PCA

October 27, 2019

[1]:
```
install.packages("BiocManager")
library(BiocManager)
BiocManager::install("DESeq2")
library("DESeq2")
```

```
Updating HTML index of packages in '.Library'
Making 'packages.html' … done
Bioconductor version 3.9 (BiocManager 1.30.9), ?BiocManager::install for help
Bioconductor version 3.9 (BiocManager 1.30.9), R 3.6.1 (2019-07-05)
Installing package(s) 'DESeq2'
Updating HTML index of packages in '.Library'
Making 'packages.html' … done
Old packages: 'askpass', 'backports', 'boot', 'callr', 'caret', 'clipr',
  'cluster', 'curl', 'data.table', 'dbplyr', 'digest', 'dplyr', 'evaluate',
  'foreach', 'foreign', 'formatR', 'fs', 'ggplot2', 'glmnet', 'gower', 'haven',
  'hexbin', 'hms', 'htmltools', 'htmlwidgets', 'httpuv', 'httr', 'ipred',
  'IRkernel', 'iterators', 'KernSmooth', 'knitr', 'later', 'lava', 'markdown',
  'MASS', 'mgcv', 'mime', 'modelr', 'nlme', 'numDeriv', 'openssl', 'pillar',
  'pkgconfig', 'processx', 'progress', 'promises', 'purrr', 'quantmod', 'Rcpp',
  'recipes', 'repr', 'reprex', 'rmarkdown', 'rvest', 'shiny', 'sys', 'tibble',
  'tidyr', 'tinytex', 'TTR', 'whisker', 'xfun', 'xml2'
Loading required package: S4Vectors
Loading required package: stats4
Loading required package: BiocGenerics
Loading required package: parallel

Attaching package: ‘BiocGenerics’

The following objects are masked from ‘package:parallel’:

    clusterApply, clusterApplyLB, clusterCall, clusterEvalQ,
    clusterExport, clusterMap, parApply, parCapply, parLapply,
    parLapplyLB, parRapply, parSapply, parSapplyLB

The following objects are masked from ‘package:stats’:

    IQR, mad, sd, var, xtabs
```

```
The following objects are masked from ‘package:base’:

    anyDuplicated, append, as.data.frame, basename, cbind, colnames,
    dirname, do.call, duplicated, eval, evalq, Filter, Find, get, grep,
    grepl, intersect, is.unsorted, lapply, Map, mapply, match, mget,
    order, paste, pmax, pmax.int, pmin, pmin.int, Position, rank,
    rbind, Reduce, rownames, sapply, setdiff, sort, table, tapply,
    union, unique, unsplit, which, which.max, which.min


Attaching package: ‘S4Vectors’

The following object is masked from ‘package:base’:

    expand.grid

Loading required package: IRanges
Loading required package: GenomicRanges
Loading required package: GenomeInfoDb
Loading required package: SummarizedExperiment
Loading required package: Biobase
Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.

Loading required package: DelayedArray
Loading required package: matrixStats

Attaching package: ‘matrixStats’

The following objects are masked from ‘package:Biobase’:

    anyMissing, rowMedians

Loading required package: BiocParallel

Attaching package: ‘DelayedArray’

The following objects are masked from ‘package:matrixStats’:

    colMaxs, colMins, colRanges, rowMaxs, rowMins, rowRanges

The following objects are masked from ‘package:base’:

    aperm, apply, rowsum
```

```
Registered S3 methods overwritten by 'ggplot2':
  method         from
  [.quosures     rlang
  c.quosures     rlang
  print.quosures rlang
```

[18]:
```
BiocManager::install("vsn")
```

```
Bioconductor version 3.9 (BiocManager 1.30.9), R 3.6.1 (2019-07-05)
Installing package(s) 'vsn'
also installing the dependencies ‘affyio’, ‘preprocessCore’, ‘affy’, ‘limma’

Updating HTML index of packages in '.Library'
Making 'packages.html' … done
Old packages: 'askpass', 'backports', 'boot', 'callr', 'caret', 'clipr',
  'cluster', 'curl', 'data.table', 'dbplyr', 'digest', 'dplyr', 'evaluate',
  'foreach', 'foreign', 'formatR', 'fs', 'ggplot2', 'glmnet', 'gower', 'haven',
  'hexbin', 'hms', 'htmltools', 'htmlwidgets', 'httpuv', 'httr', 'ipred',
  'IRkernel', 'iterators', 'KernSmooth', 'knitr', 'later', 'lava', 'markdown',
  'MASS', 'mgcv', 'mime', 'modelr', 'nlme', 'numDeriv', 'openssl', 'pillar',
  'pkgconfig', 'processx', 'progress', 'promises', 'purrr', 'quantmod', 'Rcpp',
  'recipes', 'repr', 'reprex', 'rmarkdown', 'rvest', 'shiny', 'sys', 'tibble',
  'tidyr', 'tinytex', 'TTR', 'whisker', 'xfun', 'xml2'
```

[4]:
```
library(magrittr) # this will allow us to string commands together in a UNIX␣
 ↪-pipe - like fashion using % >%

 # get the table of read counts by indicating the path to the file
readcounts <-read.table ("./raw_countstdl.tsv", header = TRUE)
head(readcounts)
str(readcounts)
```

	ctl1	ctl2	ctl3	ctl4	treat1	treat2	treat3	treat4
ENSMUSG00000102693	0	0	0	0	0	0	0	0
ENSMUSG00000064842	0	0	0	0	1	0	0	0
ENSMUSG00000051951	2	0	1	1	11	2	1	24
ENSMUSG00000102851	0	0	0	0	0	0	0	0
ENSMUSG00000103377	0	0	0	0	0	0	0	0
ENSMUSG00000104017	0	0	0	0	0	0	0	0

```
'data.frame':   45706 obs. of  8 variables:
 $ ctl1  : int  0 0 2 0 0 0 0 0 0 0 …
 $ ctl2  : int  0 0 0 0 0 0 0 0 0 1 …
 $ ctl3  : int  0 0 1 0 0 0 0 0 0 0 …
 $ ctl4  : int  0 0 1 0 0 0 0 0 0 0 …
 $ treat1: int  0 1 11 0 0 0 0 0 0 0 …
 $ treat2: int  0 0 2 0 0 0 0 0 0 0 …
 $ treat3: int  0 0 1 0 0 0 0 0 0 0 …
```

```
 $ treat4: int  0 0 24 0 0 0 0 0 0 0 …
```

[7]:
```
# make a data.frame with meta-data where row.names should match the individual␣
↪sample names
sample_info <- data.frame(condition = gsub ( "ctl[1-4]+" , "ctl" ,␣
↪names(readcounts)), row.names = names(readcounts))
sample_info
```

	condition
ctl1	ctl
ctl2	ctl
ctl3	ctl
ctl4	ctl
treat1	treat1
treat2	treat2
treat3	treat3
treat4	treat4

[10]:
```
# generate the DESeqDataSet
DESeq.ds <- DESeqDataSetFromMatrix(countData = readcounts, colData =␣
↪sample_info, design = ~condition )

 # check the result :
colData(DESeq.ds ) %>% head
assay(DESeq.ds, "counts" ) %>% head
rowData(DESeq.ds) %>% head
```

```
DataFrame with 6 rows and 1 column
       condition
        <factor>
ctl1          ctl
ctl2          ctl
ctl3          ctl
ctl4          ctl
treat1     treat1
treat2     treat2
```

	ctl1	ctl2	ctl3	ctl4	treat1	treat2	treat3	treat4
ENSMUSG00000102693	0	0	0	0	0	0	0	0
ENSMUSG00000064842	0	0	0	0	1	0	0	0
ENSMUSG00000051951	2	0	1	1	11	2	1	24
ENSMUSG00000102851	0	0	0	0	0	0	0	0
ENSMUSG00000103377	0	0	0	0	0	0	0	0
ENSMUSG00000104017	0	0	0	0	0	0	0	0

```
DataFrame with 6 rows and 0 columns
```

[12]:
```
# test what counts() returns
counts(DESeq.ds) %>% str

 # remove genes without any counts
DESeq.ds <- DESeq.ds [rowSums(counts(DESeq.ds)) > 0, ]

 # investigate different library sizes

colSums(counts(DESeq.ds)) == colSums(readcounts) # should be the same as␣
 ↪colSums ( readcounts )
```

```
 int [1:45706, 1:8] 0 0 2 0 0 0 0 0 0 0 …
 - attr(*, "dimnames")=List of 2
  ..$ : chr [1:45706] "ENSMUSG00000102693" "ENSMUSG00000064842"
"ENSMUSG00000051951" "ENSMUSG00000102851" …
  ..$ : chr [1:8] "ctl1" "ctl2" "ctl3" "ctl4" …
```

ctl1 TRUE **ctl2** TRUE **ctl3** TRUE **ctl4** TRUE **treat1** TRUE **treat2** TRUE **treat3** TRUE **treat4** TRUE

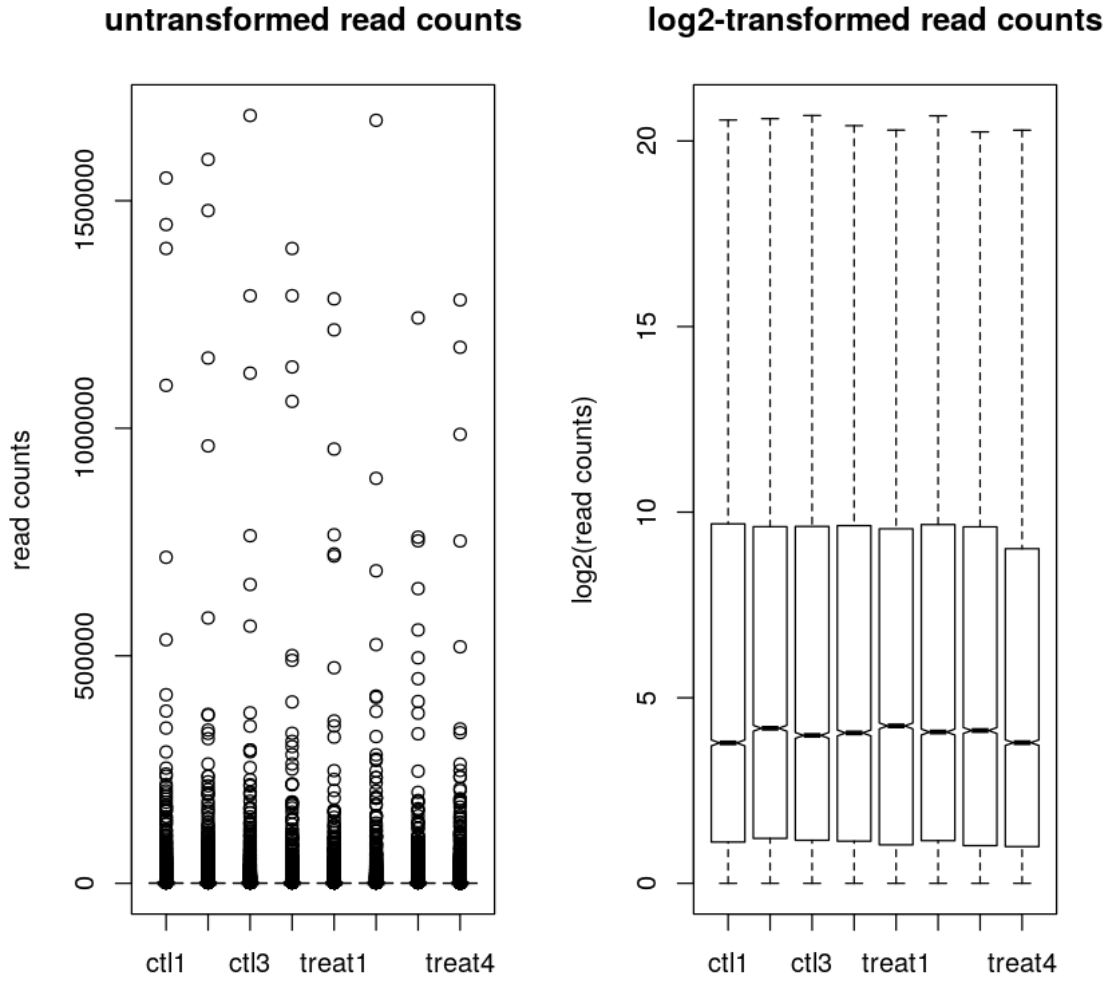
[13]:
```
#DESeq2's default method to normalize read counts to account for differences in␣
 ↪sequencing depths is imple-
#mented in estimateSizeFactors()
#1. for every gene (= row), determine the geometric mean of its read counts␣
 ↪across all samples (yielding
#the "pseudo-reference", i.e. one value per gene);
#2. divide every value of the count matrix by the corresponding␣
 ↪pseudo-reference value;
#3. for every sample (= column), determine the median of these ratios. This is␣
 ↪the size factor.

  # calculate the size factor and add it to the data set
DESeq.ds <- estimateSizeFactors(DESeq.ds)
sizeFactors(DESeq.ds)

 # if you check colData () again , you see that this now contains the␣
 ↪sizeFactors
colData (DESeq.ds)

# counts () allows you to immediately retrieve the _ normalized _ read counts
counts.sf_normalized <- counts(DESeq.ds, normalized = TRUE)
```

ctl1 0.861385212241083 **ctl2** 0.760175549092362 **ctl3** 0.808985675298092 **ctl4** 0.833769556412077 **treat1** 0.94871557100813 **treat2** 0.819283353107582 **treat3** 0.978758960142215 **treat4** 4.05397505356715

```
DataFrame with 8 rows and 2 columns
       condition        sizeFactor
```

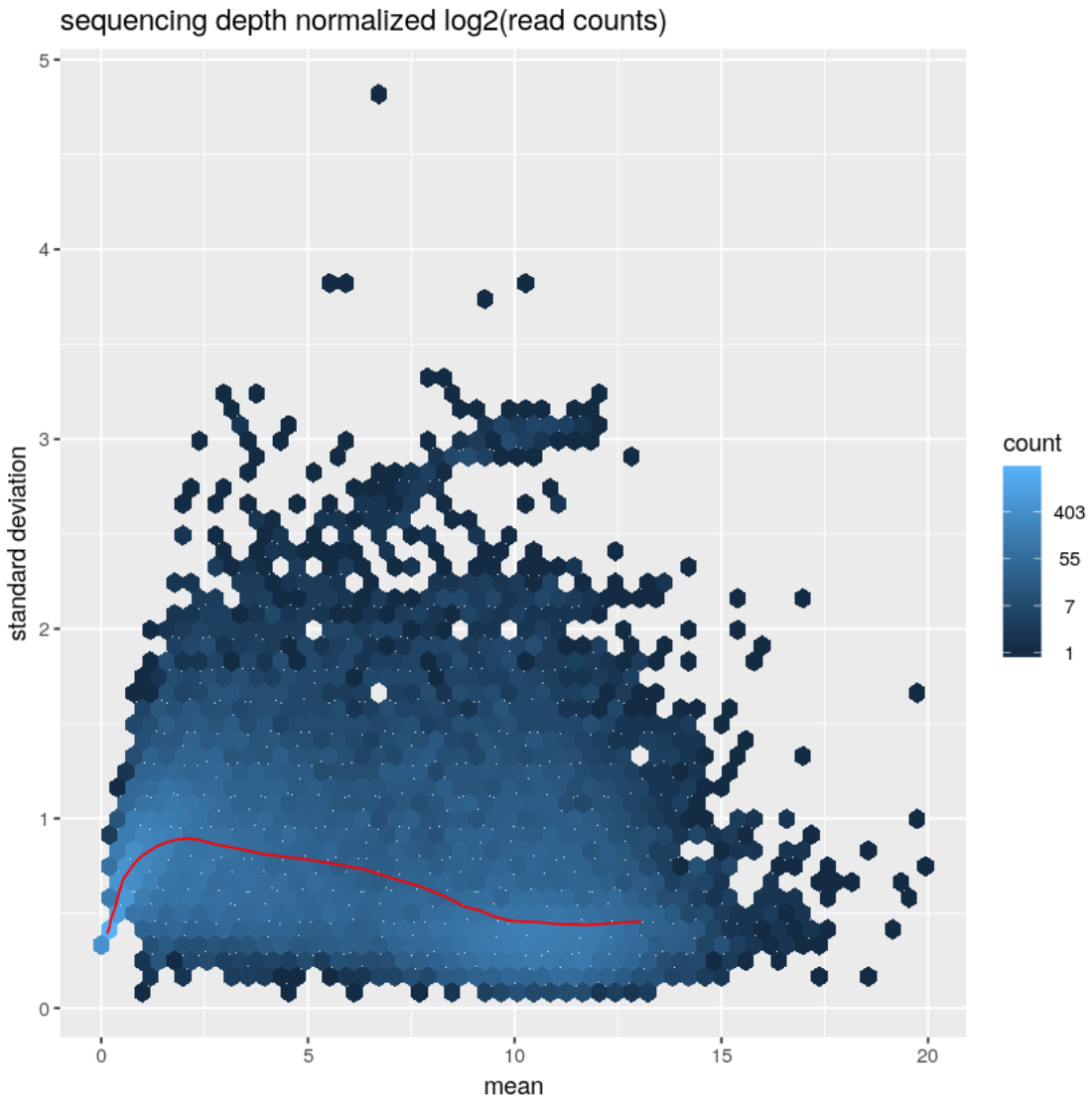
```
        <factor>         <numeric>
ctl1         ctl 0.861385212241083
ctl2         ctl 0.760175549092362
ctl3         ctl 0.808985675298092
ctl4         ctl 0.833769556412077
treat1    treat1  0.94871557100813
treat2    treat2 0.819283353107582
treat3    treat3 0.978758960142215
treat4    treat4  4.05397505356715
```

[20]:
```
#5.2
#Transformation of sequencing-depth-normalized read counts
#Due to the relatively large dynamic range of expression values that RNA-seq␣
 ↪data can cover, many down-
#stream analyses (including clustering) work much better if the read counts are␣
 ↪transformed to the log scale
#following normalization. While you will occasionally see log10 transformed␣
 ↪read counts, log2 is more com-
#monly used because it is easier to think about doubled values rather than␣
 ↪powers of 10. The transformation
#should be done in addition to sequencing depth normalization.

# transform size - factor normalized read counts to log2 scale using␣
 ↪pseudocount of 1

log.norm.counts <- log2(counts.sf_normalized + 1)

# plot the following two images underneath each other
par(mfrow = c(1,2))

# boxplots of non-transformed read counts ( one per sample )
boxplot(counts.sf_normalized, notch = TRUE , main = "untransformed read counts"␣
 ↪, ylab = "read counts")

# box plots of log2 - transformed read counts
boxplot (log.norm.counts, notch = TRUE , main = "log2-transformed read counts"␣
 ↪, ylab = "log2(read counts)")
```



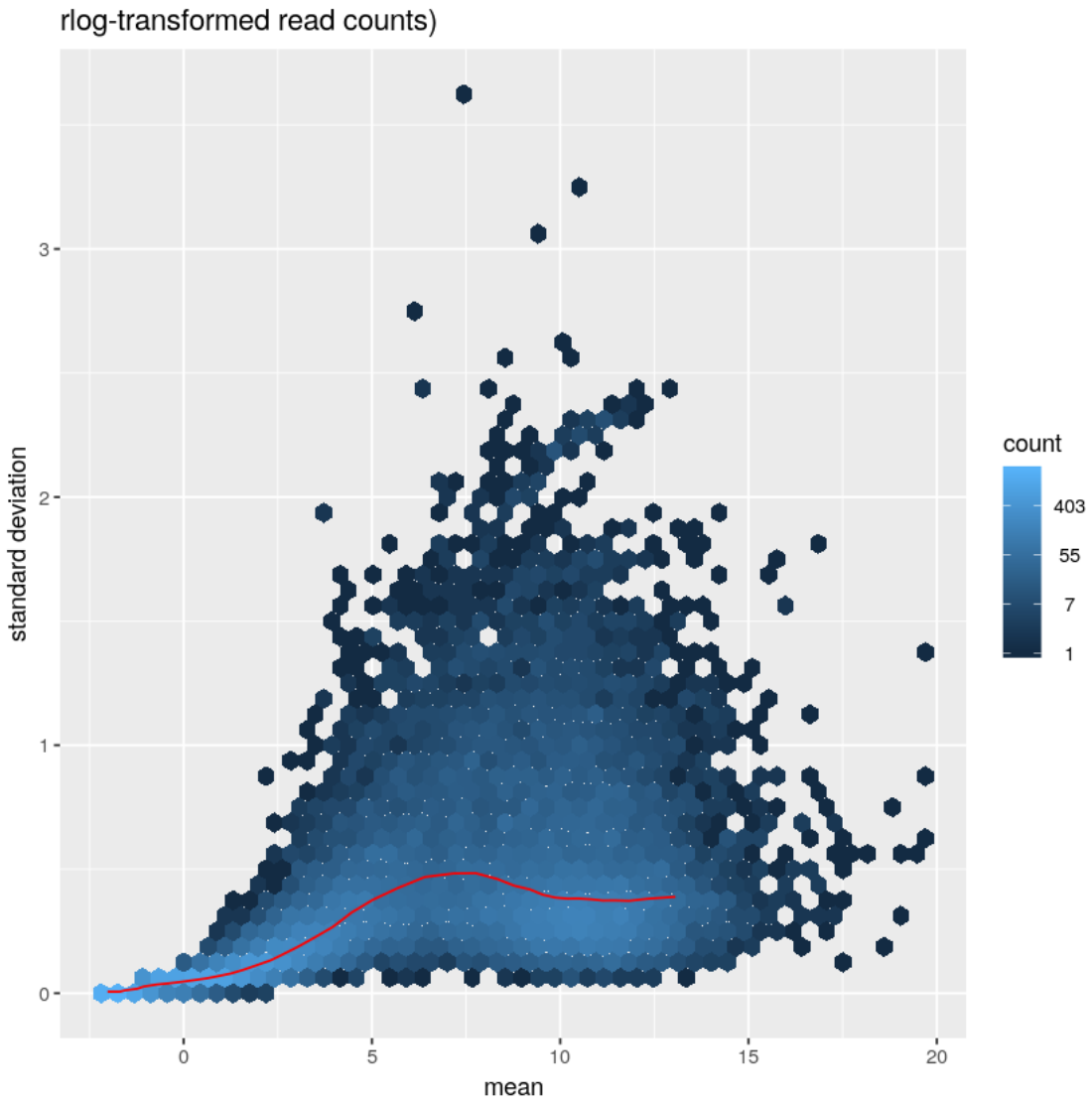
[22]:
```
#Many statistical tests and analyses assume that data is homoskedastic, i.e. that all variables have similar
#variance. However, data with large differences among the sizes of the individual observations often shows
#heteroskedastic behavior. One way to visually check for heteroskedasticity is to plot the mean vs. the standard
#deviation.

 #mean - sd plot

library(vsn)
library(ggplot2)
msd_plot <- meanSdPlot(log.norm.counts, ranks = FALSE, plot = FALSE )
```

```
msd_plot$gg +
    ggtitle("sequencing depth normalized log2(read counts)") +
    ylab("standard deviation")
```

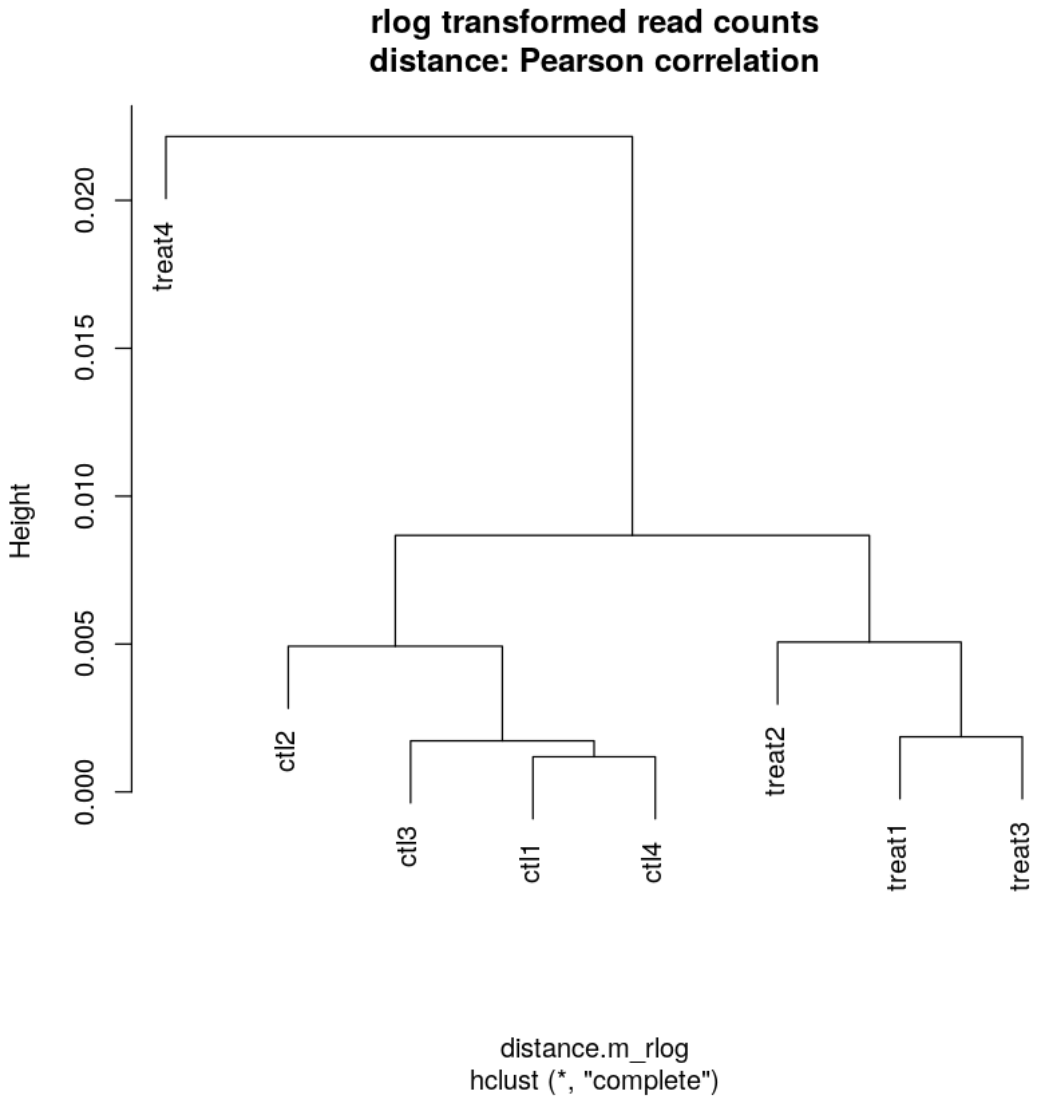


[24]:
```
#Transformation of read counts including variance shrinkage
#To reduce the amount of heteroskedasticity, DESeq2 and also edgeR offer␣
 ↪several means to shrink the variance
#of low read counts. They do this by using the dispersion-mean trend that can␣
 ↪be observed for the entire
#data set as a reference. Consequently, genes with low and highly variable read␣
 ↪counts will be assigned more
#homogeneous read count estimates so that their variance resembles the variance␣
 ↪observed for the majority
```

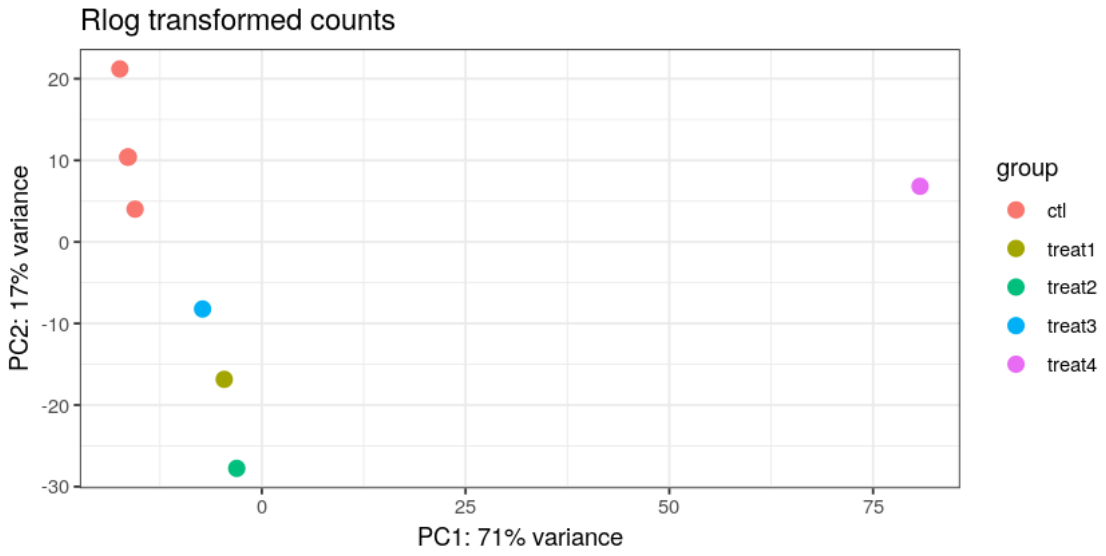
```
#of the genes (which hopefully have a more stable variance).

  # obtain regularized log - transformed values

#The rlog() function's blind parameter should be set to FALSE if the different␣
 ↪conditions lead to strong differences in a large proportion of the genes.

DESeq.rlog <- rlog(DESeq.ds , blind = FALSE )
rlog.norm.counts <- assay(DESeq.rlog)

 #mean - sd plot for rlog - transformed data

library(vsn)
library(ggplot2)
msd_plot <- meanSdPlot(rlog.norm.counts, ranks = FALSE, plot = FALSE )
msd_plot$gg +
    ggtitle("rlog-transformed read counts)") +
    ylab("standard deviation")
```



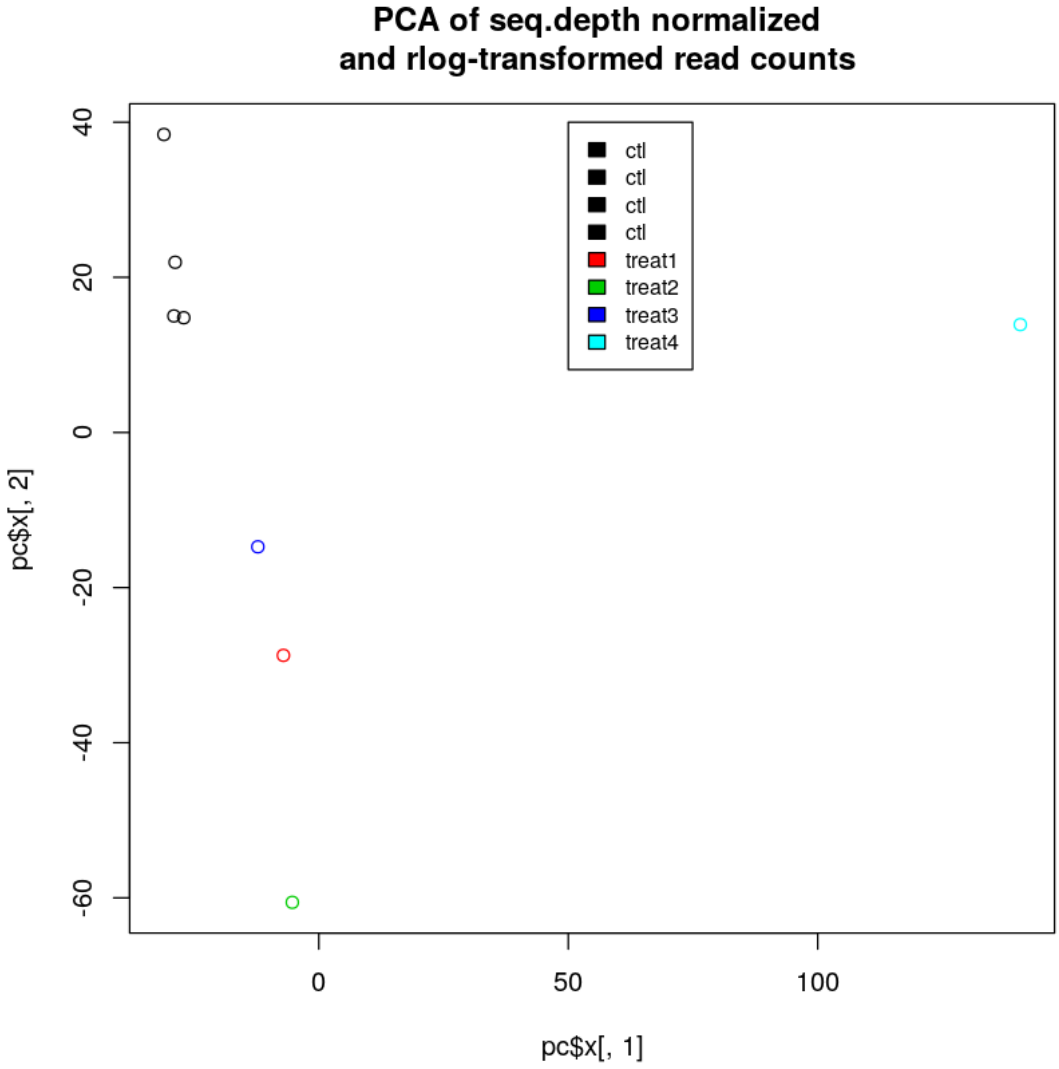
[25]:
```
#A dendrogram can be generated in R using the functions cor(), as.dist(), and␣
 ↪hclust():

# cor () calculates the correlation between columns of a matrix
distance.m_rlog <- as.dist(1 - cor(rlog.norm.counts , method = "pearson"))

# plot () can directly interpret the output of hclust ()
plot(hclust(distance.m_rlog),
    labels = colnames(rlog.norm.counts) ,
    main = "rlog transformed read counts\ndistance: Pearson correlation")
```



[30]:
```
#DESeq2 also offers a convenience function based on ggplot2 to do PCA directly␣
↪on a DESeqDataSet:

 # PCA
P <- plotPCA (DESeq.rlog)
 # plot cosmetics
P <- P + theme_bw() + ggtitle("Rlog transformed counts")
print(P)
```



[37]:
```
#PCA can be performed in base R using the function prcomp().
pc <- prcomp(t(rlog.norm.counts))

plot(pc$x[,1], pc$x[,2],
    col = colData(DESeq.ds)[,1],
    main = " PCA of seq.depth normalized\n and rlog-transformed read counts")
legend(50,40, legend = colData(DESeq.ds)[,1], col = colData(DESeq.ds)[,1], fill␣
 ↪= colData(DESeq.ds)[,1], cex = 0.8 )
```


PCA of seq.depth normalized
and rlog-transformed read counts
ctl
ctl
ctl
ctl
treat1
treat2
treat3
treat4
40
20
0
-20
-40
-60
pc$x[, 2]
0
50
100
pc$x[, 1]