

R implementation

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- 1
2 Loaded functions:

↑ Project started April 2015 -
ended June 2015

```
#source ("~/media/Data/Dropbox/humanR/01funcs.R")
rm(list=ls())
```

- 3 Load packages.

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

1 Show the length distribution of reads

- 4 Load *gff3* sequence length data for mapped QPX libraries and references. GFF3 files contain the sequence length of each contig. These contigs belong to Steve Roberts **genome v015 and 017** and **transcriptome v21** of QPX.

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

↑ Refer to github front page of
the rnaseQPX project

	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("./data/QPX_transcriptome_v2orf.gff3")
```

- 8 GFF3 counts of MME transcriptomes **MMETSP0098** and **MMETSP00992**, and the custom assembly with
9 MMETSP0098.

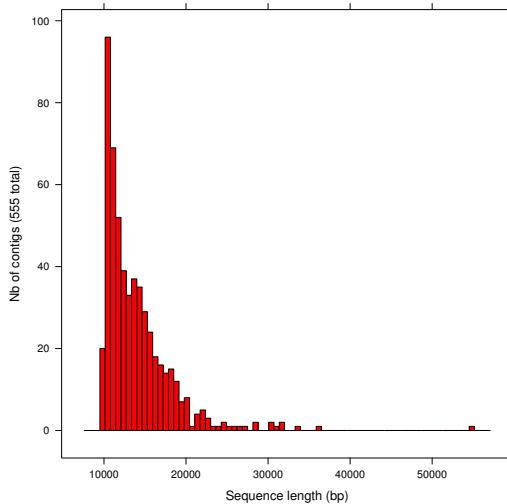
```
mme98 <- read.table("./data/MMETSP0098.gff3")
mme99 <- read.table("./data/MMETSP0099_2.gff3")
mme98c <- read.table("./data/mme98cust.gff3")
genomv015 <- read.table("./data/QPX_v015.gff3")
```

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

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

```
histogram(~ (genome$V5),
```

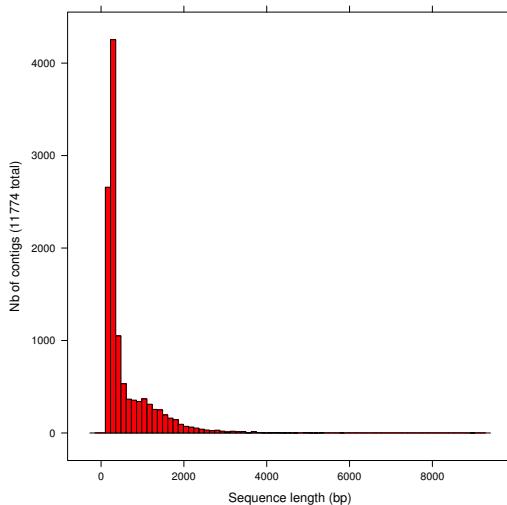
```
type= 'count',
nint = 75,
data = genome,
xlab = 'Sequence length (bp)',
ylab = 'Nb of contigs (555 total)',
col = 'red')
```



15

16 Distribution of Steve's QPX transcriptome.

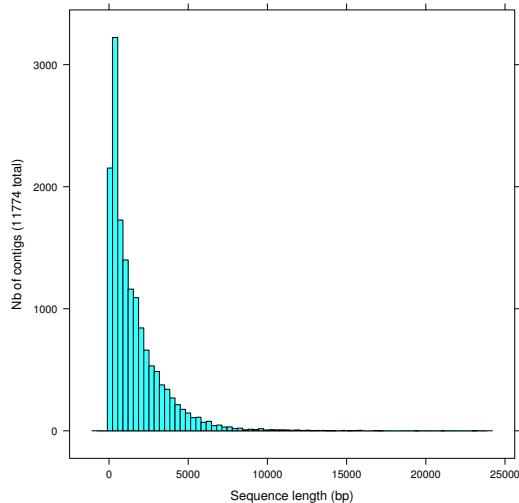
```
histogram(~ transcriptome$V5,
          type = 'count',
          col = 'red',
          data = transcriptome,
          nint = 75,
          xlab = 'Sequence length (bp)',
          ylab = 'Nb of contigs (11774 total)')
```



17

18 Distribution of length of MMETSP0098.

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



19
20 Superpose the length of contigs in:
21 • Steve's genome v017 (555 contigs)
22 • Steve's transcriptome
23 • MMEtsp0098 transcriptome
24 • MMEtsp00992 transcriptome
25 • MMEtsp0098 custom transcriptome
26 • Steve's Genome v015 (approx 22,000 contigs)
27 Merge datasets.

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

28 Plot reads length of the 6 assemblies including 2 QPX genomes.

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

↳ Assign a new column to identify contigs.

↳ A higher resolution of this chart can be found in the Supplemental Information

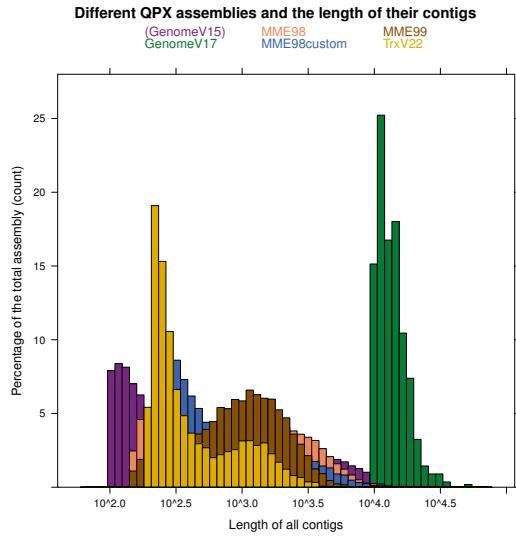
```

        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



29

30 2 Calling SNPs: Testing tools, parameters, and filters

31

32

33

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

- 34 • mmetsp0098 from New York
- 35 • mmetsp001433 from New York
- 36 • mmetsp00992 from Massachusetts
- 37 • mmetsp001002 from Virginia
- 38 • mmetsp0099 from Massachusetts
- 39 • mmetsp00100 from Virginia

40 2.1 Load data

41 Number of SNPs called with either packages were counted. Calls were done after read duplicates were
42 removed with Picard.

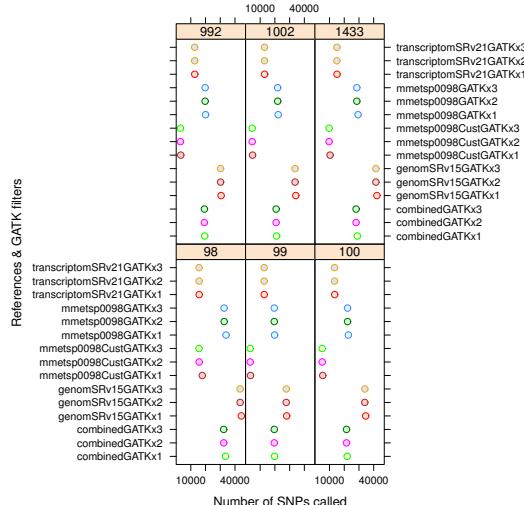
```
counts.SNP <- read.xlsx("./data/snp.counts.xlsx", sheetIndex = 1)
glimpse(counts.SNP)

Observations: 114
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...
```

43 Histogram grouped by QPX library showing difference in SNPs counts relative to the reference used for
44 mapping and the number of times GATK has been used to recalibrate calls. GATK (x1, x2, x3) represent
45 one, two or three rounds of recalibration. The recalibration is done over a list of variants called under
46 stringent parameters. SR: Steve Roberts genomes. Cust: custom assembly of mmetsp0098.

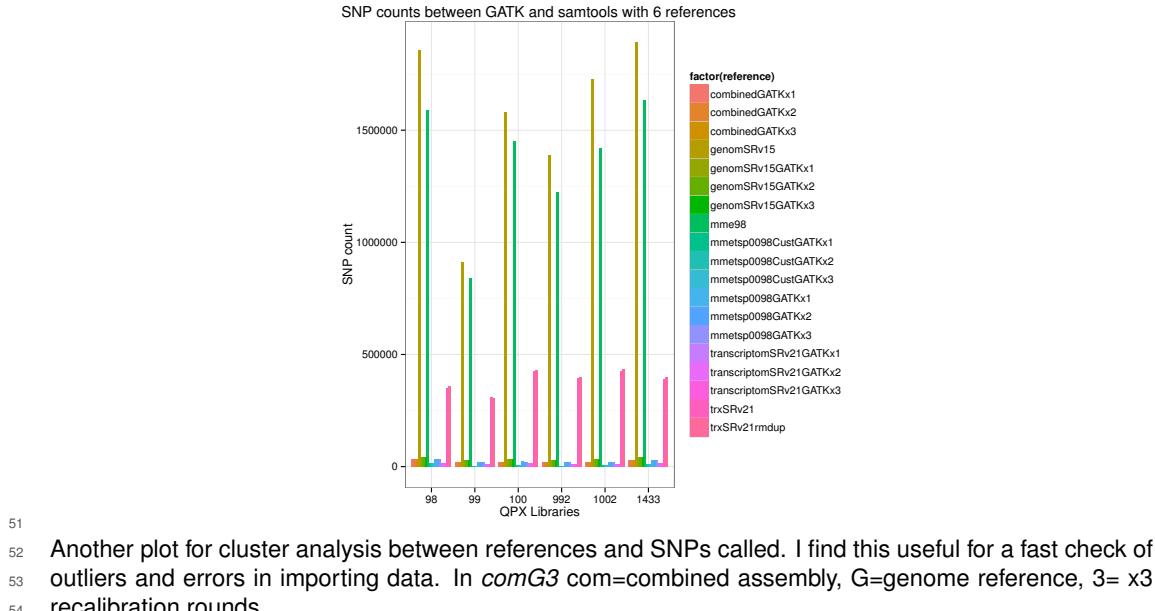
```
xyplot( factor(reference) ~ as.matrix(counts) | factor(sample),
  data = counts.SNP[-c(1:24), ],
  groups = counts.SNP$reference,
  pch = 21,
  cex = 1,
  type = c("p"),
  xlab = 'Number of SNPs called',
  ylab = 'References & GATK filters')
```

↑ Recalibration is done with
GATK. The strategy is
described in the pipeline on
github [here](#).

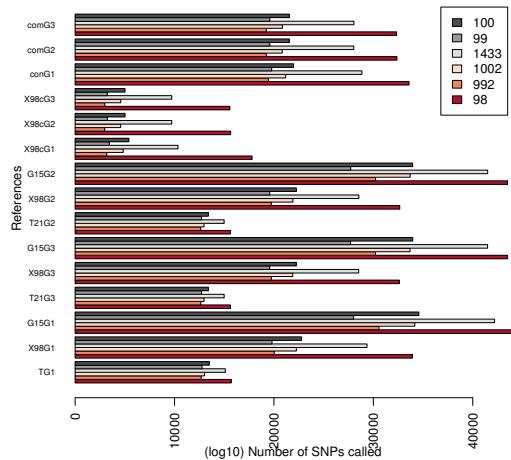


47
48 Plot the difference between QPX libraries and variant calling packages for the number of called SNPs.
49 Combined: an assembly made of the combination of mmetsp0098, 992, 1002, 1433. SR: Steve Roberts
50 genomes and transcriptomes (15 and 21 respectively). Samtools was done on trxSRv21rmdup.

```
ggplot(counts.SNP,
  aes(x = factor(sample),
    y = counts,
    fill = factor(reference))) +
  geom_bar(stat = "identity",
            position = "dodge") +
  theme_bw() +
  labs(title = "SNP counts between GATK and samtools with 6 references",
        x = "QPX Libraries",
        y = "SNP count")
```



```
dat <- read.xlsx("./data/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,
        xlab = '(log10) Number of SNPs called',
        ylab = 'References')
```



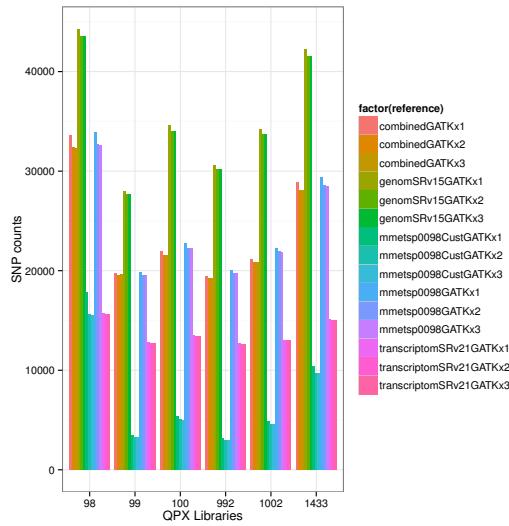
55
56 Plotting only the GATK called SNPs.

```
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() +
  labs(x = "QPX Libraries",
       y = "SNP counts")

```



57
58 Plot the difference between the number of SNPs called on the 6 QPX libraries using either the assembled
59 or custom assembled mmetsp0098 reference. Also show the variation pattern with the number of reads
60 used for calling SNPs. First, prepare SNP data.

```

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

```

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

```

ref.reads <- read.xlsx("./data/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, ]

```

63 Plot difference.

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

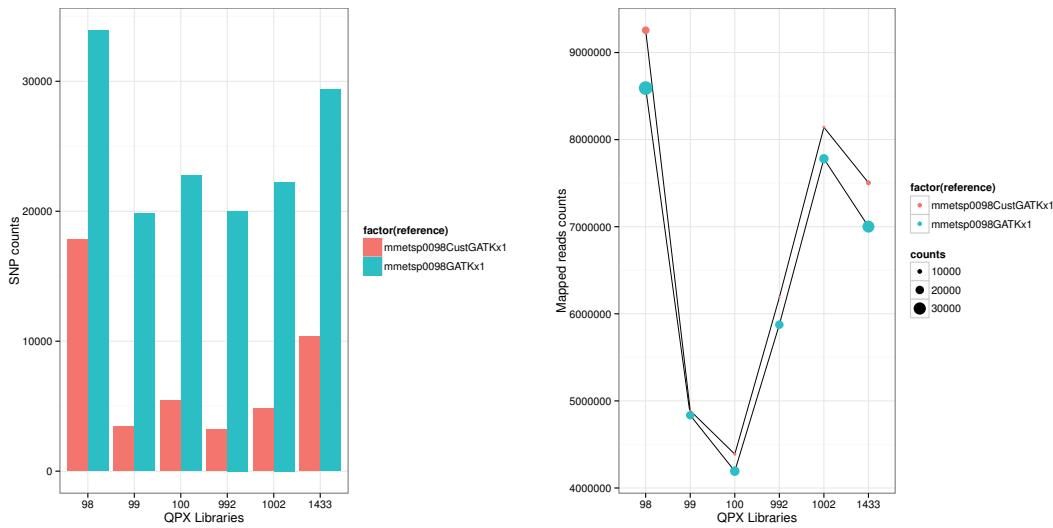
↑ A higher resolution chart of
this analysis can be found in the
Supplemental Information

```

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

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() +
  labs(x = "QPX Libraries",
       y = "Mapped reads counts")

```



64
65 Plot number of all mapped reads for each QPX 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() +
  labs(x = "QPX libraries",
       y = "Read counts")

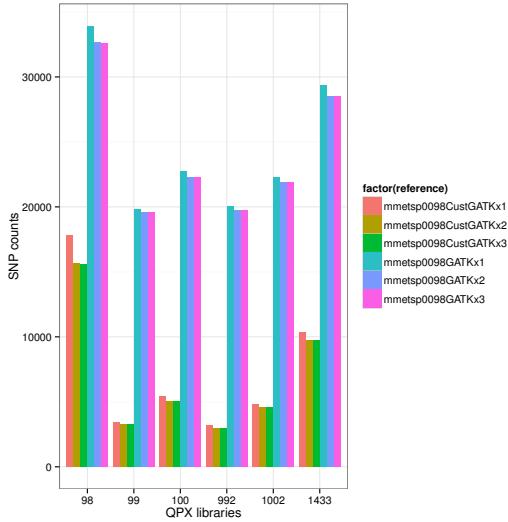
```

66
67
68

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

↑ The custom assembled *mmetsp0098* was done with trinity

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



69
70
71
72
73

Another way to show difference between GATK recalibration protocols and the way this strategy decreases the number of SNPs called by readjusting of nucleotide probabilities for each read. The plot shows also the difference in SNP called between *mmetsp0098* custom (the first 3 bars) and original (the last 3 bars) assemblies.

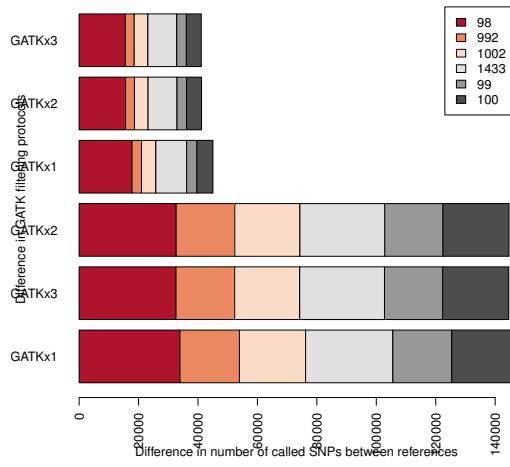
```
dat <- read.xlsx("./data/snp.counts.xlsx", sheetIndex = 3)
```

```

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 called SNPs between references",
       ylab = 'Difference in GATK filtering protocols',
       las = 2,
       legend = dat$sample)

```



74

3 Final filtering

75

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

76

77

78

3.1 Working with the combined assembly and genome v15

79

Difference in called SNPs between QPX libraries mapped to 2 different references, the combined assembly (represented by lower bar labels) and the genome v15 of S. Roberts (represented by higher bar labels). A higher resolution bar can be found in Supplemental Information.

80

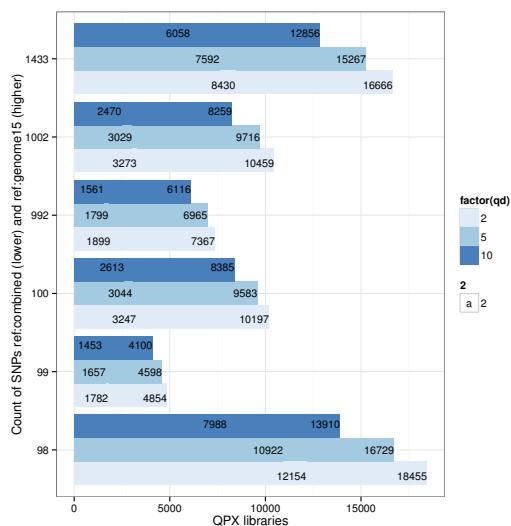
81

82

[†] Genome v15 S. Roberts is used in the remaining tests

```
dat <- read.xlsx("./data/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() +
  labs(x = "Count of SNPs ref:combined (lower) and ref:genome15 (higher)",
       y = "QPX libraries")
```



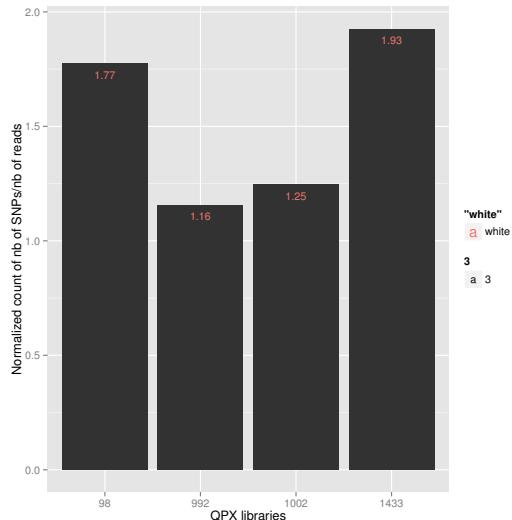
83
84 Number of SNPs per strain at $QD = 5$. SNPs called against *SR genome v15*. The number of reads
85 (approx 100 nt in size) per library has been counted and plotted previously, the data is in *refreads.xlsx*.

```

dat <- read.xlsx("./data/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)) +
  labs(x = "QPX libraries",
       y = "Normalized count of nb of SNPs/nb of reads")

```

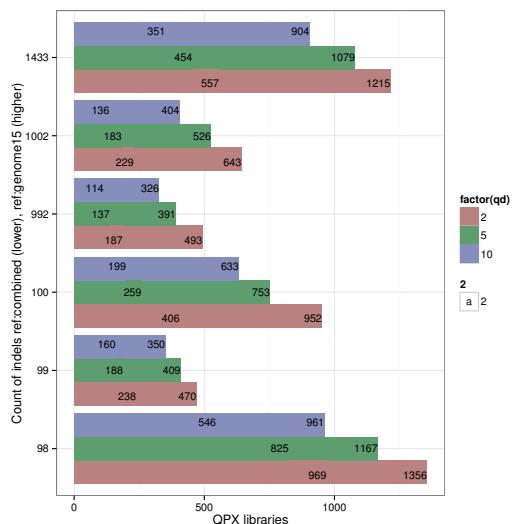


86

87

We can also do the same thing with indels.

```
dat <- read.xlsx("./data/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) +
  labs(x = "Count of indels ref:combined (lower), ref:genome15 (higher)",
       y = "QPX libraries")
```



88

89 The combined assembly is already published. It is added here with the other references because it is
90 heavily annotated and their contigs are extensively mapped. Load in new mapped data to the combined
91 reference:

```

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

Observations: 114
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, ...

```

92 Difference in SNPs called between the genome v15 of S. Roberts and the official combined assembly.
93 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), ]

```

94 Plot difference.

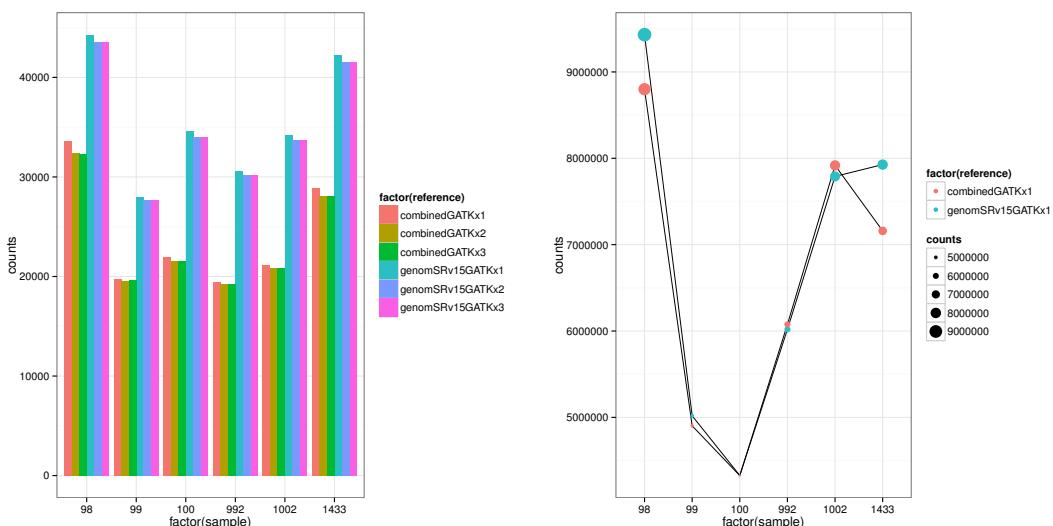
```

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()

```

[†]A higher resolution of this plot can be found in supplemental Information



95
96 **4 Descriptive stats of all processed libraries**
97 This following section shows the mean length of all sequences assembled from each library, the number of
98 base pairs per library, the identified protein features from these sequences, and the number of functional
99 enzymes identified by mapping to public libraries. It is to note the number of predicted and identified
100 rRNA features in each of these libraries is significantly low. Regress different variables on each others for
101 visualization purposes.

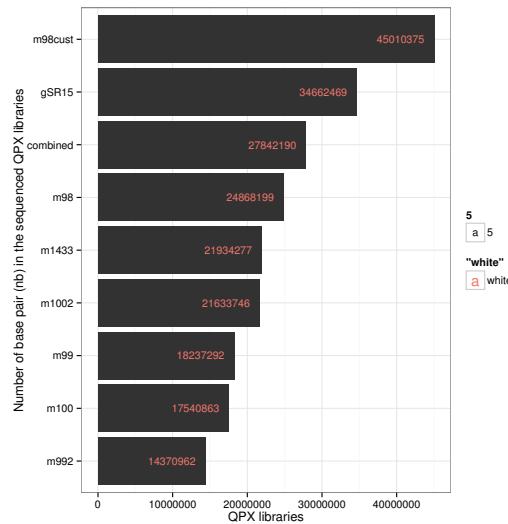
```

stats <- read.xlsx("./data/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)) +
  labs(x = "Number of base pair (nb) in the sequenced QPX libraries",
    y = "QPX libraries")

```



102
103 The plot shows the difference between QPX libraries according to the number of base pair (bp x 1000,
104 the identified protein features estimated from assembled sequences (feature), the functional sequences
105 estimated from the contigs (function), the mean length of contigs in each library, and the number of contigs
106 (sequence) assembled from raw reads after trimming and duplicate removal (all basic quality controls).

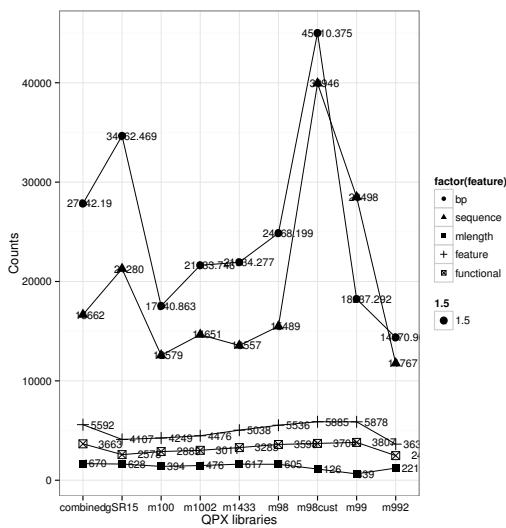
```
stats <- read.xlsx("./data/libraries.xlsx", sheetIndex = 1)
```

[†] The number of base pair must
be multiplied by 1000 in this
chart

```

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)) +
  labs(x = "QPX libraries",
       y = "Counts")

```



107

108 Principal component analysis and diagnostics.

```

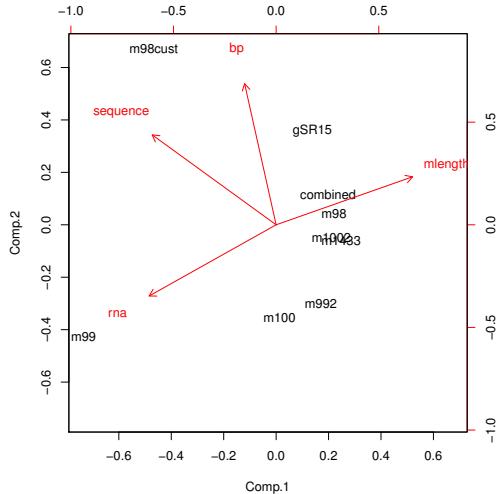
stats <- read.xlsx("./data/libraries.xlsx", sheetIndex = 1)
rownames(stats) <- stats$libraries
rstats <- stats[complete.cases(stats), -1]
rstats <- decostand(rstats, method = "range")
p = princomp(~bp + mlength + sequence + rna
             , data= rstats)
summary(p)

```

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)
```



109
110 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
```

111 5 Applied annotations, subsystem predictions, and taxonomic distribution

112 Like the title implies, identified and predicted annotations and protein features are mapped to public
113 sequence libraries.

114 Reshape data, transform columns into rows.

↑ This annotation has been
done with MG-RAST. Visit [here](#)
for a description of their fast
annotation server

```
predicted <- read.xlsx("./data/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
```

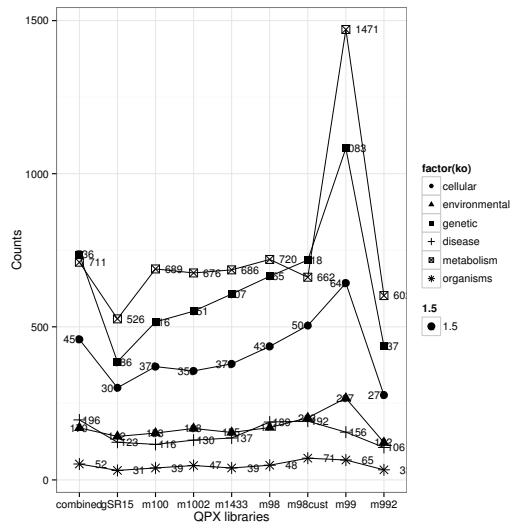
115 Plot difference in identified protein features between libraries.

```
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)) +
labs(x = "QPX libraries",
     y = "Counts")

```

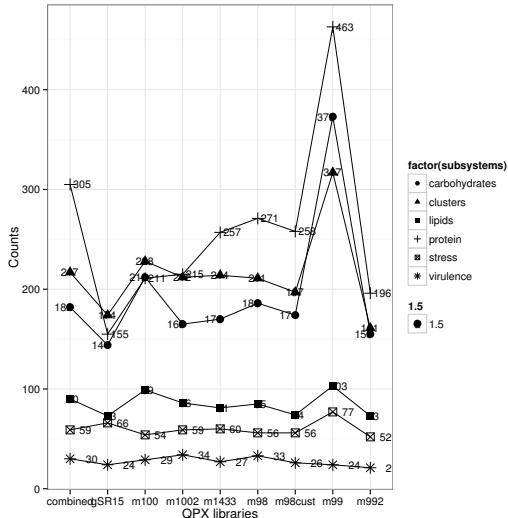


116
117 In this next snippet subsystems are discussed. Functional coupling and chromosomal clusters are shown
118 for *clustering-based subsystems* among other subsystems.

```

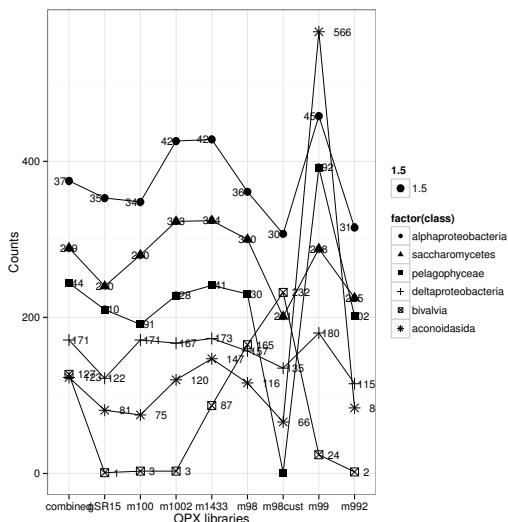
predicted <- read.xlsx("./data/libraries.xlsx", sheetIndex = 4)
predicted <- gather(predicted, "subsystems", "count", 3:8, na.rm= TRUE)
ggplot(predicted,
       aes(x = lib,
           y = count,
           group = factor(subsystems))) +
geom_line(size = .2) +
geom_point(aes(shape = factor(subsystems),
               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)) +
labs(x = "QPX libraries",
     y = "Counts")

```



119
120 Finally, a taxonomic classification on sequence similarities gives insights on sequence relatedness or
121 sample contamination. Five classes were selected, bacteria, fungi, algae, parasite, and bivalvia.

```
predicted <- read.xlsx("./data/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)) +
  labs(x = "QPX libraries",
       y = "Counts")
```



122
123 **6 Shared SNPs between libraries**
124 Shared SNPs between libraries mapped to SR genome v15. The first column shows the count of shared
125 SNPs. The next 3 columns shows the name of the QPX library (**mmetsp00** 98, 992, 1002, 1433). The
126 libraries situated on a same row share the same SNPs.

↑ A higher resolution summary
of the 4 last plots can be found
in Supplemental Information

```

shared.snps <- read.table("./data/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%)

```

127 Shared indels between libraries mapped to SR genome v15.

```

shared.indels <- read.table("./data/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%)

```

↑ A venn diagram of the shared SNPs can be found in the Manuscript

128 6.1 Component analysis and sequence closeness from MG-RAST annotation

129 Import annotated data.

```

closeness <- read.csv("./data/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)]
```

130 Principal component analysis on 5 libraries, 4 strains and the genome (v15), using an *identity score* for
131 annotating a sequence and an *alignment length score* for similarities with estimated functional features,
132 an *e-value score* for estimated functional similarities, and the *number of hits*, ie., the number of times a
133 function is identified in 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

## standardization (columns)
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)

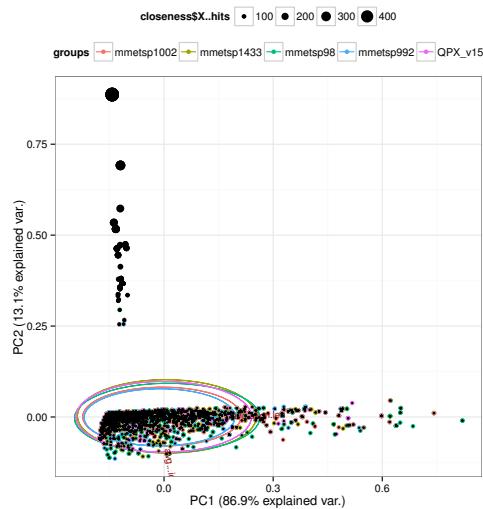
```

134 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')

```



135
136

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')
}
```

137
138

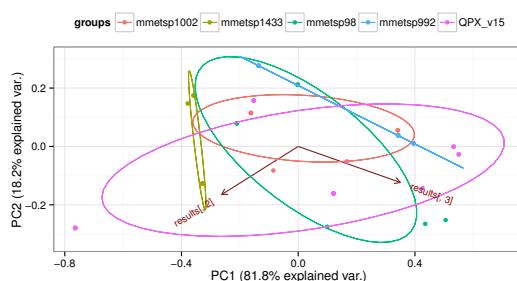
Filter sequences depending on their alignment length and the abundance of an identified function (therotically a protein).

```
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



139
140

Filter by selecting higher alignment scores only.

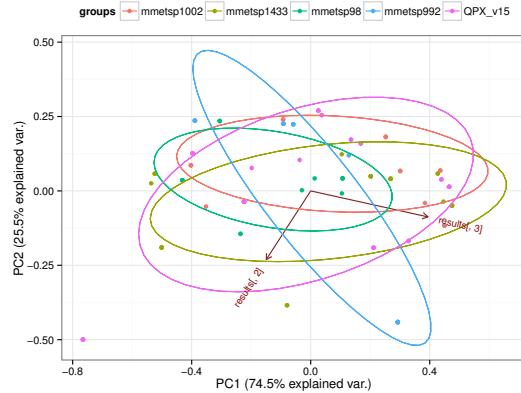
```

closenessX <- filter(closeness, avg.align.len < 60, X..hits > 2)
dim(closenessX) [1]

[1] 117

customBiplot(closenessX, method = "range")

```



141
142 Select even higher alignment similarities.

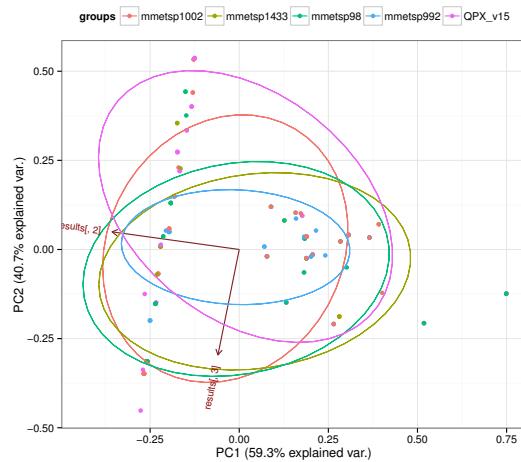
```

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

[1] 199

customBiplot(closenessX, method = "range")

```



143
144 Select on the criteria of e-Value and abundance of a functional sequence.

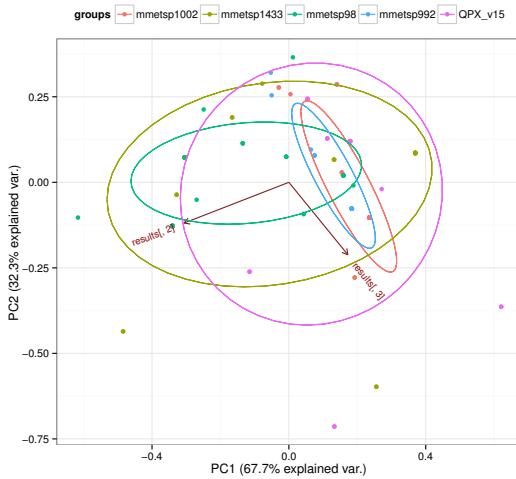
```

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

[1] 152

customBiplot(closenessX, method = "range")

```

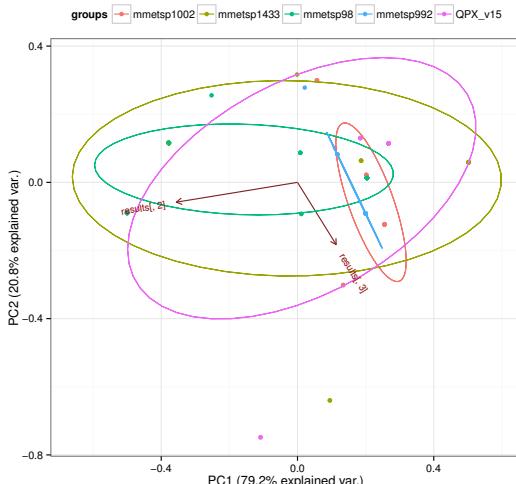


145
146

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")
```

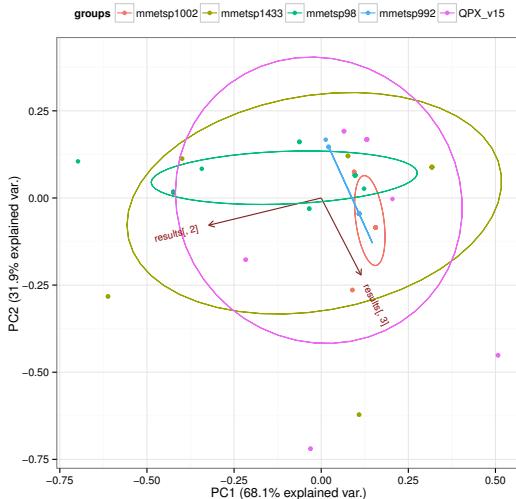


147
148

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")
```



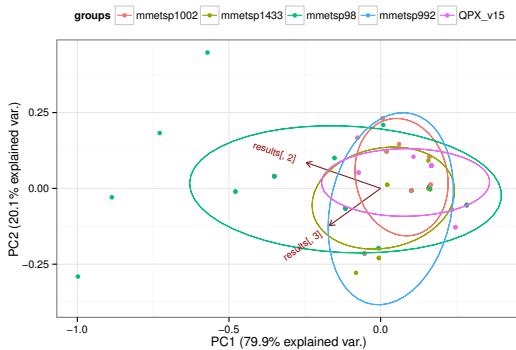
149
150 Select on the criteria of alignment length. Since SNP aggregation tests are the next step in this analysis,
151 the length of a correct alignment is technically helpful in differentiating SNP position. And abundance will
152 be more than 2 to increase probabilities of correct functional annotation.

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

[1] 100

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

↑ This final biplot represent a successful unsupervised clustering of QPX strains. Differences between strains is reflected by the nature of the assembled contigs. But also to the nature of each base pair in these sequences. Top hit contigs N=100



153
154 **7 Aggregation analysis of SNPs**
155 MMETSP libraries are already been annotated. How many contigs, peptide and cds elements are in-
156 dexed?

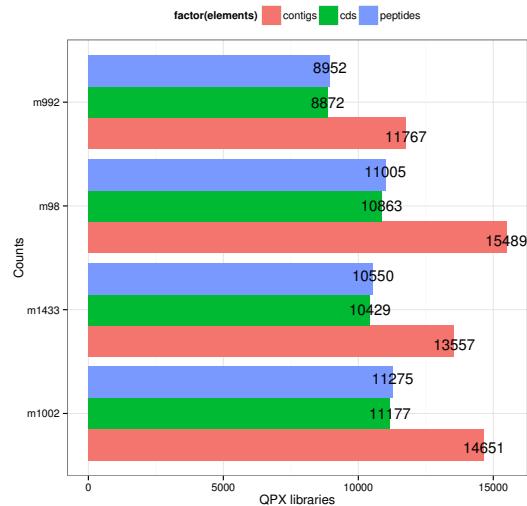
```
contigs <- read.xlsx("./data/annot.stats.xlsx", sheetIndex = 1)
```

↑ Only mmetsp 98, 992, 1002, and 1433 are used in the remaining tests

```

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)) +
  labs(x = "Counts",
       y = "QPX libraries")

```



157

7.1 Preferential substitution

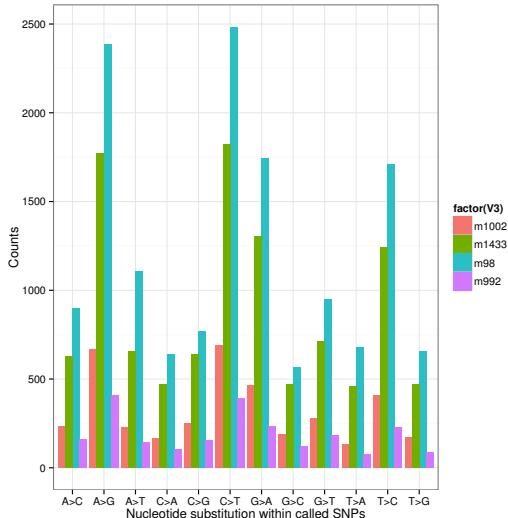
158
159
160
161
162

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

```

prefs <- read.table("./data/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() +
  labs(x = "Nucleotide substitution within called SNPs",
       y = "Counts")

```



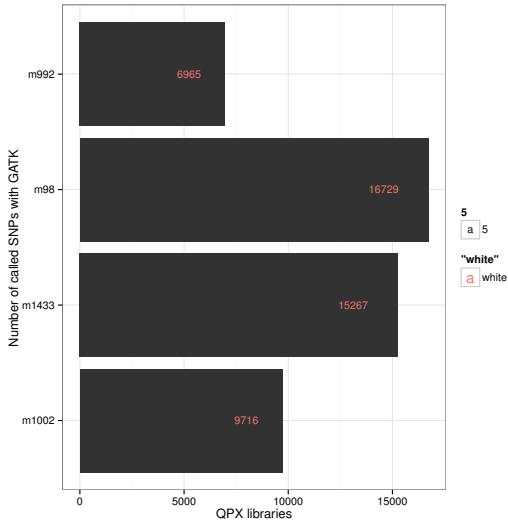
163
164 After hard filtering SNPs to the minimum from all 4 libraries, *DISCARD*-labelled SNPs were removed. The
165 remaining were imported into data frames with the following columns.

- 166 1. CHROM: number of contig
167 2. POS: SNP position on that contig
168 3. ALT: alternative SNP to the reference
169 4. AD: allelic depth for the reference and ALT alleles
170 5. DP: approximate read depth
171 6. GQ: genotype quality
172 7. PL: normalized phred scaled likelihoods

173 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)) +
  labs(x = "Number of called SNPs with GATK",
       y = "QPX libraries")
```

↑ 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.



174
175 Import SNP data: data manipulation process of removing NAs and getting the same number of SNPs
176 across all samples.

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

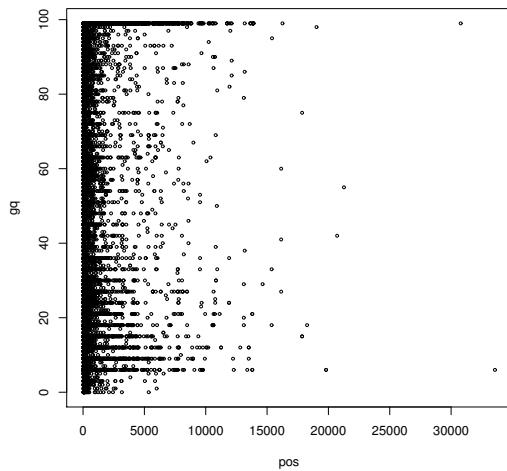
177 7.2 Distribution of SNPs in the QPX libraries

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

```
head(mall)

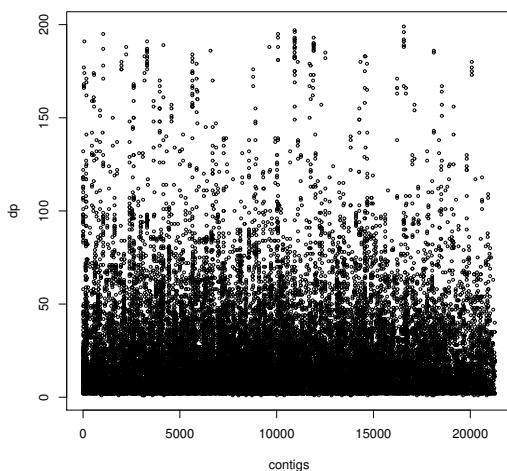
  contigs pos ad1 ad2 dp gq pl1 pl2 pl3 lib
4825      5776 244   5 18 23 99 786    0 150 m98
13214     14964  96   6  6 12 99 234    0 234 m98
6857      7810 176   3 11 14 68 392    0  68 m98
14801     17502  56   9  7 16 99 230    0 337 m98
15760     19016  80   0  7  7 21 315   21   0 m98
763       884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

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



180
181 Regression of contigs and the read depth for each SNP in those contigs. When using libraries mapped to
182 the combined assembly (as a reference transcriptome, not showing here) the plot shows that the depth of
183 coverage cat split the SNPs inside the QPX contigs into 2 separate subsets. However the regression is
184 constant when using the genome of SR v15 as a reference for mapping the libraries (as shown below).

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



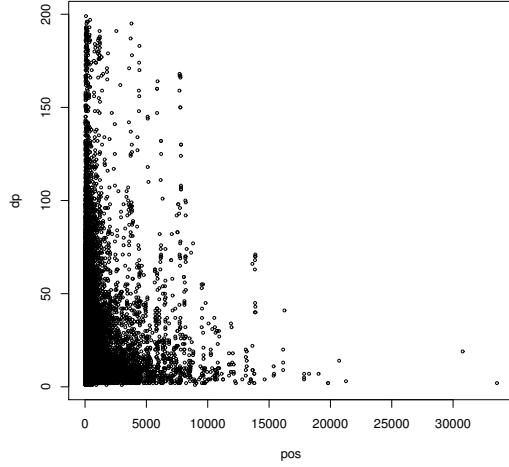
185
186 This plot shows that 11.41 % of the SNPs have a depth over 50 for the first 10 Kb QPX contigs size.

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

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

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

```
[1] 11.4
```

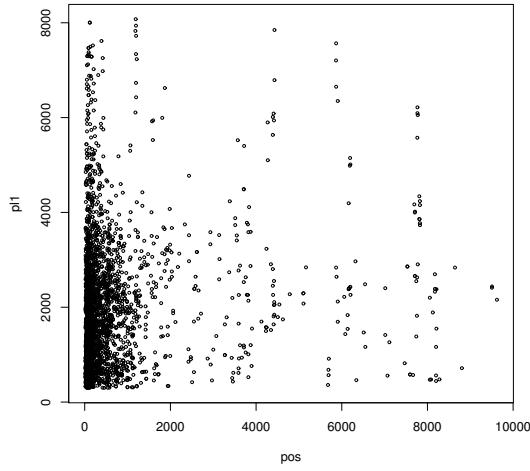


187

188 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
```



190

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:

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

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

194 Accordingly, REF/REF (pl1) is significant. Meaning the genotype we have is homozygous for the reference
195 nucleotide (not the variant), but if a variant exists, thus it represents a rare mutation (*reference needed*).
196 Therefore, the raw likelihoods must be calculated with the equation above for the picked variants and the
197 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

```

198 Lets get the variants with the highest probability that a genotype has been identified. $PL=1$ determines
 199 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

```

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

```

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

```

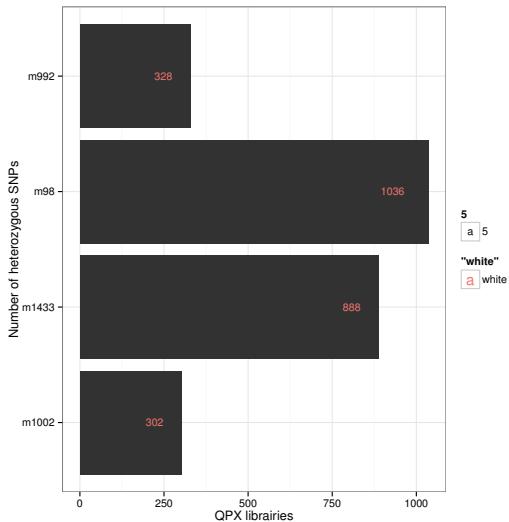
201 The original total number of SNPs was 3177 among which the total number of variants with an heterozy-
 202 gous genotype is 2554.

```

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)) +
  labs(x = "Number of heterozygous SNPs",
       y = "QPX librairies")

```

[†] A summary of the plots in this subsection can be found in the Supplemental Information



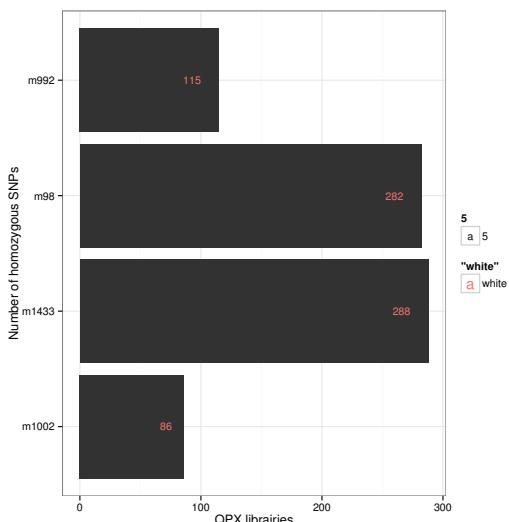
203
204 Now lets get the homozygous variants with genotype ALT/ALT with 90 %.

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

205 The total number of variants ALT/ALT is 771. Interesting thing is that using the combined assembly as a
206 reference (not showing here), m1433 had also the highest number of homozygous alleles while m98 had
207 half the number shown below.

↑ The same analysis was done twice with genome reference and the combined assembly as reference

```
dat <- as.data.frame(summary(altnall$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)) +
  labs(x = "Number of homozygous SNPs",
       y = "QPX librairies")
```



208
209 **8 Protein domain annotation**
210 Get the number of protein domains that can be predicted from the MMETSP strains. First, assembled
211 contigs must be translated into peptides. HMMER3.2b was used for annotation. Hidden Markov Models

212 were generated on Pfam database. The table below lists old and new annotations against old and new
213 Pfam v26 and v28 libraries. (> 2 years interval between versions).

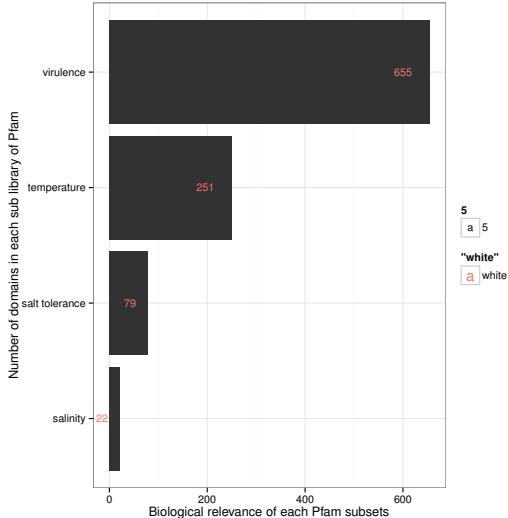
```
pfam <- read.xlsx("./data/pfam.xlsx", sheetIndex = 1)
pfam

      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
```

214 Number of domains found in Pfam v28 for :

- 215 • Virulence
216 • Temperature
217 • Salinity
218 • 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)) +
  labs(x = "Number of domains in each sub library of Pfam",
       y = "Biological relevance of each Pfam subsets")
```

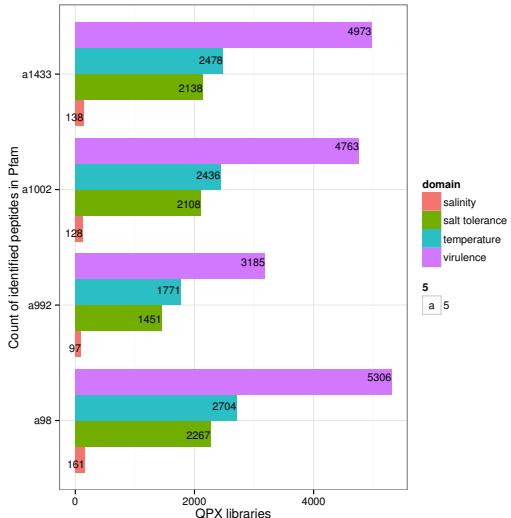


219
220 The number of domains that can be estimated from 4 QPX strains. For example if 2 totally different contigs
221 are aligned to one same domain the counter is incremented by 2.

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

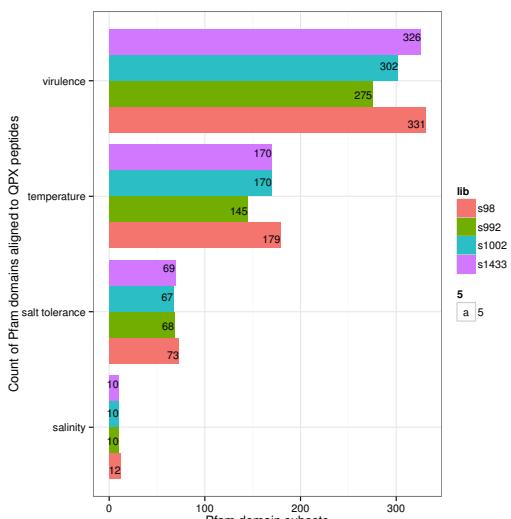
      domain pfam a98 a992 a1002 a1433 annot
1   virulence   655 5306 3185 4763 4973   new
2   temperature   251 2704 1771 2436 2478   new
3   salinity     22 161    97 128   138   new
4 salt tolerance   79 2267 1451 2108 2138   new

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)) +
  labs(x = "Count of identified peptides in Pfam",
       y = "QPX libraries")
```



222
223 The *unique* number of domains identified from the alignment. For example, if 2 totally different contigs
224 are aligned to one domain the counter is incremented by 1.

```
singlepfam <- select(pfam, contains("s"))
singlepfam <- cbind(singlepfam, X = pfam$domain, Y = pfam$annot)
singlepfam <- filter(singlepfam, Y == "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)) +
  labs(x = "Count of Pfam domains aligned to QPX peptides",
       y = "Pfam domain subsets")
```

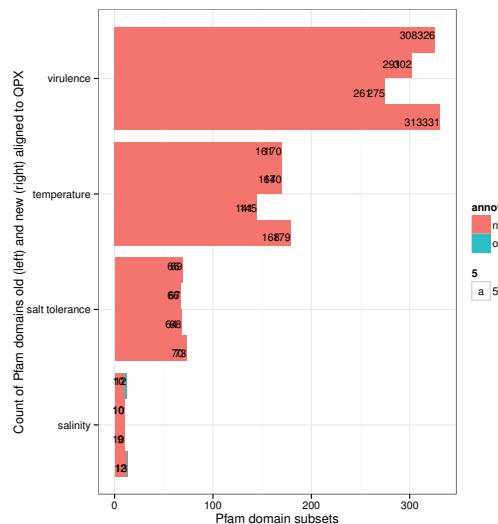


225
226 Difference in domain-peptide alignments between old and new pfam databases. Numbers on the right
227 belong to the new Pfam library. Numbers on the left belong to the old Pfam library.

```

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)) +
  labs(x = "Count of Pfam domains old (left) and new (right) aligned to QPX",
       y = "Pfam domain subsets")

```



228

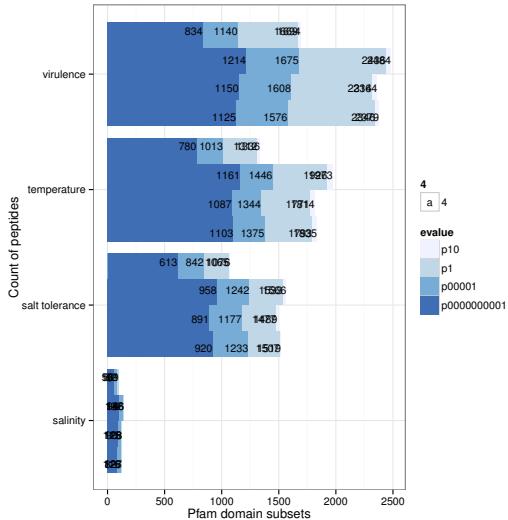
229 Get the number of peptides that match a significant e-value domain.

[†] The new Pfam library will be used for the remaining tests

```

pfam2 <- read.xlsx("./data/pfam.xlsx", sheetIndex = 2)
pfam2 <- filter(pfam2, annot == "contig")
pfam2 <- gather(pfam2, "evalue", "count", 2:5)
ggplot(pfam2,
       aes(x = pfam,
            y = count,
            fill = evalue,
            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)) +
  labs(x = "Count of peptides",
       y = "Pfam domain subsets")

```

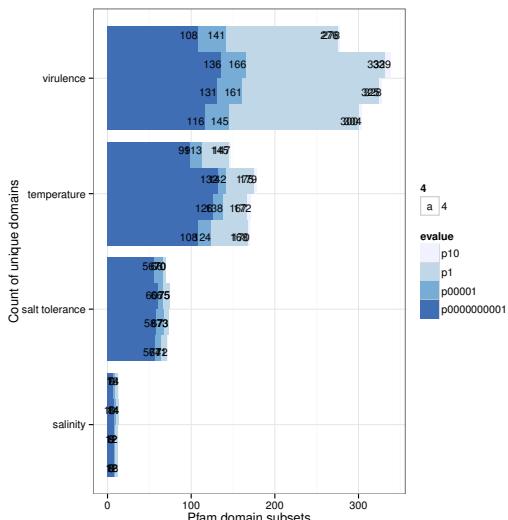


230

231 How many *unique* protein domains were found at different evalue significance.

```
pfam2 <- read.xlsx("./data/pfam.xlsx", sheetIndex = 2)
pfam2 <- filter(pfam2, annot == "domain")
pfam2 <- gather(pfam2, "evalue", "count", 2:5)
ggplot(pfam2,
  aes(x = pfam,
      y = count,
      fill = evalue,
      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)) +
  labs(x = "Count of unique domains",
       y = "Pfam domain subsets")
```

[†] A higher resolution summary of these 2 plots can be found in the Manuscript



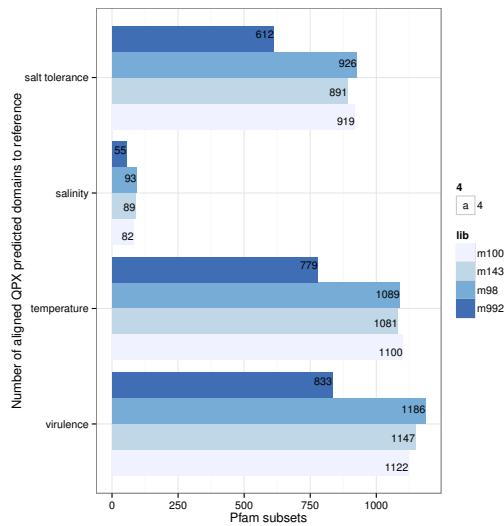
232

9 Align assembled contigs to Genome (v15)

Here is the overall stats of the BLAT of the 4 strains RNA sequenced contigs against SR. genome v15. The QPX contigs have been annotated with pfam. They are aligned to the reference genome for SNP

236 localization. Hence we can identify SNP hotspots inside and outside functional domains.

```
blat <- read.table("./data/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 = 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)) +
  labs(x = "Number of aligned QPX predicted domains to reference",
    y = "Pfam subsets")
```



237

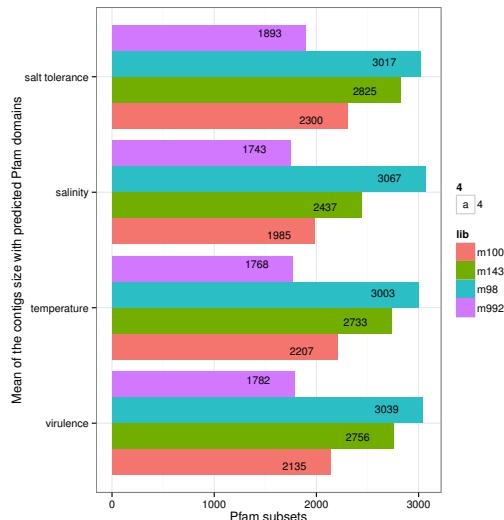
238 From the table data above the *minimum identity* of all contigs aligned is 0.9. The *mean query* is necessary
239 to choose the number of contigs mapped. Since each contig can be found multiple times in the genome (at
240 different alignment lengths of course) it is best if we choose the best contigs those that have a maximum
241 alignment length (since the PCA analysis has shown previously that a longer alignment is helpful to
242 distinguishing between strains). Contigs must be mapped/aligned once and thus, no duplicate entries
243 should be selected. For this reason choosing **an alignment length equal to the half of the mean of the**
244 **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)) +
labs(x = "Mean of the contigs size with predicted Pfam domains",
      y = "Pfam subsets")

```



245

10 Assessing SNP hotspots in 4 QPX strains

246
247
248
249
250
251
252

QPX contig assemblies by the MMETSP team were used for pfam annotation (with HMMER). SNP calling on the 4 strains used Steve Roberts reference genome v15 (called with GATK). Location of SNPs in the pfam domains was inferred after alignment of the QPX contigs (those that include a predicted functional protein domain) on the reference genome (with BLAT). Finally all data were merged in one file grouped by 4 QPX strains (2 from NY, one from each VA and MA) and 3 pfam subset pathways (Virulence, salinity/salt-tolerance, temperature).

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

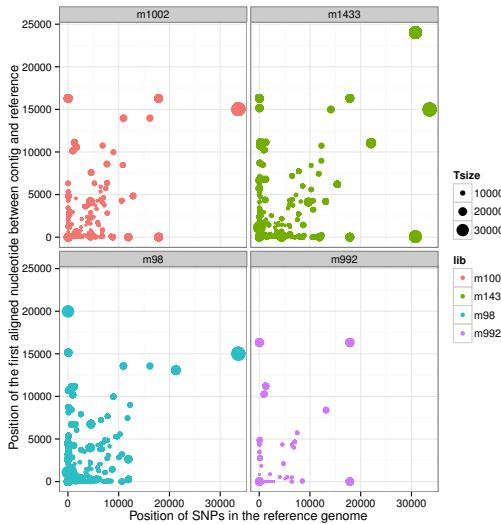
What is the correlation between a SNP position and the first reference nucleotide that align to a contig containing domain?

↑ This summary file includes SNP location on peptide domains and can be traced back to the 4 strains of QPX and the reference genome. This file can be imported into a MySQL database

```

ggplot(hotspots.raw,
       aes(x = Position,
            y = Tstart)) +
theme_bw() +
geom_point(aes(color = lib,
               size = Tsize)) +
facet_wrap(~ lib, ncol = 2) +
labs(x = "Position of SNPs in the reference genome",
      y = "Position of the first aligned nucleotide between contig and reference")

```

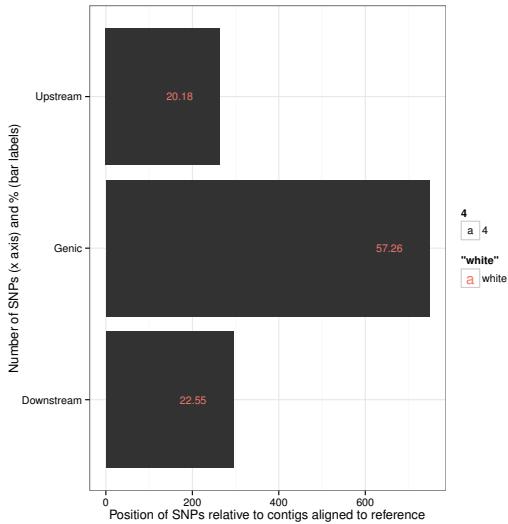


255
256
257

How many SNPs can be found inside and outside of protein domains, those of which reside in assembled QPX contigs?

[↑ A higher resolution version of this plot can be find in the Manuscript](#)

```
count <- c(264, 749, 295)
position <- c("Upstream", "Genic", "Downstream")
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")) +
  labs(x = "Number of SNPs (x axis) and % (bar labels)",
       y = "Position of SNPs relative to contigs aligned to reference")
```



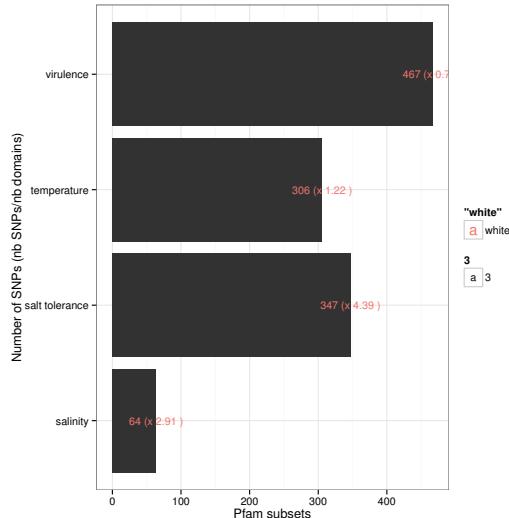
258
259
260
261
262

How are SNPs distributed between Pfam subsets? On the chart, the score between parenthesis is the normalized amount of SNPs. It has no units. It is just a score of the normalized counts of SNPs by the number of domains found in each subset. The counts are those of the position of SNPs inside the 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")) +
  labs(x = "Number of SNPs (nb SNPs/nb domains)",
       y = "Pfam subsets")

```

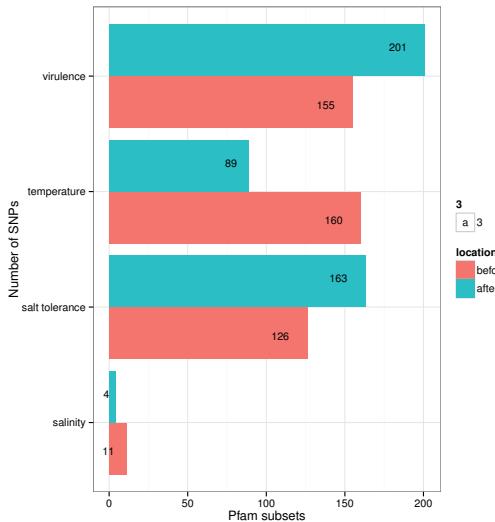


263
264 How many SNPs can be found outside of each domain? The outside SNPs can be upstream or down-
265 stream the aligned contig over the reference. The SNP position outside the domains is dependent on the
266 Reference contig length, which was selected through alignment.

```

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)) +
  labs(x = "Number of SNPs",
       y = "Pfam subsets")

```



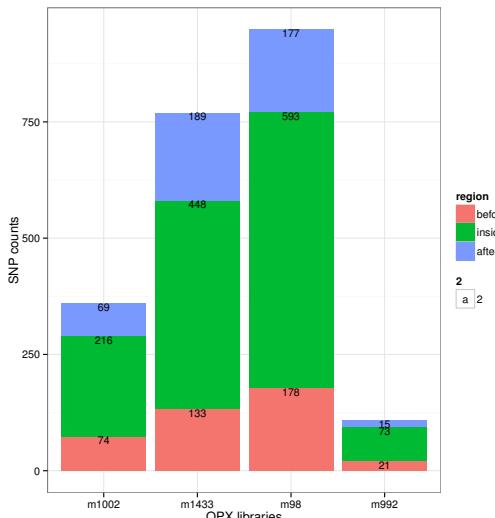
267

268 How many SNPs can be found inside and outside protein domains within each QPX strain?

```

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() +
  labs(x = "QPX libraries",
       y = "SNP counts")

```



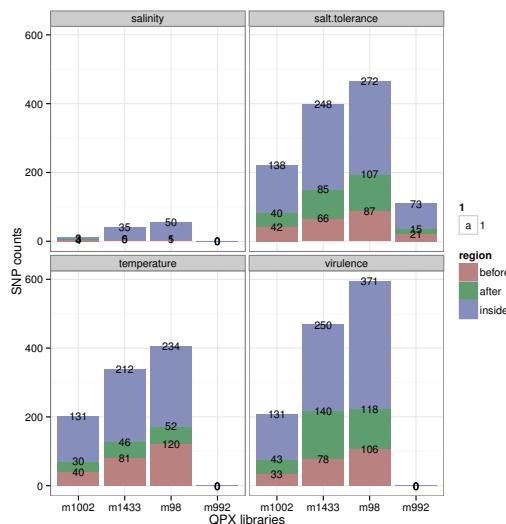
269

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

```

dat <- read.xlsx("./data/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) +
  labs(x = "QPX libraries",
       y = "SNP counts")

```



272

273 Preferential substitution inside/outside domains, per Pfam subset, and for each strain.

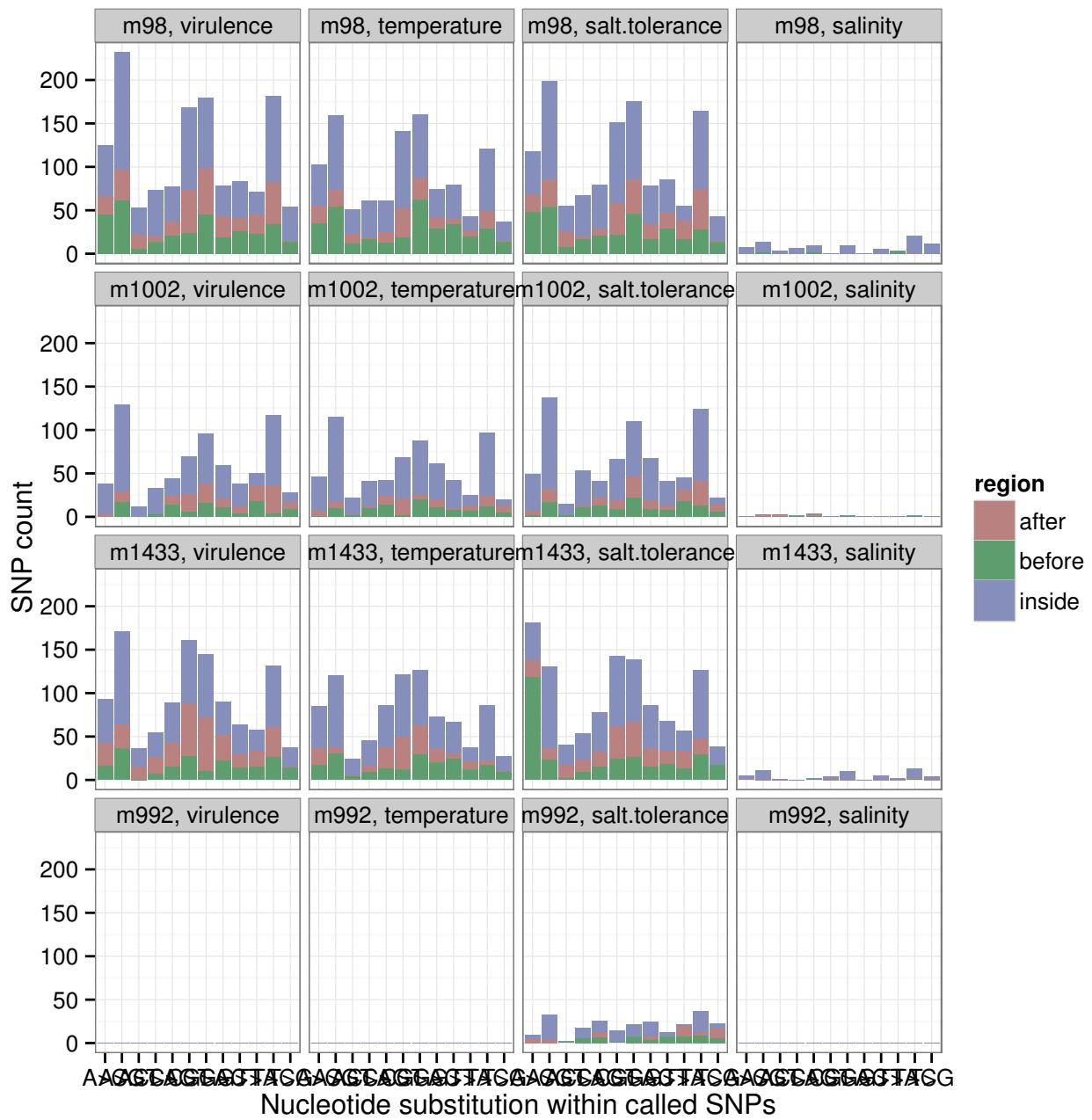
```

dat <- read.xlsx("./data/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) +
  labs(x = "Nucleotide substitution within called SNPs",
       y = "SNP count")

```



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Frequency of SNPs inside Pfam domains for each strain, per 1 Kbp. Normalized by the total size of contigs for each strain.

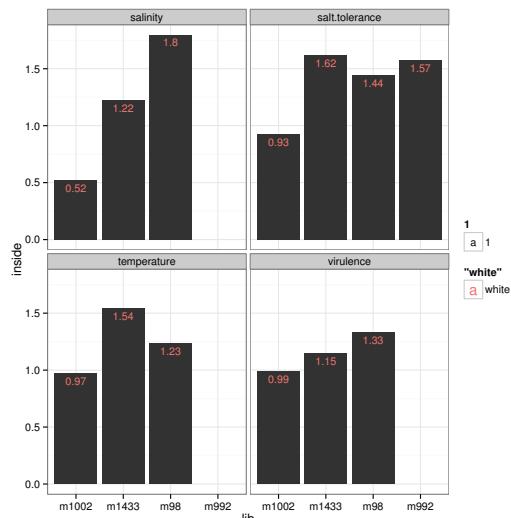
```
dat <- read.xlsx("./data/hotspots/snps.all.pfam.xlsx", sheetIndex = 1)
```

```

inside <- c(593, 73, 216, 448)
sizen <- c(425098, 46409, 242136, 337206)
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).

```



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278 11 Machine learning on SNP hotspots of 4 QPX strains

279 Lets try a support vector machine classifier to differentiate between the QPX strains using quality data
280 (above) of the variants. In progress ...

281 12 System Information

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

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

```
sessionInfo()
```

```
R version 3.2.1 (2015-06-18)
Platform: x86_64-unknown-linux-gnu (64-bit)
Running under: elementary OS Luna

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] ggbiplots_0.55    scales_0.2.5      plyr_1.8.3
[4] vegan_2.3-0       permute_0.8-4    tidyverse_0.2.0
[7] dplyr_0.4.2       latticeExtra_0.6-26 RColorBrewer_1.1-2
[10] glmnet_2.0-2     foreach_1.4.2    Matrix_1.2-1
[13] leaps_2.9        caret_6.0-47    ggplot2_1.0.1
[16] lattice_0.20-31  xlsx_0.5.7     xlsxjars_0.6.1
[19] rJava_0.9-6      knitr_1.10.5   RevoUtilsMath_3.2.1

loaded via a namespace (and not attached):
[1] Rcpp_0.11.6        compiler_3.2.1   formatR_1.2
[4] nloptr_1.0.4       highr_0.5       iterators_1.0.7
[7] tools_3.2.1        digest_0.6.8    lme4_1.1-8
[10] evaluate_0.7      nlme_3.1-121   gtable_0.1.2
[13] mgcv_1.8-6        DBI_0.3.1      parallel_3.2.1
[16] brglm_0.5-9       SparseM_1.6    proto_0.3-10
[19] cluster_2.0.2     BradleyTerry2_1.0-6 stringr_1.0.0
[22] gtools_3.5.0       nnet_7.3-10    R6_2.0.1
[25] minqa_1.2.4       reshape2_1.4.1  car_2.0-25
[28] magrittr_1.5       codetools_0.2-11 MASS_7.3-41
[31] splines_3.2.1     assertthat_0.1  pbkrtest_0.4-2
[34] colorspace_1.2-6   labeling_0.3    quantreg_5.11
[37] stringi_0.5-5     lazyeval_0.1.10 munsell_0.4.2
```