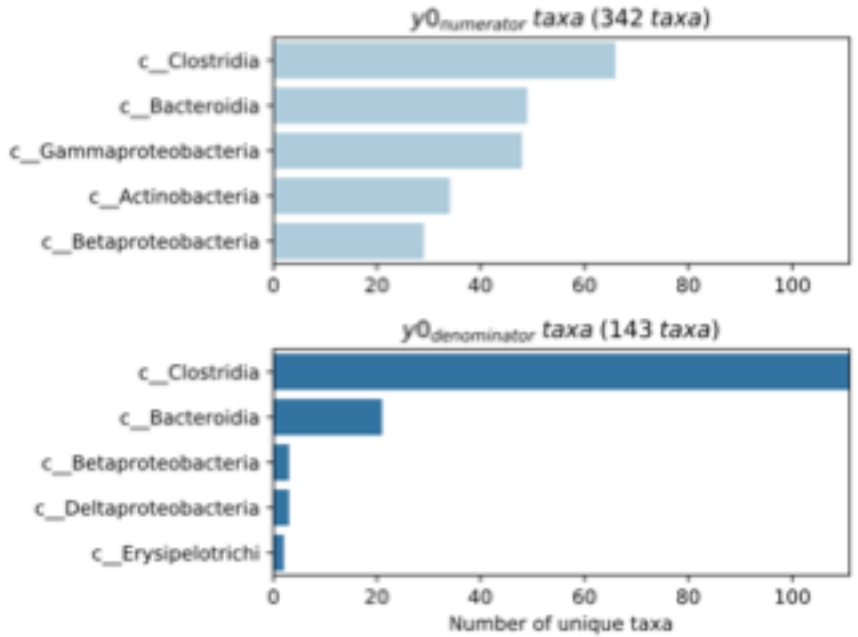
gneiss Balance Taxa Summary View



- Possible meanings
  - The taxa in the y_0 numerator on average increase in other sites compared to gut
  - The taxa in the y_0 denominator on average decrease in other sites compared to gut
  - A combination of the above occurs
  - Taxa abundances in both y_0 numerator and y_0 denominator both increase compared to gut, but taxa abundances in numerator increase more compared to denominator
  - Taxa abundances in both y_0 numerator and y_0 denominator both decrease, but taxa abundances in denominator increase more compared to numerator

gneiss Balance Taxa Summary View (cont.)

Balance Taxonomy



Numerator taxa

Download as CSV

Denominator taxa

Download as CSV

Practicum Summary

- Steps practiced
  - Importing data
  - Demultiplexing
  - Running Quality Control
  - Creating a feature table
  - Building a phylogenetic tree
  - Calculating core diversity metrics
  - Testing alpha diversity group significance and correlation
  - Performing beta diversity ordination
  - Testing beta diversity group significance
  - Assigning taxonomies
  - Performing differential abundance analysis with ANCOM and/or gneiss

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