Tutorial 3: Statistical Testing: Group Comparisons and Multiple Comparison Corrections in R

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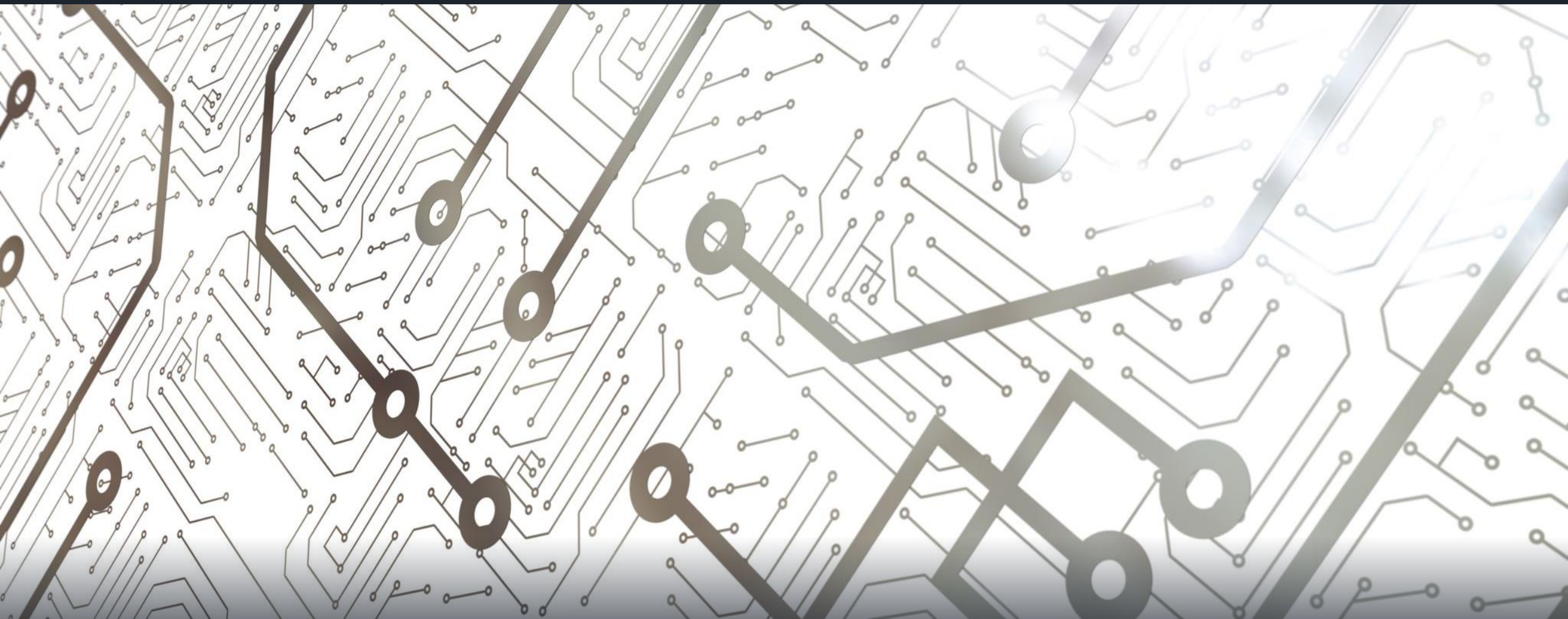
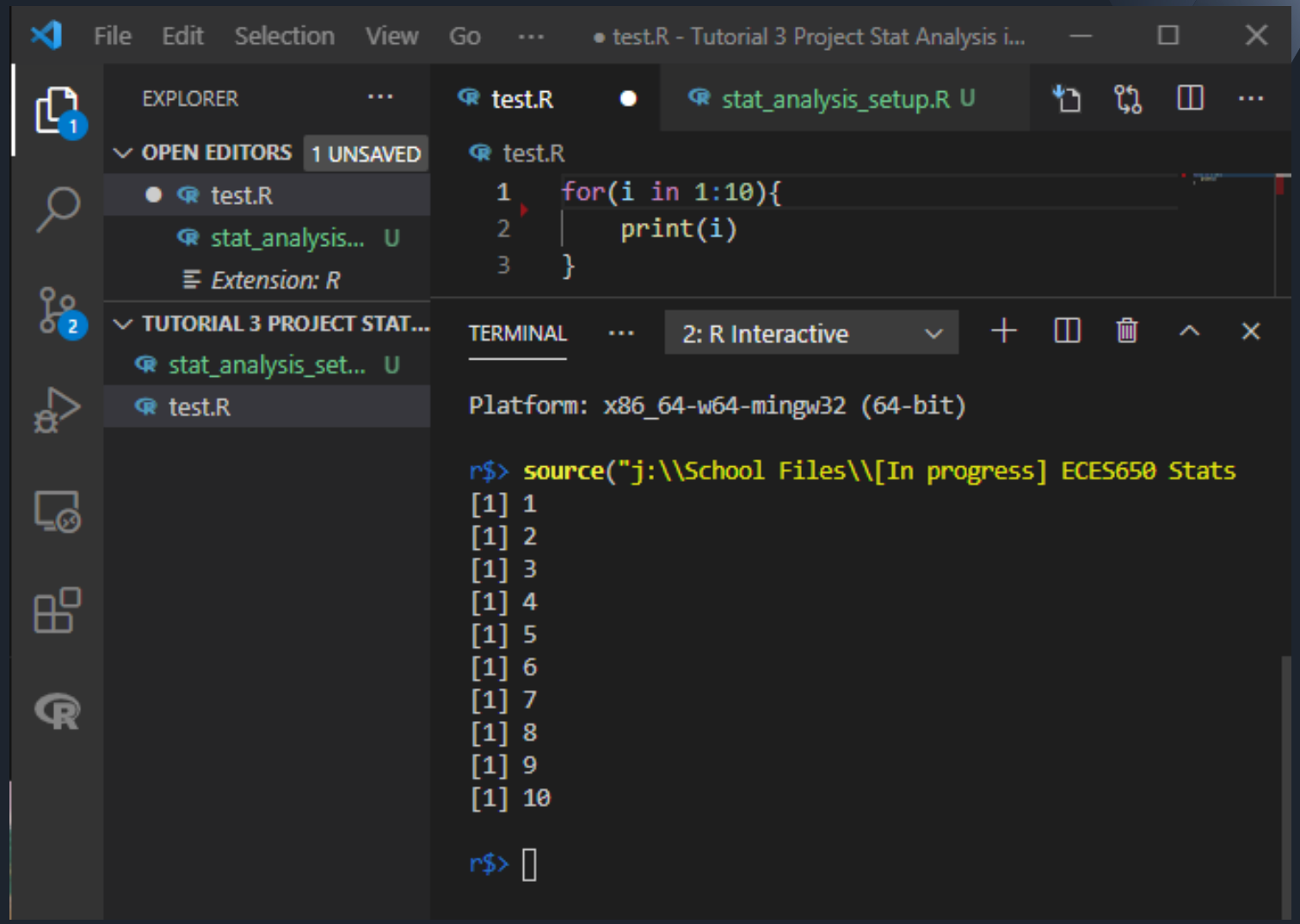


Installing R

- Choose OS from this link and install : https://cran.r-project.org/bin/
- Decide upon an environment to use (This slide is in VSCode)
  - VSCode uses two extensions for R, namely the R Extension and the R LSP Client Extension for the *radian* terminal
- Test for proper installation:



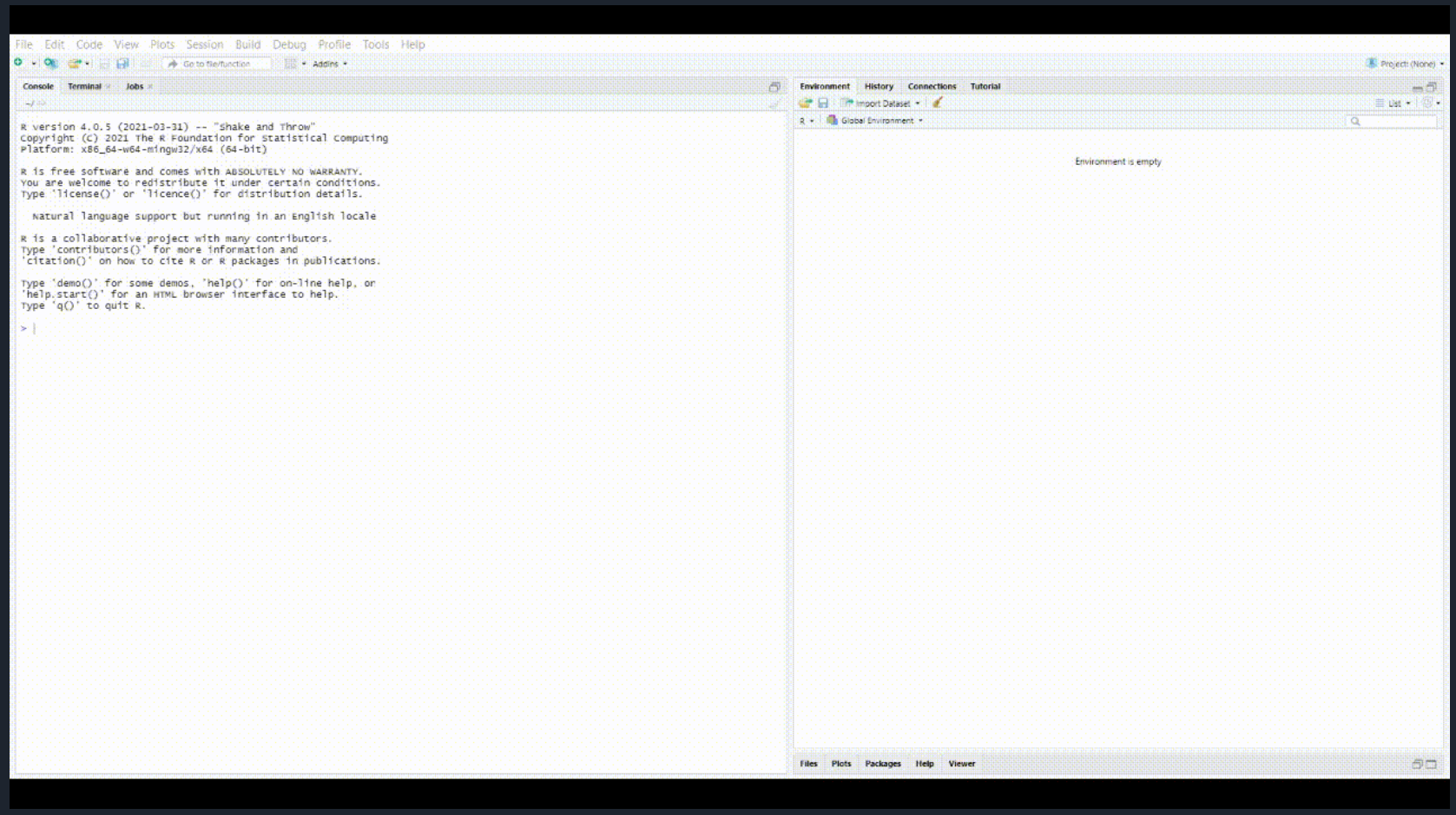
Packages for R 4.0.5

```r
## Installing packages
.cran_packages <- c("tidyverse", "cowplot", "picante", "vegan", "HMP", "dendextend", "rms", "devtools")
.bioc_packages <- c("phyloseq", "DESeq2", "microbiome", "metagenomeSeq", "ALDEx2")
.inst <- .cran_packages %in% installed.packages()
if(any(!.inst)) {
  install.packages(.cran_packages[!.inst])
}
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install(.bioc_packages, version = "3.12") #Different from the website's "3.9" since I'm using R 4.0.5
devtools::install_github("adw96/breakaway")
devtools::install_github(repo = "UVic-omics/selbal")

## Loading Libraries
library(tidyverse); packageVersion("tidyverse")
library(phyloseq); packageVersion("phyloseq")
library(DESeq2); packageVersion("DESeq2")
library(microbiome); packageVersion("microbiome")
library(vegan); packageVersion("vegan")
library(picante); packageVersion("picante")
library(ALDEx2); packageVersion("ALDEx2")
library(metagenomeSeq); packageVersion("metagenomeSeq")
library(HMP); packageVersion("HMP")
library(dendextend); packageVersion("dendextend")
library(selbal); packageVersion("selbal")
library(rms); packageVersion("rms")
library(breakaway); packageVersion("breakaway")
```

https://www.nicholas-ollberding.com/post/introduction-to-the-statistical-analysis-of-microbiome-data-in-r/

Packages for R 4.0.5

If using R Studio, to run scripts click "Source" to run the whole script

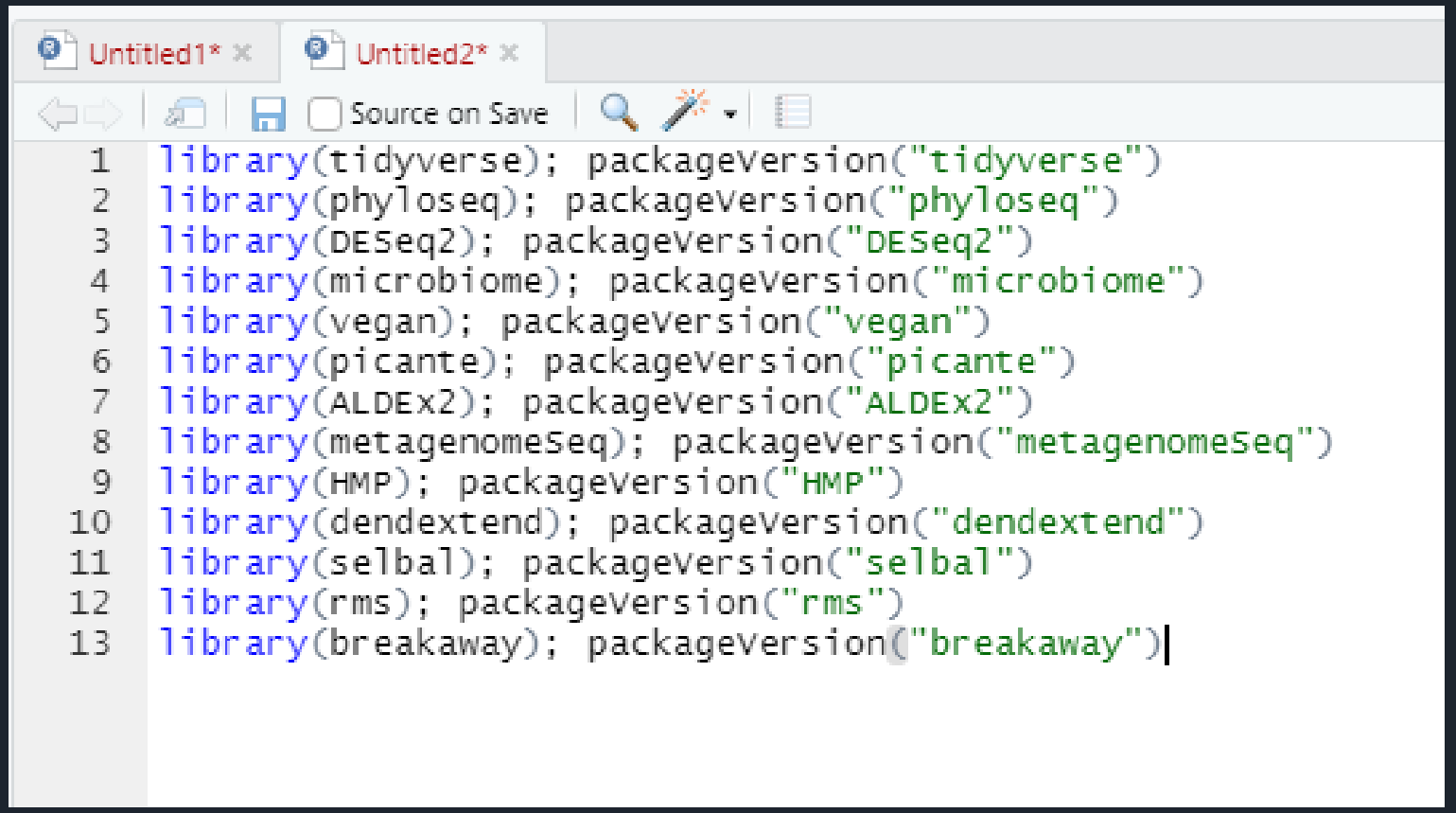


Loading & Checking Packages

It is important to check to make sure all packages were installed correctly

If a package was installed incorrectly use this command:

install.packages("*name*")



Obtaining Data Gut Microbial Data

- *ps_giloteaux_2016.rds* from https://github.com/Nick243/Create-Giloteaux-2016-Phyloseq-Object/blob/master/ps_giloteaux_2016.rds
  - Given as a *phyloseq* object
  - Place in same directory as the working environment
- Load data and sort by read count with:

```
(ps <- readRDS("ps_giloteaux_2016.rds"))
sort(phyloseq::sample_sums(ps))
```

```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 138 taxa and 87 samples ]
sample_data() Sample Data:       [ 87 samples by 22 sample variables ]
tax_table()   Taxonomy Table:    [ 138 taxa by 7 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 138 tips and 136 internal nodes ]
refseq()      DNAStringSet:      [ 138 reference sequences ]
ERR1331827 ERR1331852 ERR1331856 ERR1331869 ERR1331833 ERR1331797 ERR1331786 ERR133181
      2707       3031       3117       5083       5245       5307       5696       573
ERR1331795 ERR1331846 ERR1331811 ERR1331845 ERR1331842 ERR1331838 ERR1331855 ERR133182
      7314       7569       7665       7815       7911       8102       8115       814
ERR1331801 ERR1331841 ERR1331861 ERR1331820 ERR1331854 ERR1331863 ERR1331806 ERR133178
     11173      11442      11826      12940      13029      13094      13095      1369
ERR1331809 ERR1331828 ERR1331813 ERR1331798 ERR1331816 ERR1331830 ERR1331785 ERR133182
     16162      16494      16749      16947      17015      17457      17557      1850
ERR1331849 ERR1331860 ERR1331808 ERR1331872 ERR1331812 ERR1331850 ERR1331791 ERR133178
     21540      21553      21713      22339      22518      22639      23246      2375
ERR1331839 ERR1331794
     61206      65941
```

Phylogenetic Data in a *phyloseq* File

- Remove the samples with less than 5,000 total reads:

```
r$> (ps <- phyloseq::subset_samples(ps, phyloseq::sample_sums(ps) > 5000))
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 138 taxa and 84 samples ]
sample_data() Sample Data:       [ 84 samples by 23 sample variables ]
tax_table()   Taxonomy Table:    [ 138 taxa by 7 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 138 tips and 136 internal nodes ]
refseq()      DNAStringSet:      [ 138 reference sequences ]
```

- Remove OTUs (Operational Taxonomic Units) within the remaining samples

```
r$> (ps <- phyloseq::prune_taxa(phyloseq::taxa_sums(ps) > 0, ps))
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 138 taxa and 84 samples ]
sample_data() Sample Data:       [ 84 samples by 23 sample variables ]
tax_table()   Taxonomy Table:    [ 138 taxa by 7 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 138 tips and 136 internal nodes ]
refseq()      DNAStringSet:      [ 138 reference sequences ]
```

*OTUs – Similarly grouped samples not necessarily related via conventional taxonomy. Sometimes develop from sequencing errors.

Phylogenetic Data in a *phyloseq* File

- What's our data look like now after adding some metadata?

```
r$> phyloseq::sample_data(ps)$Status <- ifelse(phyloseq::sample_data(ps)$Subject == "Patient", "Chronic Fatigue", "Control")
    phyloseq::sample_data(ps)$Status <- factor(phyloseq::sample_data(ps)$Status, levels = c("Control", "Chronic Fatigue"))
    ps %>%
        sample_data %>%
        dplyr::count(Status)
```

```
$Status
[1] Control         Chronic Fatigue
Levels: Control Chronic Fatigue

$n
[1] 37 47

attr(,"row.names")
[1] 1 2
attr(,"class")
[1] "sample_data"
attr(,"class")attr(,"package")
[1] "phyloseq"
attr(,".S3Class")
[1] "data.frame"
```

```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 138 taxa and 84 samples ]
sample_data() Sample Data:       [ 84 samples by 23 sample variables ]
tax_table()   Taxonomy Table:    [ 138 taxa by 7 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 138 tips and 136 internal nodes ]
refseq()      DNAStringSet:      [ 138 reference sequences ]
```

- 138 taxa
- 84 samples (37 controls, 47 patients)
  - Control: without chronic fatigue
  - Patient: with chronic fatigue

Visualizing Data

- Create a phylum-level table:

```
r$> #Visualization of the data
    table(phyloseq::tax_table(ps)[, "Phylum"])
    ps_rel_abund = phyloseq::transform_sample_counts(ps, function(x){x / sum(x)})

 Actinobacteria   Bacteroidetes   Cyanobacteria   Euryarchaeota      Firmicutes    Fusobacteria  Proteobacteria     Tenericutes Verrucomicrobia
              7              11               2               1             105               1               7               2               1
```

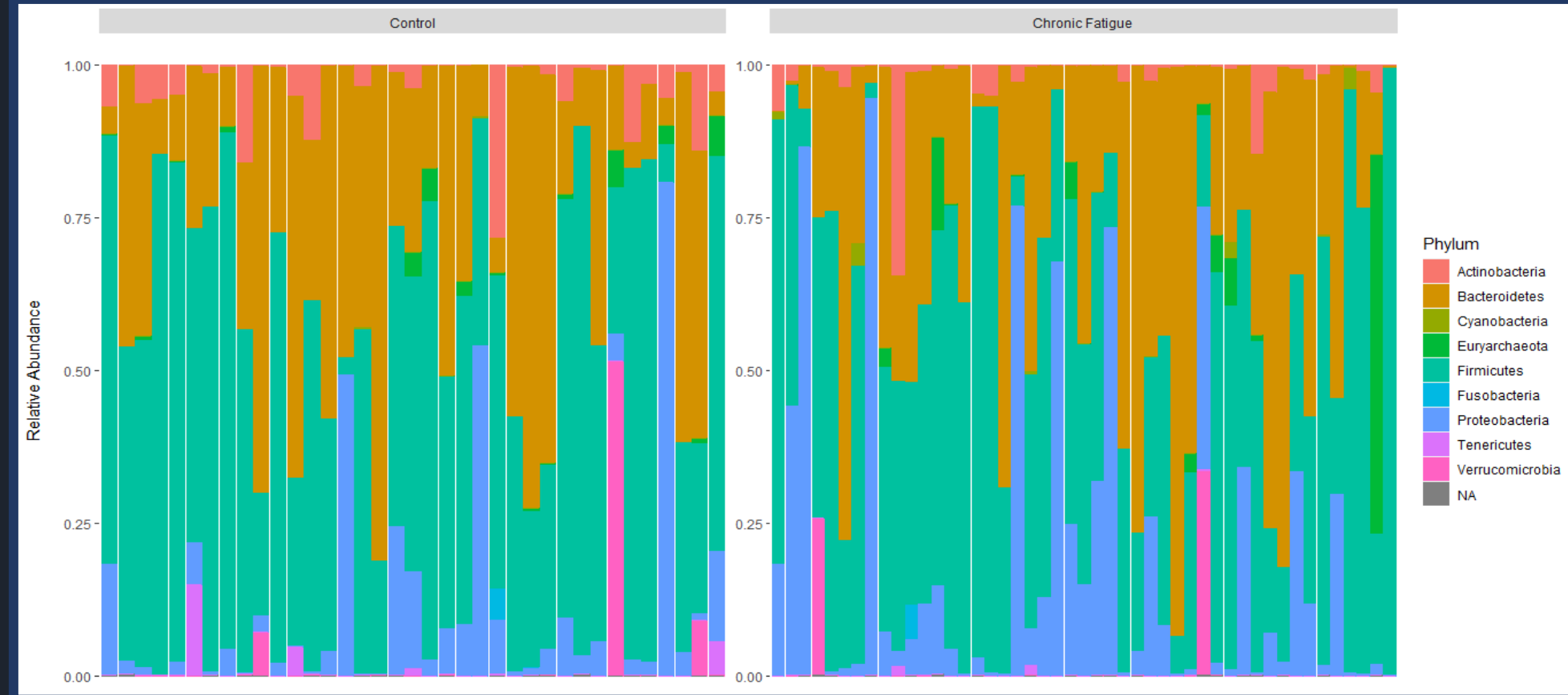
- Then view as relative abundances:

```
r$> phyloseq::otu_table(ps)[1:5, 1:5]
    phyloseq::otu_table(ps_rel_abund)[1:5, 1:5]
OTU Table:          [5 taxa and 5 samples]
                     taxa are rows
     ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851
OTU1          2        581        347        916      10498
OTU2        371         46          0        233        301
OTU3       1189         81        637        199          0
OTU4          0        172        246          0        372
OTU5        308         44        143        155        221
```

```
OTU Table:          [5 taxa and 5 samples]
                     taxa are rows
      ERR1331793  ERR1331872 ERR1331819  ERR1331794 ERR1331851
OTU1 0.0003020236 0.026008326 0.05028986 0.013891206 0.73080404
OTU2 0.0560253700 0.002059179 0.00000000 0.003533462 0.02095371
OTU3 0.1795530051 0.003625946 0.09231884 0.003017849 0.00000000
OTU4 0.0000000000 0.007699539 0.03565217 0.000000000 0.02589628
OTU5 0.0465116279 0.001969649 0.02072464 0.002350586 0.01538462
```

Visualizing Data (Now with Colors)

```
r$> phyloseq::plot_bar(ps_rel_abund, fill = "Phylum") +
      geom_bar(aes(color = Phylum, fill = Phylum), stat = "identity", position = "stack") +
      labs(x = "", y = "Relative Abundance\n") +
      facet_wrap(~ Status, scales = "free") +
      theme(panel.background = element_blank(),
            axis.text.x=element_blank(),
            axis.ticks.x=element_blank())
```



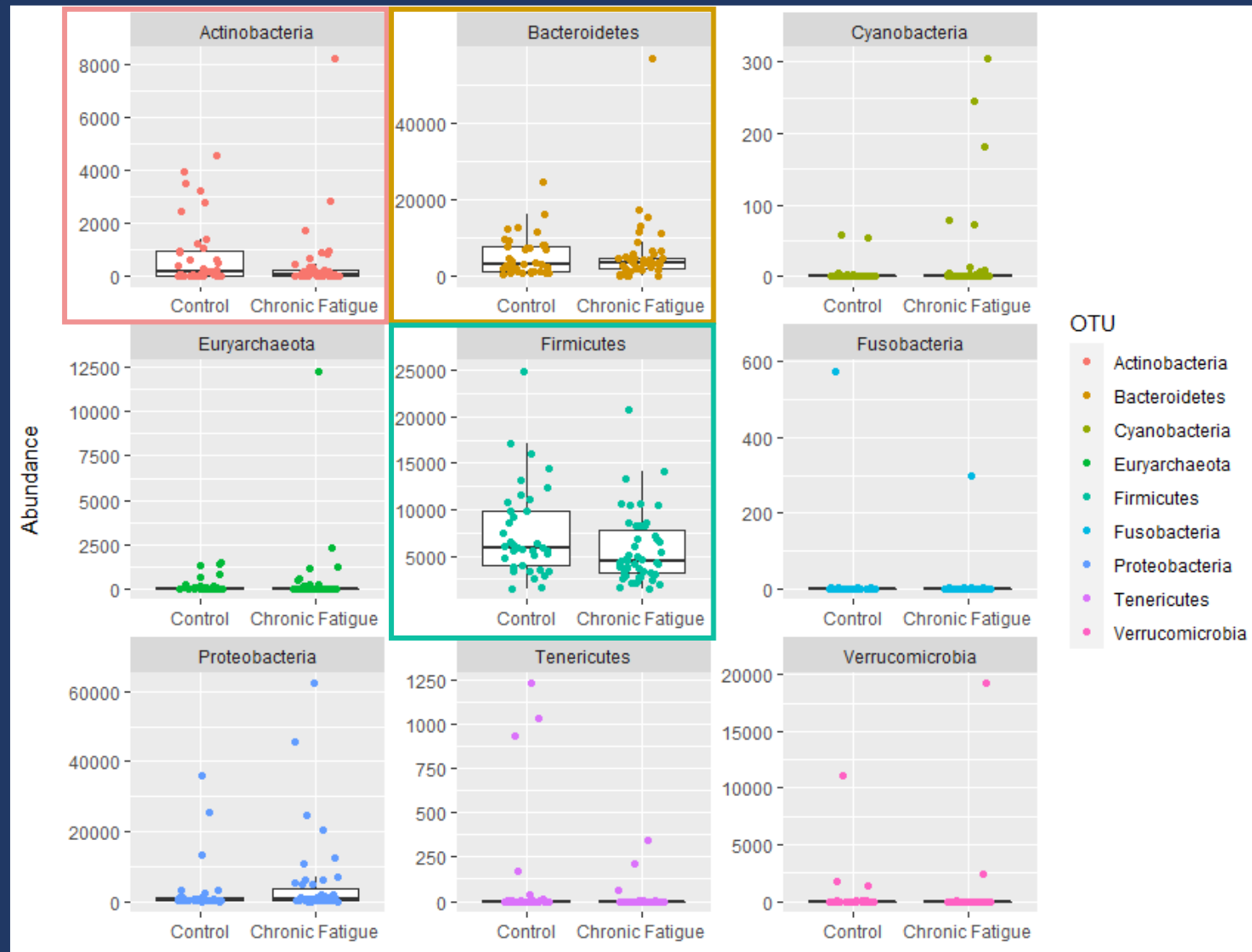
Visualizing Data (Now on the Phylum-level)

- Preparing to compare, phylum-by-phylum

```
r$> ps_phylum <- phyloseq::tax_glom(ps, "Phylum")
    phyloseq::taxa_names(ps_phylum) <- phyloseq::tax_table(ps_phylum)[, "Phylum"]
    phyloseq::otu_table(ps_phylum)[1:5, 1:5]
OTU Table:          [5 taxa and 5 samples]
                     taxa are rows
              ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851
Bacteroidetes       1903        878       1837       1969      11776
Proteobacteria       119       3315        468      62358        319
Firmicutes          4319      14429       3548       1609       2207
Actinobacteria        30        976         17          0         58
Cyanobacteria        246          0          0          0          0
```

Visualizing Data (Phylum-to-Phylum)

```
r$> phyloseq::psmelt(ps_phylum) %>%
    ggplot(data = ., aes(x = Status, y = Abundance)) +
      geom_boxplot(outlier.shape  = NA) +
      geom_jitter(aes(color = OTU), height = 0, width = .2) +
      labs(x = "", y = "Abundance\n") +
      facet_wrap(~ OTU, scales = "free")
```



- Notice the high abundances:
  - Firmicutes
  - Bacteroidetes
  - Actinobacteria

Testing for Abundance Differences (Hypothesis Testing)

- Assuming a Dirichlet-Multinomial distribution, we essentially 't-test' between all phyla (or really any taxonomic level)

```
r$> #Subset groups
    controls <- phyloseq::subset_samples(ps_phylum, Status == "Control")
    cf <- phyloseq::subset_samples(ps_phylum, Status == "Chronic Fatigue")
    #Output OTU tables
    control_otu <- data.frame(phyloseq::otu_table(controls))
    cf_otu <- data.frame(phyloseq::otu_table(cf))
    #Group rare phyla
    control_otu <- control_otu %>%
      t(.) %>%
      as.data.frame(.) %>%
      mutate(Other = Cyanobacteria + Euryarchaeota + Tenericutes + Verrucomicrobia + Fusobacteria) %>%
      dplyr::select(-Cyanobacteria, -Euryarchaeota, -Tenericutes, -Verrucomicrobia, -Fusobacteria)
    cf_otu <- cf_otu %>%
      t(.) %>%
      as.data.frame(.) %>%
      mutate(Other = Cyanobacteria + Euryarchaeota + Tenericutes + Verrucomicrobia + Fusobacteria) %>%
      dplyr::select(-Cyanobacteria, -Euryarchaeota, -Tenericutes, -Verrucomicrobia, -Fusobacteria)
    ##HMP test
    group_data <- list(control_otu, cf_otu)
    (xdc <- HMP::Xdc.sevsample(group_data))
$`Xdc statistics`
[1] 0.2769004

$`p value`
[1] 0.9980551
```


```
$`Xdc statistics`
[1] 0.2769004

$`p value`
[1] 0.9980551
```


- P-value is too high to reject the null hypothesis.

Clustering to Determine Similarity (Bray-Curtis Method)

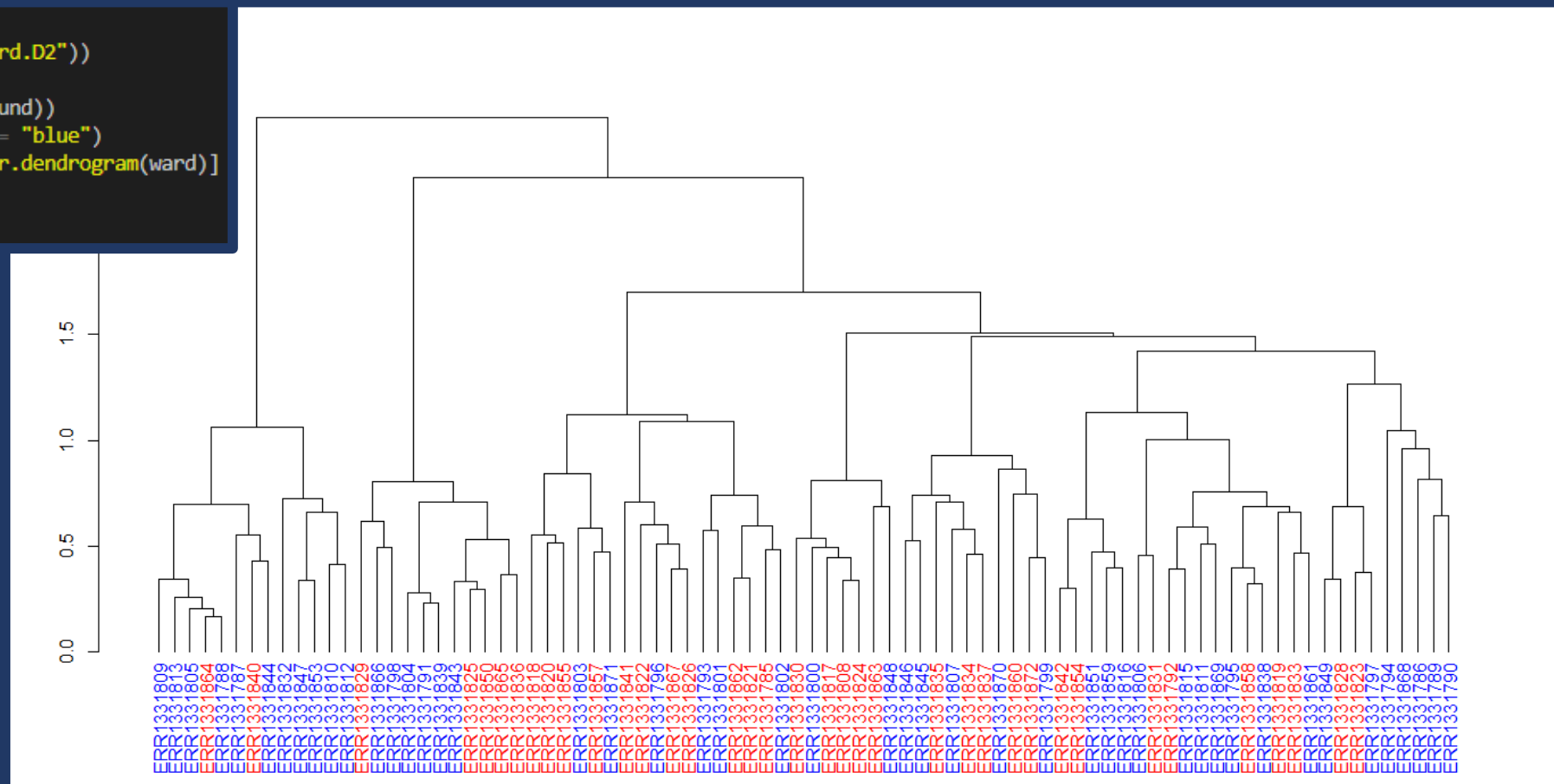
- Bray-Curtis Dissimilarity: 0 for max similarity; 1 for no shared taxa (no similarity)

```
r$> ps_rel_otu <- data.frame(phyloseq::otu_table(ps_rel_abund))
    ps_rel_otu <- t(ps_rel_otu)
    bc_dist <- vegan::vegdist(ps_rel_otu, method = "bray")
    as.matrix(bc_dist)[1:5, 1:5]
            ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851
ERR1331793  0.0000000  0.8801040  0.5975550  0.9767218  0.8684629
ERR1331872  0.8801040  0.0000000  0.7590766  0.9596181  0.9206484
ERR1331819  0.5975550  0.7590766  0.0000000  0.9556656  0.7810736
ERR1331794  0.9767218  0.9596181  0.9556656  0.0000000  0.9693291
ERR1331851  0.8684629  0.9206484  0.7810736  0.9693291  0.0000000
```

Clustering to Determine Similarity (Visualized as a Dendrogram)

- Using the distances to make a phylo-tree

```
r$> #Save as dendrogram
    ward <- as.dendrogram(hclust(bc_dist, method = "ward.D2"))
    #Provide color codes
    meta <- data.frame(phyloseq::sample_data(ps_rel_abund))
    colorCode <- c(Control = "red", `Chronic Fatigue` = "blue")
    labels_colors(ward) <- colorCode[meta$Status][order.dendrogram(ward)]
    #Plot
    plot(ward)
```

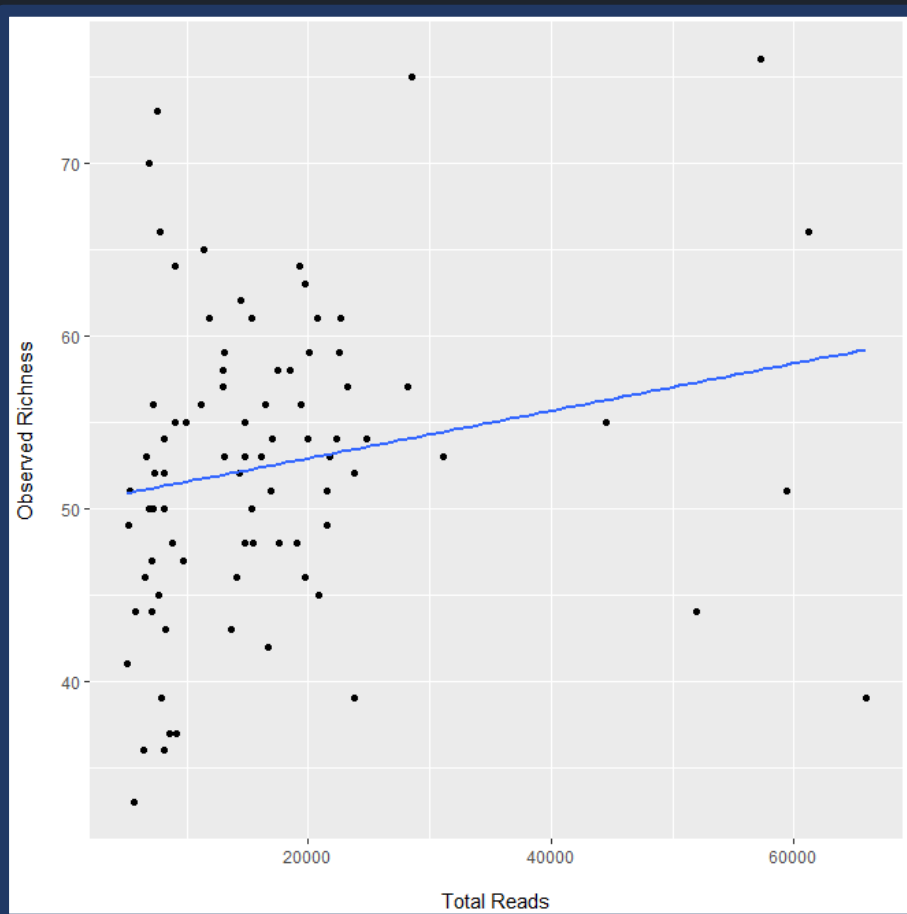


- Chronic Fatigue [Blue]
- Control Group [Red]

Alpha-diversity: Local Sample Diversity

- Testing for differences using such things as richness, Shannon diversity, and phylogenetic diversity

```
r$> #Alpha-Diversity
    ggplot(data = data.frame("total_reads" =  phyloseq::sample_sums(ps),
                             "observed" = phyloseq::estimate_richness(ps, measures = "Observed")[, 1]),
           aes(x = total_reads, y = observed)) +
      geom_point() +
      geom_smooth(method="lm", se = FALSE) +
      labs(x = "\nTotal Reads", y = "Observed Richness\n")
```



Alpha-diversity: Comparing Metrics (Table Forme)

- Split off into subsamples and cross-compare

```
r$> #Subsample reads
    (ps_rare <- phyloseq::rarefy_even_depth(ps, rngseed = 123, replace = FALSE))
    head(phyloseq::sample_sums(ps_rare))
    adiv <- data.frame(
      "Observed" = phyloseq::estimate_richness(ps_rare, measures = "Observed"),
      "Shannon" = phyloseq::estimate_richness(ps_rare, measures = "Shannon"),
      #Add include.root=FALSE
      "PD" = picante::pd(samp = data.frame(t(data.frame(phyloseq::otu_table(ps_rare)))), tree = phyloseq::phy_tree(ps_rare), include.root=FALSE)[, 1],
      "Status" = phyloseq::sample_data(ps_rare)$Status)
    head(adiv)
```

```
phyloseq-class experiment-level object
otu_table()   OTU Table:         [ 138 taxa and 84 samples ]
sample_data() Sample Data:       [ 84 samples by 23 sample variables ]
tax_table()   Taxonomy Table:    [ 138 taxa by 7 taxonomic ranks ]
phy_tree()    Phylogenetic Tree: [ 138 tips and 136 internal nodes ]
refseq()      DNAStringSet:      [ 138 reference sequences ]
ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851 ERR1331834
      5083       5083       5083       5083       5083       5083
```

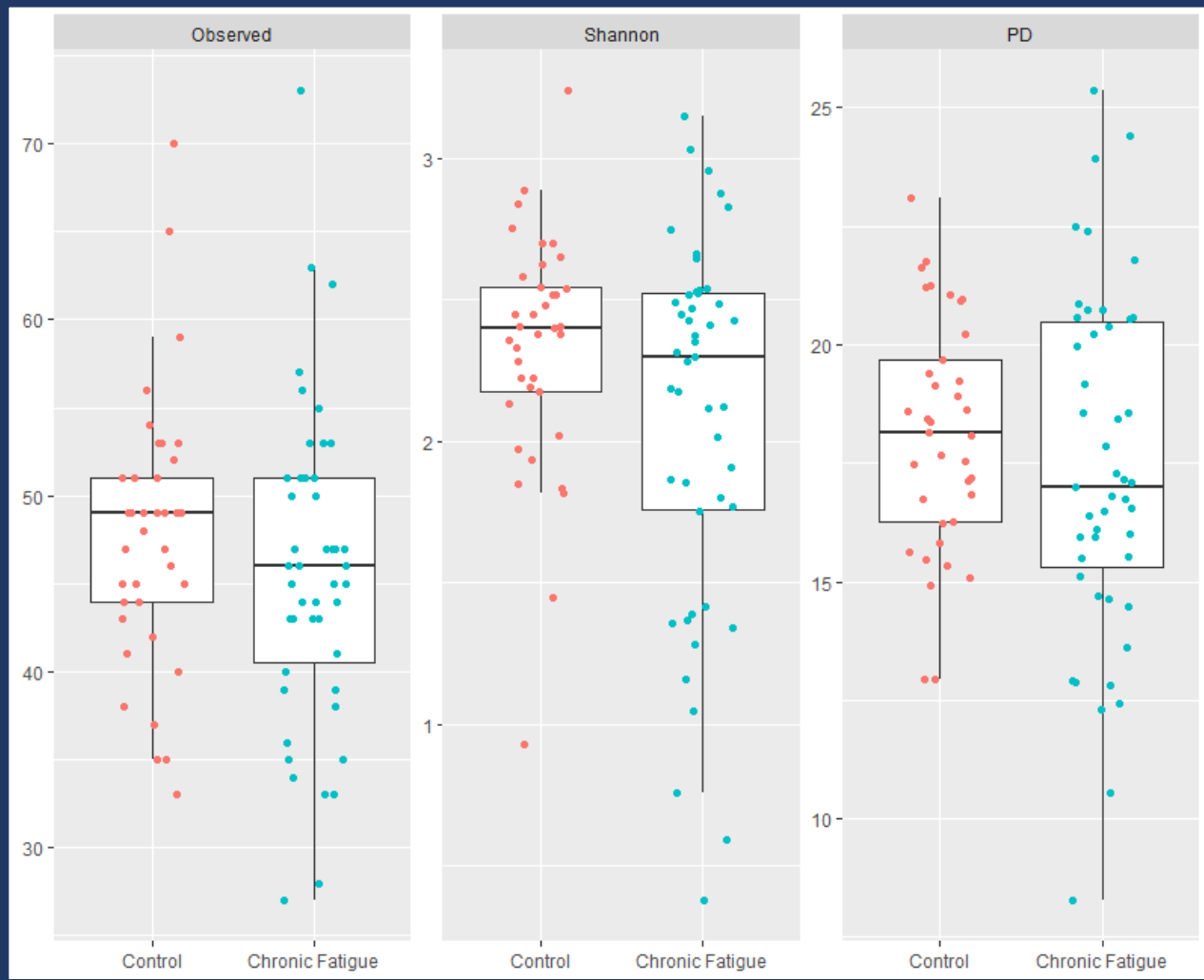
	Observed	Shannon	PD	Status
ERR1331793	53	2.7462377	20.566952	Chronic Fatigue
ERR1331872	52	2.7527053	21.258691	Control
ERR1331819	70	3.2378006	21.640313	Control
ERR1331794	27	0.3761523	8.275154	Chronic Fatigue
ERR1331851	45	1.3387308	14.649204	Chronic Fatigue
ERR1331834	54	2.8883445	20.957612	Control

Alpha-diversity: Comparing Metrics (Plot Forme)

- Lower alpha-diversity for chronic patients

```
r$> #Plot adiv measures
    adiv %>%
      gather(key = metric, value = value, c("Observed", "Shannon", "PD")) %>%
      mutate(metric = factor(metric, levels = c("Observed", "Shannon", "PD"))) %>%
      ggplot(aes(x = Status, y = value)) +
      geom_boxplot(outlier.color = NA) +
      geom_jitter(aes(color = Status), height = 0, width = .2) +
      labs(x = "", y = "") +
      facet_wrap(~ metric, scales = "free") +
      theme(legend.position="none")
```

```
r$> #Summarize
    adiv %>%
      group_by(Status) %>%
      dplyr::summarise(median_observed = median(Observed),
                median_shannon = median(Shannon),
                median_pd = median(PD))
# A tibble: 2 x 4
  Status          median_observed median_shannon median_pd
  <fct>                     <dbl>          <dbl>     <dbl>
1 Control                      49           2.40      18.1
2 Chronic Fatigue              46           2.30      17.0
```



Alpha-diversity: Comparing Metrics (Wilcoxon Rank Sum Tests)

```
r$> wilcox.test(Shannon ~ Status, data = adiv, conf.int = TRUE)

        Wilcoxon rank sum exact test

data:  Shannon by Status
W = 1037, p-value = 0.1329
alternative hypothesis: true location shift is not equal to 0
95 percent confidence interval:
 -0.04346366  0.39218192
sample estimates:
difference in location
             0.1421467
```

Fail to Reject

Shannon Entropy

```
r$> wilcox.test(PD ~ Status, data = adiv, conf.int = TRUE)

        Wilcoxon rank sum exact test

data:  PD by Status
W = 998, p-value = 0.2503
alternative hypothesis: true location shift is not equal to 0
95 percent confidence interval:
 -0.7340479  2.1737379
sample estimates:
difference in location
             0.7063854
```

Fail to Reject

PD

```
r$> wilcox.test(Observed ~ Status, data = adiv, exact = FALSE, conf.int = TRUE)

        Wilcoxon rank sum test with continuity correction

data:  Observed by Status
W = 1007.5, p-value = 0.2146
alternative hypothesis: true location shift is not equal to 0
95 percent confidence interval:
 -1.000059  5.000002
sample estimates:
difference in location
             2.000087
```

Fail to Reject

Observed

Richness using the *breakaway* Package

- Form breakaway estimates:

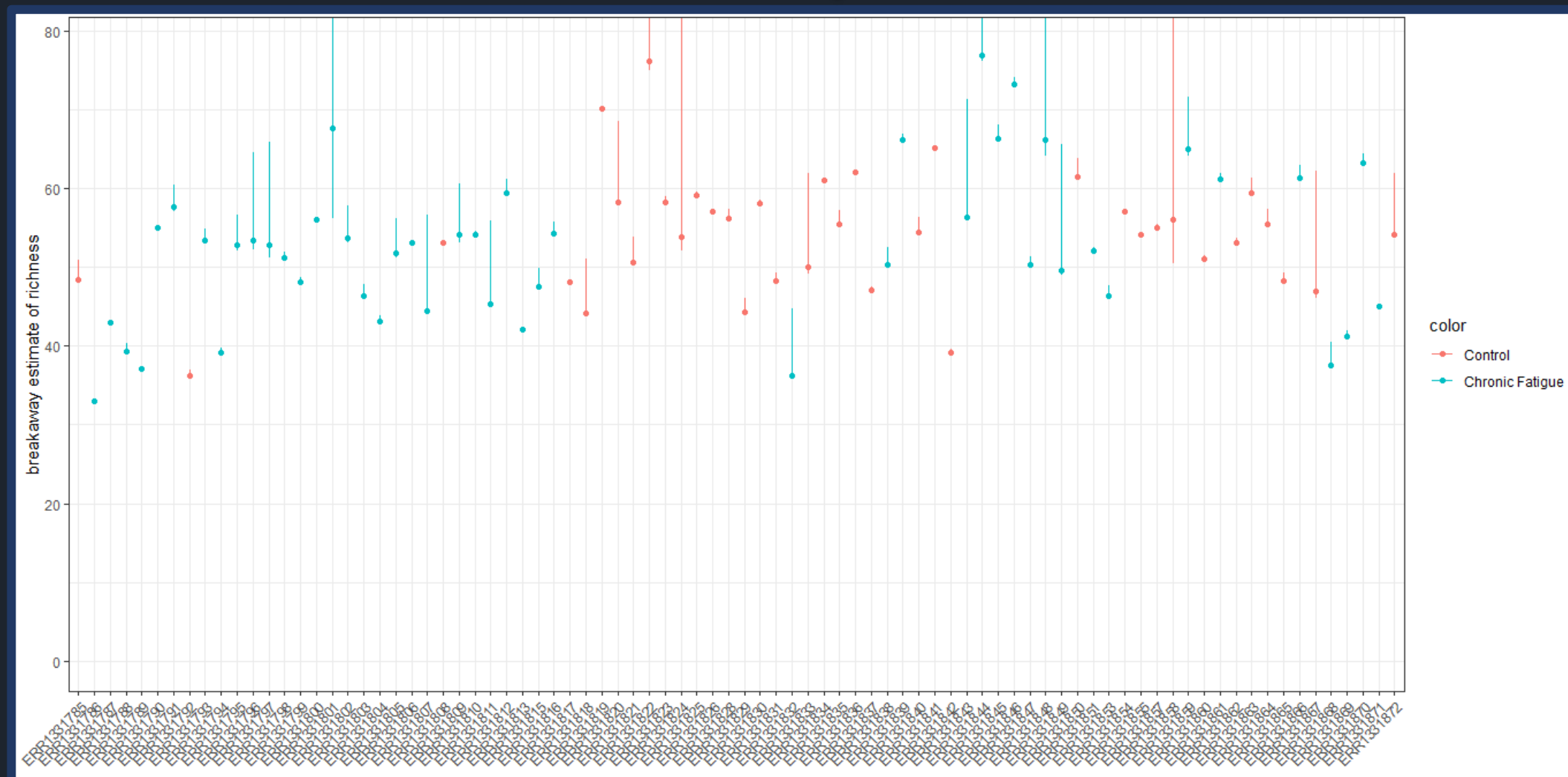
```
r$> ba_adiv <- breakaway::breakaway(ps)
    ba_adiv[1]
    #Plot estimates
$ERR1331793
Estimate of richness from method breakaway:
  Estimate is 53
 with standard error 0.6
  Confidence interval: (53, 55)
  Cutoff:  10
```

- Summary of Estimates:

```
r$> summary(ba_adiv) %>%
      add_column("SampleNames" = ps %>% otu_table %>% sample_names)
    #Test for group differnce
    bt <- breakaway::betta(summary(ba_adiv)$estimate,
                           summary(ba_adiv)$error,
                           make_design_matrix(ps, "Status"))
    bt$table
# A tibble: 84 x 8
   estimate error lower upper sample_names name      model            SampleNames
      <dbl> <dbl> <dbl> <dbl> <chr>        <chr>     <chr>            <chr>
 1     53.3 0.602  53.1  54.8 ERR1331793   breakaway Poisson          ERR1331793
 2     54.1 3.10   54.0  61.9 ERR1331872   breakaway Negative Binomial ERR1331872
 3     70.1 0.296  70.0  70.4 ERR1331819   breakaway Kemp             ERR1331819
 4     39.1 0.381  39.0  39.7 ERR1331794   breakaway Poisson          ERR1331794
 5     52.1 0.326  52.0  52.5 ERR1331851   breakaway Kemp             ERR1331851
 6     61.1 0.280  61.0  61.4 ERR1331834   breakaway Kemp             ERR1331834
 7     54.1 0.346  54.0  54.6 ERR1331810   breakaway Kemp             ERR1331810
 8     48.1 0.243  48.0  48.3 ERR1331817   breakaway Kemp             ERR1331817
 9     56.0 5.17   50.5 127.  ERR1331858   breakaway Kemp             ERR1331858
10     50.0 2.14   49.1  61.9 ERR1331833   breakaway Kemp             ERR1331833
# ... with 74 more rows
                          Estimates Standard Errors p-values
(Intercept)                54.088528       0.9817616    0.000
predictorsChronic Fatigue -2.186958       1.3085094    0.095
```

Richness using the *breakaway* Package (Plots)

```
r$> #Plot estimates
    plot(ba_adiv, ps, color = "Status")
```



Beta-diversity: Regional to Local Diversity Ratios

- Create the CLRs (centered log-ratios)

```
(ps_clr <- microbiome::transform(ps, "clr"))
```

- Untransformed values (counts):

```
r$> phyloseq::otu_table(ps)[1:5, 1:5]
OTU Table:          [5 taxa and 5 samples]
                     taxa are rows
     ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851
OTU1          2        581        347        916      10498
OTU2        371         46          0        233        301
OTU3       1189         81        637        199          0
OTU4          0        172        246          0        372
OTU5        308         44        143        155        221
```

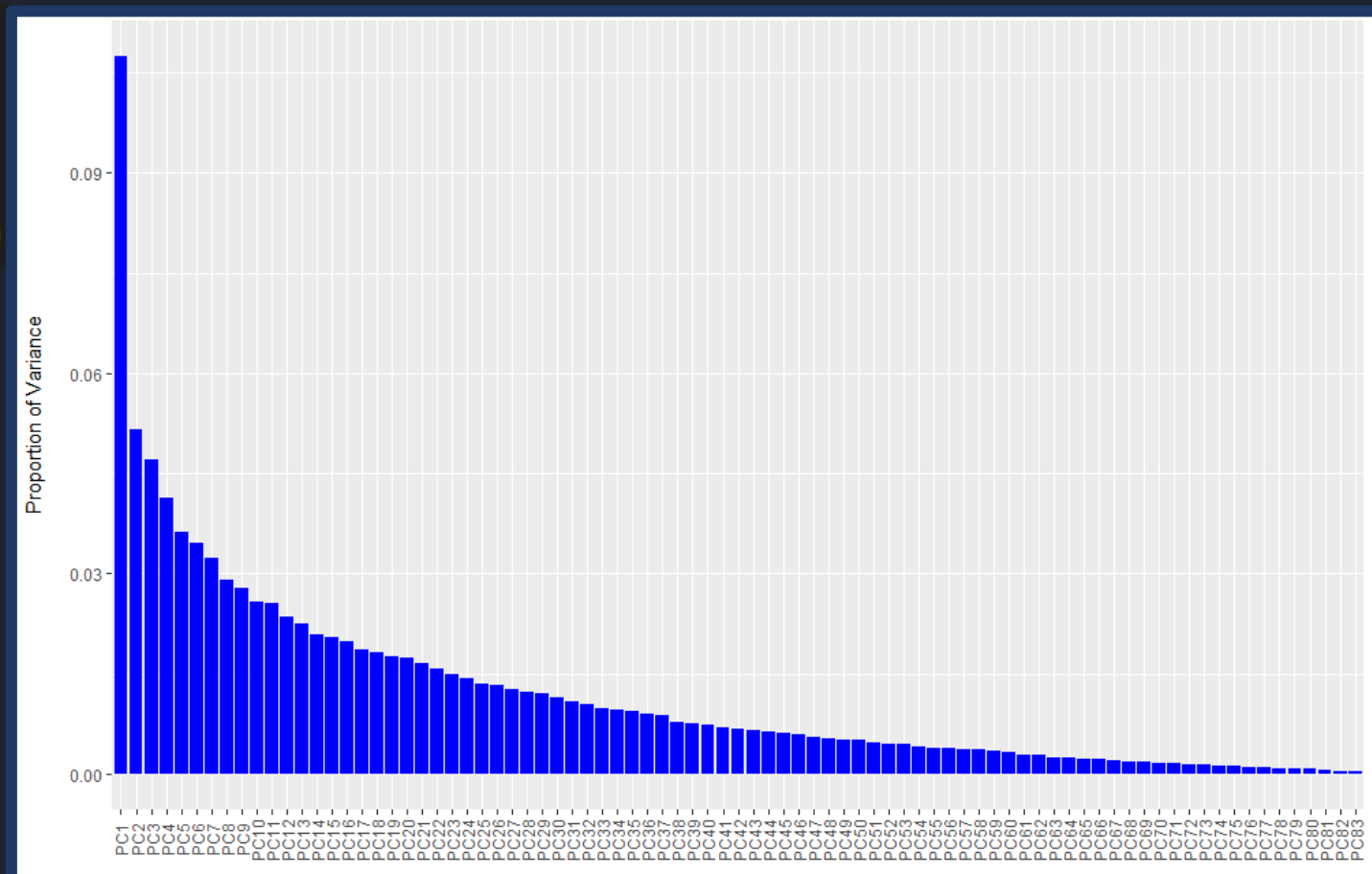
- Transformed values (log dominance):

```
r$> phyloseq::otu_table(ps_clr)[1:5, 1:5]
OTU Table:          [5 taxa and 5 samples]
                     taxa are rows
     ERR1331793 ERR1331872 ERR1331819 ERR1331794 ERR1331851
OTU1   1.289544   5.812706   5.615063   6.230204   9.467837
OTU2   6.485240   3.280355  -3.079591   4.863001   5.916398
OTU3   7.649802   3.844401   6.222432   4.705673  -1.903003
OTU4  -2.317399   4.596219   5.271139  -1.178342   6.128105
OTU5   6.299168   3.236089   4.728822   4.456584   5.607596
```

Beta-diversity: Regional to Local Diversity Ratios (continued)

- Apply PCA (Principal Component Analysis)

```
r$> #PCA via phyloseq
    ord_clr <- phyloseq::ordinate(ps_clr, "RDA")
    #Plot scree plot
    phyloseq::plot_scree(ord_clr) +
      geom_bar(stat="identity", fill = "blue") +
      labs(x = "\nAxis", y = "Proportion of Variance\n")
```



Beta-diversity: Eigenvalues and Principal Components

- Eigenvalues

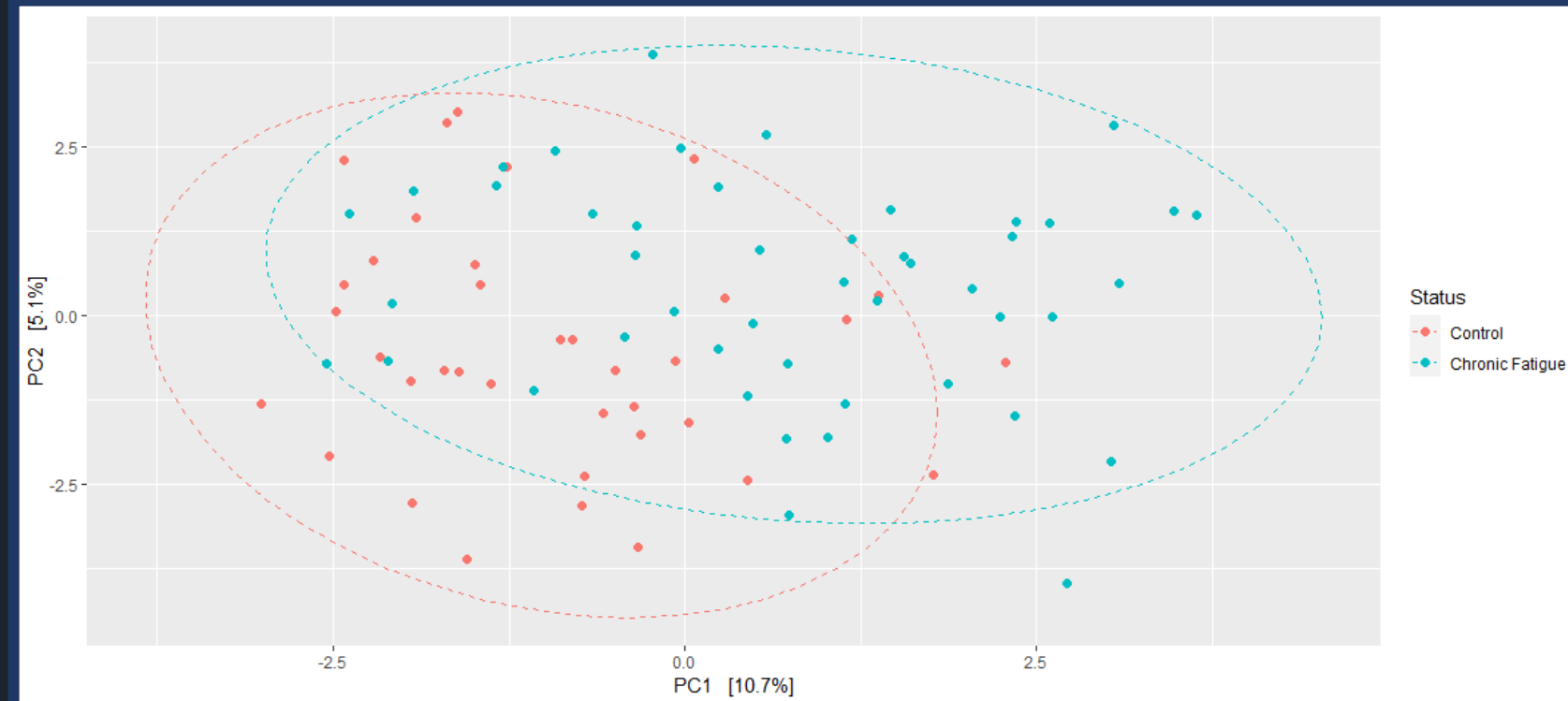
```
r$> head(ord_clr$CA$eig)
     PC1      PC2      PC3      PC4      PC5      PC6
75.69204 36.27003 33.16649 29.08833 25.52986 24.32215
```

- Proportion of Variance explained by Principal Component

```
r$> sapply(ord_clr$CA$eig[1:5], function(x) x / sum(ord_clr$CA$eig))
       PC1        PC2        PC3        PC4        PC5
0.10744095 0.05148344 0.04707812 0.04128939 0.03623832
```

Beta-diversity: The First Two Principal Components

```
r$> #Scale axes and plot ordination
    clr1 <- ord_clr$CA$eig[1] / sum(ord_clr$CA$eig)
    clr2 <- ord_clr$CA$eig[2] / sum(ord_clr$CA$eig)
    phyloseq::plot_ordination(ps, ord_clr, type="samples", color="Status") +
      geom_point(size = 2) +
      coord_fixed(clr2 / clr1) +
      stat_ellipse(aes(group = Status), linetype = 2)
```



- Notice the slight separation between the Control and the Fatigued

Beta-diversity: PERMANOVA and *adonis*

```
r$> #Generate distance matrix
    clr_dist_matrix <- phyloseq::distance(ps_clr, method = "euclidean")
    #ADONIS test
    vegan::adonis(clr_dist_matrix ~ phyloseq::sample_data(ps_clr)$Status)

Call:
vegan::adonis(formula = clr_dist_matrix ~ phyloseq::sample_data(ps_clr)$Status)

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

                                  Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
phyloseq::sample_data(ps_clr)$Status  1      2240 2240.17  3.2666 0.03831  0.001 ***
Residuals                        82     56233  685.77         0.96169
Total                            83     58473                 1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

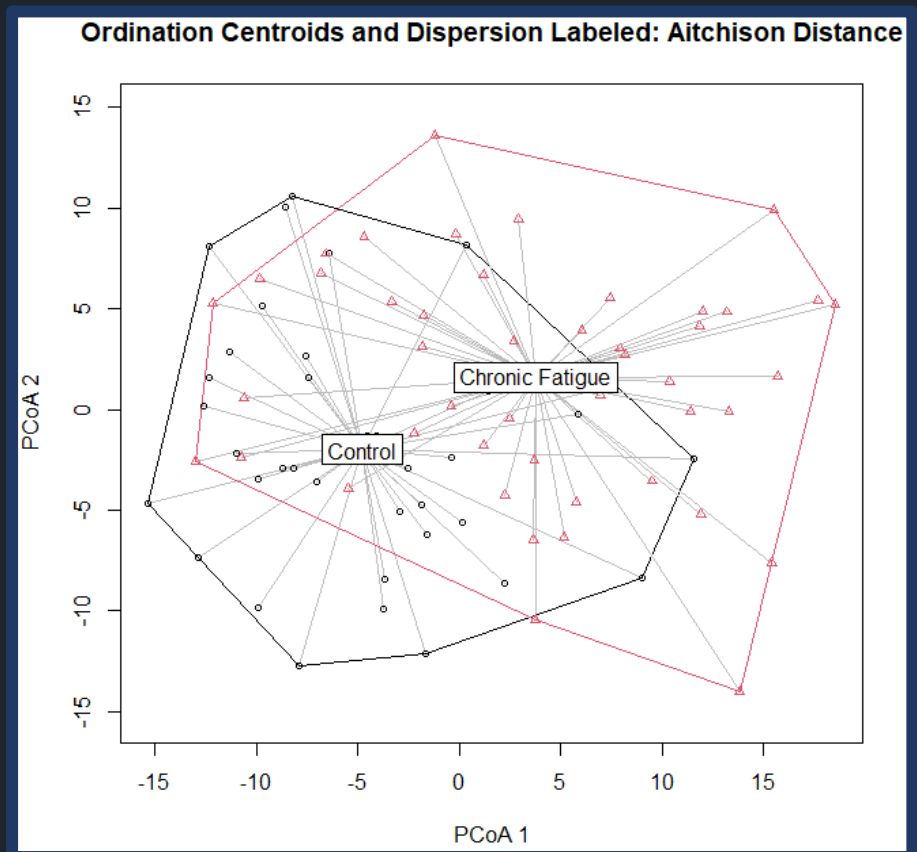
Beta-diversity: PERMANOVA and *adonis* (Plot and Dispersion Test)

- Dispersion Test

```
r$> #Dispersion test and plot
    dispr <- vegan::betadisper(clr_dist_matrix, phyloseq::sample_data(ps_clr)$Status)
    dispr

        Homogeneity of multivariate dispersions

Call: vegan::betadisper(d = clr_dist_matrix, group = phyloseq::sample_data(ps_clr)$Status)

No. of Positive Eigenvalues: 83
No. of Negative Eigenvalues: 0

Average distance to median:
        Control Chronic Fatigue
           25.1            26.2

Eigenvalues for PCoA axes:
(Showing 8 of 83 eigenvalues)
PCoA1 PCoA2 PCoA3 PCoA4 PCoA5 PCoA6 PCoA7 PCoA8
 6282  3010  2753  2414  2119  2019  1895  1693
```

- PCoA Plotted

```
r$> plot(dispr, main = "Ordination Centroids and Dispersion Labeled: Aitchison Distance", sub = "")
```



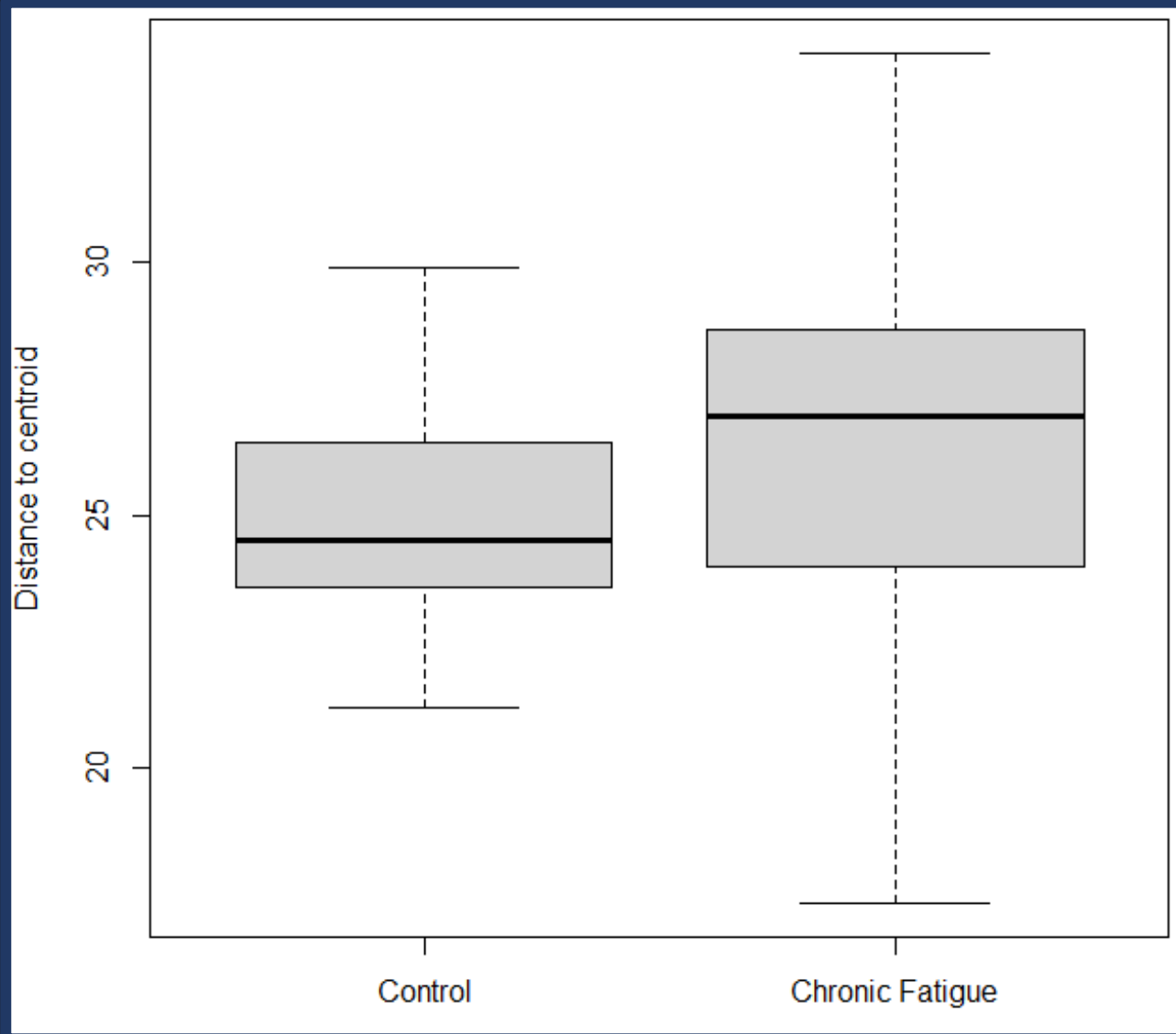
Beta-diversity: PERMANOVA and *adonis* (Box and Permutation)

- PCoA Plot as Box

```
r$> boxplot(dispr, main = "", xlab = "")
```

- Permutation Test

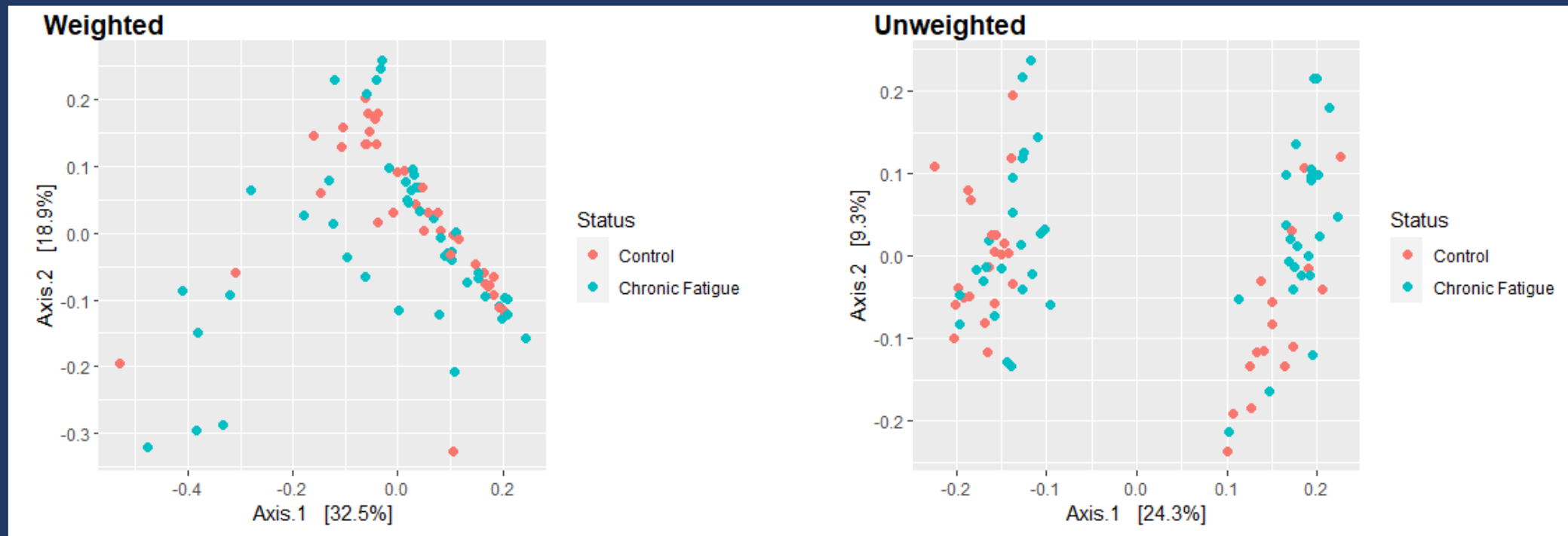
```
r$> permutest(dispr)

Permutation test for homogeneity of multivariate dispersions
Permutation: free
Number of permutations: 999

Response: Distances
          Df Sum Sq Mean Sq      F N.Perm Pr(>F)
Groups     1  24.95 24.9463 3.0491    999  0.077 .
Residuals 82 670.89  8.1816
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```



Principal Coordinate Analysis and the UniFrac Distance

- Using PCoA to computer similarity as the UniFrac distance

```
r$> #Generate distances
    ord_unifrac <- ordinate(ps_rare, method = "PCoA", distance = "wunifrac")
    ord_unifrac_un <- ordinate(ps_rare, method = "PCoA", distance = "unifrac")
    #Plot ordinations
    a <- plot_ordination(ps_rare, ord_unifrac, color = "Status") + geom_point(size = 2)
    cowplot::plot_grid(a, b, nrow = 1, ncol = 2, scale = .9, labels = c("Weighted", "Unweighted"))
```



Differential Abundance Testing

- Identifying taxa that respond the greatest to changes in some condition
  - Statistically difficult due to sampling issues
- Two Main Approaches:
  - Non-parametric Wilcoxon rank-sum test
  - Modified Wilcoxon test for NGS data
    - NGS (Next Generation Sequencing)

```
r$> ps_wilcox <- data.frame(t(data.frame(phyloseq::otu_table(ps_clr))))
    ps_wilcox$Status <- phyloseq::sample_data(ps_clr)$Status

r$> wilcox_model <- function(df){
      wilcox.test(abund ~ Status, data = df)
    }
    wilcox_pval <- function(df){
      wilcox.test(abund ~ Status, data = df)$p.value
    }

r$> wilcox_results <- ps_wilcox %>%
      gather(key = OTU, value = abund, -Status) %>%
      group_by(OTU) %>%
      nest() %>%
      mutate(wilcox_test = map(data, wilcox_model),
             p_value = map(data, wilcox_pval))
```

Differential Abundance Testing (Unmodified Wilcoxon)

- Test results including:
  - Abundance fields
  - Status fields
  - P-Values

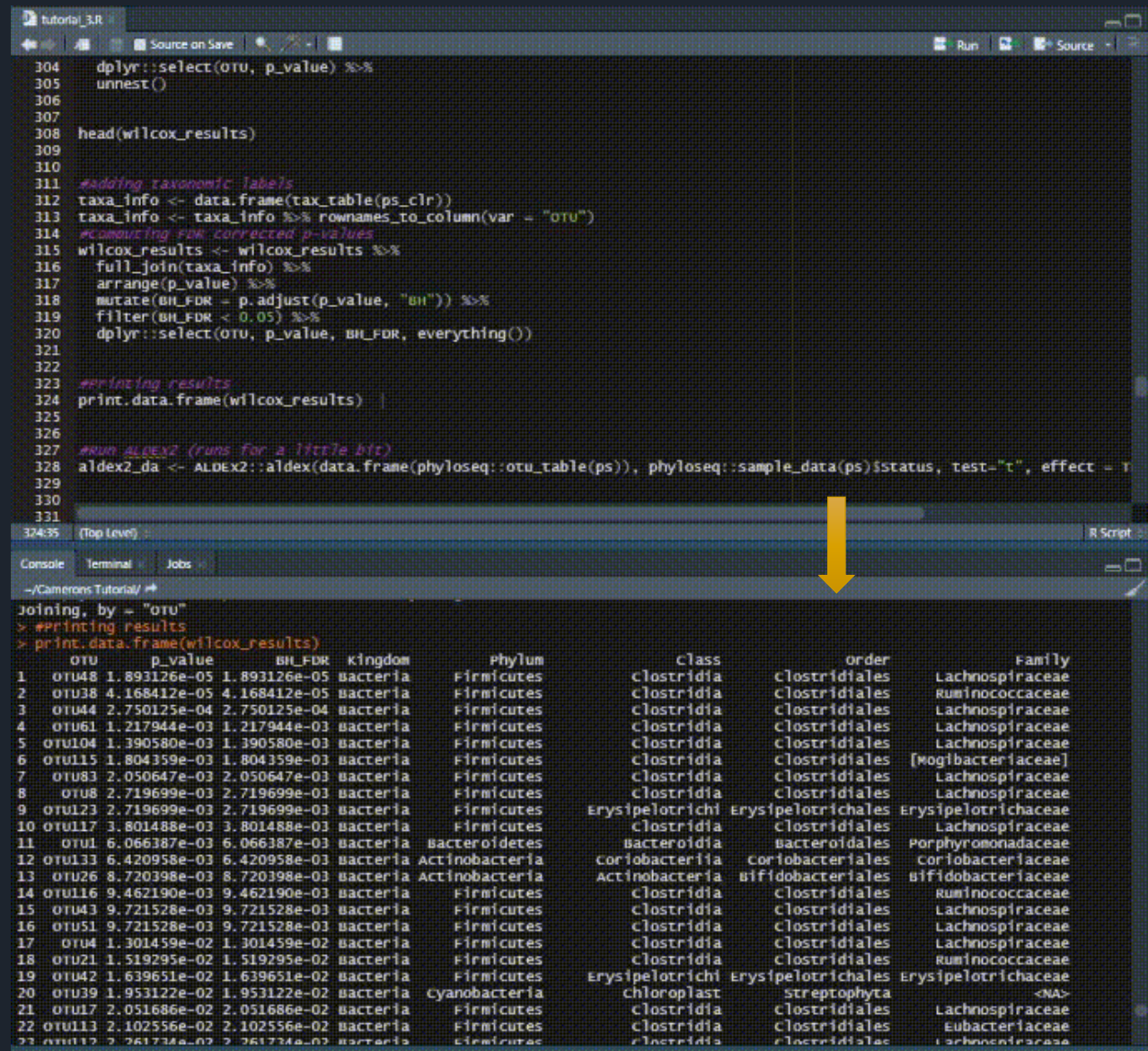
```
> head(wilcox_results)
# A tibble: 6 x 2
# Groups:   OTU [6]
  OTU   p_value
  <chr>   <dbl>
1 OTU1  0.00607
2 OTU2  0.0686
3 OTU3  0.830
4 OTU4  0.0130
5 OTU5  0.419
6 OTU6  0.258
```

```
> #Show results
> head(wilcox_results)
# A tibble: 6 x 4
# Groups:   OTU [6]
  OTU   data                    wilcox_test p_value
  <chr> <list>                  <list>      <list>
1 OTU1  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
2 OTU2  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
3 OTU3  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
4 OTU4  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
5 OTU5  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
6 OTU6  <tibble[,2] [84 x 2]> <htest>     <dbl [1]>
> head(wilcox_results$data[[1]])
# A tibble: 6 x 2
  Status           abund
  <fct>            <dbl>
1 Chronic Fatigue   1.29
2 Control           5.81
3 Control           5.62
4 Chronic Fatigue   6.23
5 Chronic Fatigue   9.47
6 Control           7.43
> wilcox_results$wilcox_test[[1]]

        Wilcoxon rank sum exact test

data:  abund by Status
W = 1172, p-value = 0.006066
alternative hypothesis: true location shift is not equal to 0

> wilcox_results$p_value[[1]]
[1] 0.006066387
```

Differential Abundance Testing (Modified Wilcoxon)

- Unpack all test values and make some more transforms

- Modified test shows many Clostridiales organisms as being 'differentially abundant'
  - In the 'Order' column of the output



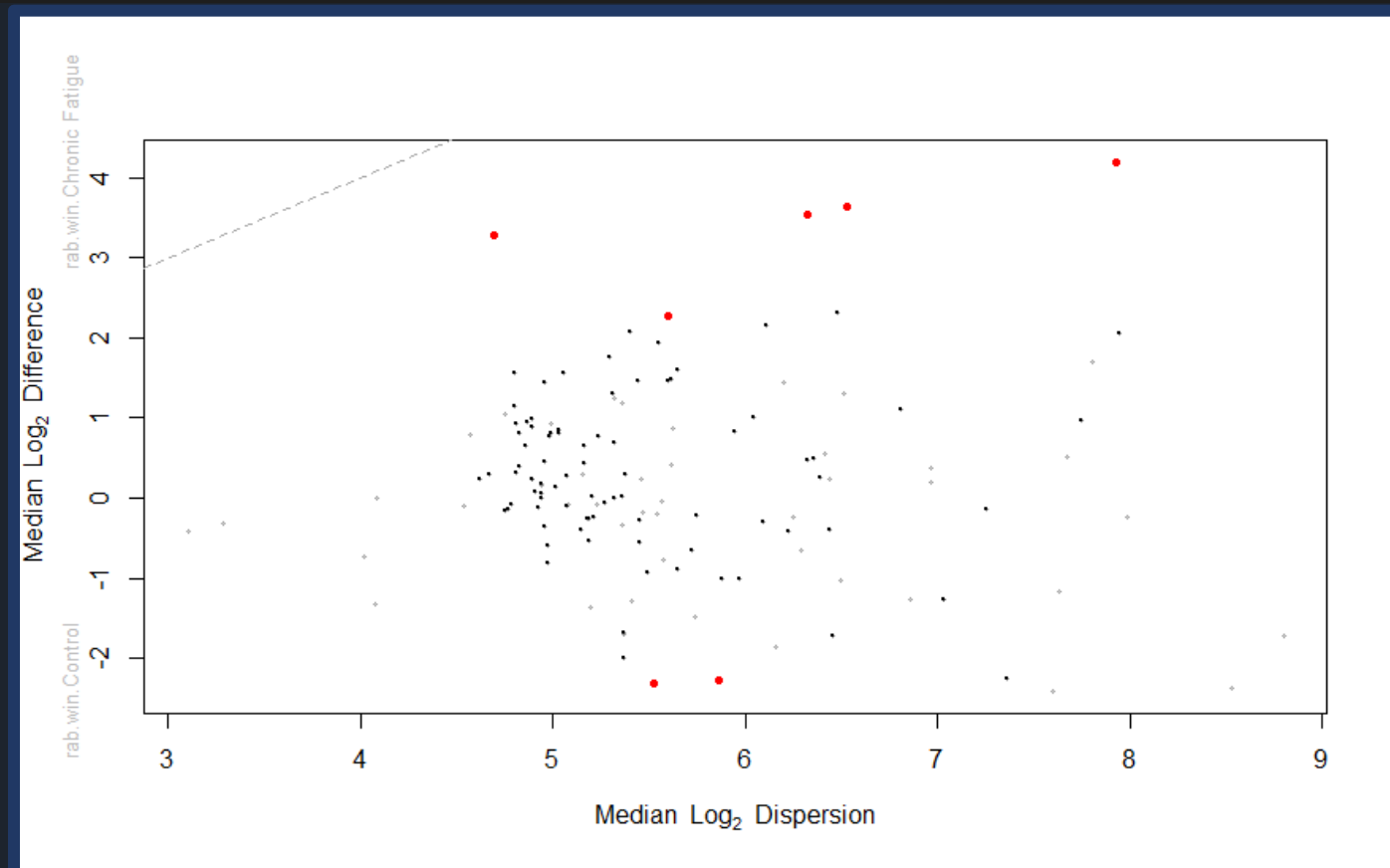
```
  dplyr::select(OTU, p_value) %>%
  unnest()


head(wilcox_results)


#adding taxonomic labels
taxa_info <- data.frame(tax_table(ps_clr))
taxa_info <- taxa_info %>% rownames_to_column(var = "OTU")
#computing FDR corrected p-values
wilcox_results <- wilcox_results %>%
  full_join(taxa_info) %>%
  arrange(p_value) %>%
  mutate(BH_FDR = p.adjust(p_value, "BH")) %>%
  filter(BH_FDR < 0.05) %>%
  dplyr::select(OTU, p_value, BH_FDR, everything())


#printing results
print.data.frame(wilcox_results)


#Run ALDEx2 (runs for a little bit)
aldex2_da <- ALDEx2::aldex(data.frame(phyloseq::otu_table(ps)), phyloseq::sample_data(ps)$status, test="t", effect = T
```

```
Joining, by = "OTU"
> #printing results
> print.data.frame(wilcox_results)
     OTU      p_value       BH_FDR  Kingdom        Phylum          Class          Order           Family
1   OTU48 1.893126e-05 1.893126e-05 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
2   OTU38 4.168412e-05 4.168412e-05 Bacteria    Firmicutes      Clostridia     Clostridiales    Ruminococcaceae
3   OTU44 2.750125e-04 2.750125e-04 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
4   OTU61 1.217944e-03 1.217944e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
5  OTU104 1.390580e-03 1.390580e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
6  OTU115 1.804359e-03 1.804359e-03 Bacteria    Firmicutes      Clostridia     Clostridiales [Mogibacteriaceae]
7   OTU83 2.050647e-03 2.050647e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
8    OTU8 2.719699e-03 2.719699e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
9  OTU123 2.719699e-03 2.719699e-03 Bacteria    Firmicutes Erysipelotrichi Erysipelotrichales Erysipelotrichaceae
10 OTU117 3.801488e-03 3.801488e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
11   OTU1 6.066387e-03 6.066387e-03 Bacteria Bacteroidetes     Bacteroidia     Bacteroidales  Porphyromonadaceae
12 OTU133 6.420958e-03 6.420958e-03 Bacteria Actinobacteria  Coriobacteriia  Coriobacteriales  Coriobacteriaceae
13  OTU26 8.720398e-03 8.720398e-03 Bacteria Actinobacteria  Actinobacteria  Bifidobacteriales  Bifidobacteriaceae
14 OTU116 9.462190e-03 9.462190e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Ruminococcaceae
15  OTU43 9.721528e-03 9.721528e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
16  OTU51 9.721528e-03 9.721528e-03 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
17   OTU4 1.301459e-02 1.301459e-02 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
18  OTU21 1.519295e-02 1.519295e-02 Bacteria    Firmicutes      Clostridia     Clostridiales    Ruminococcaceae
19  OTU42 1.639651e-02 1.639651e-02 Bacteria    Firmicutes Erysipelotrichi Erysipelotrichales Erysipelotrichaceae
20  OTU39 1.953122e-02 1.953122e-02 Bacteria Cyanobacteria      Chloroplast     Streptophyta            <NA>
21  OTU17 2.051686e-02 2.051686e-02 Bacteria    Firmicutes      Clostridia     Clostridiales    Lachnospiraceae
22 OTU113 2.102556e-02 2.102556e-02 Bacteria    Firmicutes      Clostridia     Clostridiales     Eubacteriaceae
```

Differential Abundance Testing (ALDEx2)

- ALDEx2 (ANOVA-like differential expression) does quite a few things:
  - Generate 128 Monte-Carlo posterior probabilities for all 138 taxa
  - Transform via centered log-ratio
  - Unmodified Wilcoxon for each taxa per probability
  - Determine effect size
  - Determine average p-value per taxa
  - Determine expected p-values (all instances)
  - Avoid false positives with BH-FDR (Benjamin-Hochberg False Discovery Rate)

```
r$> aldex2_da <- ALDEx2::aldex(data.frame(phyloseq::otu_table(ps)), phyloseq::sample_data(ps)$Status, test="t", effect = TRUE, denom="iqlr")

    #Plot effect sizes
    ALDEx2::aldex.plot(aldex2_da, type="MW", test="wilcox", called.cex = 1, cutoff = 0.05)
```



Differential Abundance Testing (ALDEx2)

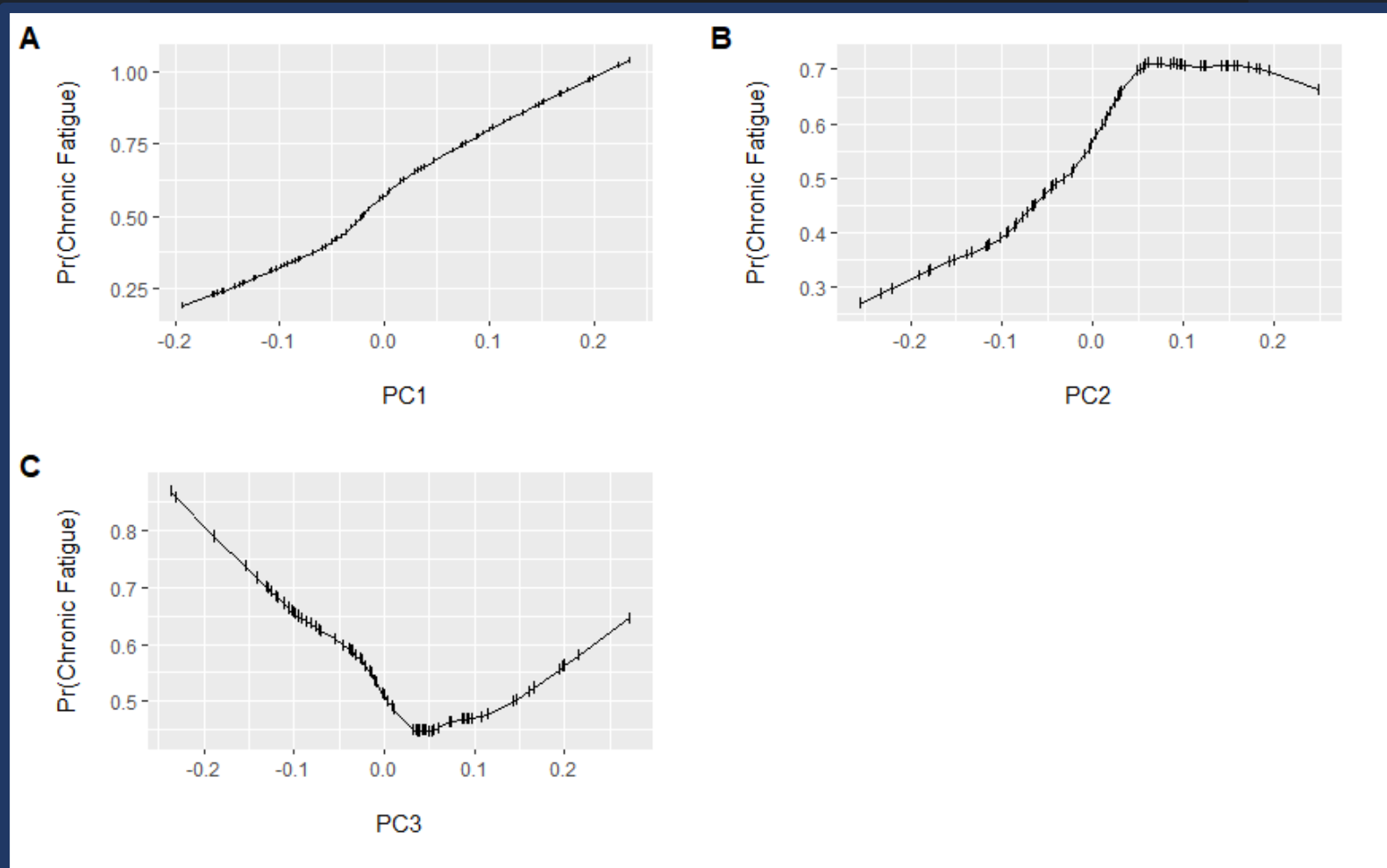
- Which is most abundant according to this test?
  - The winner is: Clostridiales again!

```
r$> #Clean up presentation
    sig_aldex2 <- aldex2_da %>%
      rownames_to_column(var = "OTU") %>%
      filter(wi.eBH < 0.05) %>%
      arrange(effect, wi.eBH) %>%
      dplyr::select(OTU, diff.btw, diff.win, effect, wi.ep, wi.eBH)
    sig_aldex2 <- left_join(sig_aldex2, taxa_info)

    sig_aldex2
Joining, by = "OTU"
    OTU  diff.btw diff.win     effect        wi.ep      wi.eBH  Kingdom     Phylum      Class          Order          Family          Genus Species
1  OTU8 -2.307035 5.522587 -0.3839105 0.0015096450 0.039837292 Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae [Ruminococcus]    <NA>
2 OTU48  3.639216 6.528139  0.5283635 0.0025403967 0.042007768 Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae    Coprococcus    <NA>
3 OTU44  3.541267 6.324375  0.5296702 0.0013725146 0.031600961 Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae [Ruminococcus]    <NA>
4 OTU38  3.277257 4.696329  0.6206553 0.0000348666 0.004124241 Bacteria Firmicutes Clostridia Clostridiales Ruminococcaceae   Oscillospira    <NA>
```

Prediction

- Plotting the Principal Component with the outcome of Chronic Fatigue verse control
- From the graph there is potential for non-linear association
- Better to fit a non-linear on a linear than vice versa as you will have much less penalties
- This specifically can be modeled with restricted cubic splines

```
> head(clr_pcs)
                pc1         pc2          pc3          Status Status_num
ERR1331793  0.02850343 -0.07709724  0.0938970408 Chronic Fatigue          1
ERR1331872 -0.08156129  0.14193568  0.1155088427         Control          0
ERR1331819 -0.19356039 -0.08436341 -0.1048722096         Control          0
ERR1331794 -0.04193714  0.09705602  0.0110912849 Chronic Fatigue          1
ERR1331851  0.09994410  0.05534786 -0.0005008101 Chronic Fatigue          1
ERR1331834 -0.15577774  0.02921040 -0.0204667015         Control          0
```



Prediction (Restricted Cubic Splines)

- Fit Restricted Cubic Splines to the model then find the optimum value for the penalty
- Can also penalty to differ for simple and complex if we want to allow complexity but down weight the impact

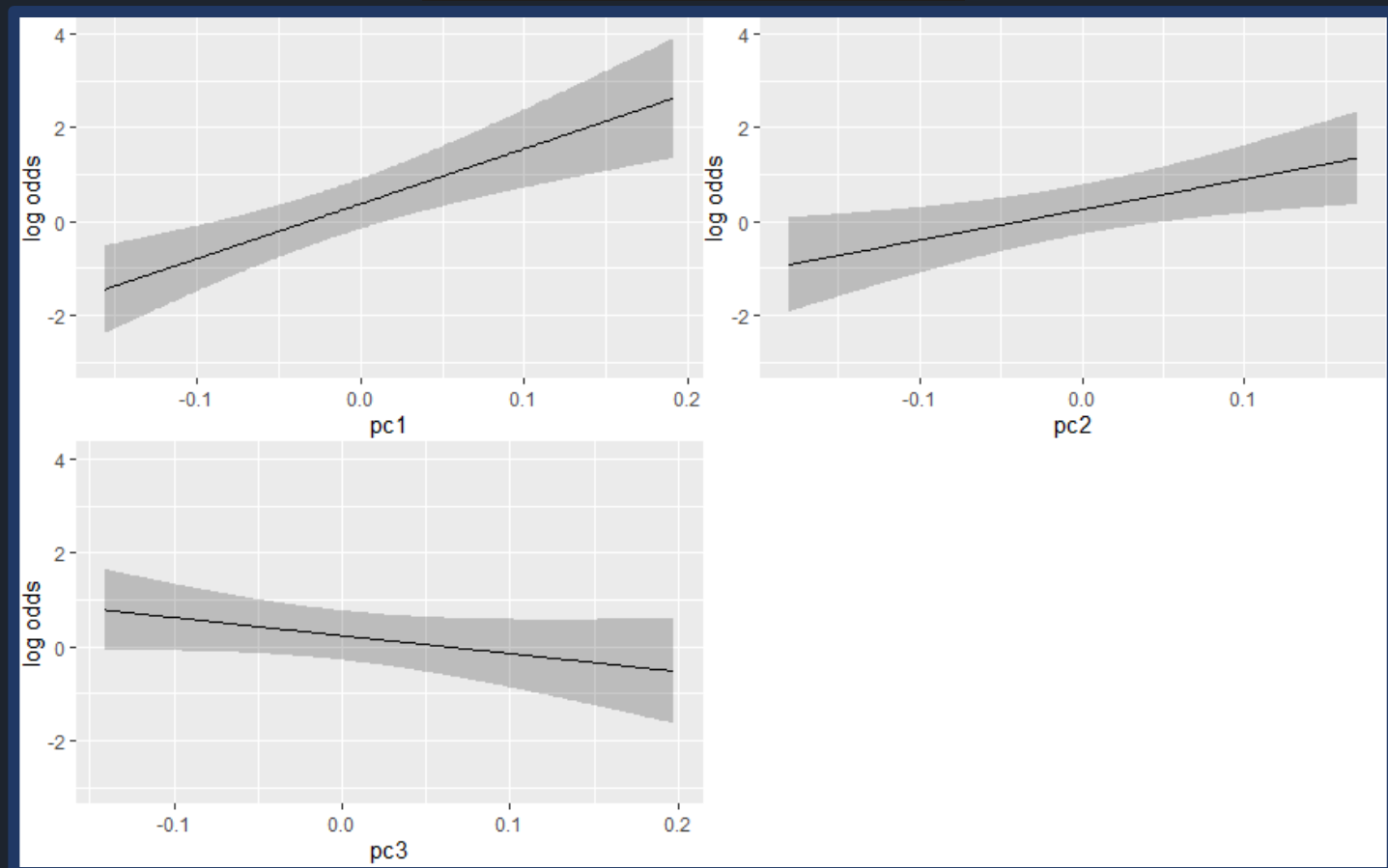
```
> #Fit full model with splines (3 knots each)
> m1 <- rms::lrm(Status_num ~ rcs(pc1, 3) + rcs(pc2, 3) + rcs(pc3, 3), data = clr_pcs, x = TRUE, y = TRUE)
> #Grid search for penalties
> pentrace(m1, list(simple = c(0, 1, 2), nonlinear = c(0, 100, 200)))

Best penalty:

 simple nonlinear       df
      1       200 2.783027

 simple nonlinear       df      aic       bic    aic.c
      0         0 6.000000 23.10845  8.523552 22.01754
      0       100 3.049209 28.21043 20.798359 27.90157
      1       100 2.810152 28.38363 21.552668 28.11659
      2       100 2.641219 28.11811 21.697792 27.87875
      0       200 3.024831 28.24577 20.892958 27.94131
      1       200 2.783027 28.42060 21.655570 28.15810
      2       200 2.611196 28.15166 21.804324 27.91706
```

```
> pen_m1 <- update(m1, penalty = list(simple = 1, nonlinear = 200))
> pen_m1
Logistic Regression Model

 rms::lrm(formula = Status_num ~ rcs(pc1, 3) + rcs(pc2, 3) + rcs(pc3,
     3), data = clr_pcs, x = TRUE, y = TRUE, penalty = list(simple = 1,
     nonlinear = 200))


 Penalty factors

  simple nonlinear interaction nonlinear.interaction
       1       200         200                   200

                      Model Likelihood     Discrimination    Rank Discrim.
                           Ratio Test           Indexes          Indexes
 Obs            84     LR chi2    33.99    R2       0.421    C        0.848
  0             37     d.f.        2.783   g        1.759    Dxy      0.695
  1             47    Pr(> chi2)<0.0001    gr       5.807    gamma    0.695
 max |deriv| 1e-12     Penalty     2.34    gp       0.322    tau-a    0.347
                                           Brier    0.159

           Coef    S.E.   Wald Z Pr(>|Z|) Penalty Scale
 Intercept  0.3458 0.2852  1.21  0.2254   0.0000
 pc1       11.6489 2.8714  4.06  <0.0001  0.1098
 pc1'       0.1202 0.9287  0.13  0.8970   1.0715
 pc2        6.4946 2.5132  2.58  0.0098   0.1098
 pc2'      -0.0015 0.7643  0.00  0.9984   1.2987
 pc3       -3.8538 2.5659 -1.50  0.1331   0.1098
 pc3'       0.0259 1.0080  0.03  0.9795   0.9856
```

Prediction (Resticted Cubic Splines Plot)

- Plot of the penalised log odds
- It can be seen that the conditional associations are quite linear
- The optimal penalties were 1 for the simple and 200 for the non-linear terms
- Effective degrees of freedom shrunk to 2.78

```
r$> #Plot Log odds
    ggplot(Predict(pen_m1))
```



Prediction (Bootstrap Resampling)

- Bootstrap resampling is done to find an out-of-sample estimate of model performance

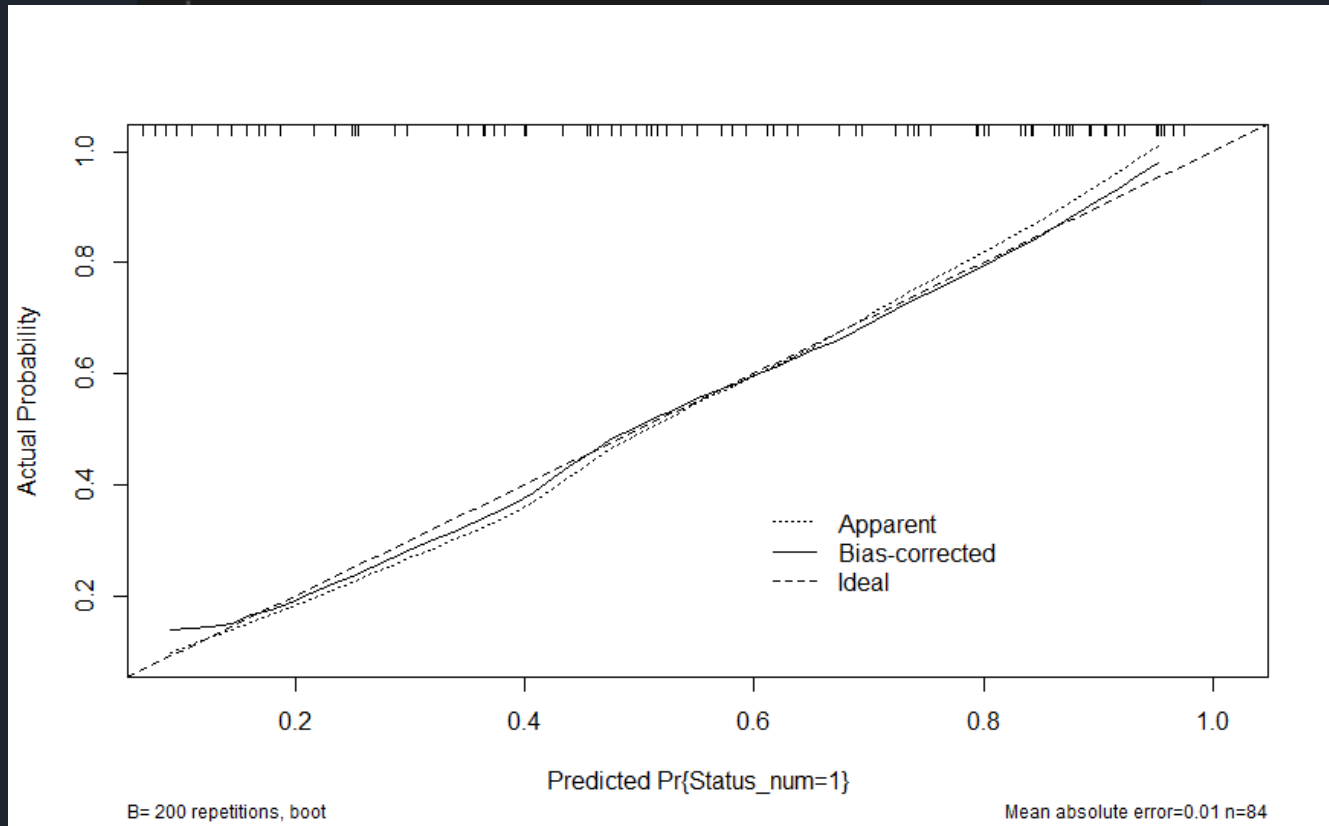
```
> #Obtain optimism corrected estimates
> (val <- rms::validate(pen_m1))
          index.orig training    test optimism index.corrected  n
Dxy           0.6952   0.7248  0.6817   0.0431          0.6521 40
R2            0.4206   0.4536  0.4295   0.0240          0.3965 40
Intercept     0.0000   0.0000 -0.0250   0.0250         -0.0250 40
Slope         1.0000   1.0000  1.0265  -0.0265          1.0265 40
Emax          0.0000   0.0000  0.0100   0.0100          0.0100 40
D             0.3927   0.4045  0.3749   0.0297          0.3630 40
U            -0.0238  -0.0238 -0.0073  -0.0165         -0.0073 40
Q             0.4165   0.4283  0.3822   0.0461          0.3704 40
B             0.1589   0.1481  0.1647  -0.0166          0.1755 40
g             1.7591   1.9083  1.9158  -0.0075          1.7666 40
gp            0.3218   0.3287  0.3363  -0.0076          0.3293 40
> #Compute corrected c-statistic
> (c_opt_corr <- 0.5 * (val[1, 5] + 1))
[1] 0.8260443
```

More on discribing this in greator detail: https://thestatsgeek.com/2014/10/04/adjusting-for-optimismoverfitting-in-measures-of-predictive-ability-using-bootstrapping/

Prediction (Resampling Plot)

- The Brier score mildly increased but the c-statistic mildy decreased with repeated resampling
- The calibration curve shows that the predictions are near the ideal across the range of predicted values
- This suggests we can expect to predict patients with chronic fatigue from healthy controls with reasonable accuracy
- Must be from similar population using top three principal components

```
> plot(cal)

n=84   Mean absolute error=0.011   Mean squared error=0.00021
0.9 Quantile of absolute error=0.022

> #Output pred. probs
> head(predict(pen_m1, type ="fitted"))
[1] 0.4560689 0.4689260 0.1137757 0.6098891 0.8683044 0.2314747
```



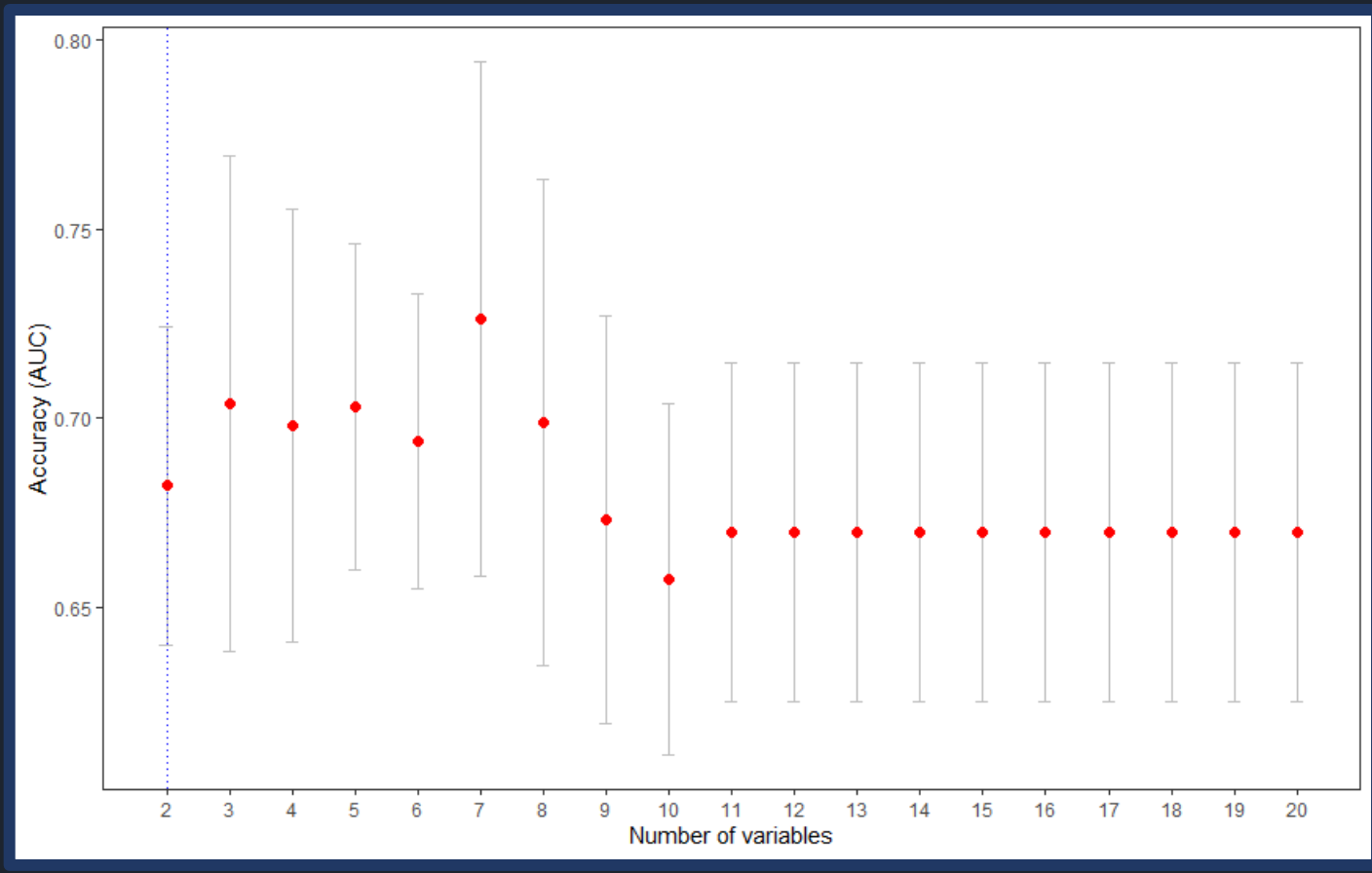
Prediction (Sebal)

- "sebal implements a forward-selection method for the identification of two groups of taxa whose relative abundance, or balance, is associated with the response variable of interest."

```
> #Run selbal
> cv_sebal <- selbal::selbal.cv(x = data.frame(t(data.frame(phyloseq::otu_table(ps_family)))),
+                               y = phyloseq::sample_data(ps_family)$Status,
+                              n.fold = 5, n.iter = 1)


###############################################################
 STARTING selbal.cv FUNCTION
###############################################################

#-------------------------------------------------------------#
# ZERO REPLACEMENT . . .


, . . . FINISHED.
#-------------------------------------------------------------#

#-------------------------------------------------------------#
# Starting the cross - validation procedure . . .
 . . . finished.
#-------------------------------------------------------------#
###############################################################

 The optimal number of variables is: 2
```

Prediction (Sebal Accuracy per Variable)

- Shows the accuracy of the system per variable
- It was already decided that two variables would be used so we focus on that for the balance graph



Prediction (Sebal Balance Graph)

- cross-validation shows two balance objects as having the relitively best rank-discrimination
- erysipelotrichaceae in the numerator and bifidobacteriaceae in the denominator
- The AUC was 0.77, but as low as AUC = 0.68 with 1 repeat of 5 fold cross-validation