

R implementation

Sleiman Bassim

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↑ Project started April 2015 -
ended June 2015

1 Loaded functions:

```
#source ("/media/Data/Dropbox/humanR/01funcs.R")
rm(list=ls())
#setwd("/media/Data/Dropbox/humanR/PD/")
#setwd("~/Dropbox/humanR/PD/")
###load("PD.Rdata", .GlobalEnv)
#lsos(pat="")
```

3 1 Load R packages

4 Load packages.

```
pkgs <- c('xlsx','lattice','latticeExtra',
         'ggplot2', 'dplyr', 'vegan', 'tidyR',
         'ggbiplot')
lapply(pkgs, require, character.only = TRUE)

[[1]]
[1] TRUE

[[2]]
[1] TRUE

[[3]]
[1] TRUE

[[4]]
[1] TRUE

[[5]]
[1] TRUE

[[6]]
[1] TRUE

[[7]]
[1] TRUE

[[8]]
[1] TRUE
```

5 1.1 Load gff3 sequence length data for all mapped libraries and references

6 GFF3 files contains the sequence length of each contig. These contigs belong to Steve Roberts genome
7 v017 and transcriptome v22 of QPX. GFF3 were generated with an in-house perl script.

```
genome <- read.table("./QPX_Genome_v017.gff3")
```

```

head(genome)

          V1 V2 V3 V4      V5 V6 V7 V8
1 QPX_v017_contig_1007 . CDS 1 15433 . . .
2 QPX_v017_contig_1043 . CDS 1 11565 . . .
3 QPX_v017_contig_1050 . CDS 1 12908 . . .
4 QPX_v017_contig_1087 . CDS 1 12852 . . .
5 QPX_v017_contig_1094 . CDS 1 10365 . . .
6 QPX_v017_contig_1128 . CDS 1 10580 . . .

          V9
1 ID=QPX_v017_contig_1007;Name=QPX_v017_contig_1007
2 ID=QPX_v017_contig_1043;Name=QPX_v017_contig_1043
3 ID=QPX_v017_contig_1050;Name=QPX_v017_contig_1050
4 ID=QPX_v017_contig_1087;Name=QPX_v017_contig_1087
5 ID=QPX_v017_contig_1094;Name=QPX_v017_contig_1094
6 ID=QPX_v017_contig_1128;Name=QPX_v017_contig_1128

transcriptome <- read.table("./QPX_transcriptome_v2orf.gff3")
head(transcriptome)

          V1 V2 V3 V4      V5 V6 V7 V8
1 QPX_transcriptome_v2_Contig_1335_1 . CDS 1 210 . . .
2 QPX_transcriptome_v2_Contig_1456_7 . CDS 1 285 . . .
3 QPX_transcriptome_v2_Contig_1465_1 . CDS 1 1107 . . .
4 QPX_transcriptome_v2_Contig_1887_1 . CDS 1 243 . . .
5 QPX_transcriptome_v2_Contig_1941_15 . CDS 1 621 . . .
6 QPX_transcriptome_v2_Contig_1952_2 . CDS 1 330 . . .

          V9
1 ID=QPX_transcriptome_v2_Contig_1335_1;Name=QPX_transcriptome_v2_Contig_1335_1
2 ID=QPX_transcriptome_v2_Contig_1456_7;Name=QPX_transcriptome_v2_Contig_1456_7
3 ID=QPX_transcriptome_v2_Contig_1465_1;Name=QPX_transcriptome_v2_Contig_1465_1
4 ID=QPX_transcriptome_v2_Contig_1887_1;Name=QPX_transcriptome_v2_Contig_1887_1
5 ID=QPX_transcriptome_v2_Contig_1941_15;Name=QPX_transcriptome_v2_Contig_1941_15
6 ID=QPX_transcriptome_v2_Contig_1952_2;Name=QPX_transcriptome_v2_Contig_1952_2

```

8 GFF3 counts of MME transcriptomes **MMETSP0098** and **MMETSP00992**, and the custom assembly that
9 I did for MMETSP0098.

```

mme98 <- read.table("./MMETSP0098.gff3")
mme99 <- read.table("./MMETSP0099_2.gff3")
mme98c <- read.table("./mme98cust.gff3")
genomv015 <- read.table("./QPX_v015.gff3")

```

10 1.2 Distribution of contig length for libraries per reference

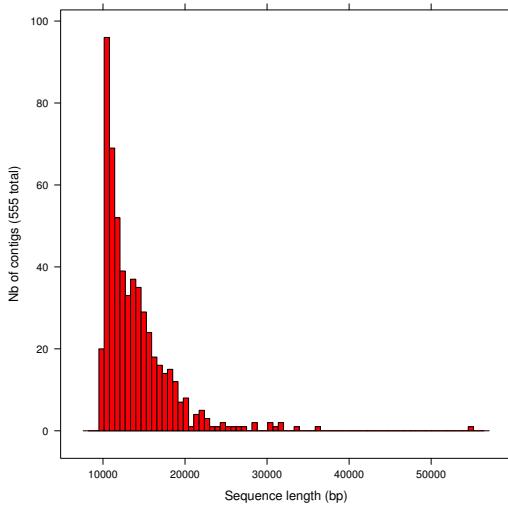
11 The number of bases has been counted and published elsewhere by the authors who assembled the
12 references and sequenced the libraries. Working through their data, we provide a distribution of contig
13 length for genome of Steve's QPX. The purpose of this analysis is to identify 2 things:

- 14 • Biases in contig length
- 15 • Comparison of parameters used for assembling the references

```

histogram(~ (genome$V5),
          type= 'count',
          nint = 75,
          data = genome,
          xlab = 'Sequence length (bp)',
          ylab = 'Nb of contigs (555 total)',
          col = 'red')

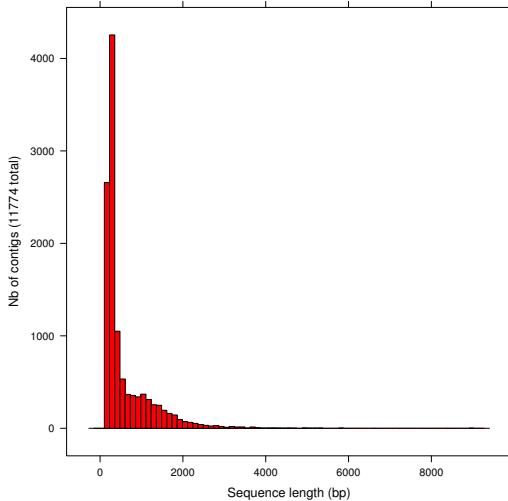
```



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Distribution of Steve's QPX transcriptome.

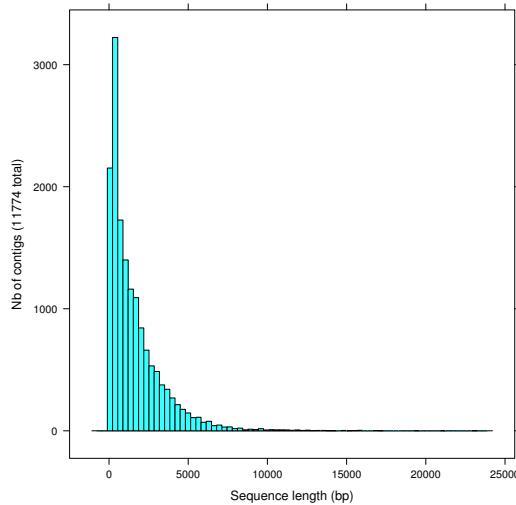
```
histogram(~ transcriptome$V5,
          type = 'count',
          col = 'red',
          data = transcriptome,
          nint = 75,
          xlab = 'Sequence length (bp)',
          ylab = 'Nb of contigs (11774 total)')
```



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Distribution of length of MMETSP0098.

```
histogram(~ mme98$V5,
          type = 'count',
          nint = 75,
          data = mme98,
          xlab = 'Sequence length (bp)',
          ylab = 'Nb of contigs (11774 total)')
```



- 20
21 Superpose length of contigs in:
22 • Steve's genome v017 (555 contigs)
23 • Steve's transcriptome
24 • MMEtsp0098 transcriptome
25 • MMEtsp00992 transcriptome
26 • MMEtsp0098 custom transcriptome
27 • Steve's Genome v015 (approx 22,000 contigs)
- 28 Merge datasets. Then add new column that designs the nature of each contig.

```
lsos()

      Type      Size PrettySize Rows Columns
genome   data.frame 128048    125 Kb   555     9
genomv015 data.frame 4840272   4.6 Mb  21280     9
mme98    data.frame 3535944   3.4 Mb  15489     9
mme98c   data.frame 8259976   7.9 Mb  39946     9
mme99    data.frame 2687328   2.6 Mb  11767     9
pkgs     character   504      504 bytes  8       NA
transcriptome data.frame 3630424   3.5 Mb  11774     9

grouping <- rbind(genome[, c(1,5)],
                     transcriptome[, c(1, 5)],
                     mme98[, c(1,5)],
                     mme99[, c(1,5)],
                     mme98c[, c(1,5)],
                     genomv015[, c(1,5)])
grouping <- data.frame(grouping,
                        y = c(rep("GenomeV17", nrow(genome)),
                              rep("TrxV22", nrow(transcriptome)),
                              rep("MME98", nrow(mme98)),
                              rep("MME99", nrow(mme99)),
                              rep("MME98custom", nrow(mme98c)),
                              rep("(GenomeV15)", nrow(genomv015)))))

dim(grouping)
[1] 100811      3

colnames(grouping)
[1] "V1" "V5" "y"
```

- 29 Plot length of the5 assembly including one genome.

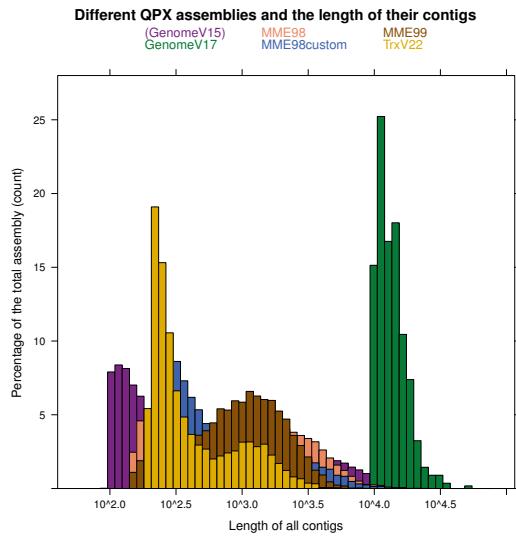
```

custom.colors <- c(col1 = "#762a83",
                   col2 = "#1b7837",
                   col3 = "#ef8a62",
                   col4 = "#2166ac",
                   col5 = "#8c510a",
                   col6 = "#e6ab02")

histogram( V1 ~ V5,
           data = grouping,
           nint = 55,
           scales = list(log = 10),
           type = "p",
           #breaks = seq(4,8,by=0.2),
           ylim = c(0,28),
           groups = grouping$y,
           panel = function(...) panel.superpose(...,
             panel.groups = panel.histogram,
             col = custom.colors,
             alpha = 1),
           auto.key=list(columns=3,
             rectangles = FALSE,
             col = custom.colors),
           main = 'Different QPX assemblies and the length of their contigs',
           xlab = 'Length of all contigs',
           ylab = 'Percentage of the total assembly (count)'
         )

Warning in histogram.formula(V1 ~ V5, data = grouping, nint = 55, scales = list(log = 10), : Can't have log Y-scale

```



30

2 Quality controls after trimming bad regions in contigs

31 Many different options are available while trimming the contigs which have been already assembled.

- 32
- 33 • Nature of PCR adapters (trueSeq2 or trueSeq3)
 - 34 • Sliding window while reading contigs
 - 35 • Crop less than a desired contig length
 - 36 • Minimum length for contigs
 - 37 • Trailing is to remove ends of contigs with bad quality

```
lsos()
```

```

      Type     Size PrettySize   Rows Columns
custom.colors character    864  864 bytes      6      NA
genome          data.frame 128048   125 Kb     555       9
genomv015       data.frame 4840272   4.6 Mb    21280       9
grouping        data.frame 9251608   8.8 Mb   100811       3
mme98           data.frame 3535944   3.4 Mb   15489       9
mme98c          data.frame 8259976   7.9 Mb   39946       9
mme99           data.frame 2687328   2.6 Mb   11767       9
pkgs            character    504  504 bytes      8      NA
transcriptome   data.frame 3630424   3.5 Mb   11774       9

trim <- read.xlsx("./Classeur1.xlsx", header = T, sheetName = "Feuill")
trim <- trim[1:3, ]
trim

  Sample. Total. default    slide    crop    slcrop default2
1     A1 30491569 30176618 29790935 30370029 30300807 30175710
2     A2 34515597 34136650 33698155 34362754 34282437 34135918
3     A3 46861893 46430064 45802956 46682292 46600781 46428610
  slide2
1 29790528
2 33697784
3 45802292

```

38 Plot the differences between nature off adapters (colors) and the combination of the other parameters
 39 (shapes).

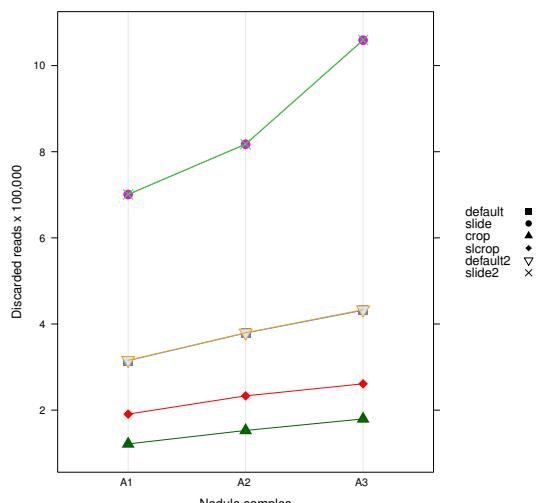
```

key.variety <- list(space = "right",
                      text = list(colnames(trim[, -c(1:2)])),
                      points = list(pch = c(15:18, 25, 4)))

dotplot(c(trim$Total-trim$default)/100000 +
          c(trim$Total-trim$slide)/100000 +
          c(trim$Total-trim$crop)/100000 +
          c(trim$Total-trim$slcrop)/100000 +
          c(trim$Total-trim$default2)/100000 +
          c(trim$Total-trim$slide2)/100000

~ trim$Sample,
  data = trim,
  type = 'o',
  pch = c(15:18, 25, 4),
  key = key.variety,
  lty = 1, cex = 1.5,
  xlab = 'Nodule samples',
  ylab = 'Discarded reads x 100,000')

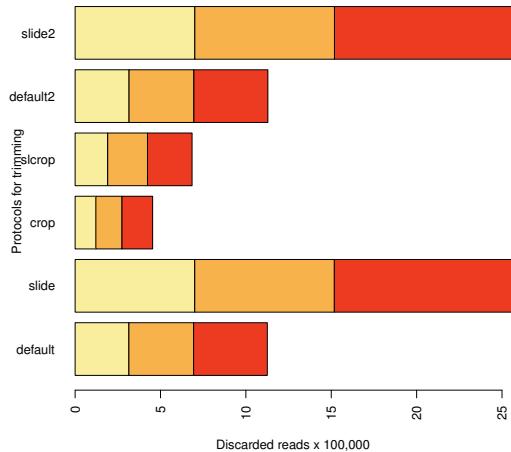
```



41 Another way to visualize the discarded reads.

```
custom.colors <- c(col1 = "#ffeda0", col2 = "#feb24c", col3 = "#f03b20")

barplot(as.matrix((trim$Total-trim[, -c(1:2)]) / 100000),
       horiz = TRUE,
       col = custom.colors,
       xlab = 'Discarded reads x 100,000',
       ylab = 'Protocols for trimming',
       las = 2)
```



42

3 Aligning contigs to reference

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Two sets of reads were mapped to 2 references. First batch from the trimmed reads with the default parameters (adapters clipping, trailing, and minimum length) and TrueSeq3 adapters (ones Bassem supplied). Second batch were also trimmed with default settings but using TrueSeq2 adapters (ones that sleiman supplied). With the second batch of adapters, more reads were trimmed and discarded. This analysis will try to show why by regressing the number of mapped reads over the length of each reference.

```
ref.genome1 <- read.table("./refGenome/A1.htseq.counts.txt")
ref.genome2 <- read.table("./refGenome/A2.htseq.counts.txt")
ref.genome3 <- read.table("./refGenome/A3.htseq.counts.txt")
ref.genome4 <- read.table("./refGenome/A1-4.htseq.counts.txt")
ref.genome5 <- read.table("./refGenome/A2-4.htseq.counts.txt")
ref.genome6 <- read.table("./refGenome/A3-4.htseq.counts.txt")
```

50 Merge all mapped reads on the genome

```
ref.genome <- data.frame(
  A1 = ref.genome1[-c(556:560), 2],
  A2 = ref.genome2[-c(556:560), 2],
  A3 = ref.genome3[-c(556:560), 2],
  A1.4 = ref.genome4[-c(556:560), 2],
  A2.4 = ref.genome5[-c(556:560), 2],
  A3.4 = ref.genome6[-c(556:560), 2],
  contigs = ref.genome1[-c(556:560), 1])
dim(ref.genome)

[1] 555    7

# just because im lazy
genome1 <- data.frame(contigs= genome[,1], length = genome$V5)
```

51 Merge length and number of mapped reads

```
ref.genome.mix <- merge(genome1, ref.genome)
```

```

head(ref.genome.mix)

  contigs length   A1   A2   A3 A1.4 A2.4 A3.4
1 QPX_v017_contig_1007 15433 117 197 249 117 197 249
2 QPX_v017_contig_1020 12397 123 164 171 123 164 171
3 QPX_v017_contig_1021 18562 335 487 596 335 488 596
4 QPX_v017_contig_1023 19919 116 198 331 116 198 331
5 QPX_v017_contig_103 10989 71 111 107 71 111 105
6 QPX_v017_contig_1034 10178 196 289 655 196 289 655

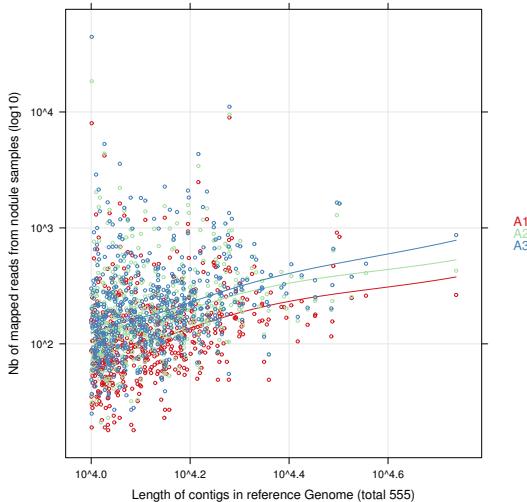
```

52 Plot the correlation between read length and number of mapped reads on the genome of QPX with the
 53 remaining reads from the default trimming with TrueSeq3 adapters.

```

custom.colors <- c('#d7191c', '#abdda4', '#2b83ba')
key.variety <- list(space = "right",
                      text = list(colnames(ref.genome.mix[, 3:5])),
                      col = custom.colors)
xyplot(A1 + A2 + A3 ~ length,
       data = ref.genome.mix,
       xlab = 'Length of contigs in reference Genome (total 555)',
       ylab = 'Nb of mapped reads from nodule samples (log10)',
       col = custom.colors,
       cex = 0.5,
       type = c("g", "p", "smooth"),
       scales = list(log = 10),
       key = key.variety)

```

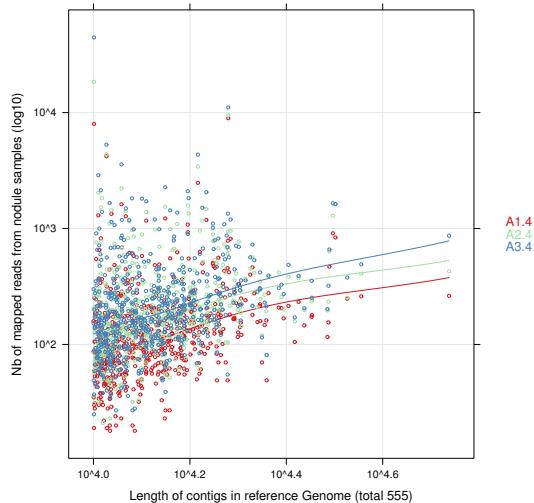


54
 55 Regression between reads and length of contigs in reference genome with adapters TrueSeq2 under
 56 default trimming settings.

```

custom.colors <- c('#d7191c', '#abdda4', '#2b83ba')
key.variety <- list(space = "right",
                      text = list(colnames(ref.genome.mix[, 6:8])),
                      col = custom.colors)
xyplot(A1.4 + A2.4 + A3.4 ~ length,
       alpha = .5,
       data = ref.genome.mix,
       xlab = 'Length of contigs in reference Genome (total 555)',
       ylab = 'Nb of mapped reads from nodule samples (log10)',
       col = custom.colors,
       cex = 0.5,
       type = c("g", "p", "smooth"),
       scales = list(log = 10),
       key = key.variety)

```



57
58 Previously i regressed the number of mapped reads of nodule samples over the reference genome of
59 QPX. Now its time to do the same thing over the reference transcriptome of QPX. Both references belong
60 to the Steve Roberts.

```
ref.transcriptome1 <- read.table("./refTranscriptome/A1.htseq.counts.txt")
ref.transcriptome2 <- read.table("./refTranscriptome/A2.htseq.counts.txt")
ref.transcriptome3 <- read.table("./refTranscriptome/A3.htseq.counts.txt")
ref.transcriptome4 <- read.table("./refTranscriptome/A1-4.htseq.counts.txt")
ref.transcriptome5 <- read.table("./refTranscriptome/A2-4.htseq.counts.txt")
ref.transcriptome6 <- read.table("./refTranscriptome/A3-4.htseq.counts.txt")
dim(ref.transcriptome1)

[1] 11779      2

tail(ref.transcriptome1)

          V1      V2
11774 QPX_transcriptome_v2_Contig_9_3     6
11775           __no_feature     0
11776           __ambiguous   568
11777           __too_low_aQual 147407
11778           __not_aligned 29746511
11779           __alignment_not_unique     0
```

61 Merge all mapped reads on the transcriptome

```
ref.transcriptome <- data.frame(
```

```

A1 = ref.transcriptome1[-c(11775:11779), 2],
A2 = ref.transcriptome2[-c(11775:11779), 2],
A3 = ref.transcriptome3[-c(11775:11779), 2],
A1.4 = ref.transcriptome4[-c(11775:11779), 2],
A2.4 = ref.transcriptome5[-c(11775:11779), 2],
A3.4 = ref.transcriptome6[-c(11775:11779), 2],
contigs = ref.transcriptome1[-c(11775:11779), 1])
dim(ref.transcriptome)
[1] 11774      7

head(ref.transcriptome)

  A1 A2 A3 A1.4 A2.4 A3.4           contigs
1  0  2  0   0   2     0 QPX_transcriptome_v2_Contig_10002_1
2  0  0  0   0   0     0 QPX_transcriptome_v2_Contig_10002_2
3  2  2  7   2   2     7 QPX_transcriptome_v2_Contig_1000_1
4  1  0  0   1   0     0 QPX_transcriptome_v2_Contig_1000_2
5  0  0  0   0   0     0 QPX_transcriptome_v2_Contig_1000_3
6 58 72 99   59  72   100 QPX_transcriptome_v2_Contig_1000_4

# just because im lazy
transcriptome1 <- data.frame(contigs= transcriptome[,1], length = transcriptome$V5)

```

62 Merge length and number of mapped reads

```

ref.transcriptome.mix <- merge(transcriptome1, ref.transcriptome)
head(ref.transcriptome.mix)

  contigs length A1 A2 A3 A1.4 A2.4 A3.4
1 QPX_transcriptome_v2_Contig_1000_1    201  2  2  7   2   2   7
2 QPX_transcriptome_v2_Contig_1000_2    258  1  0  0   1   0   0
3 QPX_transcriptome_v2_Contig_10002_1   477  0  2  0   0   2   0
4 QPX_transcriptome_v2_Contig_10002_2   264  0  0  0   0   0   0
5 QPX_transcriptome_v2_Contig_1000_3    321  0  0  0   0   0   0
6 QPX_transcriptome_v2_Contig_1000_4   1473 58 72 99   59  72  100

```

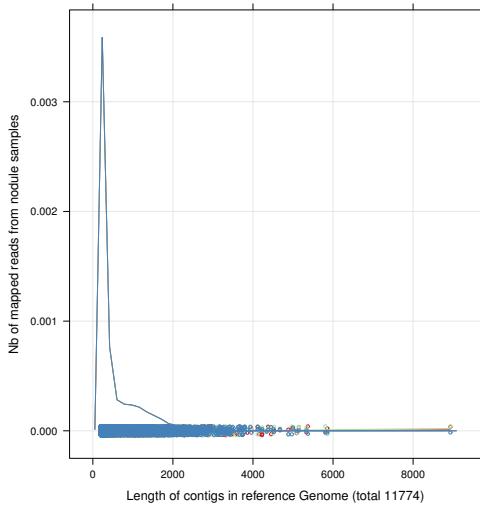
63 3.1 Concentration of contigs in different libraries

64 Plot correlation between transcriptome contigs and assembled reads of nodule samples. Trimming parameters are of default with TrueSeq3 adapters.

```

custom.colors <- c('#d7191c', '#abdd4', '#2b83ba')
key.variety <- list(space = "right",
                      text = list(colnames(ref.genome.mix[, 3:5])),
                      col = custom.colors)
densityplot(A1 + A2 + A3 ~ length,
            data = ref.transcriptome.mix,
            #           alpha = .7,
            xlab = 'Length of contigs in reference Genome (total 11774)',
            ylab = 'Nb of mapped reads from nodule samples',
            col = custom.colors,
            cex = 0.5,
            type = c("g", "p", "smooth"),
            #           scales = list(log = 10),
            key = key.variety)

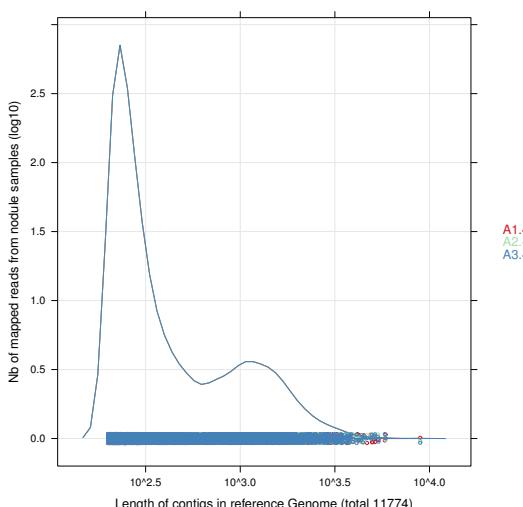
```



66
67 Plot correlation same as above but with TrueSeq2 adapters with default parameters.

```
custom.colors <- c('#d7191c', '#abdda4', '#2b83ba')
key.variety <- list(space = "right",
                      text = list(colnames(ref.genome.mix[, 6:8])),
                      col = custom.colors)
densityplot(A1.4 + A2.4 + A3.4 ~ length,
            data = ref.transcriptome.mix,
#           alpha = .9,
            xlab = 'Length of contigs in reference Genome (total 11774)',
            ylab = 'Nb of mapped reads from nodule samples (log10)',
            col = custom.colors,
            cex = 0.5,
            type = c("g", "p", "smooth"),
            scales = list(log = 10),
            key = key.variety)
```

Warning in densityplot.formula(A1.4 + A2.4 + A3.4 ~ length, data = ref.transcriptome.mix,
: Can't have log Y-scale



68
69 Load the number of mapped reads to the MMETSP0098 transcriptome before discarding duplicates.

```
ref.dupA1R3 <- read.table("./refMME98/A1.htseq.counts.txt")
ref.dupA2R3 <- read.table("./refMME98/A2.htseq.counts.txt")
ref.dupA3R3 <- read.table("./refMME98/A3.htseq.counts.txt")
```

70 Merge all mapped reads to MMETSP0098 reference transcriptome before discarding duplicates (ie, raw
71 counts).

```

ref.mme98 <- data.frame(A1 = ref.dupA1R3$V2,
                         A2 = ref.dupA2R3$V2,
                         A3 = ref.dupA3R3$V2,
                         contigs = ref.dupA1R3$V1)

```

72 Add the length values to each contig mapped to MMETsp0098. But first remove extra rows.

```

nr <- nrow(ref.mme98)
ref.mme98 <- ref.mme98[1:(nr-5), ]
tail(ref.mme98)

  A1 A2 A3           contigs
15484  0  0  0 MMETSP0098-20131031|9992
15485 12  6 17 MMETSP0098-20131031|9993
15486  0  0  0 MMETSP0098-20131031|9995
15487  3  3  3 MMETSP0098-20131031|9996
15488  0  0  0 MMETSP0098-20131031|9998
15489  2  9  5 MMETSP0098-20131031|9999

```

73 Merge length and counts.

```
#ref.mme98.mix <- merge(ref.mme98, mme98[, c(1,5)])
```

74 Load the number of mapped reads to the MMETSP0099_2 transcriptome before discarding duplicates.

```

ref.dupA1R4 <- read.table("./refMME992/A1.htseq.counts.txt")
ref.dupA2R4 <- read.table("./refMME992/A2.htseq.counts.txt")
ref.dupA3R4 <- read.table("./refMME992/A3.htseq.counts.txt")

```

75 Merge all mapped reads to MMETSP0099_2 reference transcriptome before discarding duplicates (ie, raw counts).

```

ref.mme992 <- data.frame(A1 = ref.dupA1R4$V2,
                           A2 = ref.dupA2R4$V2,
                           A3 = ref.dupA3R4$V2,
                           contigs = ref.dupA1R4$V1)

```

77 Add the length values to each contig mapped to MMETsp0099_2. But first remove extra rows.

```

nr <- nrow(ref.mme992)
ref.mme992 <- ref.mme992[1:(nr-5), ]
tail(ref.mme992)

  A1 A2 A3           contigs
11762  0  0  0 MMETSP0099_2-20121227|9994
11763  0  8  9 MMETSP0099_2-20121227|9995
11764  0  0  0 MMETSP0099_2-20121227|9996
11765  0  0  1 MMETSP0099_2-20121227|9997
11766  0  0  0 MMETSP0099_2-20121227|9998
11767  0  0  0 MMETSP0099_2-20121227|9999

```

78 Load the number of mapped reads to SR v015 genome before discarding duplicates.

```

ref.dupA1R5 <- read.table("./refGenomV015/A1.htseq.counts.txt")
ref.dupA2R5 <- read.table("./refGenomV015/A2.htseq.counts.txt")
ref.dupA3R5 <- read.table("./refGenomV015/A3.htseq.counts.txt")

```

79 Merge all mapped reads to SR v015 reference genome before discarding duplicates (ie, raw counts).

```

ref.genomv015 <- data.frame(A1 = ref.dupA1R5$V2,
                             A2 = ref.dupA2R5$V2,
                             A3 = ref.dupA3R5$V2,
                             contigs = ref.dupA1R5$V1)

```

80 Add the length values to each contig mapped to SR v015 reference genome. But first remove extra rows.

81

```

nr <- nrow(ref.genomv015)
ref.genomv015 <- ref.genomv015[1:(nr-5), ]
tail(ref.genomv015)

  A1 A2 A3      contigs
21275 0 1 2 QPX_v015_contig_9994
21276 0 0 0 QPX_v015_contig_9995
21277 1 5 3 QPX_v015_contig_9996
21278 1 2 2 QPX_v015_contig_9997
21279 3 1 2 QPX_v015_contig_9998
21280 9 5 21 QPX_v015_contig_9999

```

82 4 Removing the duplicate reads and reducing bias for better coverage

83 After aligning the reads to a reference duplicates must be removed.

84 First load the sample reads mapped to reference genome (without duplication) of Steve Roberts.

```

nodupA1R1 <- read.table("./nodupR1/A1.htseq.nodup.counts.txt")
nodupA2R1 <- read.table("./nodupR1/A2.htseq.nodup.counts.txt")
nodupA3R1 <- read.table("./nodupR1/A3.htseq.nodup.counts.txt")

```

85 Second load the sample reads mapped to reference transcriptome (withtout duplication) of Steve Roberts.

86

```

nodupA1R2 <- read.table("./nodupR2/A1.htseq.nodup.counts.txt")
nodupA2R2 <- read.table("./nodupR2/A2.htseq.nodup.counts.txt")
nodupA3R2 <- read.table("./nodupR2/A3.htseq.nodup.counts.txt")

```

87 Third load the sample reads mapped to reference transcriptome (without duplication) of MMESTO0098.

```

nodupA1R3 <- read.table("./nodupR3/A1.htseq.counts.nodup.txt")
nodupA2R3 <- read.table("./nodupR3/A2.htseq.counts.nodup.txt")
nodupA3R3 <- read.table("./nodupR3/A3.htseq.counts.nodup.txt")

```

88 Forth load of sample reads mapped to reference transcriptome MMETSP0099_2.

```

nodupA1R4 <- read.table("./nodupR4/A1.htseq.counts.nodup.txt")
nodupA2R4 <- read.table("./nodupR4/A2.htseq.counts.nodup.txt")
nodupA3R4 <- read.table("./nodupR4/A3.htseq.counts.nodup.txt")

```

89 Forth load of sample reads mapped to reference SR genome v015 with approximately 21,000 contigs.

```

nodupA1R5 <- read.table("./nodupR5/A1.htseq.counts.nodup.txt")
nodupA2R5 <- read.table("./nodupR5/A2.htseq.counts.nodup.txt")
nodupA3R5 <- read.table("./nodupR5/A3.htseq.counts.nodup.txt")

```

90 Merge mapped samples relative to the reference.

- 91 • R1 = genome of QPX (steve roberts, 555 contigs)
- 92 • R2 = transcriptome of QPX (steve roberts)
- 93 • R3 = transcriptome of QPX MMETSP0098
- 94 • R4 = transcriptome of QPX MMETSP0099_2
- 95 • R5 = genome of QPX (steve roberts v015, approx. 21,000 contigs)

```

allR1 <- data.frame(A1n = nodupA1R1$V2,

```

```

A2n = nodupA2R1$V2,
A3n = nodupA3R1$V2,
reference = rep("genomSRv017", nrow(nodupA1R1)),
contigs = nodupA1R1$V1)
allR1 <- allR1[1:555, ]

allR2 <- data.frame(A1n = nodupA1R2$V2,
A2n = nodupA2R2$V2,
A3n = nodupA3R2$V2,
reference = rep("trxSRv022", nrow(nodupA1R2)),
contigs = nodupA1R2$V1)
allR2 <- allR2[1:11774, ]

allR3 <- data.frame(A1n = nodupA1R3$V2,
A2n = nodupA2R3$V2,
A3n = nodupA3R3$V2,
reference = rep("trxMME98", nrow(nodupA1R3)),
contigs = nodupA1R3$V1)
allR3 <- allR3[1:15489, ]

allR4 <- data.frame(A1n = nodupA1R4$V2,
A2n = nodupA2R4$V2,
A3n = nodupA3R4$V2,
reference = rep("trxMME992", nrow(nodupA1R4)),
contigs = nodupA1R4$V1)
allR4 <- allR4[1:c(nrow(nodupA1R4))-5),]

allR5 <- data.frame(A1n = nodupA1R5$V2,
A2n = nodupA2R5$V2,
A3n = nodupA3R5$V2,
reference = rep("genomSRv015", nrow(nodupA1R5)),
contigs = nodupA1R5$V1)
allR5 <- allR5[1:c(nrow(nodupA1R5))-5),]

```

96 Put before /after duplicates removal in one dataset for genome of Steve Roberts.

```

genomeSR <- merge(ref.genome.mix[, 1:5], allR1)
head(genomeSR)

  contigs length A1 A2 A3 A1n A2n A3n reference
1 QPX_v017_contig_1007 15433 117 197 249 109 191 240 genomSRv017
2 QPX_v017_contig_1020 12397 123 164 171 118 157 159 genomSRv017
3 QPX_v017_contig_1021 18562 335 487 596 319 460 568 genomSRv017
4 QPX_v017_contig_1023 19919 116 198 331 109 196 326 genomSRv017
5 QPX_v017_contig_103 10989 71 111 107 68 106 104 genomSRv017
6 QPX_v017_contig_1034 10178 196 289 655 185 279 592 genomSRv017

rownames(genomeSR) <- genomeSR$contigs
genomeSR <- t(genomeSR[, -c(1, 9)])
genomeSR[, 1:3]

  QPX_v017_contig_1007 QPX_v017_contig_1020 QPX_v017_contig_1021
length          15433           12397           18562
A1              117             123             335
A2              197             164             487
A3              249             171             596
A1n             109             118             319
A2n             191             157             460
A3n             240             159             568

genomeSR <- data.frame(genomeSR,
y = c(2, rep(0, 3), rep(1, 3)))

```

97 Put the before /after duplicates removal in one dataset for transcriptome of SR.

```

transcriptomeSR <- merge(ref.transcriptome.mix[, 1:5], allR2)
head(transcriptomeSR)

      contigs length A1 A2 A3 A1n A2n A3n
1 QPX_transcriptome_v2_Contig_1000_1    201  2  2  7  2  2  7
2 QPX_transcriptome_v2_Contig_1000_2    258  1  0  0  1  0  0
3 QPX_transcriptome_v2_Contig_10002_1   477  0  2  0  0  2  0
4 QPX_transcriptome_v2_Contig_10002_2   264  0  0  0  0  0  0
5 QPX_transcriptome_v2_Contig_1000_3   321  0  0  0  0  0  0
6 QPX_transcriptome_v2_Contig_1000_4   1473 58 72 99 52 69 94
reference
1 trxSRv022
2 trxSRv022
3 trxSRv022
4 trxSRv022
5 trxSRv022
6 trxSRv022

```

98 Present difference for each sample mapped to the references. First merge all samples before /after
99 duplicates were removed.

```

allRefs <- rbind(allR1, allR2, allR3, allR4, allR5)
dim(allRefs)

[1] 60865      5

summary(allRefs$reference)

genomSRv017    trxSRv022    trxMME98    trxMME992  genomSRv015
      555        11774       15489       11767      21280

allRefs.raw <- rbind(ref.genome.mix[, 3:5],
                      ref.transcriptome.mix[, 3:5],
                      ref.mme98[, 1:3],
                      ref.mme992[, 1:3],
                      ref.genomv015[, 1:3])
dim(allRefs.raw)

[1] 60865      3

allDF <- cbind(allRefs, allRefs.raw)
allDF[sample(1:20000, 5), ]

      A1n A2n A3n reference
13661  0   0   0  trxMME98          MMETSP0098-20131031|11472  0   0
8346   4   8  12  trxSRv022  QPX_transcriptome_v2_Contig_5162_2  0   0
6367   0   4   7  trxSRv022  QPX_transcriptome_v2_Contig_3790_1 14  17
14112  0   1   0  trxMME98          MMETSP0098-20131031|12093  0   1
16724  0   0   3  trxMME98          MMETSP0098-20131031|15135  0   0

      A3
13661  0
8346   0
6367  20
14112  0
16724  3

```

100 Plot the difference before and after duplicates were discarded. The number of mapped reads to the
101 reference contigs is descriptive for any bias in contig assembly. For example in the case of SR genome
102 v017, more than 20 % of A1, A2, A3 reads align to a small set of contigs. We have to imagine that each
103 vertical bar is a different reference contig. The best distribution is a constant one.
104 Even though we did not plot length of contigs, the analyzes above demonstrate that length is linearly
105 correlated to the number of mapped reads. Therefore, peaks indicate a specific preference that reads
106 have to map to the assembled reference.

```
custom.colors <- c(col1 = "#762a83",
```

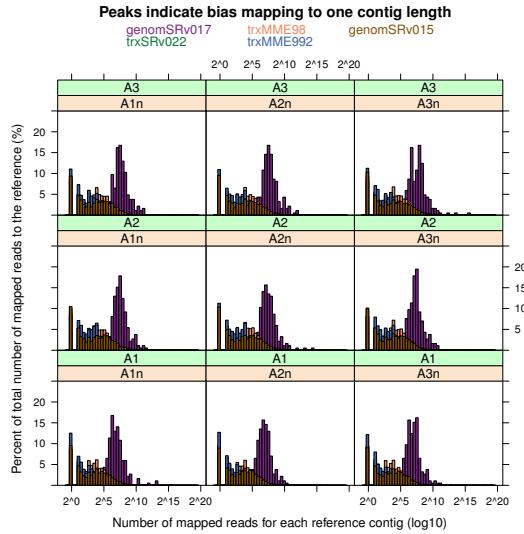
```

        col2 = "#1b7837",
        col3 = "#ef8a62",
        col4 = "#2166ac",
        col5 = "#8c510a",
        col6 = "#e6ab02")

histogram( ~ A1 + A2 + A3 | c('A1n', 'A2n', 'A3n'),
  data = allDF,
  nint = 50,
  scales = list(log = 2),
  type = "p",
  ylim = c(0,25),
  groups = allDF$reference,
  panel = function(...) panel.superpose(...,
    panel.groups = panel.histogram,
    col = custom.colors,
    alpha = 1),
  auto.key=list(columns=3,
    rectangles = FALSE,
    col = custom.colors),
  main = 'Peaks indicate bias mapping to one contig length',
  ylab = 'Percent of total number of mapped reads to the reference (%)',
  xlab = 'Number of mapped reads for each reference contig (log10)'
)

Warning in histogram.formula(~A1 + A2 + A3 | c("A1n", "A2n", "A3n"), data = allDF,
: Can't have log Y-scale
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length
Warning in pmax('c("A1n", "A2n", "A3n")' = c(FALSE, FALSE, FALSE, FALSE, : an
argument will be fractionally recycled
Warning in id & (if (is.shingle(var)) ((var >= levels(var)[[levels[i]]][1]) &
: longer object length is not a multiple of shorter object length

```



107

108 5 Calling SNPs: Testing tools, parameters, and filters

109 SNPs were called either with samtools *mpileup* function and the highest significant were selected with
 110 bcftools or they have been called with GATK. Either way SNP calling was done on each library separately.
 111 Libraries were:

- 112 • mmetsp0098
- 113 • mmetsp001433
- 114 • mmetsp00992
- 115 • mmetsp001002
- 116 • mmetsp0099
- 117 • mmetsp00100

118 5.1 Load data

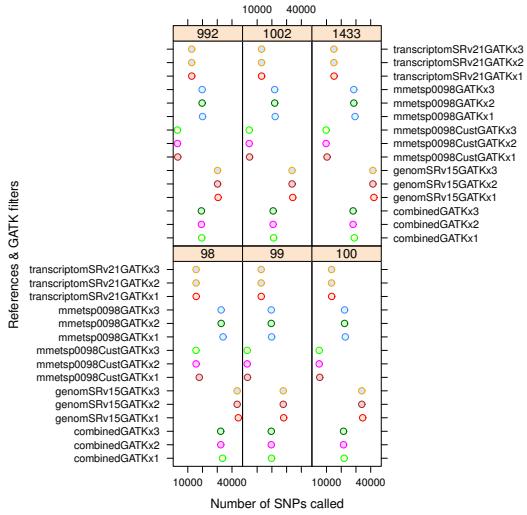
119 Number of SNPs called with either package were counted. Calls were done after read duplicates removal
 120 with Picard.

```
counts.SNP <- read.xlsx("./snp.counts.xlsx", sheetIndex = 1)
glimpse(counts.SNP)

Variables:
$ sample      (dbl) 98, 992, 1002, 1433, 99, 100, 98, 992, 1002, 14...
$ counts      (dbl) 351790, 395060, 427790, 389188, 309813, 425947, ...
$ reference   (fctr) trxSRv21, trxSRv21, trxSRv21, trxSRv21, trxSRv...
```

121 Histogram grouped by library showing difference in SNPs called relative to the reference used for mapping
 122 and the number of times GATK has been used to recalibrate calls.

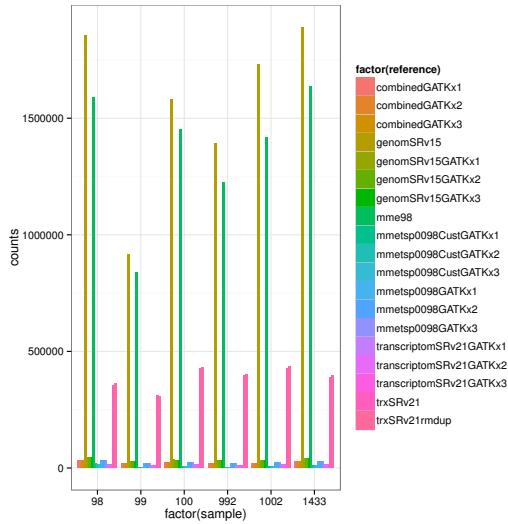
```
xyplot( factor(reference) ~ as.matrix(counts) | factor(sample),
        data = counts.SNP[-c(1:24), ],
        groups = counts.SNP$reference,
        pch = 21,
        cex = 1,
        type = c("p"),
        xlab = 'Number of SNPs called',
        ylab = 'References & GATK filters')
```



123

Plot the difference between libraries and packages for the number of called SNPs.

```
ggplot(counts.SNP,  
       aes(x = factor(sample),  
            y = counts,  
            fill = factor(reference))) +  
  geom_bar(stat = "identity",  
           position = "dodge") +  
  theme_bw()
```



125

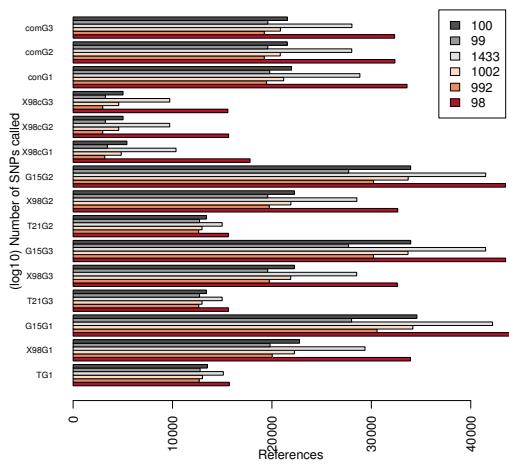
126 Another plot for cluster analysis between references and SNPs called. I find this useful for a fast check of
127 outliers and errors in importing data.

```
dat <- read.xlsx("./snp.counts.xlsx", sheetIndex = 4)
```

```

custom.colors <- c(col1 = "#b2182b",
                   col2 = "#ef8a62",
                   col3 = "#fddbc7",
                   col4 = "#e0e0e0",
                   col5 = "#999999",
                   col6 = "#4d4d4d")
barplot(as.matrix(dat[, -c(1:5)]),
       col = custom.colors,
       horiz = TRUE,
       las = 2,
       beside = T,
       legend.text = factor(dat[, 1]),
       cex.names = .7,
       ylab = '(log10) Number of SNPs called',
       xlab = 'References')

```



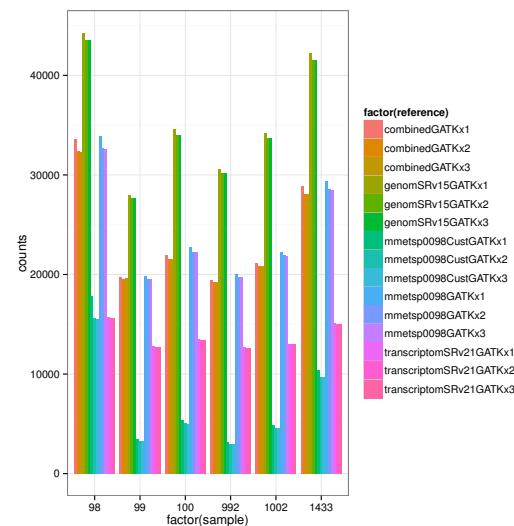
128

129 Plotting only the GATK called SNPs.

```

counts.SNP <- counts.SNP[-c(1:24), ]
ggplot(counts.SNP,
       aes(x = factor(sample),
            y = counts,
            fill = factor(reference))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw()

```



130

131 Plot the difference between the number of SNPs called on the 6 libraries using either the assembled or
 132 custom assembled mmetsp0098 reference. Also show the variation pattern with the number of reads
 133 used for calling SNPs. First, prepare SNP data.

```
x1 <- counts.SNP[counts.SNP$reference %in% "mmetsp0098GATKx1", ]
x2 <- counts.SNP[counts.SNP$reference %in% "mmetsp0098CustGATKx1", ]
```

134 Next, add the number of reads per library. This is the count of non duplicate reads that mapped to each
 135 of all the references used.

```
ref.reads <- read.xlsx("./refreads.xlsx", sheetIndex = 1)
head(ref.reads)

  sample  counts      reference
1      98 8591456 mmetsp0098GATKx1
2     992 5875110 mmetsp0098GATKx1
3    1002 7780584 mmetsp0098GATKx1
4    1433 7001081 mmetsp0098GATKx1
5      99 4835298 mmetsp0098GATKx1
6    100 4193326 mmetsp0098GATKx1

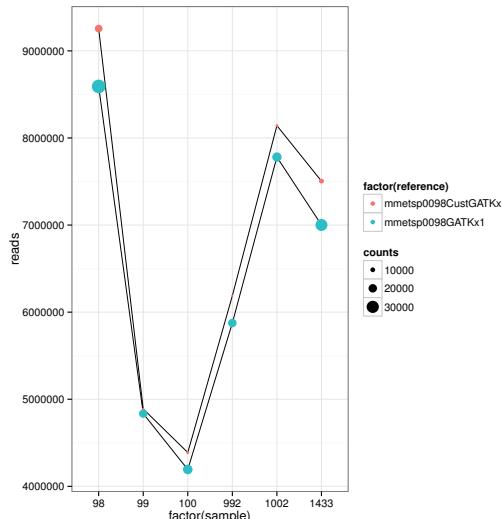
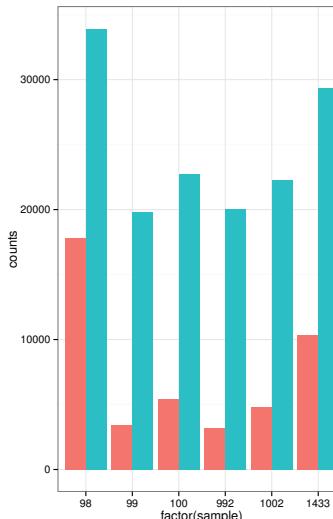
y <- ref.reads[1:12, ]
```

136 Plot difference.

```
dat <- data.frame(rbind(x1, x2), reads = y$counts)

ggplot(dat,
       aes(x = factor(sample),
            y = counts,
            fill = factor(reference))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw()

ggplot(dat,
       aes(x = factor(sample),
            y = reads,
            group = factor(reference))) +
  geom_line(size = .2) +
  geom_point(data = dat,
             aes(x = factor(sample),
                  y = reads,
                  colour = factor(reference),
                  size = counts)) +
  theme_bw()
```

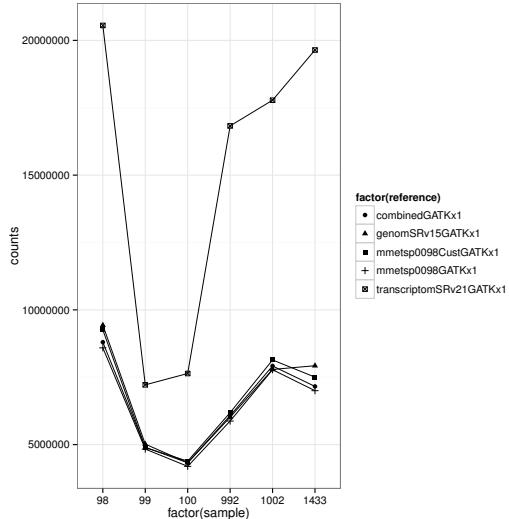


137 Plot number of all mapped reads for each library and for all 4 references.

```

ggplot(ref.reads,
       aes(x = factor(sample),
            y = counts,
            group = factor(reference))) +
  geom_line(size = .2) +
  geom_point(aes(shape = factor(reference))) +
  theme_bw()

```

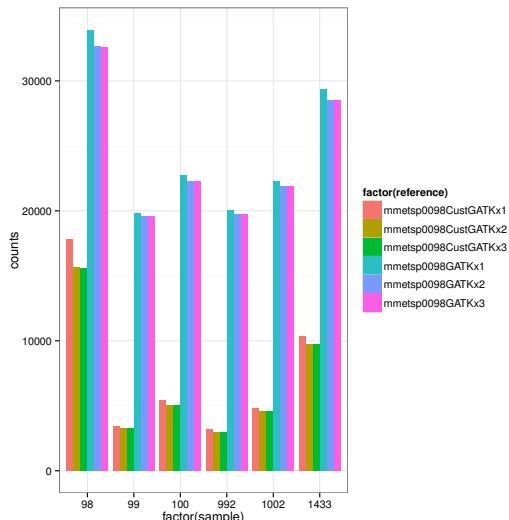


139
140 Difference in SNPs called between the already assembled and the custom assembled *mmetsp0098* reference.
141

```

dat <- read.xlsx("./snp.counts.xlsx", sheetIndex = 2)
ggplot(dat,
       aes(x = factor(sample),
            y = counts,
            fill = factor(reference))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw()

```



142
143 Another way to show difference between GATK recalibration protocols decreasing the number of SNPs
144 called after readjusting of nucleotide probabilities for each read.

```

dat <- read.xlsx("./snp.counts.xlsx", sheetIndex = 3)

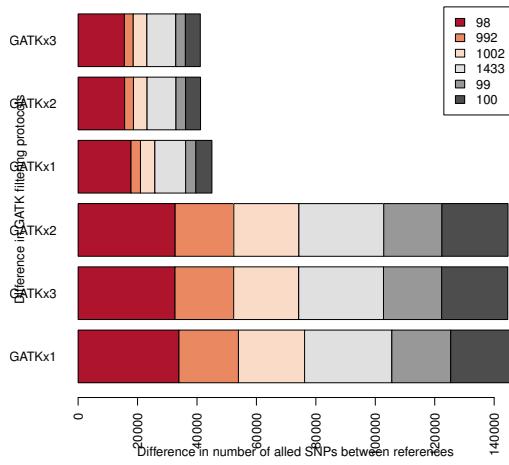
```

```

custom.colors <- c(col1 = "#b2182b",
                   col2 = "#ef8a62",
                   col3 = "#fddbc7",
                   col4 = "#e0e0e0",
                   col5 = "#999999",
                   col6 = "#4d4d4d")

barplot(as.matrix(dat[, -1]),
       horiz = TRUE,
       col = custom.colors,
       xlab = "Difference in number of alled SNPs between references",
       ylab = 'Difference in GATK filtering protocols',
       las = 2,
       legend = dat$sample)

```



145
146 **5.2 Final filtering**
147 GATK hard filtering removes SNPs with low quality or confidence. This is calculated relatively to the depth
148 of coverage. Using 3 three different thresholds for *QD* we get the number of SNPs that pass the filters.

$$QD = \frac{Confidence}{DepthCoverage} \quad (1)$$

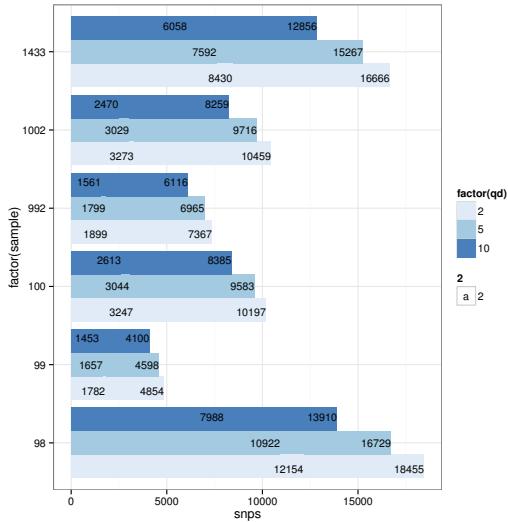
$$DepthOfCoverage = \frac{NbOfReads \times ReadLength}{AssemblySize} \quad (2)$$

149 Only one reference was used here, that is Steve Roberts' genome v15.

```

dat <- read.xlsx("./hard.snps.xlsx", sheetIndex = 1)
ggplot(dat,
       aes(x = factor(sample),
            y = snps,
            fill = factor(qd)
       #      group = factor(reference)
       )) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  geom_text(aes(x = factor(sample),
                y = snps,
                ymax = snps,
                label = snps,
                size = 2,
                hjust = 1),
            position = position_dodge(width=1)) +
  coord_flip() +
  scale_fill_brewer()

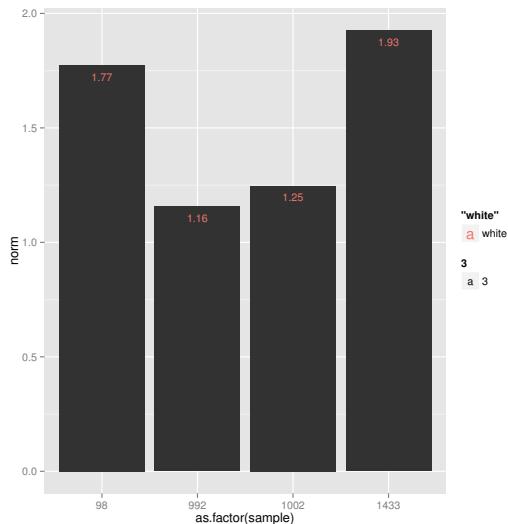
```



150
151 Number of SNPs per strain at $QD = 5$. SNPs called against *SR genome v15*. The number of reads
152 (approx 100 nt) per library has been counted and plotted above, the data is in *refreads.xlsx*.

```
dat <- read.xlsx("./hard.snps.xlsx", sheetIndex = 1)
dat <- dat[7:10, 1:2]
dat$Treads <- ref.reads[c(19, 22, 20, 21), 2]
dat$norm <- with(dat, (snps/Treads)*1000)

ggplot(dat,
       aes(x = as.factor(sample),
            y = norm)) +
  geom_bar(stat = "identity") +
  geom_text(aes(x = as.factor(sample),
                y = norm,
                ymax = norm,
                label = round(norm, digits = 2),
                color = "white",
                vjust = 2,
                size = 3))
```



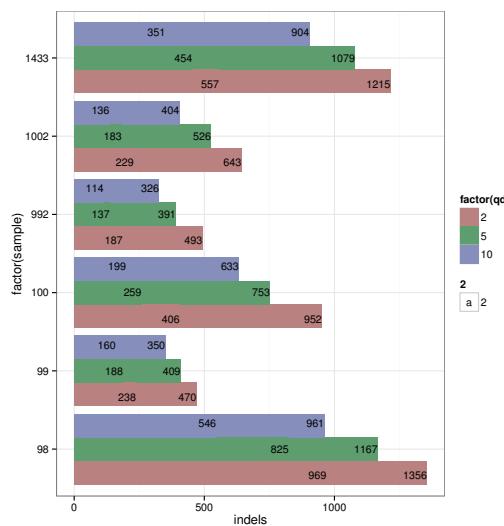
153
154 We can also do the same thing with indels.

```
dat <- read.xlsx("./hard.snps.xlsx", sheetIndex = 1)
```

```

ggplot(dat,
  aes(x = factor(sample),
      y = indels,
      fill = factor(qd))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  geom_text(aes(x = factor(sample),
                y = indels,
                ymax = indels,
                label = indels,
                size = 2,
                hjust = 1),
            position = position_dodge(width=1)) +
  coord_flip() +
  scale_fill_hue(c = 40, l = 60)

```



155

6 Working with a combined assembly

156

The combined assembly is already published. It is added here with the other references because it is heavily annotated and their contigs are extensively mapped. Future analysis of SNPs and QPX genome structure depends on good annotation data. Load in new mapped data to the combined reference:

```

combined <- read.xlsx("./snp.counts.xlsx", sheetIndex = 1)
glimpse(combined)

Variables:
$ sample     (dbl) 98, 992, 1002, 1433, 99, 100, 98, 992, 1002, 14...
$ counts     (dbl) 351790, 395060, 427790, 389188, 309813, 425947, ...
$ reference  (fctr) trxSRv21, trxSRv21, trxSRv21, trxSRv21, trxSRv...

```

160

Difference in SNPs called between the genome v15 of S. Roberts and the official combined assembly.
First extract relative rows.

```

dev <- paste("genomSRv15GATKx", seq(1,3,1), sep = "")
ser <- paste("combinedGATKx", seq(1,3,1), sep = "")
difference <- rbind(combined[combined$reference %in% dev, ],
                     combined[combined$reference %in% ser, ])

d.ref <- ref.reads[c(19:30), ]

```

162

Plot difference.

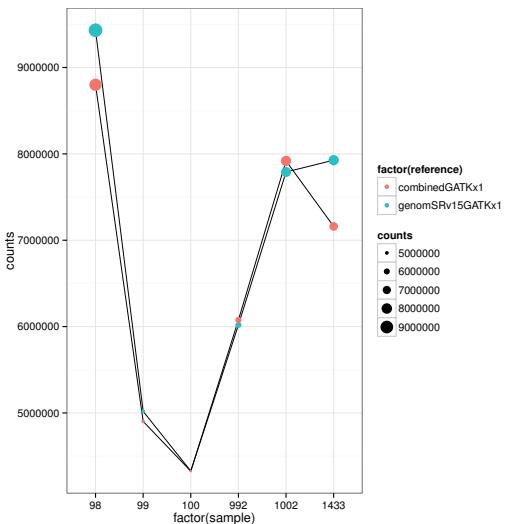
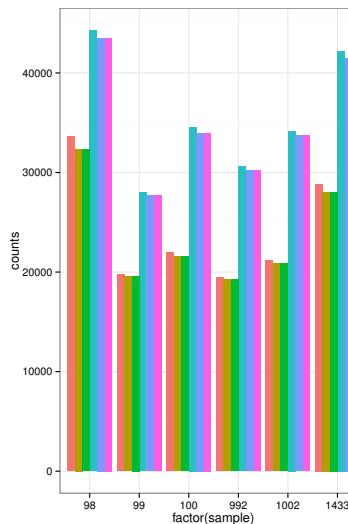
```
ggplot(difference,
```

```

aes(x = factor(sample),
    y = counts,
    fill = factor(reference))) +
geom_bar(stat = "identity",
         position = "dodge") +
theme_bw()

ggplot(d.ref,
       aes(x = factor(sample),
           y = counts,
           group = factor(reference))) +
geom_line(size = .2) +
geom_point(data = d.ref,
            aes(x = factor(sample),
                y = counts,
                colour = factor(reference),
                size = counts)) +
theme_bw()

```



163

7 Descriptive stats of all processed libraries

164
165
166
167
168
169

This following section shows the mean length of all sequences assembled from each library, the number of base pairs per library, the identified protein features from these sequences, and the number of functional enzymes identified by mapping to public libraries. It is to note the number of predicted and identified rRNA features in each of these libraries is significantly low. Regress different variables on each others for visualization purposes.

```
stats <- read.xlsx("./libraries.xlsx", sheetIndex = 1)
```

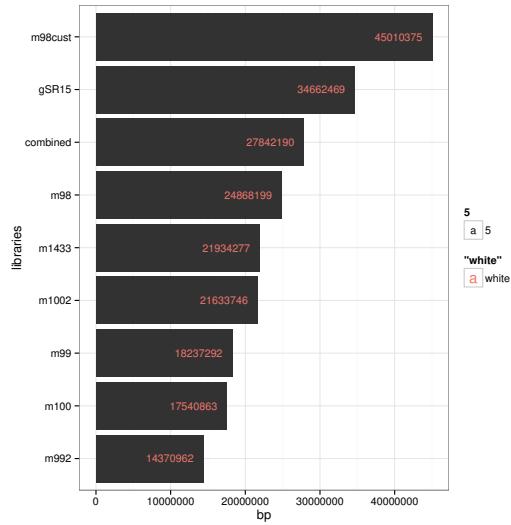
```

rstats <- stats[complete.cases(stats), ]
rownames(rstats) <- stats[, 1]

# The whole new magical script
# job: order columns
# dependecies: dplyr
rstats <- within(rstats,
  libraries <- factor(libraries,
    levels = arrange(rstats,
      bp) $libraries))

ggplot(rstats,
  aes(x = libraries,
  y = bp)) +
  geom_bar(stat = "identity") +
  theme_bw() +
  coord_flip() +
  geom_text(aes(x = libraries,
    y = bp,
    ymax = bp,
    label = bp,
    size = 5,
    color = "white",
    hjust = 1.2))

```



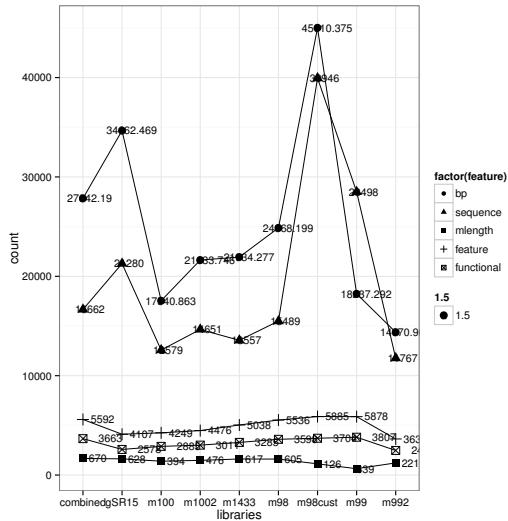
170
 171 Difference between libraries in the number of base pair (bp), which must be multiplied by 1000 bp, identi-
 172 fied protein features inside assembled sequences (feature), functional sequences in the contigs (function),
 173 the mean length in each library, and the number of contigs (sequence) assembled from raw reads after
 174 trimming and duplicate removal (all basic quality controls).

```
stats <- read.xlsx("./libraries.xlsx", sheetIndex = 1)
```

```

rstats <- stats[complete.cases(stats), ]
rstats$bp <- rstats$bp/1000
#rstats <- rename(rstats, bpx1000 = bp)
rstats <- gather(rstats, "feature", "count", c(2:4, 7:8))
ggplot(rstats,
  aes(x = libraries,
      y = count,
      group = factor(feature))) +
  geom_line(size = .2) +
  geom_point(aes(shape = factor(feature),
                 size = 1.5)) +
  theme_bw() +
  geom_text(aes(x = libraries,
                y = count,
                ymax = count,
                label = count,
                size = 1.5,
                hjust = ifelse(sign(count)>1, .5, 0)),
            position = position_dodge(width = 1))

```



175

176 Principal component analysis and diagnostics.

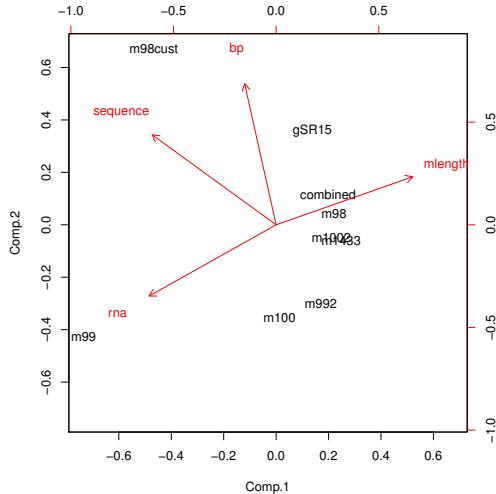
```

stats <- read.xlsx("./libraries.xlsx", sheetIndex = 1)
rownames(stats) <- stats$libraries
rstats <- stats[complete.cases(stats), -1]
rstats <- decostand(rstats, method = "range")
p = princomp(~bp + mlength + sequence + rna
             , data= rstats)
summary(p)

Importance of components:
              Comp.1   Comp.2   Comp.3   Comp.4
Standard deviation     0.459    0.382   0.1401  0.017928
Proportion of Variance 0.560    0.387   0.0522  0.000855
Cumulative Proportion  0.560    0.947   0.9991  1.000000

biplot(p)

```



177
178

Finally a summary of all sequence data.

```
stats[, -1]

      bp sequence mlength   sd mgc feature functional rna
m98     24868199    15489    1605 1765  45    5536     3598  21
m98cust 45010375    39946    1126 1505  42    5885     3704  60
m992    14370962    11767    1221  921  46    3632     2476   7
m1433   21934277    13557    1617 1677  46    5038     3285  29
m1002   21633746    14651    1476 1133  45    4476     3011   9
m99     18237292    28498     639  504  49    5878     3807 229
m100    17540863    12579    1394 1042  46    4249     2885 121
gSR15   34662469    21280    1628 2907  44    4107     2578   9
combined 27842190    16662    1670 1908  45    5592     3663  34
```

179
180
181

8 Applied annotations, subsystem predictions, and taxonomic distribution

Like the title implies, identified and predicted annotations and protein features are mapped to public sequence libraries. Reshape data, transform columns into rows.

```
predicted <- read.xlsx("./libraries.xlsx", sheetIndex = 3)
predicted <- gather(predicted, "ko", "count", 3:8, na.rm = TRUE)
summary(predicted)

  lib     chart          ko        count
combined: 6  ko:54 cellular :9  Min.   : 31
gSR15   : 6  environmental:9  1st Qu.:132
m100    : 6  genetic    :9  Median :272
m1002   : 6  disease    :9  Mean   :361
m1433   : 6  metabolism :9  3rd Qu.:589
m98     : 6  organisms  :9  Max.   :1471
(Other)  :18
```

182

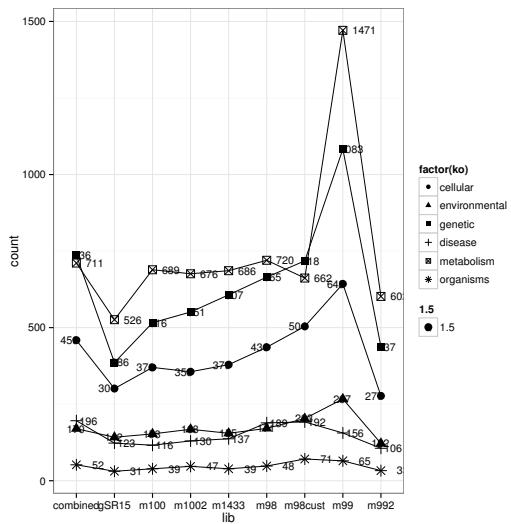
Plot difference in identified protein features between libraries.

```
ggplot(predicted,
```

```

aes(x = lib,
    y = count,
    group = factor(ko)) +
geom_line(size = .2) +
geom_point(aes(shape = factor(ko),
               size = 1.5)) +
theme_bw() +
geom_text(aes(x = lib,
              y = count,
              ymax = count,
              label = count,
              size = 1.5,
              hjust = ifelse(sign(count)>1, .5, 0)),
          position = position_dodge(width = 1))

```

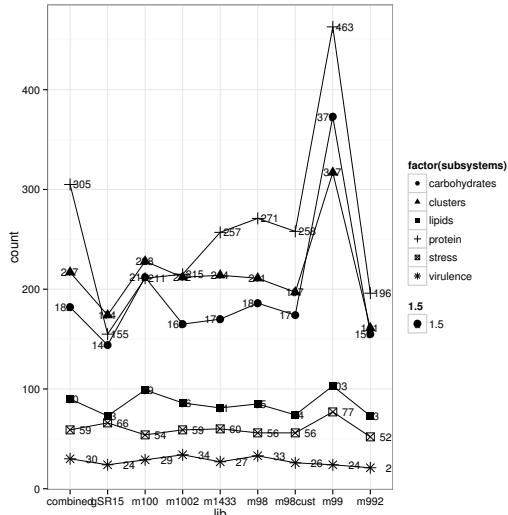


183
184 In this next snippet subsystems are discussed. **Functional coupling and chromosomal clusters** are shown
185 for *clustering-based subsystems* among other subsystems.

```

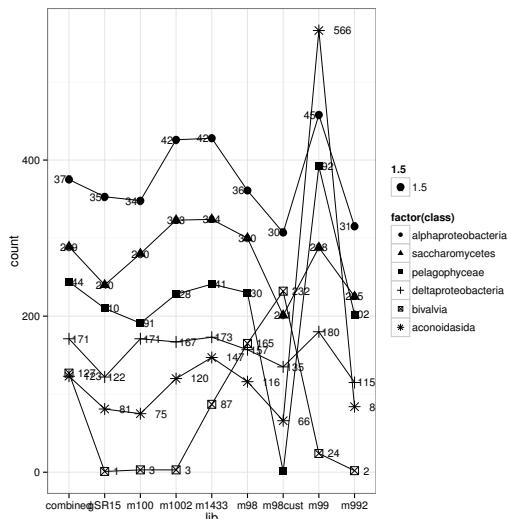
predicted <- read.xlsx("./libraries.xlsx", sheetIndex = 4)
predicted <- gather(predicted, "subsystems", "count", 3:8, na.rm= TRUE)
ggplot(predicted,
       aes(x = lib,
           y = count,
           group = factor(subsystems))) +
  geom_line(size = .2) +
  geom_point(aes(shape = factor(subsystems),
                 size = 1.5)) +
  theme_bw() +
  geom_text(aes(x = lib,
                y = count,
                ymax = count,
                label = count,
                size = 1.5,
                hjust = ifelse(sign(count)>1, .5, 0)),
            position = position_dodge(width = 1))

```



186
187 Finally, a taxonomic classification on sequence similarities gives insights on sequence relatedness or
188 sample contamination. Five classes were selected, bacteria, fungi, algae, parasite, and bivalvia.

```
predicted <- read.xlsx("./libraries.xlsx", sheetIndex = 5)
predicted <- gather(predicted, "class", "count", c(3,5:9), na.rm = TRUE)
ggplot(predicted,
  aes(x = lib,
      y = count,
      group = factor(class))) +
  geom_line(size = .2) +
  geom_point(aes(shape = factor(class),
                  size = 1.5)) +
  theme_bw() +
  geom_text(aes(x = lib,
                y = count,
                ymax = count,
                label = count,
                size = 1.5,
                hjust = ifelse(sign(count)>1, .5, 0)),
            position = position_dodge(width = 1))
```



189
190 **9 Shared SNPs between libraries**
191 Shared SNPs between libraries mapped to SR genome v15.

```
shared.snps <- read.table("./shared.snps.txt", fill =TRUE)
```

```
shared.snps
```

	X132	X1433.0.9..	X992.1.9..
200	98 (1.2%)	992 (2.9%)	
314	1002 (3.2%)	1433 (2.1%)	
328	1002 (3.4%)	1433 (2.1%)	992 (4.7%)
587	1002 (6.0%)	98 (3.5%)	992 (8.4%)
589	1433 (3.9%)	98 (3.5%)	992 (8.5%)
632	1002 (6.5%)	98 (3.8%)	
655	992 (9.4%)		
825	1002 (8.5%)	992 (11.8%)	
1679	1002 (17.3%)		
1702	1002 (17.5%)	1433 (11.1%)	98 (10.2%)
2577	1433 (16.9%)		
3394	98 (20.3%)		
3649	1002 (37.6%)	1433 (23.9%)	98 (21.8%)
992 (52.4%)			
5976	1433 (39.1%)	98 (35.7%)	

192 Shared indels between libraries mapped to SR genome v15.

```
shared.indels <- read.table("./shared.indels.txt", fill = TRUE)  
shared.indels
```

	X8	X98.0.7..	X992.2.0..
12	1433 (1.1%)	992 (3.1%)	
14	1002 (2.7%)	1433 (1.3%)	992 (3.6%)
15	1002 (2.9%)	1433 (1.4%)	
31	1002 (5.9%)	98 (2.7%)	
40	1433 (3.7%)	98 (3.4%)	992 (10.2%)
41	1002 (7.8%)	98 (3.5%)	992 (10.5%)
62	1002 (11.8%)	992 (15.9%)	
68	992 (17.4%)		
78	1002 (14.8%)	1433 (7.2%)	98 (6.7%)
139	1002 (26.4%)		
146	1002 (27.8%)	1433 (13.5%)	98 (12.5%)
992 (37.3%)			
267	1433 (24.7%)		
316	98 (27.1%)		
507	1433 (47.0%)	98 (43.4%)	

193 10 Component analysis and sequence closeness

194 Import annotated data.

```
closeness <- read.csv("./pca.csv", sep = "\t")
```

```
summary(closeness)
```

```
metagenome
mmetsp1002:1141
mmetsp1433:1278
mmetsp98 :1279
mmetsp992 :1088
QPX_v15 :1030
```

```
level.1
Carbohydrates : 848
Amino Acids and Derivatives : 785
Protein Metabolism : 769
Clustering-based subsystems : 559
Miscellaneous : 552
Cofactors, Vitamins, Prosthetic Groups, Pigments: 462
(Other) :1841
```

```
level.2
0 : 750
Plant-Prokaryote DOE project : 506
Protein biosynthesis : 499
RNA processing and modification: 346
Central carbohydrate metabolism: 326
Folate and pterines : 278
(Other) :3111
```

```
level.3
YgfZ : 120
Ribosome LSU eukaryotic and archaeal: 101
Proteasome eukaryotic : 91
Ribosome SSU eukaryotic and archaeal: 91
Serine-glyoxylate cycle : 88
tRNA modification Bacteria : 76
(Other) :5249
```

```
function.
GTP cyclohydrolase I (EC 3.5.4.16) type 1 : 70
Acetyl-CoA acetyltransferase (EC 2.3.1.9) : 55
Serine hydroxymethyltransferase (EC 2.1.2.1) : 50
Cysteine desulfurase (EC 2.8.1.7) : 48
3-ketoacyl-CoA thiolase (EC 2.3.1.16) : 40
Branched-chain amino acid aminotransferase (EC 2.6.1.42): 40
(Other) :5513
```

	abundance	avg.eValue	avg...ident	avg.align.len
1	:3943	Min. : -269	Min. :-183.0	Min. : 24
2	:1140	1st Qu.: -57	1st Qu.: 63.2	1st Qu.: 61
3	: 280	Median : -29	Median : 66.2	Median : 92
4	: 155	Mean : -41	Mean : 65.7	Mean : 118
5	: 69	3rd Qu.: -15	3rd Qu.: 70.2	3rd Qu.:156
6	: 68	Max. : 3	Max. : 95.6	Max. :544
(Other)	: 161			

```
X..hits X
Min. : 1 Min. :1
1st Qu.: 1 1st Qu.:1
Median : 1 Median :1
Mean : 4 Mean :1
3rd Qu.: 2 3rd Qu.:2
Max. :457 Max. :3
NA's :5727
```

```
closeness <- closeness[, c(1, 7:10)]
```

195 Principal component analysis on 5 libraries, 4 strains and the genome (v15), using an *identity score* for
196 annotating a sequence and an *alignment length score* for similarities with a functional feature, an *e-value*
197 *score* for functional similarities, and the *number of hits*, ie., the number of times a function is identified in
198 a library.

```

rownames(closeness) <- paste(closeness[, 1], 1:nrow(closeness), sep = ".")
x=closeness[, -1]
head(x)

    avg.eValue avg...ident avg.align.len X..hits
mmetsp1433.1      -57      74.7       142      1
QPX_v15.2          -57      74.7       142      1
mmetsp98.3          -57      74.7       142      1
mmetsp1002.4        -57      74.7       142      1
mmetsp1433.5        -60      64.7       173      1
QPX_v15.6          -60      64.7       173      1

results <- decostand(x, method = "range")
head(results)

    avg.eValue avg...ident avg.align.len X..hits
mmetsp1433.1      0.779     0.925      0.227      0
QPX_v15.2          0.779     0.925      0.227      0
mmetsp98.3          0.779     0.925      0.227      0
mmetsp1002.4        0.779     0.925      0.227      0
mmetsp1433.5        0.768     0.889      0.287      0
QPX_v15.6          0.768     0.889      0.287      0

p = princomp(~ avg...ident + avg.align.len
, data= results)
summary(p)

Importance of components:
                                         Comp.1   Comp.2
Standard deviation      0.156 0.0605
Proportion of Variance  0.869 0.1309
Cumulative Proportion   0.869 1.0000

#plot(p, type = "l")
#biplot(p, cex = .4)

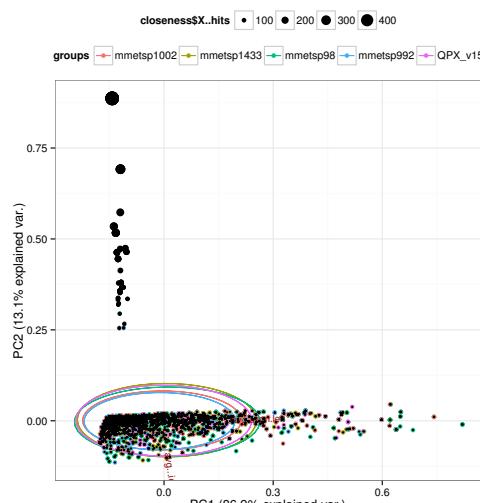
```

199 Clustering and visualization of all sequences without applying any filters.

```

ggbiplot(p, obs.scale = 1,
         var.scale = 1,
         groups = closeness$metagenome,
         ellipse = TRUE,
         circle = FALSE) +
  geom_point(aes(size = closeness$X..hits)) +
  theme_bw() +
  theme(legend.direction = 'horizontal',
        legend.position = 'top')

```



200

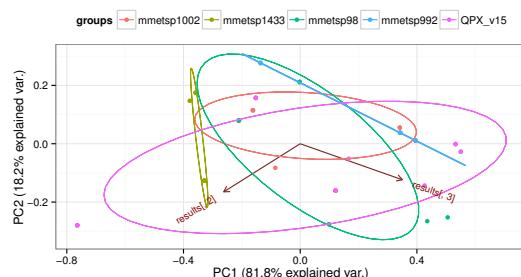
201 Build a custom PCA function for repetitive iterations.

```
customBiplot <- function(data, method) {  
  x = data[, -1]  
  results <- decostand(x, method = method)  
  p = princomp(~ results[, 2] + results[, 3]  
    , data = results)  
  ggbiplot(p, obs.scale = 1,  
    var.scale = 1,  
    groups = data$metagenome,  
    ellipse = TRUE,  
    circle = FALSE) +  
    theme_bw() +  
    theme(legend.direction = 'horizontal',  
      legend.position = 'top')  
}
```

202 Filter sequences depending on their alignment length and the abundance of a function.

```
closenessX <- filter(closeness, avg.align.len < 50, X..hits > 2)  
dim(closenessX) [1]  
  
[1] 57  
  
customBiplot(closenessX, method = "range")
```

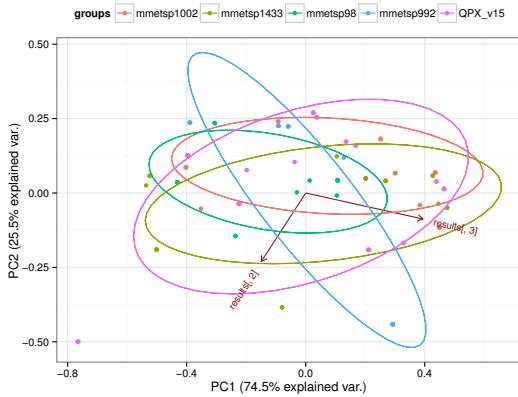
[†] results[2] = identity and
results[3] = alignment length



203

204 Select higher alignment similarities.

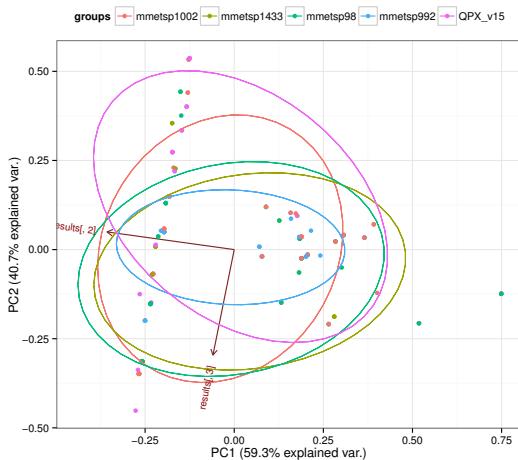
```
closenessX <- filter(closeness, avg.align.len < 60, X..hits > 2)  
dim(closenessX) [1]  
  
[1] 117  
  
customBiplot(closenessX, method = "range")
```



205
206 Select even higher alignment similarities.

```
closenessX <- filter(closeness, avg.align.len < 100, X..hits > 4)
dim(closenessX) [1]
[1] 199

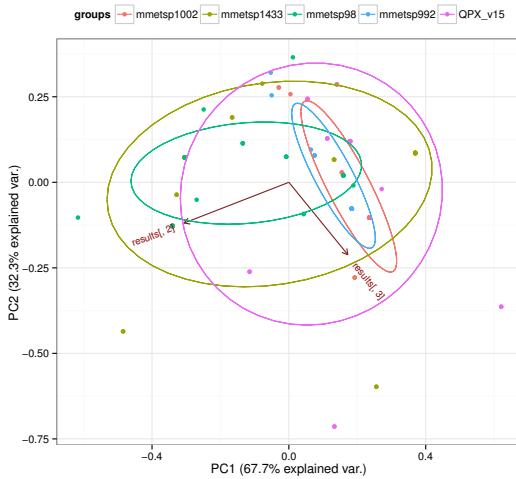
customBiplot(closenessX, method = "range")
```



207
208 Select on the criteria of e-Value and abundance of a functional sequence.

```
closenessX <- filter(closeness, avg.eValue < -40, X..hits > 2)
dim(closenessX) [1]
[1] 152

customBiplot(closenessX, method = "range")
```

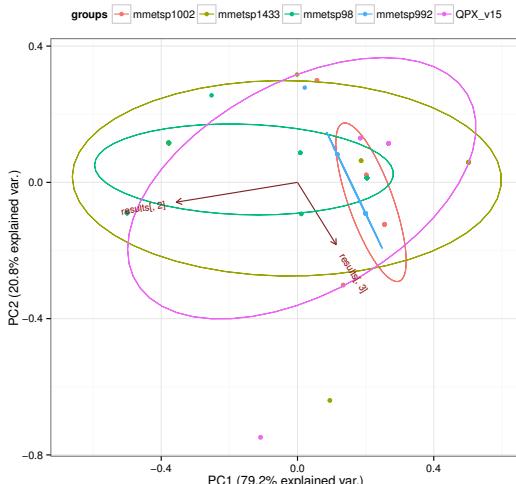


209
210

Select on the criteria of e-Value and abundance of a functional sequence.

```
closenessX <- filter(closeness, avg.eValue < -40, X..hits > 3)
dim(closenessX) [1]
[1] 126

customBiplot(closenessX, method = "range")
```

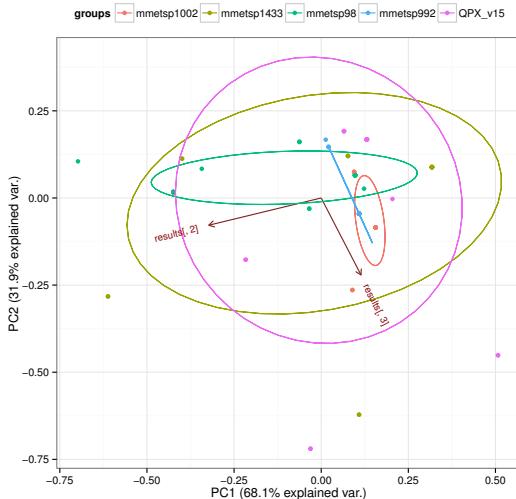


211
212

Select on the criteria of e-Value and abundance of a functional sequence.

```
closenessX <- filter(closeness, avg.eValue < -50, X..hits > 2)
dim(closenessX) [1]
[1] 109

customBiplot(closenessX, method = "range")
```

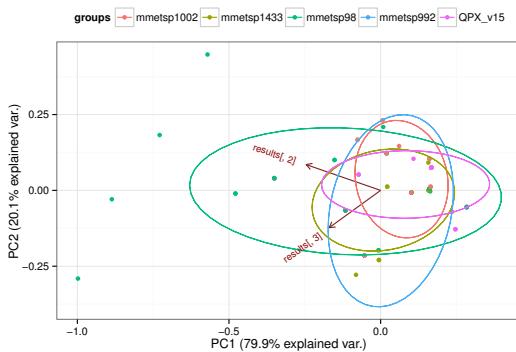


213
214 Select on the criteria of alignment length. Since SNP aggregation tests are the next step in this analysis,
215 the length of a correct alignment is technically helpful in differentiating SNP position. And abundance will
216 be more than 2 to increase probabilities of correct functional annotation.

```
closenessX <- filter(closeness, avg.align.len > 200, X..hits >= 2)
dim(closenessX) [1]

[1] 100

customBiplot(closenessX, method = "range")
```



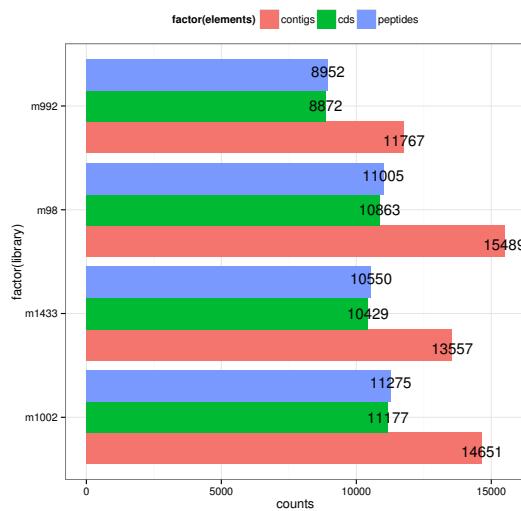
217
218 **11 Aggregation analysis of SNPs**
219 MMETSP libraries are already been annotated. How many contigs, peptide and cds elements are in-
220 dexed?

```
contigs <- read.xlsx("./annot.stats.xlsx", sheetIndex = 1)
```

```

contigs <- gather(contigs, "elements", "counts", 2:4)
ggplot(contigs,
  aes(x = factor(library),
      y = counts,
      fill = factor(elements))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  theme(legend.direction = 'horizontal',
        legend.position = 'top') +
  coord_flip() +
  geom_text(aes(x = factor(library),
                y = counts,
                ymax = counts,
                label = counts,
                hjusts = ifelse(sign(counts) > 0, 1, 0)),
            position = position_dodge(width = 1))

```



221

222 11.1 Preferential substitution

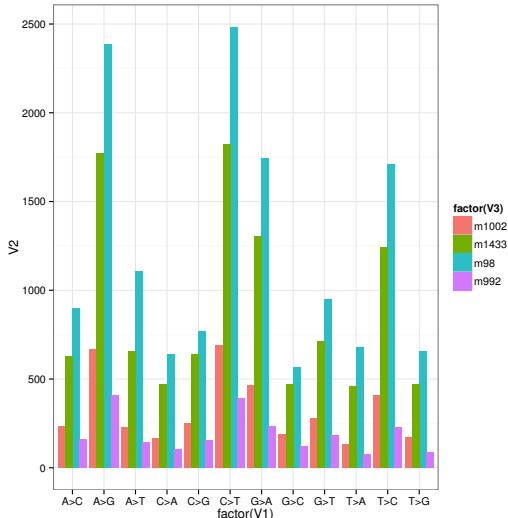
223 Preferential substitution of nucleotides. It should be noted that *mmetsp0098* and *mmetsp1433* are both
 224 bigger in library size than the others. Therefore comparison of SNPs should be done for each library
 225 separately. However there is a resemblance in substitution between libraries since the pattern is quite
 226 similar for all nucleotides.

```

prefs <- read.table("./all.stats.txt")
prefs$V3 <- c(rep("m1002", 12),
             rep("m98", 12),
             rep("m992", 12),
             rep("m1433", 12))

ggplot(prefs,
  aes(x = factor(V1),
      y = V2,
      fill = factor(V3))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw()

```



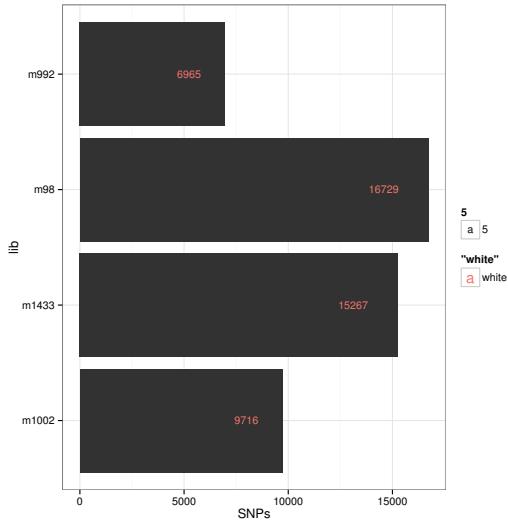
227
228 After hard filtering SNPs to the minimum from all 4 libraries, *DISCARD*-labelled SNPs were removed. The
229 remaining were imported into data frames with the following columns.

- 230 1. CHROM: number of contig
231 2. POS: SNP position on that contig
232 3. ALT: alternative SNP to the reference
233 4. AD: allelic depth for the reference and ALT alleles
234 5. DP: approximate read depth
235 6. GQ: genotype quality
236 7. PL: normalized phred scaled likelihoods

237 The structure of the data frame is similar to the *iris* data.

```
x <- c('m98', 'm1433', 'm1002', 'm992')
y <- c(16729, 15267, 9716, 6965)
dat <- data.frame(lib = x, SNPs = y)
ggplot(dat,
  aes(x = lib,
      y = SNPs)) +
  geom_bar(stat = "identity") +
  theme_bw() +
  coord_flip() +
  geom_text(aes(x = lib,
                y = SNPs,
                ymax = SNPs,
                label = SNPs,
                size = 5,
                col = "white",
                hjust = 2))
```

[†] With low number of sample it is impossible to create a f(SNP)=strain machine learning framework. To make a binary table of SNPs at least 100 samples must be used.



238
 239 Import SNP data: data manipulation process of removing NAs and getting the same number of SNPs
 240 across all samples.

```
m98 <- read.table("./m98.ml.txt", fill = NA)
m1433 <- read.table("./m1433.ml.txt", fill = NA)
m992 <- read.table("./m992.ml.txt", fill = NA)
m1002 <- read.table("./m1002.ml.txt", fill = NA)

colnames(m98) <- c('contigs', 'pos', 'ad1', 'ad2',
                     'dp', 'qq', 'p11', 'p12', 'p13', 'lib')
colnames(m1433) <- c('contigs', 'pos', 'ad1', 'ad2',
                     'dp', 'qq', 'p11', 'p12', 'p13', 'lib')
colnames(m992) <- c('contigs', 'pos', 'ad1', 'ad2',
                     'dp', 'qq', 'p11', 'p12', 'p13', 'lib')
colnames(m1002) <- c('contigs', 'pos', 'ad1', 'ad2',
                     'dp', 'qq', 'p11', 'p12', 'p13', 'lib')

m98 <- m98[complete.cases(m98), ]
m1433 <- m1433[complete.cases(m1433), ]
m992 <- m992[complete.cases(m992), ]
m1002 <- m1002[complete.cases(m1002), ]

m98 <- m98[! m98$lib != 'm98', ]
m1433 <- m1433[! m1433$lib != 'm1433', ]
m992 <- m992[! m992$lib != 'm992', ]
m1002 <- m1002[! m1002$lib != 'm1002', ]

m1433$p13 <- as.numeric(m1433$p13)

m98$lib <- factor(m98$lib, "m98")
m992$lib <- factor(m992$lib, "m992")
m1433$lib <- factor(m1433$lib, "m1433")
m1002$lib <- factor(m1002$lib, "m1002")

index <- min(dim(m98)[1], dim(m1433)[1],
             dim(m992)[1], dim(m1002)[1])
set.seed(123)
mall <- rbind(m98[sample(nrow(m98), index), ],
               m1433[sample(nrow(m1433), index), ],
               m992[sample(nrow(m992), index), ],
               m1002[sample(nrow(m1002), index), ])
dim(mall)
[1] 27844 10
```

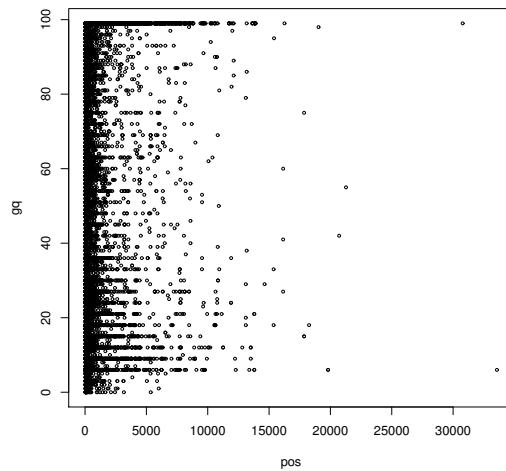
11.2 Available data

241 Regressing the genome quality of SNPs on the position of the SNPs inside a contig. This shows that
242 SNPs are concentrated in the first 10 Kb.
243

```
head(mall)

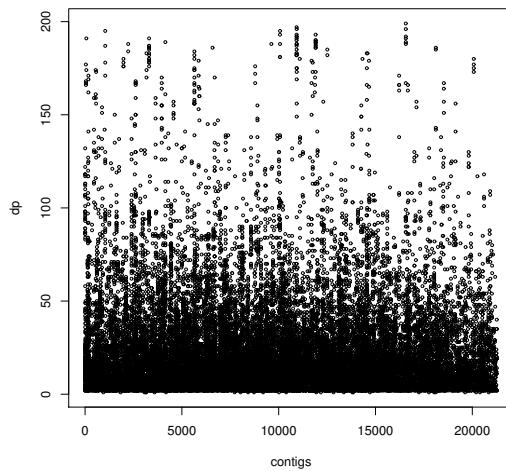
  contigs pos ad1 ad2 dp gq pl1 pl2 pl3 lib
4825      5776 244   5  18 23 99 786    0 150 m98
13214     14964  96   6   6 12 99 234    0 234 m98
6857      7810 176   3  11 14 68 392    0  68 m98
14801     17502  56   9   7 16 99 230    0 337 m98
15760     19016  80   0   7  7 21 315   21   0 m98
763       884  810  17  29 46 99 986    0 516 m98

with(mall, plot(pos, gq, cex = .5))
```



244 Regression of contigs and the read depth for each SNPs in those contigs. When using libraries mapped
245 to the combined assembly (as a reference transcriptome) the plot shows that the depth of coverage
246 distinguishes between 2 different subsets of contigs. However the regression is constant when using the
247 genome of SR v15 as a reference for mapping the libraries (as shown below).
248

```
with(mall, plot(contigs, dp, cex = .5))
submall <- filter(mall, dp > 50, pos <= 10000)
```



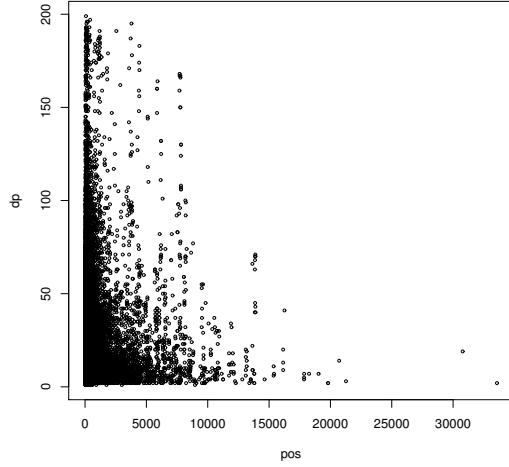
249 This plot shows that 11.41 % of the SNPs have a depth over 50 for the first 10 Kb.
250

```
with(mall, plot(pos, dp, cex = .5))
```

```
## percentage of SNPs with read depth higher than 35
```

```
(nrow(submall) / nrow(mall)) * 100
```

```
[1] 11.4
```

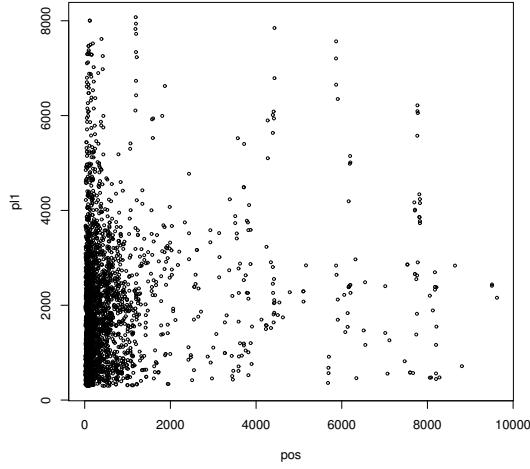


251

252 Plotting only SNPs with DP > 50 and in contigs which length <= 10 Kb, and regressing toward a phred-scaled adjusted likelihood for each variant or genotype likelihood.

```
with(submall, plot(pos, pl1, cex = .5))  
summary(submall$lib)
```

```
m98 m1433 m992 m1002  
1256 1127 417 377
```



254

Linear regression between position of the SNP and the normalized phred scaled likelihood, which on its own is an accuracy determination score. Phred likelihoods (PL) are computed for the REF/REF, REF/ALT, and ALT/ALT variants. To convert a PL to a raw likelihood L:

$$P(L|AA) = 10^{-PL/10} \quad (3)$$

255 These probabilities are adjusted with phred scores. They determine the probability of a base observed
256 given a reference genotype, an heterozygous genotype or a non-reference genotype respectively (pl1,
257 pl2, and pl3).

258 Accordingly, REF/REF (pl1) is significant. Meaning the genotype we have is homozygous for the reference
259 nucleotide (not the variant), but if a variant exists, thus it represents a rare mutation (*reference needed*).
260 Therefore, the raw likelihoods must be calculated with the equation above for the picked variants and the
261 genotype with $P=1$ is the most significant genotype at that nucleotide.

```

fit <- lm(pos~pl1, data = submall)
summary(fit)

Call:
lm(formula = pos ~ pl1, data = submall)

Residuals:
    Min      1Q  Median      3Q     Max 
-1353   -484   -344    -97   8996 

Coefficients:
            Estimate Std. Error t value Pr(>|t|)    
(Intercept) 315.2290   39.9153    7.90  3.9e-15 ***
pl1          0.1442    0.0171    8.42  < 2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 1280 on 3175 degrees of freedom
Multiple R-squared:  0.0219, Adjusted R-squared:  0.0216 
F-statistic:  71 on 1 and 3175 DF,  p-value: <2e-16

```

262 Lets get the variants with the highest probability that a genotype has been identified. $PL=1$ determines
 263 the genotype, either homozygous for REF (pl1) or ALT (pl3) or heterozygous REF/ALT (pl2).

```

submall[, 7:9] <- apply(submall[, 7:9], 2, function(x) 10^{(-x/1000)})
head(submall)

contigs pos ad1 ad2 dp gq      pl1 pl2      pl3 lib
1      6470 215 43  21  64 99 0.18113    1 0.01717908 m98
2     20921 54  88  21 109 99 0.22751    1 0.000000196 m98
3     13280 47  78  19  97 99 0.27542    1 0.00054954 m98
4     10194 378  5  49  54 63 0.01064    1 0.86496792 m98
5     19812 80  55  41  96 99 0.04027    1 0.00693426 m98
6     4446 65  15  65  80 99 0.00514    1 0.49545019 m98

```

264 Lets extract all heterozygous alleles with at least 90 % confidence.

```

heteromall <- filter(submall, pl2 >= .9)

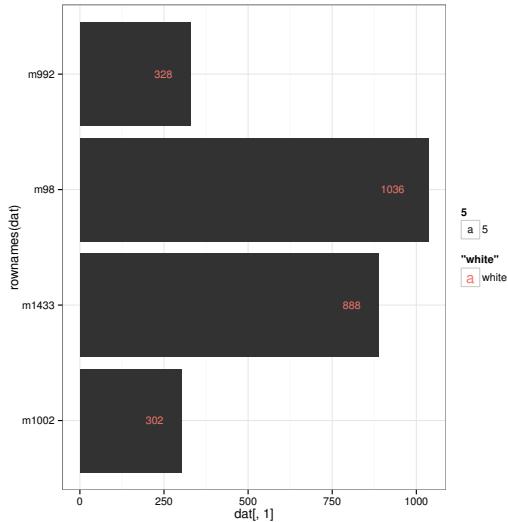
```

265 The original number of SNPs was 3177 among which the number of variants with heterozygous genotype
 266 is 2554.

```

dat <- as.data.frame(summary(heteromall$lib))
ggplot(dat,
       aes(x = rownames(dat),
           y = dat[, 1])) +
  theme_bw() +
  geom_bar(stat = "identity") +
  coord_flip() +
  geom_text(aes(x = rownames(dat),
                y = dat[, 1],
                ymax = dat[, 1],
                size = 5,
                label = dat[, 1],
                col = "white",
                hjust = 2))

```

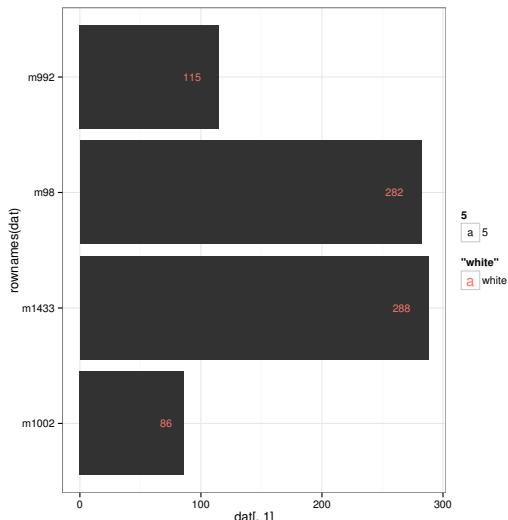


267
268 Now lets get the homozygous variants with genotype ALT/ALT with 90 %.

```
269 altmall <- filter(submall, pl3 >= .9)
```

270 The number of variants ALT/ALT is 771. Interesting thing is that using the combined assembly as a
271 reference, m1433 had also the highest number of homozygous alleles while m98 had half the number
shown below.

```
dat <- as.data.frame(summary(altmall$lib))
ggplot(dat,
       aes(x = rownames(dat),
            y = dat[, 1])) +
  theme_bw() +
  coord_flip() +
  geom_bar(stat = "identity") +
  geom_text(aes(x = rownames(dat),
                y = dat[, 1],
                ymax = dat[, 1],
                label = dat[, 1],
                size = 5,
                color = "white",
                hjust = 2))
```



272
273 **12 Protein domain annotation**
274 Get the number of protein domains from the MMETSP strains. First, contigs must be translated into pep-
275 tides. HMMER3.2b was used for annotation. Hidden Markov Models were generated on Pfam database.
276 The table below lists old and new annotations against old and new Pfam v26 and v28 libraries. (> 2 years
277 interval between versions).

```

pfam <- read.xlsx("./pfam.xlsx", sheetIndex = 1)
pfam

      domain pfam  a98 s98 a992 s992 a1002 s1002 a1433 s1433
1     virulence  655 5098 313 3075   261  4606   291  4794   308
2    temperature  251 2484 168 1680   141  2283   164  2277   161
3     salinity   22 163 13 91     9  123   10  137   12
4 salt tolerance   79 2231 70 1422   64  2097   66  2078   66
5     virulence  655 5306 331 3185   275  4763   302  4973   326
6    temperature  251 2704 179 1771   145  2436   170  2478   170
7     salinity   22 161 12 97     10  128   10  138   10
8 salt tolerance   79 2267 73 1451   68  2108   67  2138   69
  annot
1   old
2   old
3   old
4   old
5   new
6   new
7   new
8   new

```

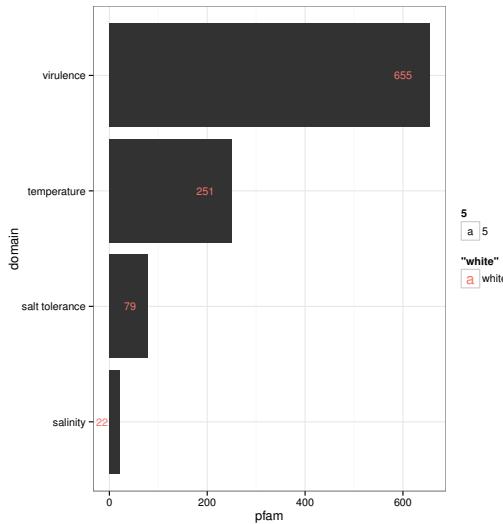
278 Number of domains found in Pfam v28 for :

- 279 • Virulence
- 280 • Temperature
- 281 • Salinity
- 282 • Salt tolerance

```

ggplot(pfam[1:4, ],
       aes(x = domain,
            y = pfam)) +
  coord_flip() +
  theme_bw() +
  geom_bar(stat = "identity") +
  geom_text(aes(x = domain,
                y = pfam,
                ymax = pfam,
                label = pfam,
                size = 5,
                color = "white",
                hjust = 2))

```



283
284 All domains of the above proteins found in the 4 strain libraries.

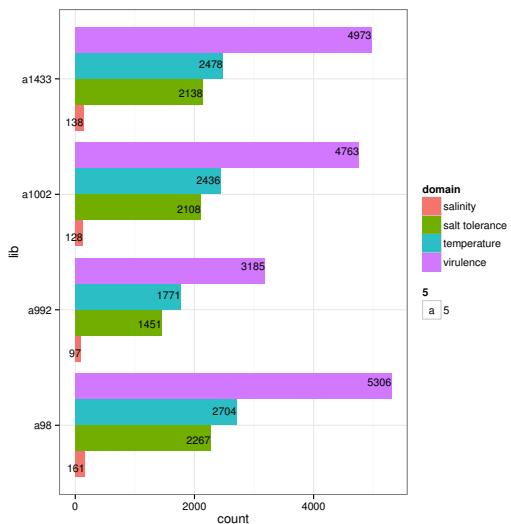
```

allpfam <- select(pfam, contains("a"))
allpfam <- filter(allpfam, annot == "new")
allpfam

      domain pfam   a98  a992  a1002  a1433 annot
1     virulence    655  5306  3185   4763   4973   new
2   temperature    251  2704  1771   2436   2478   new
3   salinity      22   161   97    128    138   new
4 salt tolerance    79  2267  1451   2108   2138   new

allpfam <- gather(allpfam[, -2], "lib", "count", 2:5)
ggplot(allpfam,
  aes(x = lib,
       y = count,
       fill = domain)) +
  theme_bw() +
  coord_flip() +
  geom_bar(stat = "identity",
            position = "dodge") +
  geom_text(aes(x = lib,
                    y = count,
                    ymax = count,
                    label = count,
                    size = 5,
                    hjust = 1),
             position = position_dodge(width = 1))

```



285

286 How many possible proteins can be found among the 4 strains.

```

singlepfam <- select(pfam, contains("s"))

```

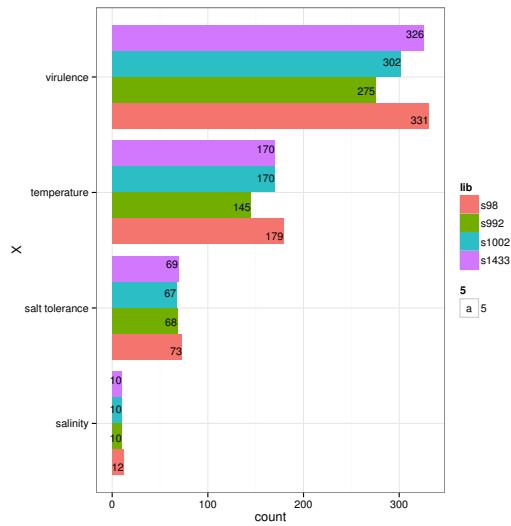
```

singlepfam <- cbind(singlepfam, X = pfam$domain, Y = pfam$annot)
singlepfam <- filter(singlepfam, Y == "new")
singlepfam

  s98 s992 s1002 s1433          X     Y
1 331 275   302   326      virulence new
2 179 145   170   170    temperature new
3 12   10    10    10      salinity new
4 73   68    67    69  salt tolerance new

singlepfam <- gather(singlepfam, "lib", "count", 1:4)
ggplot(singlepfam,
  aes(x = X,
      y = count,
      fill = lib)) +
  theme_bw() +
  coord_flip() +
  geom_bar(stat = "identity",
            position = "dodge") +
  geom_text(aes(x = X,
                y = count,
                ymax = count,
                label = count,
                size = 5,
                hjust = 1),
            position = position_dodge(width = 1))

```



287

288 Difference between old and new annotations against pfam database.

```

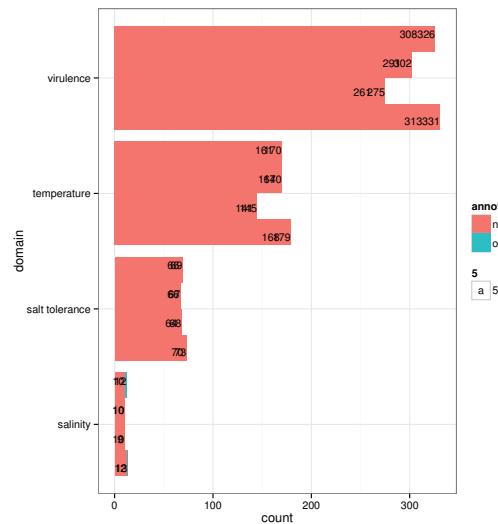
newpfam <- select(pfam, contains("s"))

```

```

newpfam <- cbind(newpfam, annot = pfam$annot, domain = pfam$domain)
newpfam <- gather(newpfam, "lib", "count", 1:4)
ggplot(newpfam,
  aes(x = domain,
      y = count,
      fill = annot,
      group = lib)) +
  theme_bw() +
  coord_flip() +
  geom_bar(stat = "identity",
            position = "dodge") +
  geom_text(aes(x = domain,
                y = count,
                ymax = count,
                label = count,
                size = 5,
                hjust = 1),
            position = position_dodge(width = 1))

```



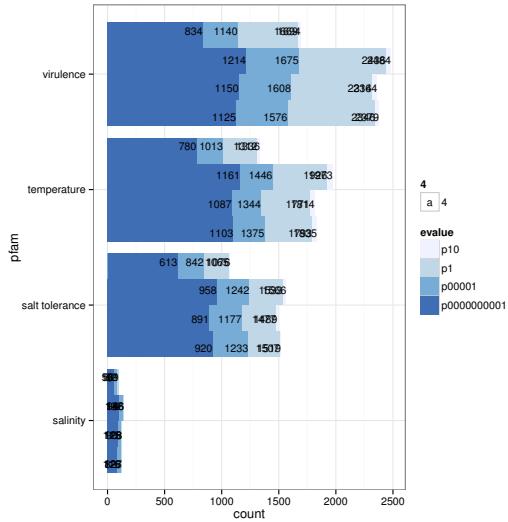
289
290 Get the number of contigs that match a significant e-value domain.

```
pfam2 <- read.xlsx("./pfam.xlsx", sheetIndex = 2)
```

```
pfam2
```

```
    lib  p10    p1 p00001 p0000000001 annot      pfam
1   m98  339  332    166      136 domain  virulence
2   m992  278  276    141      108 domain  virulence
3   m1002 304  300    145      116 domain  virulence
4   m1433 328  325    161      131 domain  virulence
5   m98 2484 2436   1675     1214 contig virulence
6   m992 1694 1669   1140     834 contig virulence
7   m1002 2379 2346   1576     1125 contig virulence
8   m1433 2364 2314   1608     1150 contig virulence
9   m98  179  175    142      132 domain  temperature
10  m992  147  145    113      99 domain  temperature
11  m1002 170  168    124     108 domain  temperature
12  m1433 172  167    138     126 domain  temperature
13  m98 1973 1926   1446     1161 contig temperature
14  m992 1336 1312   1013     780 contig temperature
15  m1002 1835 1793   1375     1103 contig temperature
16  m1433 1814 1771   1344     1087 contig temperature
17  m98   14   14     10       8 domain   salinity
18  m992   14   13     9        7 domain   salinity
19  m1002  13   12     9        8 domain   salinity
20  m1433  12   12     9        8 domain   salinity
21  m98  146  146    132     99 contig  salinity
22  m992 101   99    82      56 contig  salinity
23  m1002 127  126    116     82 contig  salinity
24  m1433 128  128    115     90 contig  salinity
25  m98   75   75     66      60 domain  salt tolerance
26  m992   70   70     66      56 domain  salt tolerance
27  m1002  72   71     64      57 domain  salt tolerance
28  m1433  73   73     67      58 domain  salt tolerance
29  m98 1566 1533   1242    958 contig  salt tolerance
30  m992 1076 1065   842     613 contig  salt tolerance
31  m1002 1519 1507   1233    920 contig  salt tolerance
32  m1433 1489 1477   1177    891 contig  salt tolerance
```

```
pfam2 <- filter(pfam2, annot == "contig")
pfam2 <- gather(pfam2, "evalute", "count", 2:5)
ggplot(pfam2,
  aes(x = pfam,
      y = count,
      fill = evalute,
      group = lib)) +
  theme_bw() +
  geom_bar(stat = "identity",
            position = "dodge") +
  scale_fill_brewer() +
  coord_flip() +
  geom_text(aes(x = pfam,
                y = count,
                ymax = count,
                label = count,
                size = 4,
                hjust = 1),
            position = position_dodge(width = 1))
```



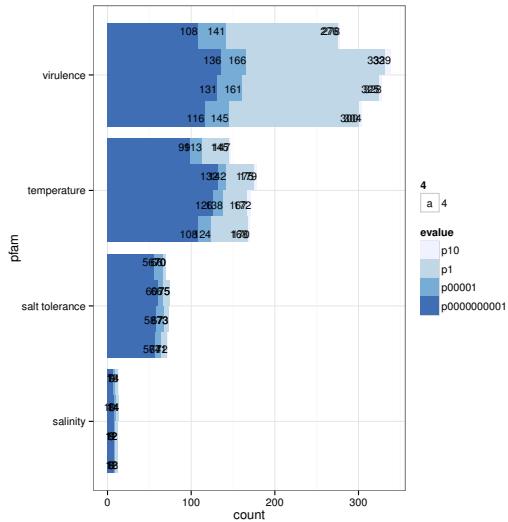
291

292 How many protein domains were found at different evaluate significance.

```

pfam2 <- read.xlsx("./pfam.xlsx", sheetIndex = 2)
pfam2 <- filter(pfam2, annot == "domain")
pfam2 <- gather(pfam2, "evaluate", "count", 2:5)
ggplot(pfam2,
  aes(x = pfam,
      y = count,
      fill = evaluate,
      group = lib)) +
  geom_bar(stat = "identity",
            position = "dodge") +
  scale_fill_brewer() +
  coord_flip() +
  theme_bw() +
  geom_text(aes(x = pfam,
                y = count,
                ymax = count,
                size = 4,
                label = count,
                hjust = 1),
            position = position_dodge(width = 1))

```



293

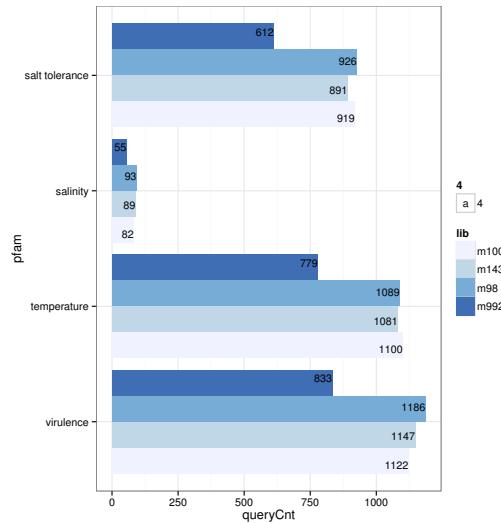
13 Map RNA contigs to Genome (v15) contigs

294 Here is the overall stats of the BLAT of the 4 strains RNA sequenced contigs against SR. genome v15.
 295 Until now the RNA contigs have been annotated with pfam, then reverse translated into DNA contigs then
 296 aligned on the reference genome for SNP localization.

```

blat <- read.table("./pfam.stats.genomics.txt", header = T)
x <- c("m98", "m992", "m1002", "m1433")
y <- gl(4, 4, 16, labels = c("virulence", "temperature", "salinity", "salt tolerance"))
blat <- data.frame(blat, lib = c(rep(x, 4)), pfam = y)
ggplot(blat,
       aes(x = pfam,
            y = queryCnt,
            fill = lib)) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  coord_flip() +
  scale_fill_brewer() +
  geom_text(aes(x = pfam,
                y = queryCnt,
                ymax = queryCnt,
                label = queryCnt,
                size = 4,
                hjust = 1),
            position = position_dodge(width = 1))

```

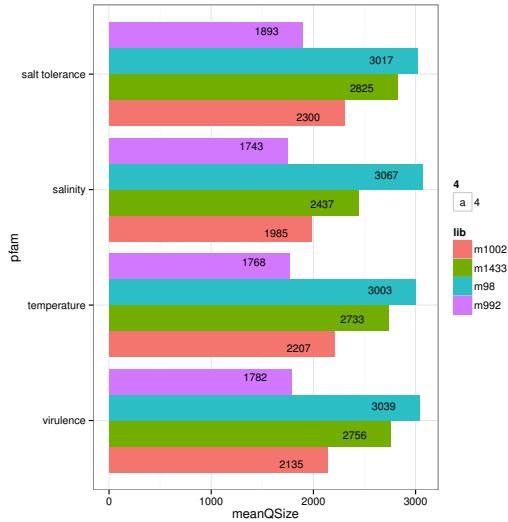


298
299 From the table data above the minimum identity of all contigs aligned is 0.9. The mean query is necessary
300 to choose the number of contigs mapped. Since each contig can be found multiple times in the genome (at
301 different alignment lengths of course) it is best if we choose the best contigs those that have a maximum
302 alignment length. For those contigs must be mapped/aligned once and thus, no duplicate entries should
303 be selected for whatever contig. For this reason choosing an alignment length equal to the half of the
304 mean of the alignment length gives the minimum number of duplicate contigs.

```

ggplot(blat,
       aes(x = pfam,
            y = meanQSize,
            fill = lib)) +
  coord_flip() +
  theme_bw() +
  geom_bar(stat = "identity",
            position = "dodge") +
  geom_text(aes(x = pfam,
                y = meanQSize,
                ymax = meanQSize,
                label = meanQSize,
                size = 4,
                hjust = 2),
            position = position_dodge(width = 1))

```



305
306 **14 Assessing SNP hotspots in 4 QPX strains**
307 QPX assemblies by MMETSP team were used for pfam annotation (with HMMER). SNP calling on the
308 4 strains used Steve Roberts reference genome v15 (called with GATK). Location of SNPs in the pfam
309 domains was inferred after alignment of the QPX contigs on the reference genome (with BLAT). Finally
310 all data were merged in one file grouped by 4 QPX strains (2 from NY, one from each VA and MA) and 3
311 pfam subset pathways (Virulence, salinity/salt-tolerance, temperature).

```
hotspots.raw <- read.table("./hotspots/all.pfam.snp.txt", header = TRUE)
```

```

head(hotspots.raw)

  row Qname          Tname match mismatch repmatch N QgapCount
1   1      5 QPX_v015_contig_5842  2327      0      0 0      0
2   2      5 QPX_v015_contig_5842  2327      0      0 0      0
3   3      5 QPX_v015_contig_5842  2327      0      0 0      0
4   4      5 QPX_v015_contig_5842  2327      0      0 0      0
5   5      5 QPX_v015_contig_5842  2327      0      0 0      0
6   6      5 QPX_v015_contig_5842  2327      0      0 0      0
  QgapBases TgapCount TgapBases Strand Qsize Qstart Qend Tsize Tstart
1       0        1        50      - 2372      0 2327 4816 104
2       0        1        50      - 2372      0 2327 4816 104
3       0        1        50      - 2372      0 2327 4816 104
4       0        1        50      - 2372      0 2327 4816 104
5       0        1        50      - 2372      0 2327 4816 104
6       0        1        50      - 2372      0 2327 4816 104
  Tend BlockCount BlockSize qStarts tStarts           pfam.x lib.x
1 2481         2 376,1951, 45,421, 104,530, salt.tolerance m98
2 2481         2 376,1951, 45,421, 104,530, salt.tolerance m98
3 2481         2 376,1951, 45,421, 104,530, salt.tolerance m98
4 2481         2 376,1951, 45,421, 104,530, salt.tolerance m98
5 2481         2 376,1951, 45,421, 104,530, temperature m98
6 2481         2 376,1951, 45,421, 104,530, temperature m98
  Position REF ALT Quality lib.y Domain accession tLen qLen evalue
1     23   C   T    88.8   m98 SH3_1 PF00018.24    48 573 2.5e-25
2     23   C   T    88.8   m98 SH3_1 PF00018.24    48 573 7.9e-26
3     23   C   T    88.8   m98 SH3_1 PF00018.24    48 573 2.5e-25
4     23   C   T    88.8   m98 SH3_1 PF00018.24    48 573 7.9e-26
5     22   G   C    88.8   m98 SH3_1 PF00018.24    48 573 2.5e-25
6     22   G   C    88.8   m98 SH3_1 PF00018.24    48 573 7.9e-26
  score2 cValue iEval score alnFrom alnTo acc description
1   81.9 2.3e-16 2.8e-14  46.5    352    396 0.96      SH3
2   81.9 1.4e-12 1.1e-10  33.3    262    309 0.86      SH3
3   81.9 2.9e-12 3.6e-10  33.3    262    309 0.86      SH3
4   81.9 1.1e-16 8.9e-15  46.5    352    396 0.96      SH3
5   81.9 2.3e-16 2.8e-14  46.5    352    396 0.96      SH3
6   81.9 1.4e-12 1.1e-10  33.3    262    309 0.86      SH3
  pfam.y lib
1   temperature m98
2   salt.tolerance m98
3   temperature m98
4   salt.tolerance m98
5   temperature m98
6   salt.tolerance m98

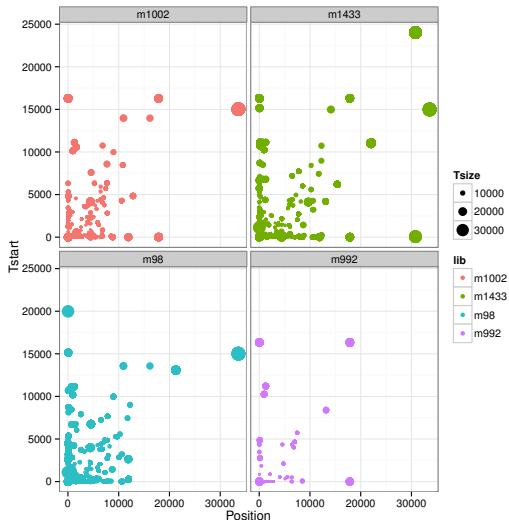
```

312 What is the correlation between a SNP position and the first reference nucleotide that align to contig
 313 containing domain?

```

ggplot(hotspots.raw,
  aes(x = Position,
      y = Tstart)) +
  theme_bw() +
  geom_point(aes(color = lib,
                 size = Tsize)) +
  facet_wrap(~ lib, ncol = 2)

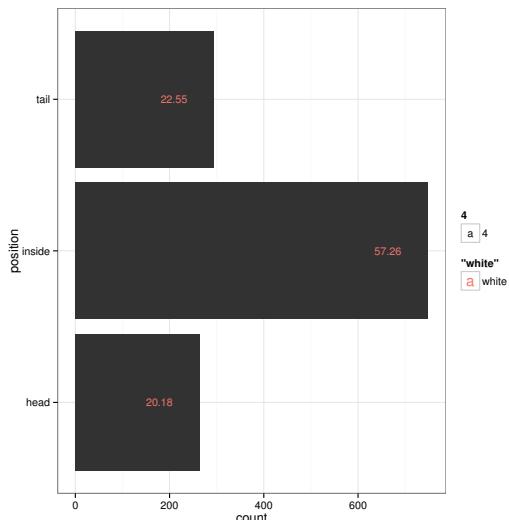
```



314

315 How many SNPs can be found inside and outside of protein domains?

```
count <- c(264, 749, 295)
position <- c("head", "inside", "tail")
dat <- data.frame(position, count)
dat$per <- round((dat$count/sum(dat[, 2]))*100, digits = 2)
ggplot(dat,
       aes(x = position,
            y = count)) +
  theme_bw() +
  geom_bar(stat = "identity") +
  coord_flip() +
  geom_text(aes(x = position,
                y = count,
                ymax = count,
                label = per,
                size = 4,
                hjust = 2,
                color = "white"))
```



316

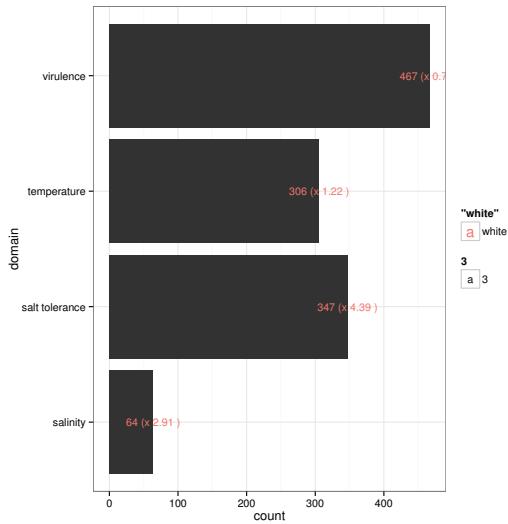
317 How are SNPs distributed between pfam domains? The between parenthesis score is an identifier for the highest SNP concentration. It has no units. It is just a score of normalized counts. The counts is relative to the position of SNPs inside the protein domains.

```
domain <- c("virulence", "temperature", "salinity", "salt tolerance")
```

```

count <- c(467, 306, 64, 347)
dat <- data.frame(domain, count)
dat$norm <- round(dat$count/pfam[1:4, 2], digits = 2)
ggplot(dat,
  aes(x = domain,
       y = count)) +
  theme_bw() +
  coord_flip() +
  geom_bar(stat = "identity") +
  geom_text(aes(x = domain,
                y = count,
                ymax = count,
                label = paste(count, "(x", norm, ")"),
                size = 3,
                hjust = .5,
                color = "white"))

```

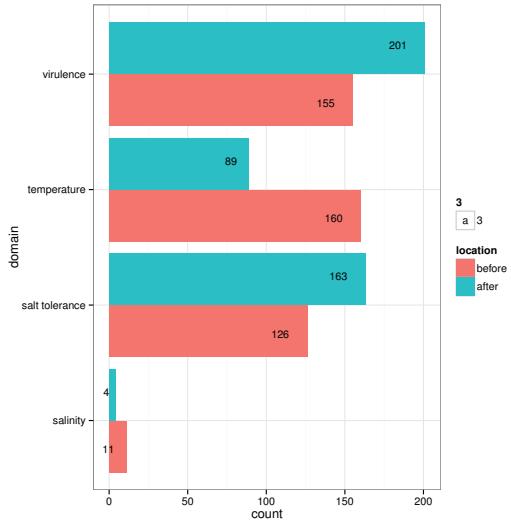


320
321 How many SNPs can be found outside of each domain? Separate analysis. The SNP position outside
322 the domains is dependent on the Reference contig length, which was selected through alignment.

```

before <- c(155, 160, 11, 126)
after <- c(201, 89, 4, 163)
dat <- data.frame(domain, before, after)
dat <- gather(dat, "location", "count", 2:3)
ggplot(dat,
  aes(x = domain,
       y = count,
       fill = location)) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  coord_flip() +
  geom_text(aes(x = domain,
                y = count,
                ymax = count,
                label = count,
                size = 3,
                hjust = 2),
            position = position_dodge(width = 1))

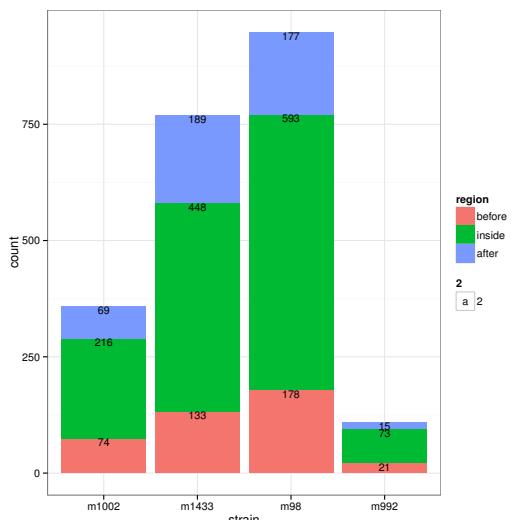
```



323

324 How many SNPs can be found inside and outside protein domains between strains?

```
before <- c(178, 21, 74, 133)
after <- c(177, 15, 69, 189)
inside <- c(593, 73, 216, 448)
strain <- c("m98", "m992", "m1002", "m1433")
dat <- data.frame(strain, before, inside, after)
dat <- gather(dat, "region", "count", 2:4)
ggplot(dat,
       aes(x = strain,
            y = count,
            fill = region)) +
  geom_bar(stat = "identity") +
  geom_text(aes(x = strain,
                y = count,
                ymax = count,
                label = count,
                vjust = 1,
                size = 2),
            position = "stack") +
  theme_bw()
```



325

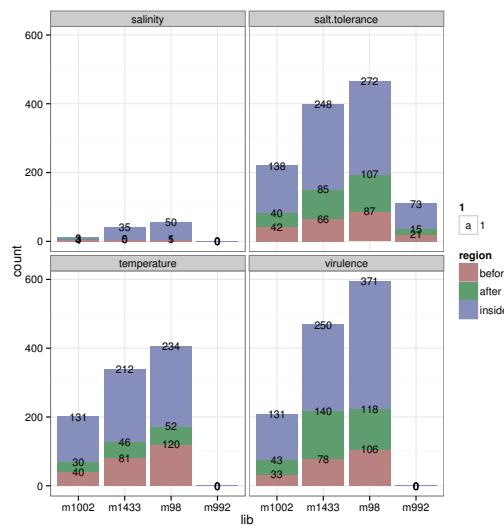
326 How many SNPs can be found inside and outside domains between virulence, temperature, salinity and
327 within strain?

```
dat <- read.xlsx("./hotspots/snps.all.pfam.xlsx", sheetIndex = 1)
```

```

dat <- gather(dat, "region", "count", 3:5)
ggplot(dat,
       aes(x = lib,
            y = count,
            fill = region)) +
  geom_bar(stat = "identity") +
  geom_text(aes(x = lib,
                y = count,
                ymax = count,
                label = count,
                size = 1,
                hjust = .5),
            position = "stack") +
  facet_wrap(~ pfam, ncol = 2) +
  theme_bw() +
  scale_fill_hue(c = 40, l = 60)

```



328

329 Preferential substitution inside/outside domains per pfam subset for each strain.

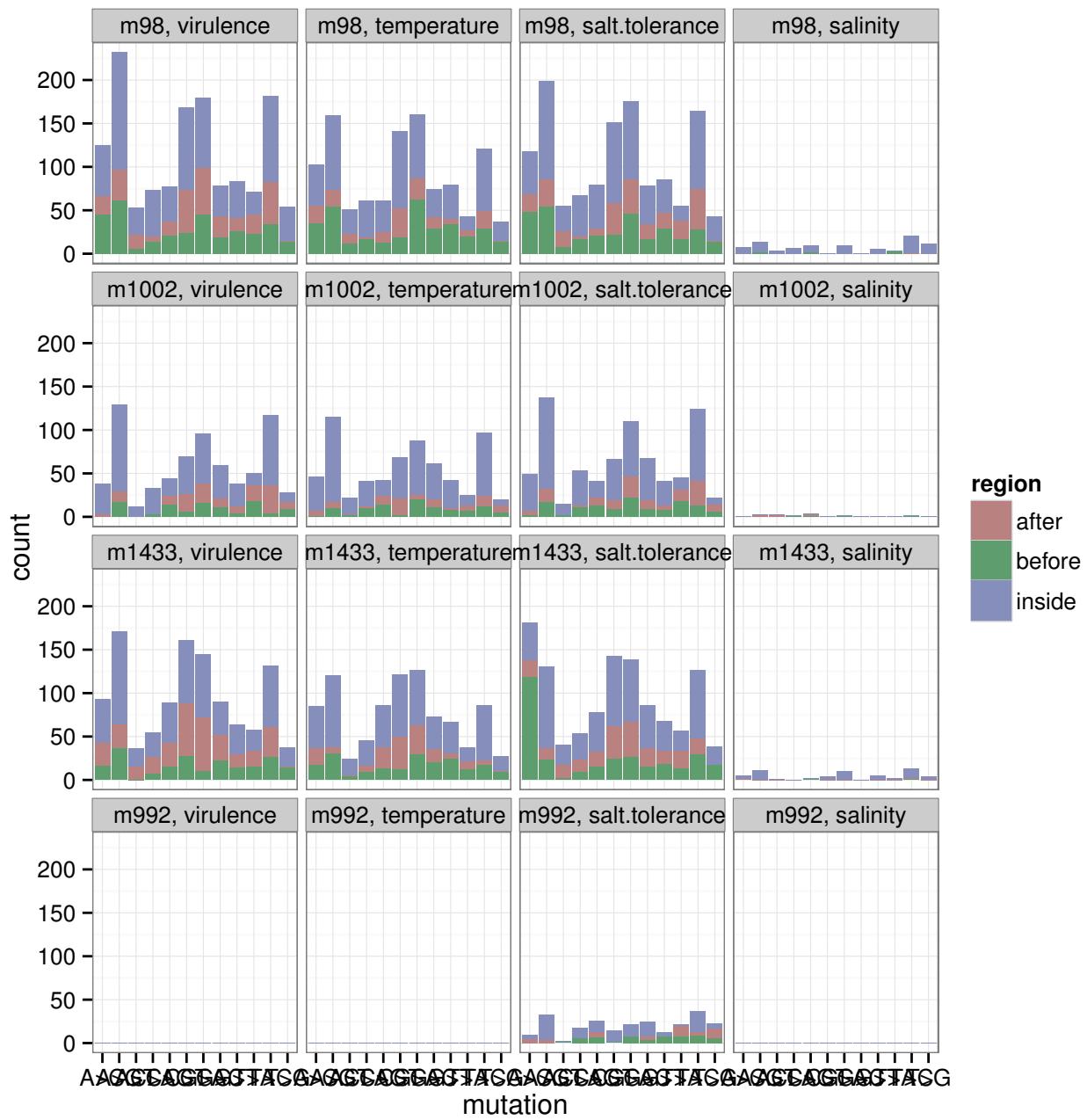
```

dat <- read.xlsx("./hotspots/snps.all.pfam.xlsx", sheetIndex = 2)
dat <- gather(dat, "mutation", "count", 3:14)
dat$mutation <- gsub(".", ">", dat$mutation, fixed = TRUE)
dat$pfam <- factor(dat$pfam, levels = c("virulence",
                                         "temperature",
                                         "salt.tolerance",
                                         "salinity"))

dat$lib <- factor(dat$lib, levels = c("m98",
                                         "m1002", "m1433", "m992"))

ggplot(dat,
       aes(x = mutation,
            y = count,
            fill = region)) +
  geom_bar(stat = "identity") +
  theme_bw() +
  facet_wrap(lib ~ pfam, ncol = 4) +
  scale_fill_hue(c = 40, l = 60)

```



330
331 How many SNPs can be found inside protein domains between strains, per 1 kb? The number showing
332 has been normalized with the total sum of sizes of contigs containing those SNPs.

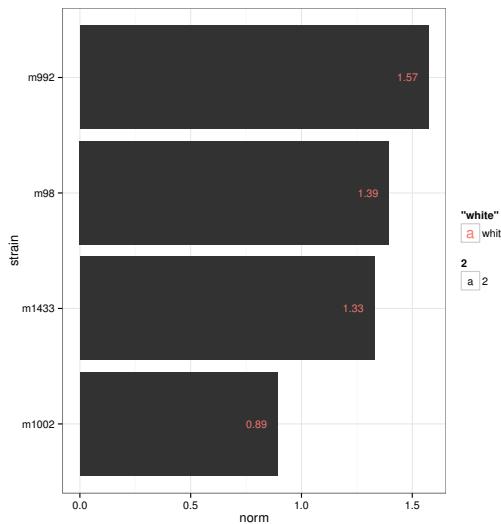
```
inside <- c(593, 73, 216, 448)
```

```

sizen <- c(425098, 46409, 242136, 337206)
strain <- c("m98", "m992", "m1002", "m1433")
dat <- data.frame(strain, inside, sizen)
dat$norm <- with(dat, (inside/sizen)*1000)

ggplot(dat,
       aes(x = strain,
            y = norm)) +
  coord_flip() +
  geom_bar(stat = "identity") +
  geom_text(aes(x = strain,
                y = norm,
                ymax = norm,
                label = round(norm, digits = 2),
                hjust = 1.5,
                color = "white",
                size = 2)) +
  theme_bw()

```



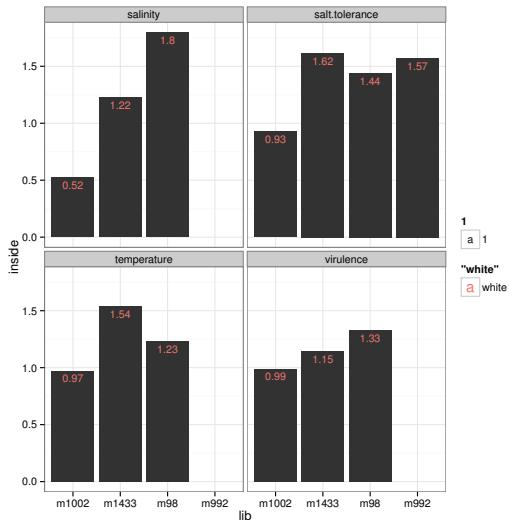
333
334 Frequency of SNPs inside pfam domains for each strain, per 1 Kb. Normalized by the total size of contigs
335 for each strain.

```

dat <- read.xlsx("./hotspots/snps.all.pfam.xlsx", sheetIndex = 1)
dat$inside <- with(dat, (inside/tsum)*1000)
ggplot(dat,
       aes(x = lib,
            y = inside)) +
  geom_bar(stat = "identity") +
  geom_text(aes(x = lib,
                y = inside,
                ymax = inside,
                label = round(inside, digits = 2),
                size = 1,
                color = "white",
                vjust = 1.5)) +
  facet_wrap(~ pfam, ncol = 2) +
  theme_bw()

```

Warning: Removed 1 rows containing missing values (position_stack).
 Warning: Removed 1 rows containing missing values (position_stack).
 Warning: Removed 1 rows containing missing values (position_stack).
 Warning: Removed 1 rows containing missing values (geom_text).
 Warning: Removed 1 rows containing missing values (geom_text).
 Warning: Removed 1 rows containing missing values (geom_text).



336

337 15 Machine learning on 4 QPX strains

338 Lets try a support vector machine classifier to differentiate between the QPX strains using quality data
339 (above) of the variants.

340 16 System Information

341 The version number of R and packages loaded for generating the vignette were:

```
## ## save(list=ls(pattern=".*/.*"), file="PD.Rdata")
sessionInfo()

R version 3.1.2 (2014-10-31)
Platform: x86_64-unknown-linux-gnu (64-bit)

locale:
[1] LC_CTYPE=en_US.UTF-8          LC_NUMERIC=C
[3] LC_TIME=en_US.UTF-8          LC_COLLATE=en_US.UTF-8
[5] LC_MONETARY=en_US.UTF-8       LC_MESSAGES=en_US.UTF-8
[7] LC_PAPER=en_US.UTF-8          LC_NAME=en_US.UTF-8
[9] LC_ADDRESS=en_US.UTF-8        LC_TELEPHONE=en_US.UTF-8
[11] LC_MEASUREMENT=en_US.UTF-8    LC_IDENTIFICATION=en_US.UTF-8

attached base packages:
[1] grid      stats     graphics   grDevices  utils      datasets 
[7] methods   base

other attached packages:
[1] glmnet_1.9-8      Matrix_1.1-4      leaps_2.9
[4] caret_6.0-37      ggbioplot_0.55    scales_0.2.4
[7] plyr_1.8.1        tidyverse_0.1     vegan_2.2-0
[10] permute_0.8-3     dplyr_0.3.0.2     ggplot2_1.0.0
[13] latticeExtra_0.6-26 RColorBrewer_1.0-5  lattice_0.20-29
[16] xlsx_0.5.7       xlsxjars_0.6.1    rJava_0.9-6
[19] knitr_1.8

loaded via a namespace (and not attached):
[1] assertthat_0.1      BradleyTerry2_1.0-5 brglm_0.5-9
[4] car_2.0-22         cluster_1.15.3      codetools_0.2-9
[7] colorspace_1.2-4    compiler_3.1.2      DBI_0.3.1
[10] digest_0.6.4       evaluate_0.5.5     foreach_1.4.2
[13] formatR_1.0        gtable_0.1.2       gtools_3.4.1
[16] highr_0.4          iterators_1.0.7    labeling_0.3
[19] lazyeval_0.1.9     lme4_1.1-7        magrittr_1.5
[22] MASS_7.3-35        mgcv_1.8-4        minqa_1.2.4
[25] munsell_0.4.2      nlme_3.1-118      nloptr_1.0.4
[28] nnet_7.3-8         parallel_3.1.2    proto_0.3-10
[31] Rcpp_0.11.3        reshape2_1.4      splines_3.1.2
[34] stringr_0.6.2      tcltk_3.1.2      tools_3.1.2
```