Applied Statistics for Bioinformatics using R

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Preface

The purpose of this book is to give an introduction into statistics in order to solve some problems of bioinformatics. Statistics provides procedures to explore and visualize data as well as to test biological hypotheses. The book intends to be introductory in explaining and programming elementary statistical concepts, thereby bridging the gap between high school levels and the specialized statistical literature. After studying this book readers have a sufficient background for *Bioconductor Case Studies* (Hahne et al., 2008) and *Bioinformatics and Computational Biology Solutions Using R and Bioconductor* (Genteman et al., 2005). The theory is kept minimal and is always illustrated by several examples with data from research in bioinformatics. Prerequisites to follow the stream of reasoning is limited to basic high-school knowledge about functions. It may, however, help to have some knowledge of gene expressions values (Pevsner, 2003) or statistics (Bain & Engelhardt, 1992; Ewens & Grant, 2005; Rosner, 2000; Samuels & Witmer, 2003), and elementary programming. To support self-study a sufficient amount of challenging exercises are given together with an appendix with answers.

The programming language R is becoming increasingly important because it is not only very flexible in reading, manipulating, and writing data, but all its outcomes are directly available as objects for further programming. R is a rapidly growing language making basic as well as advanced statistical programming easy. From an educational point of view, R provides the possibility to combine the learning of statistical concepts by mathematics, programming, and visualization. The plots and tables produced by R can readily be used in typewriting systems such as Emacs, LaTeX, or Word.

Chapter 1 gives a brief introduction into basic functionalities of R. Chapter 2 starts with univariate data visualization and the most important descriptive statistics. Chapter 3 gives commonly used discrete and continuous distributions to model events and the probability by which these occur. These distributions are applied in Chapter 4 to statistically test hypotheses from bioinformatics. For each test the statistics involved are briefly explained and its application is illustrated by examples. In Chapter 5 linear models are explained and applied to testing for differences between groups. It gives a basic approach. In Chapter 6 the three phases of analysis of microarray data (preprocessing, analysis, post processing) are briefly introduced and illustrated by many examples bringing ideas together with R scrips and interpretation of results. Chapter 7 starts with an intuitive approach into Euclidian distance

and explains how it can be used in two well-known types of cluster analysis to find groups of genes. It also explains how principal components analysis can be used to explore a large data matrix for the direction of largest variation. Chapter 8 shows how gene expressions can be used to predict the diagnosis of patients. Three such prediction methods are illustrated and compared. Chapter 9 introduces a query language to download sequences efficiently and gives various examples of computing important quantities such as alignment scores. Chapter 10 introduces the concept of a probability transition matrix which is applied to the estimation of phylogenetic trees and (Hidden) Markov Models.

R commands come after its prompt `>`, except when commands are part of the ongoing text. Input and output of R will be given in `verbatim typewriting style`. To save space sometimes not all of the original output from R is printed. The end of an example is indicated by the box □. In its Portable Document Format (PDF)[1] there are many links to the Index, Table of Contents, Equations, Tables, and Figures. Readers are encouraged to copy and paste scripts from the PDF into the R system in order to study its outcome. Apart from using the book to study application of statistics in bioinformatics, it can also be useful for statistical programming.

I would like to thank my colleges Joop Bouman, Sven Warris and Jan Peter Nap for their useful remarks on parts of an earlier draft. Many thanks also go to my students for asking questions that gave hints to improve clarity. Remarks to further improve the text are appreciated.

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Chapter 1

Brief Introduction into Using R

To get started a gentle introduction to the statistical programming language R will be given (R Development Core Team, 2009), specific for our purposes. This will solve the practical issues to follow the stream of reasoning. In particular, it is briefly explained how to install R and Bioconductor, how to obtain help, and how to perform simple calculations.

Since many computations are essentially performed on data vectors, several basic illustrations of this are given. With respect to gene expressions the data vectors are placed one beneath the other to form a data matrix with the genes as rows and the patients as columns. The idea of a data matrix is extensively explained and illustrated by several examples. A larger example consists of the classical Golub et al. (1999) data, which will be analyzed frequently to illustrate statistical procedures.

1.1 Getting R Started on your PC

You can downloaded R freely from `http://cran.r-project.org`. Click on your favorite operating system (`Windows`, `Linux` or `MacOS`) and simply follow the instructions. After a little patience you should be able to start R (Ihaka & Gentleman, 1996) after which a screen is opened with the prompt `>`. The input and output of R will be displayed in `verbatim typewriting style`.

All useful functions of R are contained in libraries which are called "packages". The standard installation of R makes basic packages available such as `base` and `stats`. From the button `Packages` at `cran.r-project.org` it can be seen that R has a huge number of packages available for a wide scale

of statistical procedures. To download a specific package you can use the following.

```
> install.packages(c("TeachingDemos"),repo="http://cran.r-project.org",
 + dep=TRUE)
```

This installs the package `TeachingDemos` developed by Greg Snow from the repository `http://cran.r-project.org`. By setting the option `dep` to `TRUE` the packages on which the `TeachingDemos` depend are also installed. This is strongly recommended! Alternatively, in the Windows application of R you can simply click on the `Packages` button at the top of your screen and follow the instructions. After installing you have to load the package in order to use its functions. For instance, to produce a nice plot of the outcome of throwing twelve times with a die, you can use the following.

```
> library(TeachingDemos)
> plot(dice(12,1))
```

In the sequel we shall often use packages from Bioconductor, a very useful open source software project for the analysis and comprehension of genomic data. To follow the book it is essential to install Bioconductor on your PC or network. Bioconductor is primarily based on R and can be installed, as follows.

```
> source("http://www.bioconductor.org/biocLite.R")
> biocLite()
```

Then to download the `ALL` package from a repository to your system, to load it, and to make the `ALL` data (Chiaretti, et. al, 2004) available for usage, you can use the following.

```
> biocLite("ALL")
> library(ALL)
> data(ALL)
```

These data will be analyzed extensively later-on in Chapter 5 and 6. General help on loaded Bioconductor packages becomes available by `openVignette()`. For further information the reader is referred to `www.bioconductor.org` or to several other URL's[1] .

[1] http://mccammon.ucsd.edu/~bgrant/bio3d/user_guide/user_guide.html
http://rafalab.jhsph.edu/software.html
http://dir.gmane.org/gmane.science.biology.informatics.conductor

In this and the following chapters we will illustrate many statistical ideas by the Golub et al. (1999) data, see also Section 1.8. The `golub` data become available by the following.[2]

```
> library(multtest)
> data(golub)
```

R is object-oriented in the sense that everything consists of objects belonging to certain classes. Type `class(golub)` to obtain the class of the object `golub` and `str(golub)` to obtain its structure or content. Type `objects()` or `ls()` to view the currently loaded objects, a list probably growing soon to be large. To prevent conflicting definitions, it is wise to remove them all at the end of a session by `rm(list=ls())`. To quit a session, type `q()`, or simply click on the cross in the upper right corner of your screen.

1.2 Getting help

All functionalities of R are well-organized in so-called packages. Use the function `library()` to see which packages are currently installed on your operating system. The packages `stats` and `base` are automatically installed, because these contain many basic functionalities. To obtain an overview of the content of a package use `ls(package:stats)` or `library(help="stats")`. Help on the purpose of specific functions can be obtained from the (package) manual by typing a question mark in front of a function. For instance, `?sum` gives details on summation. In case you are seeking help on a function which uses `if`, simply type `apropos("if")`. When you are starting with a new concept such as "boxplot", it is convenient to have an example showing output (a plot) and programming code. Such is given by `example(boxplot)`. The function `history` can be useful for collecting previously given commands.

Type `help.start()` to launch an HTML page linking to several well-written R manuals such as: "An Introduction to R", "The R Language Definition", "R Installation and Administration", and "R Data Import/Export". Further help can be obtained from `http://cran.r-project.org`. Its "contributed" page contains well-written freely available on-line books[3] and useful reference charts[4]. At http://www.r-project.org you can use `R site`

[2] Functions to read data into R are `read.table` or `read.csv`, see also the "The R Data Import/Export manual".

[3] "R for Beginners" by Emmanuel Paradis or the "The R Guide" by Jason Owen

[4] "R reference card" by Tom Short or by Jonathan Baron

`search`, `Rseek`, or other useful search engines. There are a number of useful URL's with information on R.[5]

1.3 Calculating with R

R can be used as a simple calculator. For instance, to add 2 and 3 we simply insert the following.

```
> 2+3
[1] 5
```

In many calculations the natural base $e = 2.718282$ of exponential functions is used. Such type of functions can be called as follows.

```
> exp(1)
[1] 2.718282
```

To compute $e^2 = e \cdot e$ we use `exp(2)`.[6] So, indeed, we have e^x =`exp(x)`, for any value of x.

The sum $1 + 2 + 3 + 4 + 5$ can be computed by

```
> sum(1:5)
[1] 15
```

and the product $5! = 5 \cdot 4 \cdot 3 \cdot 2 \cdot 1$ by

```
> prod(1:5)
[1] 120
```

1.4 Generating a sequence and a factor

In order to compute so-called quantiles of distributions (see e.g. Section 2.1.4) or plots of functions, we need to generate sequences of numbers. The easiest way to construct a sequence of numbers is by

```
> 1:5
[1] 1 2 3 4 5
```

[5] We mention in particular:
http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/R_BioCondManual.html

[6] The argument of functions is always placed between parenthesis `()`.

This sequence can also be produced by the function `seq`, which allows for various sizes of steps to be chosen. For instance, in order to compute percentiles of a distribution we may want to generate numbers between zero and one with step size equal to 0.1.

```
> seq(0,1,0.1)
 [1] 0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
```

For plotting and testing of hypotheses we need to generate yet another type of sequence, called a "factor". It is designed to indicate an experimental condition of a measurement or the group to which a patient belongs.[7] When, for instance, for each of three experimental conditions there are measurements from five patients, the corresponding factor can be generated as follows.

```
> factor <- gl(3,5)
> factor
 [1] 1 1 1 1 1 2 2 2 2 2 3 3 3 3 3
Levels: 1 2 3
```

The three conditions are often called "levels" of a factor. Each of these levels has five repeats corresponding to the number of observations (patients) within each level (type of disease). We shall further illustrate the idea of a factor soon because it is very useful for purposes of visualization.

1.5 Computing on a data vector

A data vector is simply a collection of numbers obtained as outcomes from measurements. This can be illustrated by a simple example on expression values of a gene. Suppose that gene expression values $1, 1.5$, and 1.25 from the persons "Eric", "Peter", and "Anna" are available. To store these in a vector we use the concatenate command `c()`, as follows.

```
> gene1 <- c(1.00,1.50,1.25)
> gene1
[1] 1.00 1.50 1.25
```

[7] See e.g. Samuals & Witmer (2003, Chap. 8) for a full explanation of experiments and statistical principles of design.

Now we have created the object `gene1` containing three gene expression values. To compute the sum, mean, and standard deviation of the gene expression values we use the corresponding built-in-functions.

```
> sum(gene1)
[1] 3.75
> mean(gene1)
[1] 1.25
> sum(gene1)/3
[1] 1.25
> sd(gene1)
[1] 0.25
> sqrt(sum((gene1-mean(gene1))^2)/2)
[1] 0.25
```

By defining $x_1 = 1.00$, $x_2 = 1.50$, and $x_3 = 1.25$, the sum of the weights can be expressed as $\sum_{i=1}^{n} x_i = 3.75$. The mathematical summation symbol $\sum$ is in R language simply `sum`. The mean is denoted by $\overline{x} = \sum_{i=1}^{3} x_i/3 = 1.25$ and the sample standard deviation as

$$s = \sqrt{\sum_{i=1}^{3}(x_i - \overline{x})^2/(3-1)} = 0.25.$$

1.6 Constructing a data matrix

In various types of spreadsheets it is custom to store data values in the form of a matrix consisting of rows and columns. In bioinformatics gene expression values (from several groups of patients) are stored as rows such that each row contains the expressions values of the patients corresponding to a particular gene and each column contains all gene expression values for a particular person. To illustrate this by a small example suppose that we have the following expression values on three genes from Eric, Peter, and Anna.[8]

```
> gene2 <- c(1.35,1.55,1.00)
> gene3 <- c(-1.10,-1.50,-1.25)
> gene4 <- c(-1.20,-1.30,-1.00)
```

[8]By the function `data.entry` you can open and edit a screen with the values of a matrix.

Before constructing the matrix it is convenient to add the names of the rows and the columns. To do so we construct the following list.

```
> rowcolnames <- list(c("gene1","gene2","gene3","gene4"),
+     c("Eric","Peter","Anna"))
```

After the last comma in the first line we give a carriage return for R to come up with a new line starting with + in order to complete a command. Now we can construct a matrix containing the expression values from our four genes, as follows.

```
> gendat <- matrix(c(gene1,gene2,gene3,gene4), nrow=4, ncol=3,
+    byrow=TRUE, dimnames = rowcolnames)
```

Here, `nrow` indicates the number of rows and `ncol` the number of columns. The gene vectors are placed in the matrix as rows. The names of the rows and columns are attached by the `dimnames` parameter. To see the content of the just created object `gendat`, we print it to the screen.

```
> gendat
        Eric Peter  Anna
gene1  1.00  1.50  1.25
gene2  1.35  1.55  1.30
gene3 -1.10 -1.50 -1.25
gene4 -1.20 -1.30 -1.00
```

A matrix such as `gendat` has two indices `[i,j]`, the first of which refers to rows and the second to columns[9]. Thus, if you want to print the second element of the first row to the screen, then type `gendat[1,2]`. If you want to print the first row, then use `gendat[1,]`. For the second column, use `gendat[,2]`.

It may be desirable to write the data to a file for using these in a later stage or to send these to a college of yours. Consider the following script.

```
> write.table(gendat,file="D:/data/gendat.Rdata")
> gendatread  <- read.table("D:/data/gendat.Rdata")
> gendatread
        Eric Peter  Anna
gene1  1.00  1.50  1.25
```

[9]Indices referring to rows, columns, or elements are always between square brackets `[]`.

```
gene2  1.35  1.55  1.30
gene3 -1.10 -1.50 -1.25
gene4 -1.20 -1.30 -1.00
```

An alternative is to use `write.csv`.[10]

1.7 Computing on a data matrix

Means or standard deviations of rows or columns are often important for drawing biologically relevant conclusions. Such type of computations on a data matrix can be accomplished by "for loops". However, it is much more convenient to use the `apply` functionality on a matrix. To do so we specify the name of the matrix, indicate rows or columns (1 for rows and 2 for columns), and the name of the function. To illustrate this we compute the mean of each person (column).

```
> apply(gendat,2,mean)
   Eric  Peter   Anna
0.0125 0.0625 0.0750
```

Similarly, the mean of each gene (row) can be computed.

```
> apply(gendat,1,mean)
    gene1     gene2     gene3     gene4
 1.250000  1.400000 -1.283333 -1.166667
```

It frequently happens that we want to re-order the rows of a matrix according to a certain criterion, or, more specifically, the values in a certain column vector. For instance, to re-order the matrix `gendat` according to the row means, it is convenient to store these in a vector and to use the function `order`.

```
> meanexprsval <- apply(gendat,1,mean)
> o <- order(meanexprsval,decreasing=TRUE)
> o
[1] 2 1 4 3
```

[10] For more see the "R Data import/Export" manual, Chapter 3 of the book "R for Beginners", or search the internet by the key "r wiki matrix".

Thus gene2 appears first because it has the largest mean 1.4, then gene1 with 1.25, followed by gene4 with -1.16 and, finally, gene3 with -1.28. Now that we have collected the order numbers in the vector o, we can re-order the whole matrix by specifying o as the row index.[11]

```
> gendat[o,]
       Eric Peter  Anna
gene2  1.35  1.55  1.30
gene1  1.00  1.50  1.25
gene4 -1.20 -1.30 -1.00
gene3 -1.10 -1.50 -1.25
```

Another frequently occurring problem is that of selecting genes with a certain property. We illustrate this by several methods to select genes with positive mean expression values. A first method starts with the observation that the first two rows have positive means and to use c(1,2) as a row index.

```
> gendat[c(1,2),]
      Eric Peter Anna
gene1 1.00  1.50 1.25
gene2 1.35  1.55 1.30
```

A second way is to use the row names as an index.

```
> gendat[c("gene1","gene2"),]
      Eric Peter Anna
gene1 1.00  1.50 1.25
gene2 1.35  1.55 1.30
```

A third and more advanced way is to use an evaluation in terms of TRUE or FALSE of logical elements of a vector. For instance, we may evaluate whether the row mean is positive.

```
> meanexprsval > 0
gene1 gene2 gene3 gene4
 TRUE  TRUE FALSE FALSE
```

Now we can use the evaluation of meanexprsval > 0 in terms of the values TRUE or FALSE as a row index.

[11] You can also use functions like sort or rank.

```
> gendat[meanexprsval > 0,]
       Eric Peter Anna
gene1 1.00  1.50 1.25
gene2 1.35  1.55 1.30
```

Observe that this selects genes for which the evaluation equals `TRUE`. This illustrates that genes can be selected by their row index, row name or value on a logical variable.

1.8 Application to the Golub (1999) data

The gene expression data collected by Golub et al. (1999) are among the classical in bioinformatics. A selection of the set is called `golub` and is contained in the `multtest` package, which is part of Bioconductor. The data consist of gene expression values of 3051 genes (rows) from 38 leukemia patients[12]. Twenty seven patients are diagnosed as acute lymphoblastic leukemia (ALL) and eleven as acute myeloid leukemia (AML). The tumor class is given by the numeric vector `golub.cl`, where ALL is indicated by 0 and AML by 1. The gene names are collected in the matrix `golub.gnames` of which the columns correspond to the gene index, ID, and Name, respectively. We shall first concentrate on expression values of a gene with manufacturer name `"M92287_at"`, which is known in biology as `"CCND3 Cyclin D3"`. The expression values of this gene are collected in row 1042 of `golub`. To load the data and to obtain relevant information from row 1042 of `golub.gnames`, use the following.

```
> library(multtest); data(golub)
> golub.gnames[1042,]
[1] "2354"            "CCND3 Cyclin D3" "M92287_at"
```

The data are stored in a matrix called `golub`. The number of rows and columns can be obtained by the functions `nrow` and `ncol`, respectively.

```
> nrow(golub)
[1] 3051
> ncol(golub)
[1] 38
```

[12] The data are pre-processed by procedures described in Dudoit et al. (2002).

So the matrix has 3051 rows and 38 columns, see also `dim(golub)`. Each data element has a row and a column index. Recall that the first index refers to rows and the second to columns. Hence, the second value from row 1042 can be printed to the screen as follows.

```
> golub[1042,2]
[1] 1.52405
```

So 1.52405 is the expression value of gene CCND3 Cyclin D3 from patient number 2. The values of the first column can be printed to the screen by the following.

```
> golub[,1]
```

To save space the output is not shown. We may now print the expression values of gene CCND3 Cyclin D3 (row 1042) to the screen.

```
> golub[1042,]
 [1]  2.10892  1.52405  1.96403  2.33597  1.85111  1.99391  2.06597  1.81649
 [9]  2.17622  1.80861  2.44562  1.90496  2.76610  1.32551  2.59385  1.92776
[17]  1.10546  1.27645  1.83051  1.78352  0.45827  2.18119  2.31428  1.99927
[25]  1.36844  2.37351  1.83485  0.88941  1.45014  0.42904  0.82667  0.63637
[33]  1.02250  0.12758 -0.74333  0.73784  0.49470  1.12058
```

To print the expression values of gene CCND3 Cyclin D3 to the screen only for the ALL patients, we have to refer to the first twenty seven elements of row 1042. A possibility to do so is by the following.

```
> golub[1042,1:27]
```

However, for the work ahead it is much more convenient to construct a factor indicating the tumor class of the patients. This will turn out useful e.g. for separating the tumor groups in various visualization procedures. The factor will be called `gol.fac` and is constructed from the vector `golub.cl`, as follows.

```
> gol.fac <- factor(golub.cl, levels=0:1, labels = c("ALL","AML"))
```

In the sequel this factor will be used frequently. Obviously, the labels correspond to the two tumor classes. The evaluation of `gol.fac=="ALL"` returns `TRUE` for the first twenty seven values and `FALSE` for the remaining eleven. This is useful as a column index for selecting the expression values of the ALL patients. The expression values of gene CCND3 Cyclin D3 from the ALL patients can now be printed to the screen, as follows.

```
> golub[1042,gol.fac=="ALL"]
```

For many types of computations it is very useful to combine a factor with the `apply` functionality. For instance, to compute the mean gene expression over the ALL patients for each of the genes, we may use the following.

```
> meanALL <- apply(golub[,gol.fac=="ALL"], 1, mean)
```

The specification `golub[,gol.fac=="ALL"]` selects the matrix with gene expressions corresponding to the ALL patients. The 3051 means are assigned to the vector `meanALL`.

After reading the classical article by Golub et al. (1999), which is strongly recommended, one becomes easily interested in the properties of certain genes. For instance, gene CD33 plays an important role in distinguishing lymphoid from myeloid lineage cells. To perform computations on the expressions of this gene we need to know its row index. This can obtained by the `grep` function.[13]

```
> grep("CD33",golub.gnames[,2])
[1] 808
```

Hence, the expression values of antigen CD33 are available at `golub[808,]` and further information on it by `golub.gnames[808,]`.

1.9 Running scripts

It is very convenient to use a plain text writer like Notepad, Kate, Emacs, or WinEdt for the formulation of several consecutive R commands as separated lines (scripts). Such command lines can be executed by simply using copy and paste into the command line editor of R. Another possibility is to execute a script from a file. To illustrate the latter consider the following.

```
> library(multtest); data(golub)
> gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
> mall <- apply(golub[,gol.fac=="ALL"], 1, mean)
> maml <- apply(golub[,gol.fac=="AML"], 1, mean)
> o <- order(abs(mall-maml), decreasing=TRUE)
> print(golub.gnames[o[1:5],2])
```

[13] Indeed, several functions of R are inspired by the Linux operating system.

```
[1] "CST3 Cystatin C (amyloid angiopathy and cerebral hemorrhage)"
[2] "INTERLEUKIN-8 PRECURSOR"
[3] "Interleukin 8 (IL8) gene"
[4] "DF D component of complement (adipsin)"
[5] "MPO Myeloperoxidase"
```

The row means of the expression values per patient group are computed and stored in the object `mall` and `maml`, respectively. The absolute values of the differences in means are computed and their order numbers (from large to small) are stored in the vector `o`. Next, the names of the five genes with the largest differences in mean are printed to the screen.

After saving the script under e.g. the name `meandif.R` in the directory `D:\\Rscripts\\meandif.R`, it can be executed by using `source("D:\\Rscripts\\meandif.R")`. Once the script is available for a typewriter it is easy to adapt it and to re-run it.

Readers are strongly recommended to trial-and-error with respect to writing programming scripts. To run these it is very convenient to have your favorite word processor available and to use, for instance, the copy-and-paste functionality.

1.10 Overview and concluding remarks

It is easy to install R and Bioconductor. R has many convenient built-in-functions for statistical programming. Help and illustrations on many topics are available from various sources. With the reference charts, R manuals, (on-line) books and R Wiki at hand you have various sources of information to help you along with practical issues. Although there recently became several GUI's available, we shall concentrate on the command line editor because its range of possibilities is much larger.

The above introduction is of course very brief. A more extensive introduction into R, assuming some background on biomedical statistics, is given by Dalgaard (2002). There are book length treatments combining R with statistics (Venables, & Ripley, 2002; Everitt & Hothorn, 2006). Other treatments go much deeper into programming aspects (Becker, Chambers, & Wilks, 1988; Venables & Ripley, 2000; Gentleman, 2008).

For the sake of illustration we shall work frequently with the data kindly provided by Golub et al. (1999) and Chiaretti et al. (2004). The corre-

sponding scientific articles are freely available from the web. Having these available may further motivate readers for the computations ahead.

1.11 Exercises

1. Some questions to orientate yourself.

   (a) Use the function `class` to find the class to which the following objects belong: `golub`, `golub[1,1]`,`golub.cl`, `golub.gnames`, `apply`, `exp`, `gol.fac`, `plot`, `ALL`.

   (b) What is the meaning of the following abbreviations: `rm`, `sum`, `prod`, `seq`, `sd`, `nrow`.

   (c) For what purpose are the following functions useful: `grep`, `apply`, `gl`, `library`, `source`, `setwd`, `history`, `str`.

2. `gendat` Consider the data in the matrix `gendat`, constructed in Section 1.6. Its small size has the advantage that you can check your computations even by a pocket calculator. [14]

   (a) Use `apply` to compute the standard deviation of the persons.

   (b) Use `apply` to compute the standard deviation of the genes.

   (c) Order the matrix according to the gene standard deviations.

   (d) Which gene has the largest standard deviation?

3. Computations on gene means of the Golub data.

   (a) Use `apply` to compute the mean gene expression value.

   (b) Order the data matrix according to the gene means.

   (c) Give the names of the three genes with the largest mean expression value.

   (d) Give the biological names of these genes.

4. Computations on gene standard deviations of the Golub data.

   (a) Use `apply` to compute the standard deviation per gene.

[14] Obtaining some routine with the apply functionality is quite helpful for what follows.

(b) Select the expression values of the genes with standard deviation larger than two.

(c) How many genes have this property?

5. Oncogenes in Golub data.

(a) How many oncogenes are there in the dataset? Hint: Use `grep`.

(b) Find the biological names of the three oncogenes with the largest mean expression value for the ALL patients.

(c) Do the same for the AML patients.

(d) Write the gene probe ID and the gene names of the ten genes with largest mean gene expression value to a `csv` file.

6. Constructing a factor. Construct factors that correspond to the following setting.

(a) An experiment with two conditions each with four measurements.

(b) Five conditions each with three measurements.

(c) Three conditions each with five measurements.

7. Gene means for B1 patients. Load the `ALL` data from the `ALL` library and use `str` and `openVignette()` for a further orientation.

(a) Use `exprs(ALL[,ALL$BT=="B1"]` to extract the gene expressions from the patients in disease stage B1. Compute the mean gene expressions over these patients.

(b) Give the gene identifiers of the three genes with the largest mean.

Chapter 2

Data Display and Descriptive Statistics

A few essential methods are given to display and visualize data. It quickly answers questions like: How are my data distributed? How can the frequencies of nucleotides from a gene be visualized? Are there outliers in my data? Does the distribution of my data resemble that of a bell-shaped curve? Are there differences between gene expression values taken from two groups of patients?

The most important central tendencies (mean, median) are defined and illustrated together with the most important measures of spread (standard deviation, variance, inter quartile range, and median absolute deviation).

2.1 Univariate data display

To observe the distribution of data various visualization methods are made available. These are frequently used by practitioners as well as by experts.

2.1.1 Frequency table

Discrete data occur when the values naturally fall into categories. A frequency table simply gives the number of occurrences within a category.

Example 1. A gene consists of a sequence of nucleotides $\{A, C, G, T\}$. The number of each nucleotide can be displayed in a frequency table. This

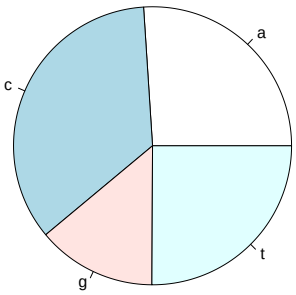
will be illustrated by the Zyxin gene which plays an important role in cell adhesion (Golub et al., 1999). The accession number (X94991.1) of one of its variants can be found in a data base like NCBI (UniGene). The code below illustrates how to read the sequence "X94991.1" of the species homo sapiens from GenBank, , to construct a pie from a frequency table of the four nucleotides.

```
install.packages(c("ape"),repo="http://cran.r-project.org",dep=TRUE)
library(ape)
table(read.GenBank(c("X94991.1"),as.character=TRUE))
pie(table(read.GenBank(c("X94991.1"))))
```

From the resulting frequencies in Table 2.1 it seems that the nucleotides are not equally likely. A nice way to visualize a frequency table is by plotting a pie. □

Table 2.1: A frequency table and its pie of Zyxin gene.

A	C	G	T
410	789	573	394



2.1.2 Plotting data

An elementary method to visualize data is by using a so-called stripchart, by which the values of the data are represented as e.g. small boxes. Often,

it is useful in combination with a factor that distinguishes members from different experimental conditions or patients groups.

Example 1. Many visualization methods will be illustrated by the Golub et al. (1999) data. We shall concentrate on the expression values of gene `"CCND3 Cyclin D3"`, which are collected in row 1042 of the data matrix `golub`. To plot the data values one can simply use `plot(golub[1042,])`. In the resulting plot in Figure 2.1 the vertical axis gives the size of the expression values and the horizontal axis the index of the patients. It can be observed that the values for patient 28 to 38 are somewhat lower, but, indeed, the picture is not very clear because the groups are not plotted separately.

To produce two adjacent stripcharts one for the ALL and one for the AML patients, we use the factor called `gol.fac` from the previous chapter.

```
data(golub, package = "multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
stripchart(golub[1042,] ~ gol.fac, method="jitter")
```

From the resulting Figure 2.2 it can be observed that the CCND3 Cyclin D3 expression values of the ALL patients tend to have larger expression values than those of the AML patients. □

2.1.3 Histogram

Another method to visualize data is by dividing the range of data values into a number of intervals and to plot the frequency per interval as a bar. Such a plot is called a histogram.

Example 1. A histogram of the expression values of gene `"CCND3 Cyclin D3"` of the acute lymphoblastic leukemia patients can be produced as follows.

```
> hist(golub[1042, gol.fac=="ALL"])
```

The function `hist` divides the data into 5 intervals having width equal to 0.5, see Figure 2.3. Observe from the latter that one value is small and the other are more or less symmetrically distributed around the mean. □

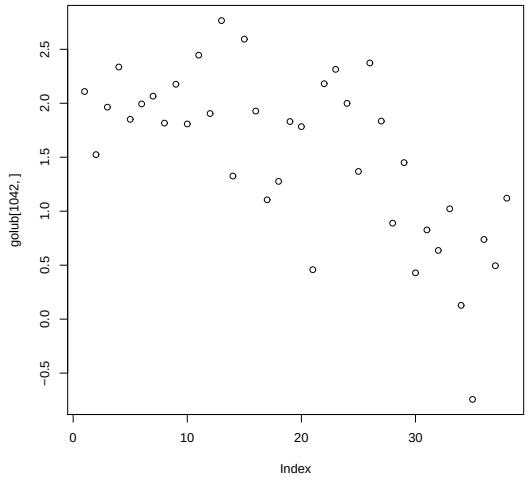


Figure 2.1: Plot of gene expression values of CCND3 Cyclin D3.

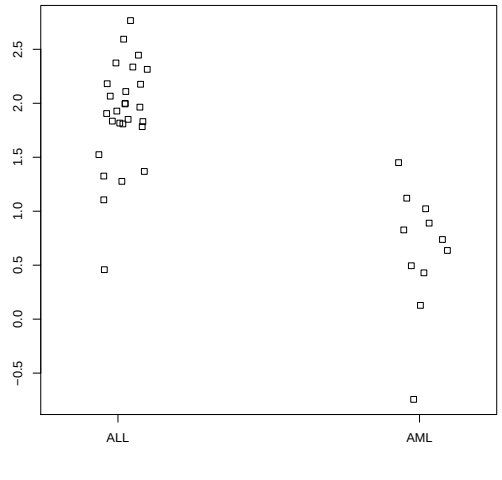


Figure 2.2: Stripchart of gene expression values of CCND3 Cyclin D3 for ALL and AML patients.

2.1.4 Boxplot

It is always possible to sort n data values to have increasing order $x_1 \leq x_2 \leq \cdots \leq x_n$, where x_1 is the smallest, x_2 is the first-to-the smallest, etc. Let $x_{0.25}$ be a number for which it holds that 25% of the data values $x_1, \cdots, x_n$ is smaller. That is, 25% of the data values lay on the left side of the number $x_{0.25}$, reason for which it is called the first *quartile* or the 25th *percentile*. The second quartile is the value $x_{0.50}$ such that 50% of the data values are smaller. Similarly, the third quartile or 75th percentile is the value $x_{0.75}$ such that 75% of the data is smaller. A popular method to display data is by drawing a box around the first and the third quartile (a bold line segment for the median), and the smaller line segments (whiskers) for the smallest and the largest data values. Such a data display is known as a box-and-whisker plot.

Example 1. A vector with gene expression values can be put into increasing order by the function `sort`. We shall illustrate this by the ALL

expression values of gene `"CCND3 Cyclin D3"` in row 1042 of `golub`.

```
> x <- sort(golub[1042, gol.fac=="ALL"], decreasing = FALSE)
> x[1:5]
[1] 0.458 1.105 1.276 1.326 1.368
```

The second command prints the first five values of the sorted data values to the screen, so that we have $x_1 = 0.458$, $x_2 = 1.105$, etc. Note that the mathematical notation x_i corresponds exactly to the R notation `x[i]` □

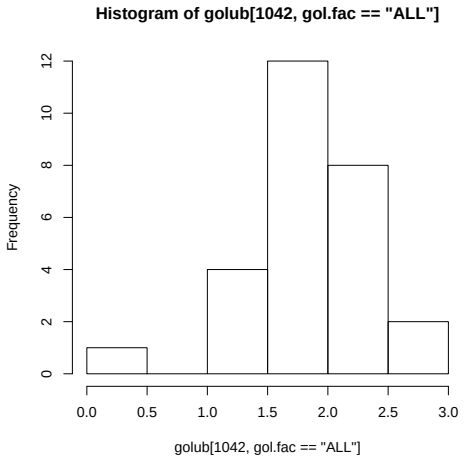


Figure 2.3: Histogram of ALL expression values of gene CCND3 Cyclin D3.

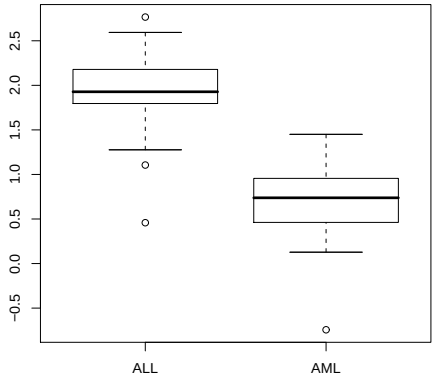


Figure 2.4: Boxplot of ALL and AML expression values of gene CCND3 Cyclin D3.

Example 2. A view on the distribution of the expression values of the ALL and the AML patients on gene CCND3 Cyclin D3 can be obtained by constructing two separate boxplots adjacent to one another. To produce such a plot the factor `gol.fac` is again very useful.

```
> boxplot(golub[1042,] ~  gol.fac)
```

From the position of the boxes in Figure 2.4 it can be observed that the gene expression values for ALL are larger than those for AML. Furthermore, since the two sub-boxes around the median are more or less equally wide, the data are quite symmetrically distributed around the median.

To compute exact values for the quartiles we need a sequence running from 0.00 to 1.00 with steps equal to 0.25. To construct such a sequence the function `seq` is useful.

```
> pvec <- seq(0,1,0.25)
> quantile(golub[1042, gol.fac=="ALL"],pvec)
   0%   25%   50%   75%  100%
0.458 1.796 1.928 2.179 2.766
```

The first quartile $x_{0.25} = 1.796$, the second $x_{0.50} = 1.928$, and the third $x_{0.75} = 2.179$. The smallest observed expression value equals $x_{0.00} = 0.458$ and the largest $x_{1.00} = 2.77$. The latter can also be obtained by the function `min(golub[1042, gol.fac=="ALL"])` and `max(golub[1042, gol.fac=="ALL"])`, or more briefly by `range(golub[1042, gol.fac=="ALL"])`. □

Outliers are data values laying far apart from the pattern set by the majority of the data values. The implementation in R of the (modified) `boxplot` draws such *outlier* points separately as small circles. A data point x is defined as an outlier point if

$$x < x_{0.25} - 1.5 \cdot (x_{0.75} - x_{0.25}) \quad \text{or} \quad x > x_{0.75} + 1.5 \cdot (x_{0.75} - x_{0.25}).$$

From Figure 2.4 it can be observed that there are outliers among the gene expression values of ALL patients. These are the smaller values 0.45827 and 1.10546, and the largest value 2.76610. The AML expression values have one outlier with value -0.74333.

To define *extreme outliers*, the factor 1.5 is raised to 3.0. Note that this is a descriptive way of defining outliers instead of statistically testing for the existence of an outlier.

2.1.5 Quantile-Quantile plot

A method to visualize the distribution of gene expression values is by the so-called quantile-quantile (Q-Q) plot. In such a plot the quantiles of the gene expression values are displayed against the corresponding quantiles of the normal (bell-shaped). A straight line is added representing points which correspond exactly to the quantiles of the normal distribution. By observing the extent in which the points appear on the line, it can be evaluated to what degree the data are normally distributed. That is, the closer the gene

expression values appear to the line, the more likely it is that the data are normally distributed.

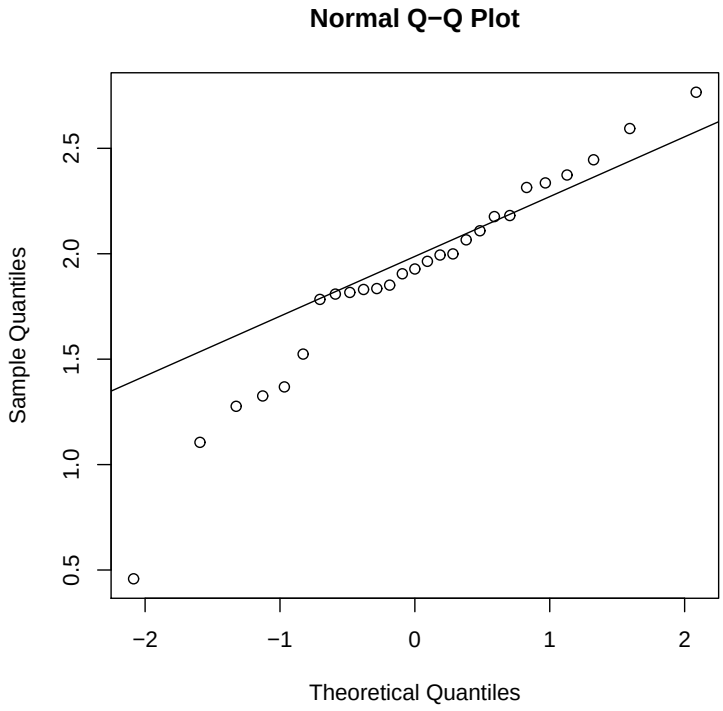


Figure 2.5: Q-Q plot of ALL gene expression values of CCND3 Cyclin D3.

Example 1. To produce a Q-Q plot of the ALL gene expression values of CCND3 Cyclin D3 one may use the following.

```
qqnorm(golub[1042, gol.fac=="ALL"])
qqline(golub[1042, gol.fac=="ALL"])
```

From the resulting Figure 2.5 it can be observed that most of the data points are on or near the straight line, while a few others are further away. □

The above example illustrates a case where the degree of non-normality is moderate so that a clear conclusion cannot be drawn. By making the

exercises below, the reader will gather more experience with the degree in which gene expression values are normally distributed.

2.2 Descriptive statistics

There exist various ways to describe the central tendency as well as the spread of data. In particular, the central tendency can be described by the mean or the median, and the spread by the variance, standard deviation, interquartile range, or median absolute deviation. These will be defined and illustrated.

2.2.1 Measures of central tendency

The most important descriptive statistics for central tendency are the mean and the median. The sample mean of the data values $x_1, \cdots, x_n$ is defined as

$$\overline{x} = \frac{1}{n}\sum_{i=1}^{n} x_i = \frac{1}{n}(x_1 + \cdots + x_n).$$

Thus the sample *mean* is simply the average of the n data values. Since it is the sum of all data values divided by the sample size, a few extreme data values may largely influence its size. In other words, the mean is not robust against outliers.

The *median* is defined as the second quartile or the 50th percentile, and is denoted by $x_{0.50}$. When the data are symmetrically distributed around the mean, then the mean and the median are equal. Since extreme data values do not influence the size of the median, it is very robust against outliers. Robustness is important in bioinformatics because data are frequently contaminated by extreme or otherwise influential data values.

Example 1. To compute the mean and median of the ALL expression values of gene CCND3 Cyclin D3 consider the following.

```
> mean(golub[1042, gol.fac=="ALL"])
[1] 1.89
> median(golub[1042, gol.fac=="ALL"])
[1] 1.93
```

Note that the mean and the median do not differ much so that the distribution seems quite symmetric. □

2.2.2 Measures of spread

The most important measures of spread are the standard deviation, the interquartile range, and the median absolute deviation. The *standard deviation* is the square root of the *sample variance*, which is defined as

$$s^2 = \frac{1}{n-1}\sum_{i=1}^{n}(x_i - \overline{x})^2 = \frac{1}{n-1}\left((x_1 - \overline{x})^2 + \cdots + (x_n - \overline{x})^2\right).$$

Hence, it is the average of the squared differences between the data values and the sample mean. The sample standard deviation s is the square root of the sample variance and may be interpreted as the distance of the data values to the mean. The variance and the standard deviation are not robust against outliers.

The *interquartile range* is defined as the difference between the third and the first quartile, that is $x_{0.75} - x_{0.25}$. It can be computed by the function `IQR(x)`. More specifically, the value `IQR(x)/1.349` is a robust estimator of the standard deviation. The *median absolute deviation* (MAD) is defined as a constant times the median of the absolute deviations of the data from the median (e.g. Jurečková & Picek, 2006, p. 63). In R it is computed by the function `mad` defined as the median of the sequence $|x_1 - x_{0.50}|, \cdots, |x_n - x_{0.50}|$ multiplied by the constant 1.4826. It equals the standard deviation in case the data come from a bell-shaped (normal) distribution (see Section 3.2.1). Because the interquartile range and the median absolute deviation are based on quantiles, these are robust against outliers.

Example 1. These measures of spread for the ALL expression values of gene CCND3 Cyclin D3 can be computed as follows.

```
> sd(golub[1042, gol.fac=="ALL"])
[1] 0.491
> IQR(golub[1042, gol.fac=="ALL"]) / 1.349
[1] 0.284
> mad(golub[1042, gol.fac=="ALL"])
[1] 0.368
```

Due to the three outliers (cf. Figure 2.4) the standard deviation is larger than the interquartile range and the mean absolute deviation. That is, the absolute differences with respect to the median are somewhat smaller, than the root of the squared differences. □

2.3 Overview and concluding remarks

Data can be stored as a vector or a data matrix on which various useful functions are defined. In particular, it is easy to produce a pie, histogram, boxplot, or Q-Q plot of a vector of data. These plots give a useful first impression of the degree of (non)normality of gene expression values.

To construct the histogram used the default method to compute the number of bars or breaks. If the data are distributed according to a bell-shaped curve, then this is often a good strategy. The number of bars can be chosen by the `breaks` option of the function `hist`. Optimal choices for this are discussed by e.g. Venables and Ripley (2002).

2.4 Exercises

Since the majority of the exercises are based on the Golub et al. (1999) data, it is essential to make these available and to learn to work with it. To stimulate self-study the answers are given at the end of the book.

1. Illustration of mean and standard deviation.
   (a) Compute the mean and the standard deviation for $1, 1.5, 2, 2.5, 3$.
   (b) Compute the mean and the standard deviation for $1, 1.5, 2, 2.5, 30$.
   (c) Comment on the differences.
2. Comparing normality for two genes. Consider the gene expression values in row 790 and 66 of the Golub et al. (1999) data.
   (a) Produce a boxplot for the expression values of the ALL patients and comment on the differences. Are there outliers?

(b) Produce a QQ-plot and formulate a hypothesis about the normality of the genes.

(c) Compute the mean and the median for the expression values of the ALL patients and compare these. Do this for both genes.

3. Effect size. An important statistic to measure the effect size which is defined for a sample as $\overline{x}/s$. It measures the mean relative to the standard deviation, so that is value is large when the mean is large and the standard deviation small.

(a) Determine the five genes with the largest effect size of the ALL patients from the Golub et al. (1999) data. Comment on their size.

(b) Invent a robust variant of the effect size and use it to answer the previous question.

4. Plotting gene expressions `"CCND3 Cyclin D3"`. Use the gene expressions from `"CCND3 Cyclin D3"` of Golub et al. (1999) collected in row 1042 of the object `golub` from the `multtest` library. After using the function `plot` you produce an object on which you can program.

(a) Produce a so-called stripchart for the gene expressions separately for the ALL as well as for the AML patients. Hint: Use a `factor` for appropriate separation.

(b) Rotate the plot to a vertical position and keep it that way for the questions to come.

(c) Color the ALL expressions red and AML blue. Hint: Use the `col` parameter.

(d) Add a title to the plot. Hint: Use `title`.

(e) Change the boxes into stars. Hint: Use the `pch` parameter.
Hint: Store the final script you like the most in your typewriter in order to be able to use it efficiently later on.

5. Box-and-Whiskers plot of `"CCND3 Cyclin D3"`. Use the gene expressions `"CCND3 Cyclin D3"` of Golub et al. (1999) from row 1042 of the object `golub` of the `multtest` library.

(a) Construct the boxplot in Figure 2.6.

(b) Add text to the plot to explain the meaning of the upper and lower part of the box.

(c) Do the same for the wiskers.

(d) Export your plot to `eps` format.

Hint 1: Use `locator()` to find coordinates of the position of the plot.
Hint 2: Use `xlim` to make the plot somewhat wider.
Hint 3: Use `arrows` to add an arrow.
Hint 4: Use `text` to add information at a certain position.

6. Box-and-wiskers plot of persons of Golub et al. (1999) data.

   (a) Use `boxplot(data.frame(golub))` to produce a box-and-wiskers plot for each column (person). Make a screen shot to save it in a word processor. Describe what you see. Are the medians of similar size? Is the inter quartile range more or less equal. Are there outliers?

   (b) Compute the mean and medians of the persons. What do you observe?

   (c) Compute the range (minimal and maximum value) of the standard deviations, the IQR and MAD of the persons. Comment of what you observe.

7. Oncogenes of Golub et al. (1999) data.

   (a) Select the oncogens by the `grep` facility and produce a box-and-wiskers plot of the gene expressions of the ALL patients.

   (b) Do the same for the AML patients and use `par(mfrow=c(2,1))` to combine the two plots such that the second is beneath the first. Are there genes with clear differences between the groups?

8. Descriptive statistics for the ALL gene expression values of the Golub et al. (1999) data.

   (a) Compute the mean and median for gene expression values of the ALL patients, report their range and comment on it.

   (b) Compute the SD, IQR, and MAD for gene expression values of the ALL patients, report their range and comment on it.

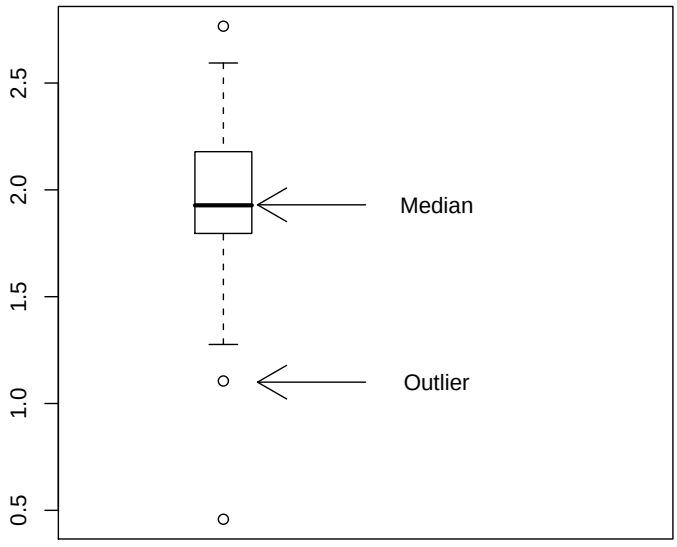


Figure 2.6: Boxplot with arrows and explaining text.

Chapter 3

Important Distributions

Questions that concern us in this chapter are: What is the probability to find fourteen purines in a microRNA of length twenty two? If expressions from ALL patients of gene CCND3 Cyclin D3 are normally distributed with mean 1.90 and standard deviation 0.5, what is the probability to observe expression values larger than 2.4?

To answer such type of questions we need to know more about statistical distributions (e.g. Samuels & Witmer, 2003). In this chapter several important distributions will be defined, explained, and illustrated. In particular, the discrete distribution binomial and the continuous distributions normal, T, F, and chi-squared will be elaborated. These distributions have a wealth of applications to statistically testing biological hypotheses. Only when deemed relevant, the density function, the distribution function, the mean μ (mu), and the standard deviation σ (sigma), are explicitly defined.

3.1 Discrete distributions

The binomial distribution is fundamental and has many applications in medicine and bioinformatics.

3.1.1 Binomial distribution

The binomial distribution fits to repeated trials each with a dichotomous outcome such as succes-failure, healthy-disease, heads-tails, purine-pyrimidine, etc. When there are n trials, then the number of ways to obtain k successes

out of n is given by the binomial coefficient

$$\frac{n!}{k!(n-k)!},$$

where $n! = n \cdot (n-1) \cdots 1$ and $0! = 1$ (Samuels & Witmer, 2003). The binomial probability of k successes out of n consists of the product of this coefficient with the probability of k successes and the probability of $n - k$ failures. Let p be the probability of succes in a single trial and X the (random) variable denoting the number of successes. Then the probability P of the event $(X = k)$ that k successes occur out of n trails can be expressed as

$$P(X = k) = \frac{n!}{k!(n-k)!} p^k (1-p)^{n-k}, \qquad \text{for} \quad k = 0, \cdots, n. \tag{3.1}$$

The collection of these probabilities is called the probability density function. [1]

Example 1. To visualize the Binomial distribution, load the `TeachingDemos` package and use the command `vis.binom()`. Click on "Show Normal Approximation" and observe that the approximation improves as n increases, taking p for instance near 0.5. □

Example 2. If two carriers of the gen for albinism marry, then each of the children has probability of 1/4 of being albino. What is the probability for one child out of three to be albino? To answer this question we take $n = 3$, $k = 1$, and $p = 0.25$ into Equation (3.1) and obtain

$$P(X = 1) = \frac{3!}{1!(3-1)!} 0.25^1 0.75^2 = 3 \cdot 0.140625 = 0.421875.$$

An elementary manner to compute this in R is by

```
> choose(3,1)* 0.25^1* 0.75^2
```

where `choose(3,1)` computes the binomial coefficient. It is more efficient to compute this by the built-in-density-function `dbinom(k,n,p)`, for instance to print the values of the probabilities.

[1] For a binomially distributed variable np is the mean, $np(1-p)$ the variance, and $\sqrt{np(1-p)}$ the standard deviation.

```
> for (k in 0:3) print(dbinom(k,3,0.25))
```

Changing `d` into `p` yields the so-called distribution function with the cumulative probabilities. That is, the probability that the number of Heads is lower than or equal to two $P(X \leq 2)$ is computed by `pbinom(2,3,0.25)`. The values of the density and distribution function are summarized in Table 3.1. From the table we read that the probability of no albino child is 0.4218 and the probability that all three children are albino equals 0.0156. □

Table 3.1: Discrete density and distribution function values of S_3, with $p = 0.6$.

number of Heads	$k = 0$	$k = 1$	$k = 2$	$k = 3$
density $P(X = k)$	0.4218	0.4218	0.1406	0.0156
distribution $P(X \leq k)$	0.4218	0.843	0.9843	1

Example 3. RNA consists of a sequence of nucleotides A, G, U, and C, where the first two are purines and the last two are pyrimidines. Suppose, for the purpose of illustration, that the length of a certain micro RNA is 22, that the probability of a purine equals 0.7, and that the process of placing purines and pyrimidines is binomially distributed. The event that our microRNA contains 14 purines can be represented by $X = 14$. The probability of this event can be computed by

$$P(X = 14) = \frac{22!}{14!(22-14)!} 0.7^{14} 0.3^{8} = \texttt{dbinom}(14, 22, 0.7) = 0.1423.$$

This is the value of the density function at 14. The probability of the event of less than or equal to 13 purines equals the value of the distribution function at value 13, that is

$$P(X \leq 13) = \texttt{pbinom}(13, 22, 0.7) = 0.1865.$$

The probability of strictly more than 10 purines is

$$P(X \geq 11) = \sum_{k=11}^{22} P(S_{22} = k) = \texttt{sum}(\texttt{dbinom}(11 : 22, 22, 0.7)) = 0.9860.$$

The binomial density function can be plotted by:

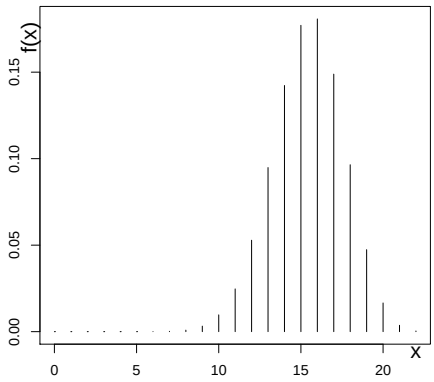


Figure 3.1: Binomial probabilities with $n = 22$ and $p = 0.7$

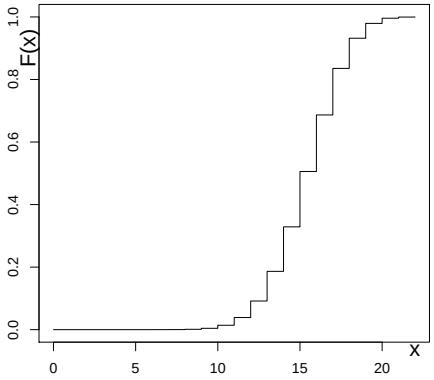


Figure 3.2: Binomial cumulative probabilities with $n = 22$ and $p = 0.7$.

```
> x <- 0:22
> plot(x,dbinom(x,size=22,prob=.7),type="h")
```

By the first line the sequence of integers $\{1, 2, \cdots, 22\}$ is constructed and by the second the density function is plotted, where the argument `h` specifies pins. From Figure 3.1 it can be observed that the largest probabilities occur near the expectation 15.4. The graph in Figure 3.2 illustrates that the distribution is an increasing step function, with x on the horizontal axis and $P(X \leq x)$ on the vertical.

A random sample of size 1000 from the binomial distribution with $n = 22$ and $p = 0.7$ can be drawn by the command `rbinom(1000,22,0.7)`. This simulates the number of purines in 1000 microRNA's each with purine probability equal to 0.7 and length 22. □

3.2 Continuous distributions

The continuous distributions normal, T, F, and chi-squared will be defined, explained and illustrated.

3.2.1 Normal distribution

The normal distribution is of key importance because it is assumed for many (preprocessed) gene expression values. That is, the data values $x_1, \cdots, x_n$ are seen as realizations of a random variable X having a normal distribution. Equivalently one says that the data values are members of a normally distributed population with mean μ (mu) and variance σ^2 (sigma squared). It is good custom to use Greek letters for population properties and $N(\mu, \sigma^2)$ for the normal distribution. The value of the distribution function is given by $P(X \leq x)$, the probability of the population to have values smaller than or equal to x. Various properties of the normal distribution are illustrated by the examples below.

Example 1. To view members of the normal distribution load the `TeachingDemos` package and give the command `vis.normal()` to launch an interactive display of bell-shaped curves. These bell-shaped curves are also called normal densities. The curves are symmetric around μ and attain a unique maximum at $x = \mu$. If x moves further away from the mean μ, then the curves moves to zero so that extreme values occur with small probability. Move the `Mean` and the `Standard Deviation` from the left to the right to explore their effect on the shape of the normal distribution. In particular, when the mean μ increases, then the distribution moves to the right. If σ is small/large, then the distribution is steep/flat. □

Example 2. Suppose that the expression values of gene CCND3 Cyclin D3 can be represented by X which is distributed as $N(1.90, 0.5^2)$. From the graph of its density function in Figure 3.3, it can be observed that it is symmetric and bell-shaped around $\mu = 1.90$. A density function may very well be seen as a histogram with arbitrarily small bars (intervals). The probability that the expression values are less then 1.4 is

$$P(X < 1.4) = \texttt{pnorm}(1.4, 1.9, 0.5) = 0.1586.$$

Figure 3.4 illustrates the value 0.16 of the distribution function at $x = 1.4$. It corresponds to the area of the blue colored surface below the graph of the density function in Figure 3.3. The probability that the expression values are larger than 2.4 is

$$P(X \geq 2.4) = 1 - \texttt{pnorm}(2.4, 1.9, 0.5) = 0.1586.$$

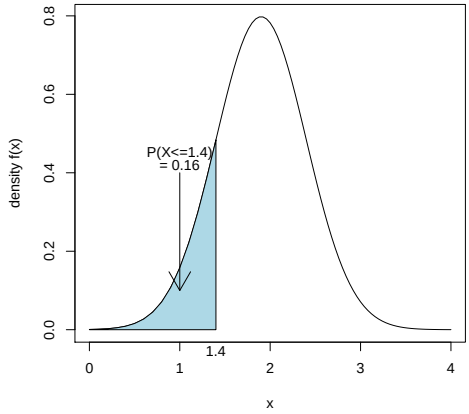


Figure 3.3: Graph of normal density with mean 1.9 and standard deviation 0.5.

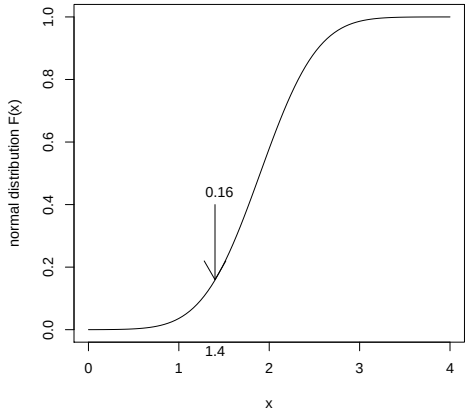


Figure 3.4: Graph of normal distribution with mean 1.9 and standard deviation 0.5.

The probability that X is between 1.4 and 2.4 equals

$$P(1.4 \leq X \leq 2.4) = \texttt{pnorm}(2.4, 1.9, 0.5) - \texttt{pnorm}(1.4, 1.9, 0.5) = 0.9545.$$

The graph of the distribution function in Figure 3.4 illustrates that it is strictly increasing. The exact value for the quantile $x_{0.025}$ can be computed by

```
> qnorm(0.025,1.9,0.5)
[1] 0.920018
```

That is, the quantile $x_{0.025} = 0.920018$. Hence, it holds that the probability of values smaller than 0.920018 equals 0.025, that is $P(X \leq 0.920018) = 0.025$, as can be verified by `pnorm(0.920018, 1.9, 0.5)`. When X is distributed as $N(1.90, 0.5^2)$, then the population mean is 1.9 and the population standard deviation 0.5. To verify this we draw a random sample of size 1000 from this population by

```
> x <- rnorm(1000,1.9,0.5)
```

The estimate `mean(x)=1.8862` and `sd(x)=0.5071` are close to their population values $\mu = 1.9$ and $\sigma = 0.5$. [2] □

[2] Use the function `round` to print the mean in a desired number a decimal places.

For X distributed as $N(\mu, \sigma^2)$, it holds that $(X-\mu)/\sigma = Z$ is distributed as $N(0,1)$. Thus by subtracting μ and dividing the result with σ any normally distributed variable can be *standardized* into a standard normally distributed Z having mean zero and standard deviation one.

3.2.2 Chi-squared distribution

The chi-squared distribution plays an important role in testing hypotheses about frequencies, see Chapter 4. To define it, let $\{Z_1, \cdots, Z_m\}$ be independent and standard normally distributed random variables. Then the sum of squares

$$\chi^2_m = Z_1^2 + \cdots + Z_m^2 = \sum_{i=1}^{m} Z_i^2,$$

is the so-called chi-squared distributed (random) variable with m degrees of freedom.

Example 1. To view various members of the χ^2 distribution load the `TeachingDemos` package. Use the command `vis.gamma()` to open an interactive display of various distributions. Click on "Visualizing the gamma", "Visualizing the Chi-squared", and adapt "Xmax". Move the "Shape" button to the right to increase the degrees of freedom. Observe that the graphs of chi-squared densities change from heavily skew to the right into more bell-shaped normal as the degrees of freedom increases. □

Example 2. Let's consider the chi-squared variable with 5 degrees of freedom; $\chi^2_5 = Z_1^2 + \cdots + Z_5^2$. To compute the probability of values smaller than eight we use the function `pchisq`, as follows.

$$P\left(\chi^2_5 \leq 8\right) = \texttt{pchisq}(8, 5) = 0.8437644.$$

This yields the value of the distribution function at $x = 8$ (see Figure 3.6). This value corresponds to the area of the blue colored surface below the graph of the density function in Figure 3.5. Often we are interested in the value for the quantile $x_{0.025}$, where $P(\chi^2_5 \leq x_{0.025}) = 0.025$. [3] Such can be computed by

[3] If the distribution function is strictly increasing, then there exists an exact and unique solution for the quantiles.

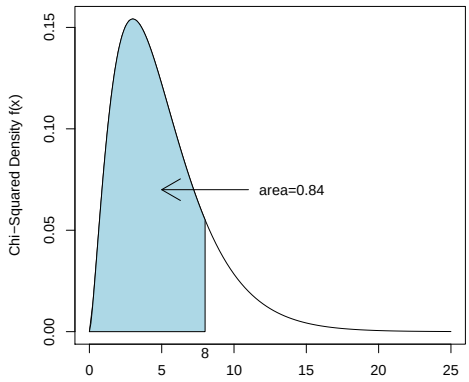


Figure 3.5: χ^2_5-density.

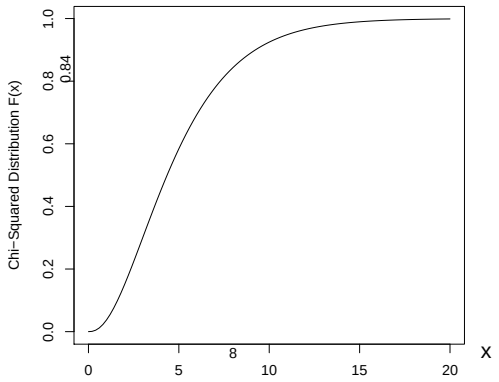


Figure 3.6: χ^2_5 distribution.

```
> qchisq(0.025, 5, lower.tail=TRUE)
[1]  0.8312
```

□

Example 3. The chi-squared distribution is frequently used as a so-called goodness of fit measure. With respect to the Golub et. al. (1999) data we may hypothesize that the expression values of gene CCND3 Cyclin D3 for the ALL patients are distributed as $N(1.90, 0.50^2)$. If this indeed holds, then the sum of squared standardized values equals their number and the probability of larger values is about 1/2. In particular, let $x_1, \cdots, x_{27}$ be the gene expression values. Then the standardized values are $z_i = (x_i - 1.90)/0.50$ and their sum of squares $\sum_1^{27} z_i^2 = 25.03312$. The probability of larger values is $P(\chi^2_{27} \geq 25.03312) = 0.5726$, which indicates that this normal distribution fits the data well. Hence, it is likely that the specified normal distribution is indeed correct. Using R the computations are as follows.

```
library(multtest); data(golub)
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
x <- golub[1042,gol.fac=="ALL"]
z <- (x-1.90)/0.50
sum(z^2)
pchisq(sum(z^2),27, lower.tail=FALSE)
```

□

3.2.3 T-Distribution

The T-distribution has many useful applications for testing hypotheses about means of gene expression values, in particular when the sample size is lower than thirty. If the data are normally distributed, then the values of $\sqrt{n}(\overline{x} - \mu)/s$ follow a T-distribution with $n-1$ degrees of freedom. The T-distribution is approximately equal to the normal distribution when the sample size is thirty.

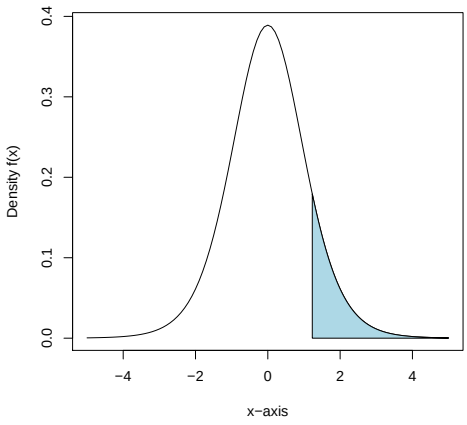


Figure 3.7: Density of T_{10} distribution.

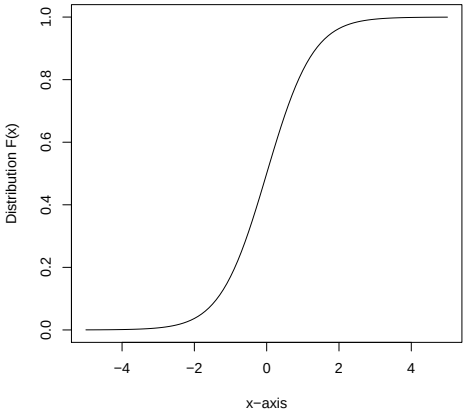


Figure 3.8: Distribution function of T_{10}.

Example 1. Load the `TeachingDemos` and give `vis.t()` to explore a visualization of the T-distribution. Click on "Show Normal Distribution" and increase the number of degrees of freedom to verify that df equal to thirty is sufficient for the normal approximation to be quite precise. □

Example 2. A quick NCBI scan makes it reasonable to assume that the gene Gdf5 has no direct relation with leukemia. For this reason we take $\mu = 0$. The expression values of this gene are collected in row 2058 of the `golub` data. To compute the sample t-value $\sqrt{n}(\overline{x} - \mu)/s$ use

```
n <- 11
x <- golub[2058, gol.fac=="AML"]
```

```
t.value <- sqrt(n)*(mean(x)-0)/sd(x)
t.value
[1] 1.236324
```

From the above we know that this has a T_{10} distribution. The probability that T_{10} is greater than 1.236324 can be computed, as follows.

$$P(T_{10} \geq 1.236324) = 1 - P(T_{10} \leq 1.236324) = 1 - \texttt{pt}(1.236324, 10) = 0.12.$$

This probability corresponds to the area of the blue colored surface below of the graph of the density function in Figure 3.7. The T distribution function with ten degrees of freedom is illustrated in Figure 3.8. The probability that the random variable T_{10} is between -2 and 2 equals

$$P(-2 \leq T_{11} \leq 2) = \texttt{pt}(2, 10) - \texttt{pt}(-2, 10) = 0.926612.$$

The 2.5% quantile can be computed by `qt(0.025,n-1)=-2.228139`. □

3.2.4 F-Distribution

The F-distribution is important for testing the equality of two variances. It can be shown that the ratio of variances from two independent sets of normally distributed random variables follows an F-distribution. More specifically, if the two population variances are equal ($\sigma_1^2 = \sigma_2^2$), then s_1^2/s_2^2 follows an F-distribution with $n_1 - 1, n_2 - 1$ degrees of freedom, where s_1^2 is the variance of the first set, s_2^2 that of the second, and n_1 is the number of observations in the first and n_2 in the second.[4]

Example 1. For equal population variances the probability is large that that the ratio of sample variances is near one. With respect to the Golub et. al. (1999) data it is easy to compute the ratio of the variances of the expression values of gene CCND3 Cyclin D3 for the ALL patients and the AML patients.

```
> var(golub[1042,gol.fac=="ALL"])/var(golub[1042,gol.fac=="AML"])
[1] 0.7116441
```

[4]It is more correct to define S_1^2/S_2^2 for certain random variables S_1^2 and S_2^2, we shall , however, not border.

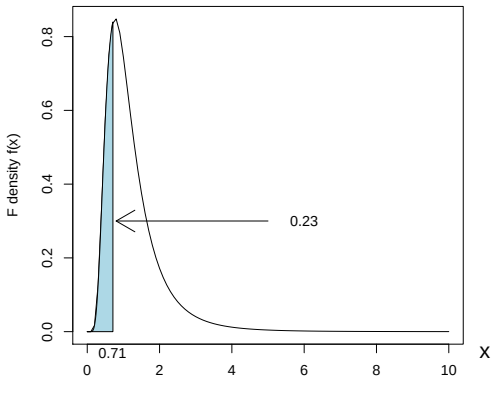


Figure 3.9: Density of $F_{26,10}$.

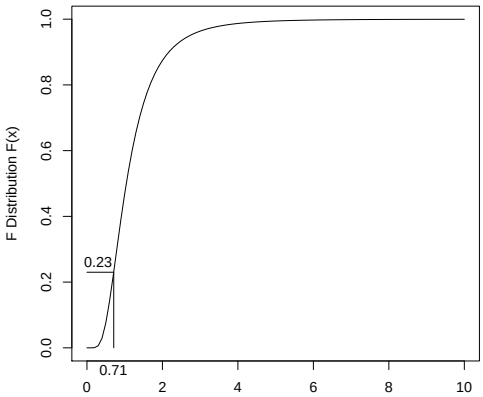


Figure 3.10: Distribution of $F_{26,10}$.

Since $n_1 = 27$ and $n_2 = 11$ this ratio is a realization of the $F_{26,10}$ distribution. Then, the probability that the ratio attains values smaller than 0.7116441 is

$$P(X \leq 0.7116441) = \texttt{pf}(\texttt{0.7116441}, \texttt{26}, \texttt{10}) = 0.2326147.$$

Figure 3.9 illustrates that this value corresponds to the area of the blue colored surface below the graph of the density function. Figure 3.10 gives the distribution function. To find the quantile $x_{0.025}$ use `qf(.025,26,10)`=0.3861673. This subject is taken further in Section 4.1.5. □

3.2.5 Plotting a density function

[5] A convenient manner to plot a density function in by using the corresponding built-in-function. For instance to plot the bell-shaped density from the normally distributed variable use the function `dnorm`, as follows.

```
> f<-function(x){dnorm(x,1.9,0.5)}
> plot(f,0,4,xlab="x-axis",ylab="density f(x)")
```

[5] This subsection is solemly on plotting and can be skipped without loss of continuity.

This produces the graph of the density function in Figure 3.3. The specification `0,4` defines the interval on the horizontal axis over which f is plotted. The vertical axis is adapted automatically. We can give the surface under f running x from 0 to 1.4 a nice blue color by using the following.

```
plot(f,0,4,xlab="x-axis",ylab="density f(x)")
x<-seq(0,1.4,0.01)
polygon(c(0,x,1.4), c(0,f(x),0), col="lightblue")
```

The basic idea of plotting is to start with a plot and next to add colors, text, arrows, etc. In particular, the command `polygon` is used to give the surface below the graph the color `"lightblue"`. The polygon (surface enclosed by many angles) is defined by the sequence of points defined as `x` and `f(x)`.

3.3 Overview and concluding remarks

For practical computations R has built-in-functions for the binomial, normal, t, F, χ^2-distributions, where `d` stands for density, `p` for (cumulative) probability distribution, `q` for quantiles, and `r` for drawing random samples, see Table 3.2. The density, expectation, and variance of most the distributions in this chapter are summarized in Table 3.3.

Table 3.2: Built-in-functions for random variables used in this chapter.

Distribution	parameters	density	distribution	quantiles	random sampling
Bin	n, p	`dbinom`(x, n, p)	`pbinom`(x, n, p)	`qbinom`(α, n, p)	`rbinom`$(10, n, p)$
Normal	μ, σ	`dnorm`(x, μ, σ)	`pnorm`(x, μ, σ)	`qnorm` (α, μ, σ)	`rnorm`$(10, \mu, \sigma)$
Chi-squared	m	`dchisq`(x, m)	`pchisq`(x, m)	`qchisq`(α, m)	`rchisq`$(10, m)$
T	m	`dt`(x, m)	`pt`(x, m)	`qt`(α, m)	`rt`$(10, m)$
F	m,n	`df`(x, m, n)	`pf`(x, m, n)	`qf`(α, m, n)	`rf`$(10, m, n)$

Although for a first introduction the above distributions are without doubt among the most important, there are several additional distributions available such as the Poisson, Gamma, beta, or Dirichlet. Obviously, these can also be programmed by yourself. The freeware encyclopedia wikipedia often gives a useful first, though technical, orientation. Note that a distribution acts as a population from which a sample can be drawn. Hence, distributions

can be seen as models of data generating procedures. For a more thorough treatment of distribution we refer the reader to Bain & Engelhardt (1992), Johnson et al. (1992), and Miller & Miller (1999).

Table 3.3: Density, mean, and variance of distributions used in this chapter.

Distribution	parameters	density	expectation	variance
Binomial	n, p	$\frac{n!}{k!(n-k)!}p^k(1-p)^{n-k}$	np	$np(1-p)$
Normal	μ, σ	$\frac{1}{\sigma\sqrt{2\pi}}\exp(-\frac{1}{2}(\frac{x-\mu}{\sigma})^2)$	μ	σ^2
Chi-squared	df=m		m	$2m$

3.4 Exercises

It is importance to obtain some routine with the computation of probabilities and quantiles.

1. Binomial Let X be binomially distributed with $n = 60$ and $p = 0.4$. Compute the following.
   (a) $P(X = 24)$, $P(X \leq 24)$, and $P(X \geq 30)$.
   (b) $P(20 \leq X \leq 30)$, $P(20 \leq X)$.
   (c) $P(20 \leq X$ or $X \geq 40)$, and $P(20 \leq X$ and $X \geq 10)$.
   (d) Compute the mean and standard deviation of X.
   (e) The quantiles $x_{0.025}$, $x_{0.5}$, and $x_{0.975}$.
2. Standard Normal. Compute the following probabilities and quantiles.
   (a) $P(1.6 < Z < 2.3)$.
   (b) $P(Z < 1.64)$.
   (c) $P(-1.64 < Z < -1.02)$.
   (d) $P(0 < Z < 1.96)$.
   (e) $P(-1.96 < Z < 1.96)$.
   (f) The quantiles $z_{0.025}$, $z_{0.05}$, $z_{0.5}$, $z_{0.95}$, and $z_{0.975}$.
3. Normal. Compute for X distributed as $N(10, 2)$ the following probabilities and quantiles.

(a) $P(X < 12)$.

(b) $P(X > 8)$.

(c) $P(9 < X < 10, 5)$.

(d) The quantiles $x_{0.025}$, $x_{0.5}$, and $x_{0.975}$.

4. T-distribution. Verify the following computations for the T_6 distribution.

(a) $P(T_6 < 1)$.

(b) $P(T_6 > 2)$.

(c) $P(-1 < T_6 < 1)$.

(d) $P(-2 < T_6 < -2)$.

(e) The quantiles $t_{0.025}$, $t_{0.5}$, and $t_{0.975}$.

5. F distribution. Compute the following probabilities and quantiles for the $F_{8,5}$ distribution.

(a) $P(F_{8,5} < 3)$.

(b) $P(F_{8,5} > 4)$.

(c) $P(1 < F_{8,5} < 6)$.

(d) The quantiles $f_{0.025}$, $f_{0.5}$, and $f_{0.975}$.

6. Chi-squared distribution. Compute the following for the chi-squared distribution with 10 degrees of freedom.

(a) $P(\chi^2_{10} < 3)$.

(b) $P(\chi^2_{10} > 4)$.

(c) $P(1 < \chi^2_{10} < 6)$.

(d) The quantiles $g_{0.025}$, $g_{0.5}$, and $g_{0.975}$.

7. MicroRNA. Suppose that for certain microRNA of size 20 the probability of a purine is binomially distributed with probability 0.7.

(a) What is the probability of 14 purines?

(b) What is the probability of less than or equal to 14 purines?

(c) What is the probability of strictly more than 10 purines?

(d) By what probability is of the number of purines between 10 and 15?

(e) How many purines do you expect? In other words: What is the mean of the distribution?

(f) What is the standard deviation of the distribution?

8. Zyxin. The distribution of the expression values of the ALL patients on the Zyxin gene are distributed according to $N(1.6, 0.4^2)$.

(a) Compute the probability that the expression values are smaller than 1.2?

(b) What is the probability that the expression values are between 1.2 and 2.0?

(c) What is the probability that the expression values are between 0.8 and 2.4?

(d) Compute the exact values for the quantiles $x_{0.025}$ and $x_{0.975}$.

(e) Use `rnorm` to draw a sample of size 1000 from the population and compare the sample mean and standard deviation with that of the population.

9. Some computations on Golub et al. (1999) data.

(a) Take $\mu = 0$ and compute the t-values for the ALL gene expression values. Find the three genes with largest absolute t-values.

(b) Compute per gene the ratio of the variances for the ALL and the AML patients. How many are between 0.5 and 1.5?

10. Extreme value investigation. This (difficult!) question aims to teach the essence of an extreme value distribution! An interesting extreme value distribution is given by Pevsner (2003, p.103). Take the maximum of a sample (with size 1000) from the standard normal distribution and repeat this 1000 times. So that you sampled 1000 maxima. Next, subtract from these maxima `an` and divide by `bn`, where

```
an <- sqrt(2*log(n)) - 0.5*(log(log(n))+log(4*pi))*(2*log(n))^(-1/2)
bn <- (2*log(n))^(-1/2)
```

Now plot the density from the normalized maxima and add the extreme value function $f(x)$ from Pevsner his book, and add the density (`dnorm`) from the normal distribution. What do you observe?

Chapter 4

Estimation and Inference

Questions that we deal with in this chapter are related to statistically testing biological hypothesis. Does the mean gene expression over ALL patients differ from that over AML patients? That is, does the mean gene expression level differ between experimental conditions? Is the mean gene expression different from zero? To what extent are gene expression values normally distributed? Are there outliers among a sample of gene expression values? How can an experimental effect be defined? How can genes be selected with respect to an experimental effect? Other important questions are: How can it be tested whether the frequencies of nucleotide sequences of two genes are different? How can it be tested whether outliers are present in the data? What is the probability of a certain micro RNA to have more than a certain number of purines?

In the foregoing chapters many population parameters were used to define families of theoretical distributions. In any research (empirical) setting the specific values of such parameters are unknown so that these must be estimated. Once estimates are available it becomes possible to statistically test biologically important hypotheses. The current chapter gives several basic examples of statistical testing and some of its background. Robust type of testing is briefly introduced as well as an outlier test.

4.1 Statistical hypothesis testing

Let μ_0 be a number representing the hypothesized population mean by a researcher on the basis of experience and knowledge from the field. With

respect to the population mean the null hypothesis can be formulated as $H_0 : \mu = \mu_0$ and the alternative hypothesis as $H_1 : \mu \neq \mu_0$. These are two statements of which the latter is the opposite of the first: Either H_0 or H_1 is true. The alternative hypothesis is true if $H_1 : \mu < \mu_0$ or $H_1 : \mu > \mu_0$ holds true. This type of alternative hypothesis is called "two-sided". In case $H_1 : \mu > \mu_0$, it is called "one-sided".

Such a null hypothesis will be statistically tested against the alternative using a suitable distribution of a statistic (e.g. standardized mean). After conducting the experiment, the value of the statistic can be computed from the data. By comparing the value of the statistic with its distribution, the researcher draws a conclusion with respect to the null hypothesis: H_0 is rejected or it is not. The probability to reject H_0, given the truth of H_0, is called the *significance level* which is generally denoted by α. We shall follow the habit in statistics to use $\alpha = 0.05$, but it will be completely clear how to adapt the procedure in case other significance levels are desired.

4.1.1 The Z-test

The Z-test applies to the situation where we want to test $H_0 : \mu = \mu_0$ against $H_1 : \mu \neq \mu_0$ and the standard deviation σ is known. Assuming that the gene expression values $(x_1, \cdots, x_n)$ are from a normal distribution we compute the standardized value $z = \sqrt{n}(\overline{x} - \mu_0)/\sigma$. Next we define the so-called p-value as the standard normal probability of Z attaining values being more extreme than $|z|$, that is occurring to the left of $-|z|$ or to the right of $|z|$.[1] Accordingly, the p-value equals

$$P(Z \leq -|z|) + P(Z \geq |z|) = 2 \cdot P(Z \leq -|z|).$$

The conclusion from the test is now as follows: If the p-value is larger than the significance level α, then H_0 is not rejected and if it is smaller than the significance level, then H_0 is rejected.

Example 1. To illustrate the Z-test we shall concentrate on the Gdf5 gene from the Golub et al. (1999) data[2]. The corresponding expression values are contained in row 2058. A quick search through the NCBI site

[1] Recall from a calculus course that $|-2| = 2$ and $|2| = 2$.

[2] We will work with `golub` throughout this chapter, so it is essential to load these data and to define the factor `gol.fac`.

makes it likely that this gene is not directly related to leukemia. Hence, we may hypothesize that the population mean of the ALL expression values equals zero. Accordingly, we test $H_0 : \mu = 0$ against $H_1 : \mu \neq 0$. For the sake of illustration we shall pretend that the standard deviation σ is known to be equal to 0.25. The z-value (=0.001116211) can be computed as follows.

```
data(golub, package = "multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
sigma <- 0.25; n <- 27; mu0 <- 0
x <- golub[2058,gol.fac=="ALL"]
z.value <- sqrt(n)*(mean(x) - mu0)/sigma
```

The p-value can now be computed as follows.

```
> 2*pnorm(-abs(z.value),0,1)
[1] 0.9991094
```

Since it is clearly larger than 0.05, we conclude that the null hypothesis of mean equal to zero is not rejected (accepted). □

Note that the above procedure implies rejection of the null hypothesis when z is highly negative or highly positive. More precisely, if z falls in the region $(-\infty, z_{0.025}]$ or $[z_{0.975}, \infty)$, then H_0 is rejected. For this reason these intervals are called "rejection regions". If z falls in the interval $(z_{0.025}, z_{0.975})$, then H_0 is not rejected and consequently this region is called "acceptance region". The situation is illustrated in Figure 4.1.

The interval $(z_{0.025}, z_{0.975})$ is often named "confidence interval", because if the null hypothesis is true, then we are 95% confident that the observed z-value falls in it. It is custom to rework the confidence interval into an interval with respect to μ (Samuels & Witmer, 2003, p. 186). In particular, the 95% confidence interval for the population mean μ is

$$\left(\overline{x} + z_{0.025}\frac{\sigma}{\sqrt{n}}, \overline{x} + z_{0.975}\frac{\sigma}{\sqrt{n}}\right). \tag{4.1}$$

That is, we are 95% certain[3] that the true mean falls in the confidence interval. Such an interval is standard output of statistical software.

[3] If we would repeat the procedure sufficiently often

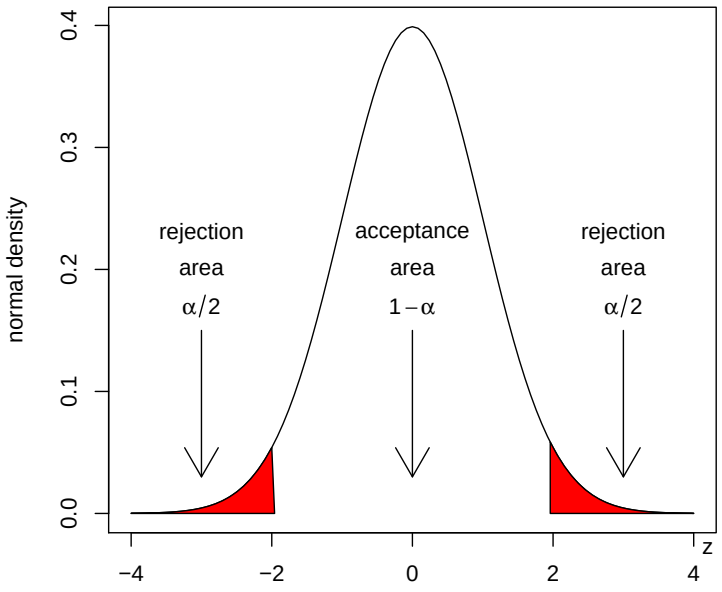


Figure 4.1: Acceptance and rejection regions of the Z-test.

Example 2. Using the data from Example 1, the 95% confidence interval given by Equation 4.1 can be computed as follows.[4]

```
> mean(x)+qnorm(c(0.025),0,1)*sigma/sqrt(n)
[1] -0.0942451
> mean(x)+qnorm(c(0.975),0,1)*sigma/sqrt(n)
[1] 0.09435251
```

Hence, the rounded estimated 95% confidence interval is $(-0.094, 0.094)$. Since $\mu_0 = 0$ falls within this interval, H_0 is not rejected. It is instructive and convenient to run the Z-test from the `TeachingDemos` package, as follows.

[4]These computations only work together with those of Example 1, especially the definition of `x`.

```
> library(TeachingDemos)
> z.test(x,mu=0,sd=0.25)

        One Sample z-test

data:  x
z = 0.0011, n = 27.000, Std. Dev. = 0.250, Std. Dev. of the sample mean
= 0.048, p-value = 0.9991
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
 -0.09424511  0.09435251
sample estimates:
  mean of x
5.37037e-05
```

From the z-value, the p-value, and the confidence interval, the conclusion is not to reject the null-hypothesis of mean equal to zero. This illustrates that testing by either of these procedures yields equivalent conclusions. □

Example 3. To develop intuition with respect to confidence intervals load the package `TeachingDemos` and give the following command.

```
>  ci.examp(mean.sim =0, sd = 1, n = 25, reps = 100,
+    method = "z", lower.conf=0.025, upper.conf=0.975)
```

Then 100 samples of size 25 from the $N(0, 1)$ distribution are drawn and for each of these the confidence interval for the population mean is computed and represented as a line segment. Apart from sampling fluctuations, the confidence level corresponds to the percentage of intervals containing the true mean (colored in black) and that the significance level corresponds to intervals not containing it (colored in red or blue). □

4.1.2 One Sample t-Test

Indeed, in almost all research situations with respect to gene expression values, the population standard deviation σ is unknown so that the above test is not applicable. In such cases t-tests are very useful for testing $H_0 : \mu = \mu_0$ against $H_1 : \mu \neq \mu_0$. The test is based on the t-value defined by $t =$

$\sqrt{n}(\overline{x} - \mu_0)/s$. The corresponding p-value is defined by $2 \cdot P(T_{n-1} \leq -|t|)$. Similar to the above, H_0 is not rejected if the p-value is larger than the significance level and H_0 is rejected if the p-value is smaller than the significance level. Equivalently, if t falls in the acceptance region $(t_{0.025,n-1}, t_{0.975,n-1})$, then H_0 is not rejected and otherwise it is. For $n = 6$ the acceptance and rejection regions are illustrated in Figure 4.2. The 95% confidence interval for the population mean is given by $(\overline{x} + t_{0.025} \cdot s/\sqrt{n}, \overline{x} + t_{0.975} \cdot s/\sqrt{n})$, where the expression $s/\sqrt{n}$ gives the so-called "standard error of the mean".

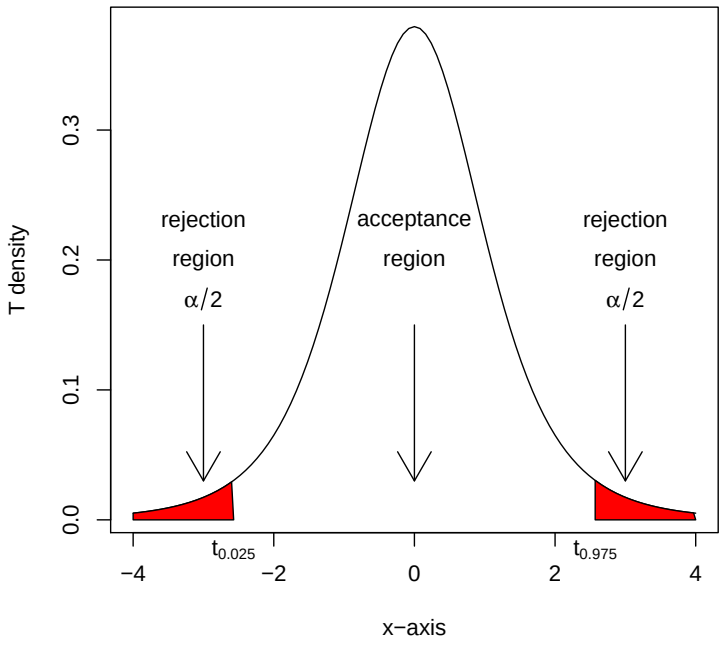


Figure 4.2: Acceptance and rejection regions of the T_5-test.

Example 1. Let's test $H_0 : \mu = 0$ against $H_1 : \mu \neq 0$ for the ALL population mean of the Gdf5 gene expressions. The latter are collected in row 2058 of the `golub` data. The t-value is computed as follows.

```
> x <- golub[2058,gol.fac=="ALL"]; mu0 <- 0; n <- 27
> t.value<-sqrt(n)*(mean(x) - mu0)/sd(x)
> t.value
[1] 0.001076867
```

The corresponding p-value can be computed by

$$2 \cdot P(T_{26} \leq -0.0010) = 2 * \texttt{pt}(-0.0010, 26) = 0.9991 > \alpha,$$

so that the conclusion is not to reject the null hypothesis of mean equal to zero.

To see whether the observed t-value belongs to the 95% confidence interval, we compute

$$(t_{0.025,26}, t_{0.975,26}) = (\texttt{qt}(0.025, \texttt{n}-1), \texttt{qt}(0.975, \texttt{n}-1)) = (-2.055, 2.055).$$

Since this interval does contain the t-value, we do not reject the hypothesis that μ equals zero. The left boundary of the 95% confidence interval for the population mean can be computed, as follows.

```
> mean(x)+qt(0.025,26)*sd(x)/sqrt(n)
[1] -0.1024562
```

The 95% confidence interval equals $(-0.1025, 0.1025)$. Since it contains zero, we do not reject the null-hypothesis.

In daily practice it is much more convenient to use the built-in-function `t.test`. We illustrate it with the current testing problem.

```
> t.test(x,mu=0)

        One Sample t-test

data:  x
t = 0.0011, df = 26, p-value = 0.9991
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
 -0.1024562  0.1025636
sample estimates:
  mean of x
5.37037e-05
```

This yields by one command line the observed t-value, the p-value, and the 95% confidence interval for μ_0. □

In the previous example the test is two-sided because H_1 holds true if $\mu < \mu_0$ or $\mu > \mu_0$. If, however, the researcher desires to test $H_0 : \mu = \mu_0$ against $H_1 : \mu > \mu_0$, then the alternative hypothesis is *one-sided* and this makes the procedure slightly different: H_0 is accepted if $P(T_n \geq t) > \alpha$ and it is rejected if $P(T_n \geq t) < \alpha$. We shall illustrate this by a variant of the previous example.

Example 2. In Chapter 2 a box-and-whiskers plot revealed that the ALL gene expression values of CCND3 Cyclin D3 are positive. Hence, we test $H_0 : \mu = 0$ against $H_1 : \mu > 0$ by the built-in-function `t-test`. Recall that the corresponding gene expression values are collected in row 1042 of the `golub` data matrix (load it if necessary).

```
> t.test(golub[1042,gol.fac=="ALL"],mu=0, alternative = c("greater"))

        One Sample t-test

data:  golub[1042, gol.fac == "ALL"]
t = 20.0599, df = 26, p-value < 2.2e-16
alternative hypothesis: true mean is greater than 0
95 percent confidence interval:
 1.732853      Inf
sample estimates:
mean of x
 1.893883
```

The large t-value indicates that, relative to its standard error, the mean differs largely from zero. Accordingly, the p-value is very close to zero, so that the conclusion is to reject H_0. □

4.1.3 Two-sample t-test with unequal variances

Suppose that gene expression data from two groups of patients (experimental conditions) are available and that the hypothesis is about the difference between the population means μ_1 and μ_2. In particular, $H_0 : \mu_1 = \mu_2$ is to

be tested against $H_1 : \mu_1 \neq \mu_2$. These hypotheses can also be formulated as $H_0 : \mu_1 - \mu_2 = 0$ and $H_1 : \mu_1 - \mu_2 \neq 0$. Suppose that gene expression data from the first group are given by $\{x_1, \cdots x_n\}$ and that of the second by $\{y_1, \cdots, y_m\}$. Let $\overline{x}$ be the mean of the first and $\overline{y}$ that of the second, and s_1^2 the variance of the first and s_2^2 that of the second. Then the t-statistic can be formulated as

$$t = \frac{(\overline{x} - \overline{y}) - (\mu_1 - \mu_2)}{\sqrt{s_1^2/n + s_2^2/m}}. \tag{4.2}$$

The decision procedure with respect to the null-hypothesis is completely similar to the above tests. Note that the t-value is large if the difference between $\overline{x}$ and $\overline{y}$ is large[5], the standard deviations s_1 and s_2 are small, and the sample sizes are large. This test is known as the Welch two-sample t-test (Lehmann, 1999).

Example 1. Golub et al. (1999) argue that gene CCND3 Cyclin D3 plays an important role with respect to discriminating ALL from AML patients. The boxplot in Figure 2.4 suggests that the ALL population mean differs from that of AML. The null hypothesis of equal means can be tested by the function `t.test` and the appropriate factor and specification `var.equal=FALSE`.

```
> t.test(golub[1042,] ~  gol.fac, var.equal=FALSE)

        Welch Two Sample t-test

data:  golub[1042, ] by gol.fac
t = 6.3186, df = 16.118, p-value = 9.87e-06
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 0.8363826 1.6802008
sample estimates:
mean in group ALL mean in group AML
        1.8938826         0.6355909
```

The t-value is quite large, indicating that the two means $\overline{x}$ and $\overline{y}$ differ largely from zero relative to the corresponding standard error (denominator in Equation 4.2). Since the p-value is extremely small, the conclusion is to reject the null-hypothesis of equal means. The data provide strong evidence that the

[5] Assuming $\mu_1 - \mu_2 = 0$.

population means do differ. □

When the first group is an experimental group and the second a control group, then $\mu_1 - \mu_2$ is the experimental effect in the population and $\overline{x} - \overline{y}$ that in the sample. The t-value is the experimental effect in the sample relative to the standard error. The size of the effect is measured by the p-value in the sense that it is smaller for larger effects.

If the two population variances are equal, then the testing procedure simplifies considerably. This is the subject of the next paragraph.

4.1.4 Two sample t-test with equal variances

Suppose exactly the same setting as in the previous paragraph, but now the variances σ_1^2 and σ_2^2 for the two groups are known to be equal. To test $H_0 : \mu_1 = \mu_2$ against $H_1 : \mu_1 \neq \mu_2$, there is a t-test which is based on the so-called pooled sample variance s_p^2. The latter is defined by the following weighted sum of the sample variances s_1^2 and s_2^2, namely

$$s_p^2 = \frac{(n-1)s_1^2 + (m-1)s_2^2}{n+m-2}.$$

Then the t-value can be formulated as

$$t = \frac{\overline{x} - \overline{y} - (\mu_1 - \mu_2)}{s_p\sqrt{\frac{1}{n} + \frac{1}{m}}}.$$

Example 1. The null hypothesis for gene CCND3 Cyclin D3 that the mean of the ALL differs from that of AML patients can be tested by the two-sample t-test using the specification `var.equal=TRUE`.

```
> t.test(golub[1042,] ~  gol.fac, var.equal = TRUE)

        Two Sample t-test

data:  golub[1042, ] by gol.fac
t = 6.7983, df = 36, p-value = 6.046e-08
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 0.8829143 1.6336690
```

```
sample estimates:
mean in group ALL mean in group AML
        1.8938826         0.6355909
```

From the p-value $6.046 \cdot 10^{-8}$, the conclusion is to reject the null hypothesis of equal population means. Note that the p-value is slightly smaller than that of the previous test. □

In case of any uncertainty about the validity of the assumption of equal population variances, one may want to test this.

4.1.5 F-test on equal variances

The assumption of the above t-test it that the two population variances are equal. Such an assumption can serve as a null hypothesis. That is, we desire to test $H_0 : \sigma_1^2 = \sigma_2^2$ against $H_0 : \sigma_1^2 \neq \sigma_2^2$. This can be accomplished by the so-called F-test, as follows. From the sample variances s_1^2 and s_2^2, the f-value $f = s_1^2/s_2^2$ can be computed, which is F_{n_1-1,n_2-1} distributed with $n_1 - 1$ and $n_2 - 1$ degrees of freedom. If $P(F_{n_1-1,n_2-1} < f) \geq \alpha/2$ for $f < 1$ or $P(F_{n_1-1,n_2-1} > f) \geq \alpha/2$ for $f > 1$, then H_0 is not rejected and otherwise it is rejected.

Example 1. The null hypothesis for gene CCND3 Cyclin D3 that the variance of the ALL patients equals that of the AML patients can be tested by the built-in-function `var.test`, as follows.

```
> var.test(golub[1042,] ~  gol.fac)

        F test to compare two variances

data:  golub[1042, ] by gol.fac
F = 0.7116, num df = 26, denom df = 10, p-value = 0.4652
alternative hypothesis: true ratio of variances is not equal to 1
95 percent confidence interval:
 0.2127735 1.8428387
sample estimates:
ratio of variances
         0.7116441
```

From the p-value 0.4652, the null-hypothesis of equal variances is not rejected. □

4.1.6 Binomial test

Suppose that for a certain micro RNA a researcher wants to test the hypothesis that the probability of a purine equals a certain value p_0. However, another researcher has reason to believe that this probability is larger. In such a setting we want to test the null-hypothesis $H_0 : p = p_0$ against the one-sided alternative hypothesis $H_1 : p > p_0$. Suppose that sequencing reveals that the micro RNA has k purines out of a total n. Assuming that the binomial distribution holds, the null-hypothesis can be tested by computing the p-value $P(X \geq k)$. If it is larger than the significance level $\alpha = 0.05$, then H_0 is not rejected and otherwise it is.

Example 1. A micro RNA of length 22 contains 18 purines. The null hypothesis $H_0 : p = 0.7$ is to be tested against the one-sided $H_1 : p > 0.7$. From

$$P(X \geq 18) = 1 - \texttt{pbinom}(\texttt{17}, \texttt{22}, \texttt{0.7}) = 0.1645 \geq 0.05 = \alpha,$$

the conclusion follows not to reject the null-hypothesis. This test can also be conducted by the function `binom.test` as follows.

```
>  binom.test(18, 22, p = 0.7,  alternative = c("greater"),
+ conf.level = 0.95)
        Exact binomial test
data:  18 and 22
number of successes = 18, number of trials = 22, p-value = 0.1645
alternative hypothesis: true probability of success is greater than 0.7
95 percent confidence interval:
 0.6309089 1.0000000
sample estimates:
probability of success
             0.8181818
```

The p-value 0.1645 is larger than the significance level 0.05, so that the null hypothesis is not rejected. □

4.1.7 Chi-squared test

It often happens that we want to test a hypothesis with respect to more than one probability. That is, the $H_0 : (\pi_1, \cdots, \pi_m) = (p_1, \cdots, p_m)$ against $H_1 : (\pi_1, \cdots, \pi_m) \neq (p_1, \cdots, p_m)$, where p_1 to p_m are given numbers corresponding to the hypothesis of a researcher. By multiplying the probabilities with the total number of observations we obtain the expected number of observations ($e_i = n \cdot p_i$). Now we can compute the statistic $q = \sum_{i=1}^{m} (o_i - e_i)^2 / e_i$, where o_i is the i-th observed and e_i the i-th expected frequency. This statistic is chi-squared (χ^2_{m-1}) distributed with $m - 1$ degrees of freedom. The p-value of the chi-squared test is defined as $P(\chi^2_{m-1} \geq q)$. If it is larger than the significance level, then the null hypothesis is not rejected, and otherwise it is.

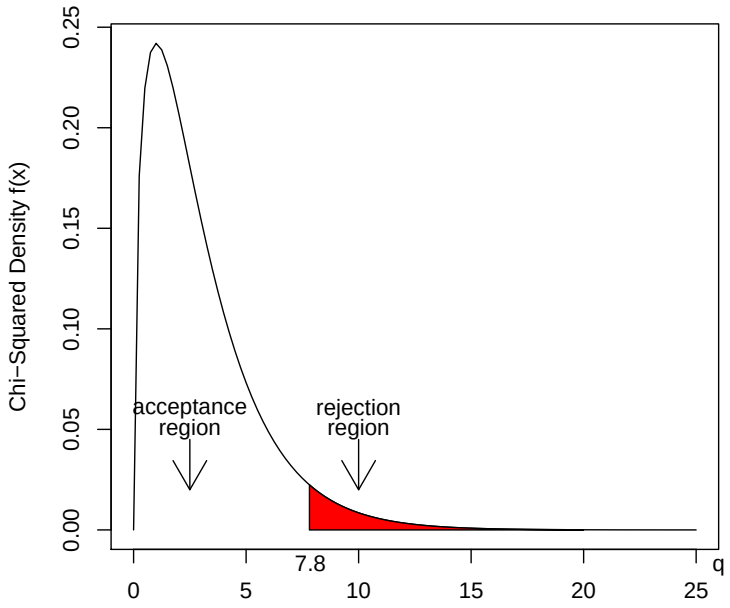


Figure 4.3: Rejection region of χ^2_3-test.

Example 1. Suppose we want to test the hypothesis that the nucleotides of Zyxin have equal probability. Let the probability of $\{A, C, G, T\}$ to occur in the sequence be given by $(\pi_1, \pi_2, \pi_3, \pi_4)$. Then the null hypothesis to be tested is $(\pi_1, \pi_2, \pi_3, \pi_4) = (1/4, 1/4, 1/4, 1/4)$. In particular, for the sequence "X94991.1" from Table 1.1 the total number of nucleotides is $n = 2166$, so that the expected frequencies e_i are equal to $2166/4 = 541.5$. Then, the q-value equals $\sum_{i=1}^{4}(o_i - e_i)^2/e_i =$

$$\frac{(410-541.5)^2}{541.5}+\frac{(789-541.5)^2}{541.5}+\frac{(573-541.5)^2}{541.5}+\frac{(394-541.5)^2}{541.5} = 187.0674$$

Since, $P(\chi^2[3] \geq 187.0674)$ is close to zero, the null hypothesis is clearly rejected. The nucleotides of Zyxin do not occur with equal probability.

A more direct manner to perform the test is by using the built-in-function `chisq.test`, as follows.

```
> library(ape)
> zyxinfreq <- table(read.GenBank(c("X94991.1"),as.character=TRUE))
> chisq.test(zyxinfreq)

        Chi-squared test for given probabilities

data:  zyxinfreq
X-squared = 187.0674, df = 3, p-value < 2.2e-16
```

The package `ape` is loaded, the Zyxin sequence `"X94991.1"` is downloaded, and the frequency table is constructed. The observed frequencies are given as input to `chisq.test` which has equal probabilities as the default option. The q-value equals `X-squared` and the degrees of freedom `df = 3`. From the corresponding p-value, the conclusion is to reject the null hypothesis of equal probabilities. The testing situation is illustrated in Figure 4.3, where the red colored surface corresponds to the rejection region $(7.81, \infty)$. Remember from the previous chapter that the left bound of this rejection interval can by found by `qchisq(0.95, 3)`. The observed $q = 187.0674$ obviously falls far into the right hand side of the rejection region, so that the corresponding p-value is very close to zero. □

Example 2. In the year 1866 Mendel observed in large number of experiments frequencies of characteristics of different kinds of seed and their offspring. In particular, this yielded the frequencies 5474, 1850 the seed shape

of ornamental sweet peas. A crossing of B and b yields off spring BB, Bb and bb with probability 0.25, 0.50, 0.25. Since Mendel could not distinguish Bb from BB, his observations theoretically occur with probability 0.75 (BB and Bb) and 0.25 (bb). To test the null hypothesis $H_0 : (\pi_1, \pi_2) = (0.75, 0.25)$ against $H_1 : (\pi_1, \pi_2) \neq (0.75, 0.25)$, we use the chi-squared test[6], as follows.

```
> pi <- c(0.75,0.25)
> x <-c(5474, 1850)
> chisq.test(x, p=pi)

        Chi-squared test for given probabilities

data:  x
X-squared = 0.2629, df = 1, p-value = 0.6081
```

From the p-value 0.6081, we do not reject the null hypothesis. □

To further illustrate the great flexibility of the chi-squared test another example is given.

Example 3. Given certain expression values for a healthy control group and an experimental group with a disease, we may define a certain cut off value and classify e.g. smaller values to be healthy and larger ones to be infected. In such a manner cut-off values can serve as a diagnostic instrument. The classification yields true positives (correctly predicted disease), false positives (incorrectly predicted disease), true negatives (correctly predicted healthy) and false negatives (incorrectly predicted healty). For the sake of illustration suppose that among twenty patients there are 5 true positives (tp), 5 false positives (fp), 5 true negatives (tn), and 5 false negatives (fn). These frequencies can be put is a two-by-two table giving the frequencies on two random variables: the true state of the persons and the predicted state of the persons (by the cut off value). In the worst case the prediction by the cut-off value is independent of the disease state of the patient. The null hypothesis of independence, can be tested by a chi-square test, as follows.

```
> dat <- matrix(c(5,5,5,5),2,byrow=TRUE)
> chisq.test(dat)
```

[6]For the sake of clarity the code is somewhat unelegant in using the symbol `pi`, the constant representing the ratio of a circle's circumference to its diameter.

```
        Pearson's Chi-squared test with Yates' continuity correction

data:  dat
X-squared = 0.2, df = 1, p-value = 0.6547
```

Since the p-value is larger than the significance level, the null hypothesis of independence is not rejected.

Suppose that for another cutoff value we obtain 8 true positives (tp), 2 false positives (fp), 8 true negatives (tn), and 2 false negatives (fn). Then testing independence yields the following.

```
> dat <- matrix(c(8,2,2,8),2,byrow=TRUE)
> chisq.test(dat)

        Pearson's Chi-squared test with Yates' continuity correction

data:  dat
X-squared = 5, df = 1, p-value = 0.02535
```

Since the p-value is smaller than the significance level, the null hypothesis of independence is rejected. □

	significant genes	non-significant genes
Chromosome 1	100	2000
genome	300	6000

Example 4. A related and frequently applied test in Bioinformatics is the Fisher exact test. In a two by two table with frequencies f_{11}, f_{22}, (f_{12}, and f_{21}), this test is based on the so-called odds ratio $f_{11}f_{22}/(f_{12}f_{21})$. Suppose that the number of significant onco type of genes in Chromosome 1 is $f_{11} = 100$ out of a total of $f_{12} = 2000$ and the number of significant genes in the whole genome is $f_{21} = 300$ out of a total of $f_{22} = 6000$. Then the odds ratio equals $100 \cdot 6000/(2000 \cdot 300) = 1$ and the number of significant oncogenes in Chromosome 1 is exactly proportional to that in the genome.

The null-hypothesis of the Fisher test is that the odds ratio equals 1 and the alternative hypothesis that it differs from 1. Suppose that the frequencies

of significant oncogenes for Chromosome 1 equals $f_{11} = 300$ out of a total of $f_{12} = 500$ and for the genome $f_{21} = 3000$ out of $f_{22} = 6000$. The hypothesis that the odd ratio equals one can now be tested as follows.

```
> dat <- matrix(c(300,500,3000,6000),2,byrow=TRUE)
> fisher.test(dat)

        Fisher's Exact Test for Count Data

data:  dat
p-value = 0.01912
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 1.029519 1.396922
sample estimates:
odds ratio
  1.199960
```

Since the p-value is smaller than the significance level, the null hypothesis of odds ratio equal to one is rejected. There are more significant oncogenes in Chromosome 1 compared to that in the genome. Other examples of the Fisher test will be given in Chapter 6. □

4.1.8 Normality tests

Various procedures are available to test the hypothesis that a data set is normally distributed. The Shapiro-Wilk test is based on the degree of linearity in a Q-Q plot (Lehmann, 1999, p.347) and the Anderson-Darling test is based on the distribution of the data (Stephens, 1986, p.372).

Example 1. To test the hypothesis that the ALL gene expression values of CCND3 Cyclin D3 from Golub et al. (1999) are normally distributed, the Shapiro-Wilk test can be used as follows.

```
> shapiro.test(golub[1042, gol.fac=="ALL"])

        Shapiro-Wilk normality test
```

```
data:  golub[1042, gol.fac == "ALL"]
W = 0.947, p-value = 0.1774
```

Since the p-value is greater than 0.05, the conclusion is not to reject the null hypothesis that CCND3 Cyclin D3 expression values follow from a normal distribution. The Anderson-Darling test is part of the `nortest` package which probably needs to be installed and loaded first. Running the test on our CCND3 Cyclin D3 gene expression values comes down to the following.

```
> library(nortest)
> ad.test(golub[1042,gol.fac=="ALL"])

        Anderson-Darling normality test

data:  scale(golub[1042, gol.fac == "ALL"])
A = 0.5215, p-value = 0.1683
```

Hence, the same conclusion is drawn as from the Shapiro-Wilk test. Note that the p-values from both tests are somewhat low. This confirms our observation in Section 2.1.5 based on the Q-Q plot that the distribution resembles the normal. From the normality tests the conclusion is that the differences in the left tail are not large enough to reject the null-hypothesis that the CCND3 Cyclin D3 expression values are normally distributed. □

4.1.9 Outliers test

When gene expression values are not normally distributed, then outliers may appear with large probability. The appearance of outliers in gene expression data may influence the value of a (non-robust) statistic to a large extent. For this reason it is useful to be able to test whether a certain set of gene expression values is contaminated by an outlier or not. Accordingly, the null-hypothesis to be tested is that a set of gene expression values does not contain an outlier and the alternative is that it is contaminated with at least one outlier. Under the assumption that the data are realizations of one and the same distribution, such a hypothesis can be tested by the Grubbs (1950) test. This test is based on the statistic $g = |\text{suspect value} - \overline{x}|/s$, where the suspect value is included for the computation of the mean $\overline{x}$ and the standard

deviation s.

Example 1. From Figure 2.4 we have observed that expression values of gene CCND3 Cyclin D3 may contain outliers with respect to the left tail. This can actually be tested by the function `grubbs.test` of the `outliers` package, as follows.

```
> library(outliers)
> grubbs.test(golub[1042, gol.fac=="ALL"])

        Grubbs test for one outlier

data:  golub[1042, gol.fac == "ALL"]
G = 2.9264, U = 0.6580, p-value = 0.0183
alternative hypothesis: lowest value 0.45827 is an outlier
```

Since the p-value is smaller than 0.05, the conclusion is to reject the null-hypothesis of no outliers. □

In case the data are normally distributed, the probability of outliers is small. Hence, extreme outliers indicate that the data are non-normally distributed with large probability. Outliers may lead to such an increase of the standard error that a true experimental effect remains uncovered (false negatives). In such cases a robust test based on ranks may be preferred as a useful alternative.

4.1.10 Wilcoxon rank test

In case the data are normally distributed with equal variance, the t-test is an optimal test for testing $H_0 : \mu_1 = \mu_2$ against $H_1 : \mu_1 \neq \mu_2$ (Lehmann, 1999). If, however, the data are not normally distributed due to skewness or otherwise heavy tails, then this optimality does not hold anymore and there is no guarantee that the significance level of the test equals the intended level α (Lehmann, 1999). For this reason rank type of tests are developed for which on beforehand no specific distributional assumptions need to be made. In the below we shall concentrate on the two-sample Wilcoxon test because of its relevance to bioinformatics. We sustain with a brief description of the basic idea and refer the interested reader to the literature on non-parametric

testing (e.g. Lehmann, 2006). To broaden our view we switch from hypotheses about means to those about distributions. An alternative hypothesis may then be formulated as that the distribution of a first group lays to the left of a second. To set the scene let the gene expression values of the first group (x_1 to x_m) have distribution F and those of the second group (y_1 to y_n) distribution G. The null hypothesis is that both distributions are equal ($H_0 : F = G$) and the alternative that these are not. For example that the x's are smaller (or larger) than the y's. By the two-sample Wilcoxon test the data $x_1, \cdots, x_m, y_1, \cdots, y_n$ are ranked and the rank numbers of the x's are summed to form the statistic W after a certain correction (Lehmann, 2006). The idea is that if the ranks of x's are smaller than those of the y's, then the sum is small. The distribution of the sum of ranks is known so that a p-value can be computed on the basis of which the null hypothesis is rejected if it is smaller than the significance level α.

Example 1. The null hypothesis that the expression values for gene CCND3 Cyclin D3 are equally distributed for the ALL patients and the AML patients can be tested by the built-in-function `wilcox.test`, as follows.

```
> wilcox.test(golub[1042,] ~  gol.fac)

        Wilcoxon rank sum test

data:  golub[1042, ] by gol.fac
W = 284, p-value = 6.15e-07
alternative hypothesis: true location shift is not equal to 0
```

Since the p-value is much smaller than $\alpha = 0.05$, the conclusion is to reject the null-hypothesis of equal distributions. □

4.2 Application of tests to a whole set gene expression data

Various tests are applied in the above to a single vector of gene expressions. In daily practice, however, we want to analyze a set of thousands of (row) vectors with gene expression values which are collected in a matrix. Such

can conveniently be accomplished by taking advantage of the fact that R stores the output of a test as an object in such a manner that we can extract information such as p-values. Recall that the smaller the p-value the larger the experimental effect. Hence, by collecting p-values in a vector we can select genes with large differences between patient groups. This and testing for normality will be illustrated by two examples.

Example 1. Having a data matrix with gene expression values, a question one might ask is: What is the percentage of genes that passes a normality test? Such can be computed as follows.

```
> data(golub,package="multtest")
> gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
> sh <- apply(golub[,gol.fac=="ALL"], 1, function(x) shapiro.test(x)$p.value)
> sum(sh > 0.05)/nrow(golub) * 100
[1] 58.27598
```

Hence, according to the Shapiro-Wilk test, 58.27% of the ALL gene expression values is normally distributed (in the sense of non-rejection). For the AML expression values this is 60.73%. It can be concluded that about forty percent of the genes do not pass the normality test. □

Example 2. In case the gene expression data are non-normally distributed the t-test may indicate conclusions different from those of the Wilcoxon test. Differences between these can be investigated by collecting the p-values from both tests and seeking for the largest differences.

```
> data(golub, package = "multtest");
> gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
> pt <- apply(golub, 1, function(x) t.test(x ~  gol.fac)$p.value)
> pw <- apply(golub, 1, function(x) wilcox.test(x ~  gol.fac)$p.value)
> resul <- data.frame(cbind(pw,pt))
> resul[pw<0.05 & abs(pt-pw)>0.2,]
             pw        pt
456  0.04480288 0.2636088
1509 0.03215830 0.4427477
```

The p-value is extracted from the output of the `t.test` function and stored in the vector `pt`. The logical operator `&` is used to select genes for which the

Wilcoxon p-value is smaller than 0.05 and the absolute difference with the p-value from the t-test is larger than 0.2. Since there are only two such genes we can draw the reassuring conclusion that the tests give similar results. □

4.3 Overview and concluding remarks

Statistical hypothesis testing consists of hypotheses, distributional assumptions, and decisions (conclusions). The hypotheses pertain to the outcome of a biological experiment and are always formulated in terms of population values of parameters. Statistically, the outcomes of experiments are seen as realizations of random variables. The latter are assumed to have a certain suitable distribution which is seen as a statistical model for outcomes of an experiment. Then a statistic is formulated (e.g. a t-value) which is treated both as a function of the random variables and as a function of the data values. By comparing the distribution of the statistic with the value of the statistic, the p-value is computed and compared to the level of significance. A large p-value indicates that the model fits the data well and that the assumptions as well as the null-hypothesis are correct with large probability. However, a low p-value indicates, under the validity of the distributional assumptions, that the outcome of the experiment is so unlikely that this causes a sufficient amount of doubt to the researcher to reject the null hypothesis.

The quality of a test is often expressed in terms of efficiency, which is usually directly related to the (asymptotic) variance of an estimator. The relative efficiency is the ratio of the asymptotic variances. For Wilcoxon's test versus the t-test this equals .955, which means that in the optimal situation where the (gene expression) data are normally distributed, Wilcoxon's test is only a little worse than the t-test. In case, however, of a few outliers or a slightly heavier tail, the Wicoxon test can be far more efficient than the t-test (Lehmann, 1999, p.176). Efficiency is directly related to power; the probability to reject a false hypothesis. The probability of drawing correct conclusions can always be improved by increasing the sample size.

These considerations set the scene for making some recommendations, which obviously should not be followed blindly. If gene expression data pass a normality test, then the Welch type of t-test provides a general test with good power properties (Ramsey, 1980; Wang, 1971). In case normality does not hold and the sample size per group is at least least four, the Wilcoxon

test is recommended.

Because the Wilcoxon p-values are based on ranks many of these are equal for different genes, so that it is less suitable for ordering in case of small sample size. On the other hand, it is obviously questionable whether extremely small differences in p-values produced by the t-test contribute to biologically relevant gene discrimination. That is, extremely small differences should not be over-interpreted.

4.4 Exercises

1. Gene CD33. Use `grep` to find the index of the important gene CD33 among the list of characters `golub.gnames`. For each test below formulate the null hypothesis, the p-value and your conclusion.

   (a) Test the normality of the ALL and AML expression values.

   (b) Test for the equality of variances.

   (c) Test for the equality of the means by an appropriate t-test.

   (d) Is the experimental effect strong?

2. Gene "MYBL2 V-myb avian myeloblastosis viral oncogene homolog-like 2" has its expression values in row 1788.

   (a) Use a boxplot to construct a hypothesis about the experimental effect.

   (b) Test for the equality of means by an appropriate t-test.

3. HOXA9. Gene "HOXA9 Homeo box A9" with expression values in row 1391, can cause leukemia (Golub et al., 1999).

   (a) Test the normality of the expression values of the ALL patients.

   (b) Test for the equality of means by an appropriate t-test.

4. Zyxin. On NCBI there are various cDNA clones of zyxin.

   (a) Find the accession number of cDNA clone with IMAGE:3504464.

   (b) Test whether the frequencies of the nucleotides are equal for each nucleic acid.

(c) Test whether the frequencies of "X94991.1" can be predicted by the probabilities of the cDNA sequence "BC002323.2".

5. Gene selection. Select the genes from the `golub` data with smallest two-sample t-test values for which the ALL mean is greater than the AML mean. Report the names of the best ten. Scan the Golub (1999) article for genes among the ten you found and discuss their biological function briefly.

6. Antigenes. Antigenes play an important role in the development of cancer. Order the antigenes according to their p-values from the Welch two-sample t-test with respect to gene expression values from the ALL and AML patients of the Golub et al. (1999) data.

7. Genetic Model. A certain genetic model predicts that four phenotypes occur in ration 9:3:3:1. In a certain experiment the offspring is observed with frequencies 930, 330, 290, 90. Do the data confirm the model?

8. Comparing two genes. Consider the gene expression values in row 790 and 66 of the Golub et al. (1999) data.

   (a) Produce a boxplot for the ALL expression values and comment on the differences. Are there outliers?

   (b) Compute the mean and the median for the ALL gene expression values for both genes. Do you observed difference between genes?

   (c) Compute three measures of spread for the ALL expression values for both genes. Do you observe difference between genes?

   (d) Test by Shapiro-Wilk and Anderson-Darling the normality for the ALL gene expression values for both genes.

9. Normality tests for gene expression values of the Golub et al. (1999) data. Perform the Shapiro-Wilk normality test separately for the ALL and AML gene expression values. What percentage passed the normality test separately for the ALL and the AML gene expression values? What percentage passes both testes?

10. Two-sample tests on gene expression values of the Golub et al. (1999) data.

(a) Perform the two-sample Welch t-test and report the names of the ten genes with the smallest p-values.

(b) Perform the Wilcoxon rank test and report the names of the ten genes with the smallest p-values.

11. Biological hypotheses. Suppose that the probability to reject a biological hypothesis by the results of a certain experiment is 0.05. Suppose that the experiment is repeated 1000 times.

(a) How many rejections do you expect.

(b) What is the probability of less than 10 rejections?

(c) What is the probability of more than 5 rejections?

(d) What is the probability that the number of rejections is between two and eight?

12. Programming some tests.

(a) Program the two-sample t-test with equal variances and illustrate it with the expression values of row 1024 the of Golub et al. (1999) data.

(b) The value of W in the two-sample Wilxoxon test equals the sum of the ranks of Group 1 minus $n(n+1)/2$, where n is the number of gene expression values in Group 1. Program this and illustrate it with the expression values of row 1024 of Golub et al. (1999) data.

(c) The value of W in the two-sample Wilxoxon test equals the number of values $x_i > y_j$, where x_i, y_j are values from Group 1 and 2, respectively. Program this and illustrate it with the expression values of row 1024 of Golub et al. (1999) data.

Chapter 5

Linear Models

We have seen that the t-test can be used to discover genes with different means in the population with respect to two groups of patients. In case, however, there are three groups of patients the question arises how genes can be selected having the largest differential expressions between group means (experimental effect)? A technique making this possible is an application of the linear model and is called analysis of variance. It is frequently applied bioinformatics.

The validity of the technique is based on the assumption that the gene expression values are normally distributed and have equal variance across groups of patients. It is of importance to investigate these assumptions because it either reassures our confidence in the conclusions or it indicates that alternative tests should be used.

In this chapter the linear model will briefly be explained. The main focus, however, is on application of the linear model for testing the hypothesis that three or more group means are equal. Several illustrations of analyzing gene expression data will be given. It will be explained how the assumptions about normality and equal variances (homogeneity) can be investigated and what alternatives can be used in case either of these does not hold. The somewhat technical concepts of "model matrix" and "contrast matrix" are explained because these are useful for several applications in the next chapter.

5.1 Definition of linear models

Given a gene expression Y_i, a basic form of the linear model is

$$Y_i = x_i\beta + \varepsilon_i, \quad \text{for} \quad i = 1, \cdots, n,$$

where Y_i is an observable variable, x_i a fixed number, β an unknown weight, ε_i a unobservable error variable. The fixed number x_i follows from a statistical "design", as we shall see. The x_i value is part of the predictor, Y_i the criterion, and ε_i the error of the model. The systematical part of the model $x_i\beta$ equals the mean of the gene expression Y_i. The model is called "linear" because the degree of the coefficient β is one. For a linear model to be a statistical model there must be some assumption with respect to the distribution of the error variables. Frequently, it is assumed that the error variables $\varepsilon_1, \cdots, \varepsilon_n$ are independent and normally distributed with zero mean, that is, according to $N(0, \sigma^2)$. Then the mean of Y_i equals $x_i\beta$ and its variance σ^2.

Example 1. A common manner to introduce the linear model is by writing

$$Y_i = \beta_1 + x_i\beta_2 + \varepsilon_i, \quad \text{for} \quad i = 1, \cdots, n,$$

so that the model part represents a straight line with intercept β_1 and slope β_2. Given data points $y_1, \cdots, y_n$ and $x_1, \cdots, x_n$, a best fitting line through the data can easily be computed by least squares estimation of the intercept and slope. A nice application to explore this is by the function `put.points.demo()` from the `TeachingDemos` package. It allows points to be added and deleted to a plot which interactively computes estimates for the slope and the intercept given the data. By choosing the points more or less on a horizontal line, the slope will be near zero. By choosing the points nearly vertical, the slope will be large. By choosing a few gross errors in the data it can be observed that the estimates are not robust against outliers. □

In order to handle gene expression data for three or more groups of patients we need to extend the model. The idea simply is to increase the number of weights to the number of groups k, so that, we obtain the weights $\beta_1, \cdots, \beta_k$ and the corresponding design values $x_{i1}, \cdots, x_{ik}$. The systematic part of the model consists of a weighted sum of these design values:

$x_{i1}\beta_1 + \cdots + x_{ik}\beta_k$. By adding measurement error to this systematic part we obtain the linear model

$$Y_i = \sum_{j=1}^{k} x_{ij}\beta_j + \varepsilon_i.$$

The design values x_{ij} for Patient i in Group j are collected in the so-called "design" matrix denoted by $\boldsymbol{X}$. In particular, the design value x_{ij} is chosen to be equal to 1 if Patient i belongs to Group j and zero if (s)he does not. By this choice it becomes possible to use linear model estimation for testing hypotheses about group means. This will be illustrated by an example.

Example 2. Suppose we have the following artificial gene expressing values 2,3,1,2, of Group 1, 8,7,9,8 of Group 2, and 11,12,13,12 of Group 3. We may assign these to a vector `y`, as follows.

```
> y <- c(2,3,1,2, 8,7,9,8, 11,12,13,12)
```

Next, we construct a factor indicating to which group each expression value belongs. In particular, the first four belong to Group 1, the second four to Group 2, and the third four to Group 3. We conveniently use the function `gl` to define the corresponding factor.

```
> a <- gl(3,4)
> a
 [1] 1 1 1 1 2 2 2 2 3 3 3 3
Levels: 1 2 3
```

The design matrix $\boldsymbol{X}$ is also called "model matrix". It is illuminating to print it to the screen.

```
> model.matrix(y ~ a - 1)
   a1 a2 a3
1   1  0  0
2   1  0  0
3   1  0  0
4   1  0  0
5   0  1  0
6   0  1  0
7   0  1  0
8   0  1  0
```

```
9   0  0  1
10  0  0  1
11  0  0  1
12  0  0  1
```

The notation `y~a-1` represents a model equation, where `-1` means to skip the intercept or general constant.[1] In this situation, the weights $(\beta_1, \beta_2, \beta_3)$ of the model specialize to the population means (μ_1, μ_2, μ_3). The model for the first gene expression value of Group 1 is $Y_1 = \mu_1 + \varepsilon_1$, for the second expression value of Group 1 it is $Y_2 = \mu_1 + \varepsilon_2$, for the first member of Group 2 it is $Y_5 = \mu_2 + \varepsilon_5$, and for the first member of Group 3 it is $Y_9 = \mu_3 + \varepsilon_9$.

Recall that population means are generally estimated by sample means. Similarly, in the current setting, estimation of the linear model comes down to estimation of group means for which there are one-sample t-type of tests available (see e.g. Rao & Toutenburg, 1995; Samuels & Witmer, 2003). To illustrate this we employ the estimation function `lm` and ask for a summary.

```
> summary(lm(y ~ a - 1))

Coefficients:
   Estimate Std. Error t value Pr(>|t|)
a1   2.0000     0.4082   4.899 0.000849 ***
a2   8.0000     0.4082  19.596 1.09e-08 ***
a3  12.0000     0.4082  29.394 2.98e-10 ***
```

The output in the first column gives the estimated mean per group. The second gives the standard error of each mean, the third the t-value (the estimate divided by the standard error), and the last the corresponding p-values. From the p-values the conclusion follows to reject the null hypotheses $H_0 : \mu_j = 0$ for Group index j running from 1 to 3. □

Using the above design matrix, the model for the gene expression values from different groups can be written as

$$Y_{ij} = \mu_j + \varepsilon_{ij}, \qquad \text{where} \quad \varepsilon_{ij} \quad \text{is distributed as} \quad N(0, \sigma^2),$$

and Y_{ij} is the expression of Person i in Group j, μ_j the mean of Group j, and the ε_{ij} the error of Person i in Group j. The error is assumed to be normally distributed with zero mean and variance equal for different persons.

[1] See also Chapter 11 of the manual "An Introduction to R".

The above illustrates that the linear model is useful for testing hypotheses about group means. In bioinformatics the linear model is applied to many sets of gene expressions, so that it is of great importance to have an overall test for the equality of means.

5.2 One-way analysis of variance

A frequent problem is that of testing the null hypothesis that three or more population means are equal. By comparing two types of variances, this is made possible by a technique called analysis of variance (ANOVA). To set the scene, let three groups of patients be available with measurements in the form of gene expression values. The null-hypothesis to be tested is $H_0 : \mu_1 = \mu_2 = \mu_3$. In statistical language such groups are called levels of a factor. Let the data for Group 1 be represented by $y_{11}, y_{21}, \cdots, y_{n1}$ those of Group 2 by $y_{12}, y_{22}, \cdots, y_{n2}$ and those of Group 3 by $y_{13}, y_{23}, \cdots, y_{n3}$, where n is the number of expression values in each group. The three sample means per patient group can be expressed by

$$\overline{y}_1 = \frac{1}{n}\sum_{i=1}^{n} y_{i1}, \quad \overline{y}_2 = \frac{1}{n}\sum_{i=1}^{n} y_{i2}, \quad \text{and} \quad \overline{y}_3 = \frac{1}{n}\sum_{i=1}^{n} y_{i3}.$$

The total number of measurements $N = 3n$, so that the overall mean $\overline{y}$ is equal to

$$\overline{y} = \frac{1}{N}\left(\sum_{i=1}^{n} y_{i1} + \sum_{i=1}^{n} y_{i2} + \sum_{i=1}^{n} y_{i3}\right).$$

For the definition of the overall test on the equality of means there are two sums of squares of importance. The *sum of squares within* (SSW) is the sum of the squared deviation of the measurements to their group mean, that is

$$SSW = \sum_{j=1}^{g}\sum_{i=1}^{n}(y_{ij} - \overline{y}_j)^2,$$

where g is the number of groups. The *sum of squares between* (SSB) is the sum of squares of the deviances of the group mean with respect to the total mean, that is

$$SSB = \sum_{j=1}^{g}\sum_{i=1}^{n}(\overline{y}_j - \overline{y})^2 = n\sum_{j=1}^{g}(\overline{y}_j - \overline{y})^2.$$

Now the f-value is defined by

$$f = \frac{SSB/(g-1)}{SSW/(N-g)}.$$

If the data are normally distributed, then this f-value follows the $F_{g-1,N-g}$ distribution, where $g-1$ and $N-g$ are the degrees of freedom (Rao, 1973, p.245). If $P(F_{g-1,N-g} > f) \geq \alpha$, then $H_0 : \mu_1 = \mu_2 = \mu_3$ is not rejected, and, otherwise it is. The idea behind the test is that, under the null-hypothesis of equal group means, the value for SSB will tend to be small, so that the observed f-value will be small and H_0 is accepted.

Example 1. Let's continue with the data from the previous example. Recall that the data of Group 1 are 2, 3, 1, 2, those of Group 2 are 8, 7, 9, 8, and of Group 3 are 11, 12, 13, 12. The number of expression values per group $n = 4$, the total number of data values $N = 12$, and the number of groups $g = 3$.

To load the data, to construct the corresponding factor, and to compute the group means one may use the following.

```
> y <- c(2,3,1,2, 8,7,9,8, 11,12,13,12)
> a <- gl(3,4)
> gm <- as.numeric(tapply(y, a, mean))
> gm
[1]  2  8 12
```

Thus we find that $\overline{y}_1 = 2$, $\overline{y}_2 = 8$, and $\overline{y}_3 = 12$. These group means are now collected in the vector `gm`. The grand mean $\overline{y}$ can be computed by `mean(y)=7.333333`. An elementary manner to compute the sums of squares between `SSB` is by

```
gm <- as.numeric(tapply(y, a, mean))
g <- 3; n <- 4; N <-12; ssb <- 0
for (j in 1:g) {ssb  <-  ssb + (gm[j]- mean(y))^2}
SSB <- n*ssb
```

This results in $SSB = 202.6667$. In a similar manner the sums of squares within (SSW) and the f-value can be computed, as follows.

```
> SSW <- 0
> for (j in 1:g) {SSW  <-  SSW + sum((y[a==j]-gm[j])^2)}
> f <- (SSB/(g-1))/(SSW/(N-g))
```

This results in $SSW = 6$ and an observed f-value equal to 152. Hence, the overall p-value is

$$P(F_{2,9} > 152) = 1 - P(F_{2,9} < 152) = \texttt{1 - pf(152, 2, 9)} = 1.159156 \cdot 10^{-7}.$$

Since this is smaller than the significance level 0.05, the conclusion is to reject the null hypothesis of equal means.

The built-in-function `anova` can be used to extract the so-called analysis of variance table from an `lm` object.

```
> anova(lm(y ~ a))
Analysis of Variance Table

Response: x
          Df  Sum Sq Mean Sq F value    Pr(>F)
fact       2 202.667 101.333     152 1.159e-07 ***
Residuals  9   6.000   0.667
```

This gives the degrees of freedom $g - 1 = 2$ and $N - g = 9$, the sums of squares between (202.667), the sums of squares within (6.0), the f-value 152 and the overall p-value $1.159 \cdot 10^{-7}$. □

Example 2. By the previous analysis of variance it is concluded that there are differences in population means. It is, however, not clear which of the means differ. A way to clarify this is by estimating the mean of Group 1 (Level 1) and then computing the difference between Group 2 and Group 1, and the difference between Group 3 and Group 1. Such corresponds to the following contrast matrix

$$\boldsymbol{C} = \begin{bmatrix} 1 & 1 & 1 \\ 0 & -1 & 0 \\ 0 & 0 & -1 \end{bmatrix}.$$

This contrast matrix is by default implemented by the model specification `y~a`, as follows.

```
> summary(lm(y ~ a))
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   2.0000     0.4082   4.899 0.000849 ***
```

```
factLevel 2   6.0000     0.5774  10.392 2.60e-06 ***
factLevel 3  10.0000     0.5774  17.321 3.22e-08 ***

Residual standard error: 0.8165 on 9 degrees of freedom
Multiple R-Squared: 0.9712,     Adjusted R-squared: 0.9649
F-statistic:   152 on 2 and 9 DF,  p-value: 1.159e-07
```

The estimated intercept is the mean of Group 1 (Level 1). The `factLevel 2` is the difference in means between Group 2 (Level 2) and Group 1 and `factLevel 3` is the difference in means between Group 3 and Group 1. By t-tests the null-hypothesis is tested that the mean of Group 1 is zero, the difference in means between Group 2 and Group 1 is zero and the difference in means between Group 3 and Group 1 is zero. That is, the null-hypotheses are $H_0 : \mu_1 = 0$, $H_0 : \mu_2 - \mu_1 = 0$, and $H_0 : \mu_3 - \mu_2 = 0$. Since the p-values that correspond to the t-values are smaller than the significance level 0.05, all null-hypotheses are rejected. The last line of the output gives the f-value, the degrees of freedom, and the corresponding overall p-value. The latter equals that of ANOVA. □

Before we analyze real gene expression data it seems well to give an example where the means do not differ.

Example 3. Let's sample data from the normal distribution with mean 1.9 and standard deviation 0.5 corresponding to three groups of patients that do not possess any type of differences between groups.

```
> y <- rnorm(12,1.9,0.5)
> round(x,2)
 [1] 1.75 1.82 1.35 1.61 2.08 1.27 2.50 2.40 2.13 0.71 2.80 2.00
> a <- gl(3,4)
> anova(lm(y ~ a))$Pr[1]
[1] 0.6154917
```

Note that by the `$Pr[1]` operator extracts the p-value from the list generated by the `anova` function. The p-value implies the conclusion not to reject the null-hypotheseis of equal means, which is consistent with the data generation process. □

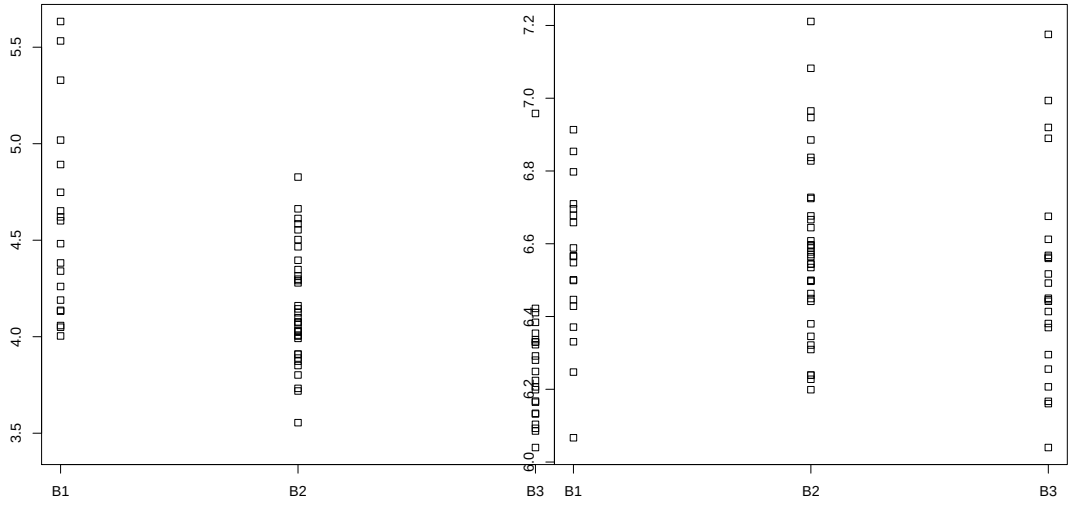


Figure 5.1: Plot of SKI-like oncogene expressions for three patient groups.

Figure 5.2: Plot of Ets2 expression values for three patient groups.

Example 4. B-cell ALL: `1866_g_at`. To illustrate analysis of variance by real data we shall use the `ALL` data from the `ALL` package, see Section 1.1. Specifically, expression levels from B-cell ALL patients in stage B1, B2, and B3 are selected with row name `1866_g_at`, which refers to an SKI-like oncogene related to oncoproteins. From the plot of the data in Figure 5.1 it can be observed that the expression levels differ between the disease stages. The null hypothesis is tested that the expression means in each stage are equal or in other words that there are no experimental effects. It is briefly indicated how the data are loaded.

```
> library(ALL);data(ALL)
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1866_g_at",]
> summary(lm(y ~ ALLB123$BT))

             Estimate Std. Error t value Pr(>|t|)
(Intercept)   4.58222    0.08506  53.873  < 2e-16 ***
```

```
ALLB123$BTB2 -0.43689    0.10513  -4.156 8.52e-05 ***
ALLB123$BTB3 -0.72193    0.11494  -6.281 2.00e-08 ***

Residual standard error: 0.3707 on 75 degrees of freedom
Multiple R-squared: 0.3461,     Adjusted R-squared: 0.3287
F-statistic: 19.85 on 2 and 75 DF,  p-value: 1.207e-07
```

From the overall p-value $1.207 \cdot 10^{-7}$ of the f-test the conclusion follows to reject the hypothesis of equal means. From the t-tests we conclude that the mean of B1 differs from zero and the differences between B2 and B1 as well as between B3 and B2 are unequal to zero. That is, the population means of Group B1, B2, and B3 do differ. □

Example 5. B-cell ALL: `1242_at`. To illustrate a case where the means do not differ we selected the expression values for probe `1242_at` of the B-cell ALL patients in stage B1, B2, and B3 from the `ALL` data. This probe corresponds to the Ets2 repressor factor which plays a role in telomerase regulation in human cancer cells. From the plot of the data in Figure 5.2, however, it can be observed that the expression values hardly differ between disease stages. The data are extracted from the `ALL` object and collected in the vector `y`. The corresponding factor is given by `ALLB123$BT`.

```
> library(ALL); data(ALL)
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1242_at",]
> summary(lm(y ~ ALLB123$BT))

             Estimate Std. Error t value Pr(>|t|)
(Intercept)   6.55083    0.05673 115.483   <2e-16 ***
ALLB123$BTB2  0.03331    0.07011   0.475    0.636
ALLB123$BTB3 -0.04675    0.07665  -0.610    0.544

Residual standard error: 0.2473 on 75 degrees of freedom
Multiple R-squared: 0.01925,    Adjusted R-squared: -0.006898
F-statistic: 0.7362 on 2 and 75 DF,  p-value: 0.4823
```

From the overall p-value 0.4823, the conclusion is not to reject the null hypothesis of equal means. More specifically, the null-hypotheses $H_0 : \mu_1 = 0$ is rejected, but from the p-value 0.636 the $H_0 : \mu_2 - \mu_1 = 0$ is not rejected,

and from p-value 0.544 the $H_0 : \mu_3 - \mu_2 = 0$ is not rejected either. □

Example 6. An interesting question is of course for how many genes of the ALL data the hypothesis of equal means is rejected by the overall ANOVA p-value? Such can be answered by collecting the p-values in a vector.

```
> pano <- apply(exprs(ALLB123),1,function(x) anova(lm(x~ALLB123$BT))$Pr[1])
> sum(pano<0.05)
[1] 2526
```

Thus the hypothesis of equal means is rejected for 2526 out of a total of 12625 genes (probes). □

5.3 Two-way analysis of variance

Having some experience with one way analysis of variance, the question may arise whether the model for means of groups can be extended from one factor to more factors. This is indeed possible. The model would then be equal to

$$Y_{ijk} = \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where α_i is the mean of Group i indicated by the first factor, β_j the mean of Group j indicated by the second factor, $(\alpha\beta)_{ij}$ the interaction effect and ε_{ijk} the error which is distributed according to $N(0, \sigma^2)$. If the means of the i groups differ, then there is a main effect of the first factor which is expressed in a p-value smaller than 0.05. Similarly, in case the means of the j groups differ, there is a main effect of the second factor, expressed in a p-value smaller than 0.05. Two-way analysis of variance will briefly be illustrated.

Example 5. A two-way approach. It case of the ALL data from Chiaretty et al. (2004) we may aggregate the B cell patients into two groups: B, B1 and B2 in the first and B3 and B4 in the second. For the second group we select from the molecular biology of the patients assigned to BCR/ABL and NEG. We shall perform the analysis on the expression values of NEDD4 binding protein 1 with probe id 32069_at. This can be computed as follows.

```
library("ALL"); data(ALL)
ALLBm <- ALL[,which(ALL$BT %in% c("B","B1","B2","B3","B4") & ALL$mol.biol %in% c("BCR
```

```
facmolb <- factor(ALLBm$mol.biol)
facB <- factor(ceiling(as.integer(ALLBm$BT)/3),levels=1:2,labels=c("B012","B34
> anova(lm(exprs(ALLBm)["32069_at",] ~ facB * facmolb))
Analysis of Variance Table

Response: exprs(ALLBm)["32069_at", ]
             Df  Sum Sq Mean Sq F value    Pr(>F)
facB          1  1.1659  1.1659  4.5999 0.0352127 *
facmolb       1  3.2162  3.2162 12.6891 0.0006433 ***
facB:facmolb  1  1.1809  1.1809  4.6592 0.0340869 *
Residuals    75 19.0094  0.2535
```

First the patients are selected with B-cell ALL and assigned molecular biology of the cancer to BCR/ABL or NEG. Next the factors are constructed to group the patients. From the p-values in the analysis of variance table it can be concluded that there two main effects as well as an interaction effect.

One may also ask for a summary of the individual effects.

```
> summary(lm(exprs(ALLBm)["32069_at",] ~ facB * facmolb))

Call:
lm(formula = exprs(ALLBm)["32069_at", ] ~ facB * facmolb)

Coefficients:
                   Estimate Std. Error t value Pr(>|t|)
(Intercept)          6.7649     0.1073  63.026  < 2e-16 ***
facBB34             -0.5231     0.1686  -3.103   0.0027 **
facmolbNEG          -0.6020     0.1458  -4.128  9.4e-05 ***
facBB34:facmolbNEG   0.5016     0.2324   2.159   0.0341 *

Residual standard error: 0.5034 on 75 degrees of freedom
Multiple R-squared: 0.2264,     Adjusted R-squared: 0.1954
F-statistic: 7.316 on 3 and 75 DF,  p-value: 0.0002285
```

In bioinformatics the question often arises how many probes there are with have significant effects. In this case we may compute the number of probes with significant main as well as interaction effects.

```
> pval <- apply(exprs(ALLBm), 1, function(x) anova(lm(x ~ facB * facmolb))$Pr[
```

```
> pvalt <- data.frame(t(pval))
> colnames(pvalt) <- c("maineffectB","maineffectmolbiol","interaction")
> sum(pvalt$maineffectB < 0.05 & pvalt$maineffectmolbiol < 0.05 & pvalt$interaction <
[1] 47
```

The three p-values per probe are collected in a matrix. This matrix is transposed so that the columns correspond to the p-values and the rows to the probes. Using the logical AND (`&`) operator and summing the TRUE values yield 47 probes with significant main and interaction effects. □

5.4 Checking assumptions

When the linear model is applied for analysis of variance there are in fact two assumptions made. First, the errors are assumed to be independent and normally distributed, and, second, the error variances are assumed to be equal for each level (patient group). The latter is generally known as the homoscedasticity assumption. The normality assumption can be tested as a null hypothesis by applying the Shapiro-Wilk test on the residuals. The homoscedasticity assumption can be tested as a hypothesis by the Breusch and Pagan (1979) test on the residuals. This latter test may very well be seen as a generalization of the F-test for equal variances.

Example 1. Testing normality of the residuals. From Figure 5.1 it can be observed that there are outliers being far apart from the bulk of the other expression values. This raises the question whether the normality assumption holds. The normality of the residuals from the estimated linear model on the B-cell ALL data from `1866_g_at`, can be tested as follows.

```
> data(ALL,package="ALL");library(ALL)
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1866_g_at",]
> shapiro.test(residuals(lm(y ~ ALLB123$BT)))

        Shapiro-Wilk normality test

data:  residuals(lm(y ~ ALLB123$BT))
W = 0.9346, p-value = 0.0005989
```

From the p-value 0.0005989, the conclusion is to reject the null-hypothesis of normally distributed residuals. □

Example 2. Testing homoscedasticity of the residuals. From Figure 5.1 it can be observed that the spread of the expression values around their mean differs between groups of patients. In order to test the homoscedasticity assumption we use the function `bptest` from the `lmtest` package.

```
> library(ALL); data(ALL); library(lmtest)
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1866_g_at",]
> bptest(lm(y ~ ALLB123$BT),studentize = FALSE)

        Breusch-Pagan test

data:  lm(y ~ ALLB123$BT)
BP = 8.7311, df = 2, p-value = 0.01271
```

From the p-value 0.01271, the conclusion follows to reject the null hypothesis of equal variances (homoscedasticity). □

5.5 Robust tests

In case departures from normality or homoscedasticity are large enough to cause concern with respect to the actual significance level or to the power of the test, an alternative testing procedure is called for. In case only homoscedasticity is violated, we are in a situation quite similar to that of t-testing with unequal variances. That is, the null hypothesis $H_0 : \mu_1 = \mu_2 = \mu_3$ of equal means can be tested without assuming equal variances by a test proposed by Welch (1951).

Example 1. In Example 2 of the previous section the hypothesis of equal variances was rejected. To apply analysis of variance without assuming equal variances (homoscedasticity) one may use the function `oneway.test`, as follows.

```
> data(ALL,package="ALL");library(ALL)
```

```
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1866_g_at",]
> oneway.test(y ~ ALLB123$BT)

        One-way analysis of means (not assuming equal variances)

data:  y and ALLB123$BT
F = 14.1573, num df = 2.000, denom df = 36.998, p-value = 2.717e-05
```

From the p-value $2.717 \cdot 10^{-5}$, the conclusion follows to reject the hypothesis of equal means. □

In case normality is violated a rank type of test is more appropriate. In particular, to test the null-hypothesis of equal distributions of groups of gene expression values, the Kruskal-Wallis rank sum test is recommended. This test can very well be seen as a generalization of the Wilcoxon test for testing the equality of two distributions. Because it is based on ranking the data, it is highly robust against non-normality, it, however, does not estimate the size of experimental effects.

Example 2. In Example 1 of the previous section we rejected the hypothesis of normally distributed residuals. We use the function `kruskal.test` to perform a non-parametric test.

```
> data(ALL,package="ALL");library(ALL)
> ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
> y <- exprs(ALLB123)["1866_g_at",]
> kruskal.test(y ~ ALLB123$BT)

        Kruskal-Wallis rank sum test

data:  y by ALLB123$BT
Kruskal-Wallis chi-squared = 30.6666, df = 2, p-value = 2.192e-07
```

From the p-value $2.192 \cdot 10^{-7}$, the null-hypothesis of equal distributions of expression values between patient groups is rejected. □

By the `apply` functionality the p-values can easily be computed for all 12625 gene expression values of the `ALL` data.

5.6 Overview and concluding remarks

By applying the above normality and homogeneity tests to complete sets of gene expression values it can quickly be seen to what extent the assumptions for the classical analysis of variance test are violated. Based on these it can be decided to add rank type of testing in order to reduce the amount of false positives and false negatives. Here, false positives are significant p-values for equal populations means and false negatives are non-significant p-values for unequal populations means.

In the next chapter it will briefly be indicated how to combine two factors into a single analysis of variance. For instance, one may want to combine B-cell stage with age groups of persons. The interested reader is referred to Faraway (2004) and Venables & Ripley (2002) for more information on using linear models in R and for a general treatment of linear models to Rao & Toutenburg (1995).

The p-values from overall tests of equality of means or distributions are important tools to order genes according to their experimental effect with respect to different patient groups. More examples are given in the next chapter where several functionalities of Bioconductor will be used for the analysis of microarray data.

5.7 Exercises

1. Analysis of gene expressions of B-cell ALL patients.
   (a) Construct a data frame containing the expression values for the B-cell ALL patients in stage B, B1, B2, B3, B4 from the `ALL` data.
   (b) How many patients are in each group.
   (c) Test the normality of the residuals from the linear model used for analysis of variance for all gene expression values. Collect the p-values in a vector.
   (d) Do the same for the homoscedasticity assumption.
   (e) How many gene expressions are normally distributed and how many homoscedastic? For how many do both hold?
2. Further analysis of gene expressions of B-cell ALL patients. Continue with the previous data frame containing the expression values for the

B-cell ALL patients in stage B, B1, B2, B3, B4 from the `ALL` data.

(a) Collect the overall p-values from ANOVA in a vector.

(b) Use `featureNames()` to report the affymetrix id's of the genes with smaller p-values than 0.000001.

(c) Collect the overall p-values from the Kruskal-Walles test in a vector.

(d) Use `featureNames()` to report the affymetrix id's of the genes with smaller p-values than 0.000001.

(e) Briefly comment on the differences you observe. That is, how many genes have p-values smaller than 0.001 from both ANOVA and Krusal-Wallis? How many only from one type of test? Hint: Collect TRUE/FALSES in logical vectors and use `table`.

3. Finding the ten best best genes among gene expressions of B-cell ALL patients. Continue with the previous data frame containing the expression values for the B-cell ALL patients in stage B, B1, B2, B3, B4 from the `ALL` data.

(a) Print the p-values and the corresponding (affimetrix) gene identifiers of the ten best from ANOVA.

(b) Do the same for the p-values from the Kruskal-Wallis test.

(c) Use the function `intersect` to find identifiers in both sets.

4. A simulation study on gene expression values.

(a) Construct a data matrix with 10000 rows and 9 columns with data from the normal distribution with mean zero and variance equal to one. Such a matrix simulates gene expressions without differences between groups (sometimes called negatives).

(b) Construct a factor for three groups each with three values.

(c) How many p-values are smaller than the significance level $\alpha = 0.05$?

(d) If the p-value is smaller than the significance level, then the conclusion is that there an experimental effect (a positive). How many false positives do you expect and how many did you observe?

(e) Construct a matrix with 10000 rows and 9 columns with normally distributed data with mean zero, one and two and variance equal to one. Assume again that there three groups each with three data values. This data matrix simulates gene expressions with differences between groups (sometimes called positives). Use ANOVA and kruskal-Wallis to find the number of significant genes (true positives). report the number of true positives and false negatives.

Chapter 6

Micro Array Analysis

The analysis of gene expression values is of key importance in bioinformatics. The technique makes it possible to give an initial answer to many important genetic type of questions. In this chapter you learn how to preprocess probe data, filter genes, to program various visualizations, to use gene ontology identifiers, to load public available gene expression data, as well as how to summarize results in html output. [1]

6.1 Probe data

The microarray technique takes advantage of hybridization properties of nucleic acids. That is, to give a rough idea, complementary molecules are attached and labeled on a solid surface in order for a specialized scanner measure the intensity of target molecules. Per gene there are about twenty such measures obtained for each probe (gene). Per probe these measures come in pairs. The intensity of the perfect match (PM) intends to measure the amount of transcripts from the gene. The intensity of the mismatch (MM) is related to non-specific binding and is often seen as a background type of noise.

The raw data from the Affymetrix scanner is stored in so-called DAT files, which are processed to so-called CEL files, where we will work with. The package `affy` has facilities to read data from a vector specifying several CEL files produced by the Affymetrix scanner.

[1] It may be convenient to explore the possibilities of the `limmaGUI`. Our approach, however, will be to concentrate on the programming aspects using the commandline.

Example 1. We will start with a built-in data set called `MLL.B` from the `ALLMLL` package. To load it and to retrieve basic information use

```
> library(affy)
> data(MLL.B, package = "ALLMLL")
> MLL.B
```

It is very useful to print the structure of the object `str(MLL.B)` and its slot names.

```
> slotNames(MLL.B)
[1] "cdfName"           "nrow"              "ncol"
[4] "assayData"         "phenoData"         "featureData"
[7] "experimentData"    "annotation"        ".__classVersion__"
```

Additional information become available from `str(MLL.B)`. The raw probe intensities are available from `exprs(MLL.B)`, which extracts the probe intensities from the `MLL.B` object. The number of rows and columns of the expression values of MLL.B can be obtained by the `dim` function.

```
> dim(exprs(MLL.B))
[1] 506944     20
```

The annotation can be extracted as follows.

```
> annotation(MLL.B)
[1] "hgu133b"
```

To print the first 10 names of the probes use

```
> probeNames(MLL.B)[1:10]
 [1] "200000_s_at" "200000_s_at" "200000_s_at" "200000_s_at" "200000_s_at"
 [6] "200000_s_at" "200000_s_at" "200000_s_at" "200000_s_at" "200000_s_at"
```

Note that the probe names are the same as those obtained by `geneNames`. The PM and MM values are collected by the functions `pm` and `mm`. To print the PM values of the first four out of the sixteen rows of the probe with identifier `200000_s_at` we may use the following.

```
> pm(MLL.B,"200000_s_at")[1:4,1:3]
           JD-ALD009-v5-U133B.CEL JD-ALD051-v5-U133B.CEL JD-ALD052-v5-U133B.CEL
200000_s_at1                661.5                  321.5                  312.5
200000_s_at2                838.8                  409.3                  395.3
200000_s_at3                865.3                  275.5                  341.3
200000_s_at4                425.8                  253.5                  196.8
```

By function `matplot` a quick view on the variability of the data within and between probes can be obtained.

```
> matplot(pm(MLL.B,"200000_s_at"),type="l", xlab="Probe No.",
 + ylab="PM Probe intensity")
```

From the resulting plot in Figure 6.1 it can be observed that the variability is substantial.

Density plots of the log of the probe values can be obtained by `hist(MLL.B)`. From the density plot of the log of the intensity data in Figure 6.2 it can be seen that these are quite skew to the right. The script to program such plots

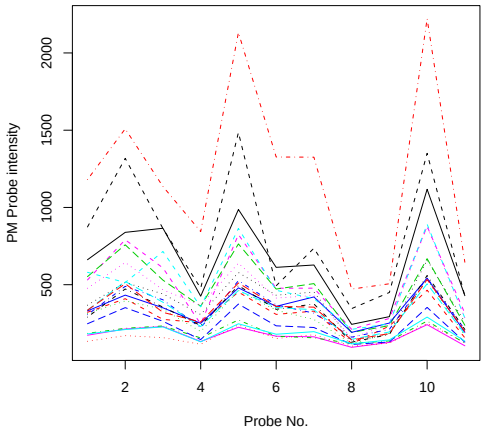


Figure 6.1: Mat plot of intensity values for a probe of `MLL.B`.

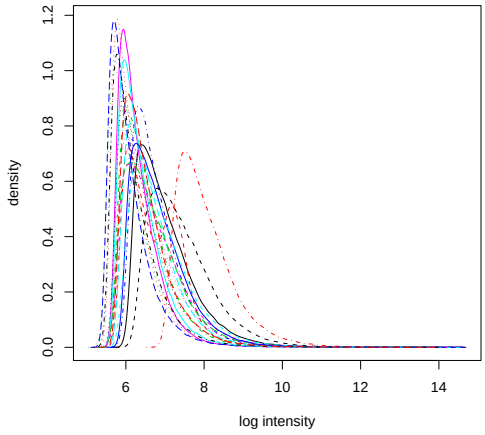


Figure 6.2: Density of `MLL.B` data.

is quite brief.

```
> MAplot(MLL.B,pairs=TRUE, plot.method= "smoothScatter")
> image(MLL.B)
```

6.2 Preprocessing methods

From various visualization methods it is clear that preprocessing of probe intensities is necessary for making biologically relevant conclusions. Bioconductor gives facilities for various preprocessing methods. Here we will only sketch what the main methods are and how these can be implemented. It should be noted that the topic of optimal preprocessing currently is a field of intense research (probably for the coming years), so that definitive recommendations are not mandatory. Preprocessing consists of three major steps: Background correction, normalization, and summarization. To obtain the available background and pm correction methods use the following.

```
> bgcorrect.methods
[1] "mas"  "none" "rma"  "rma2"
```

The mas background is part of the MAS Affymetrix software and is based on the 2% lowest probe values. RMA uses only the PM values, neglects the MM values totally, and is based on conditional expectation and the normality assumption of probes values. There are also a number of correction methods available for the PM values:

```
> pmcorrect.methods
[1] "mas"        "pmonly"     "subtractmm"
```

The following normalization methods are available:

```
> normalize.methods(MLL.B)
[1] "constant"         "contrasts"        "invariantset"     "loess"
[5] "qspline"          "quantiles"        "quantiles.robust"
```

Constant is a scaling method equivalent to linear regression on a reference array although without intercept term. More general are the non-linear normalization methods such as loess, qspline, quantiles, and robust quantiles. Loess is a nonlinear method based on local regression of MA plots. The methods of contrasts is based on loess regression. Quantile normalization is an inverse transformation of the empirical distribution with respect to an averaged sample quantile in order to impose one and the same distribution to each array. The method `qspline` uses quantiles from each array and a target array to fit a system of cubic splines. The target should be the mean (geometric) or median of each probe, but could also be the name of a particular group.

The final step of preprocessing is to aggregate multiple probe intensities into a gene expression value. The available methods are:

```
> express.summary.stat.methods
[1] "avgdiff" "liwong"  "mas" "medianpolish" "playerout"
```

The first is the simplest as it is based on averaging.

There is no single best method for all preprocessing problems. It seems, however, wise to use methods robust against outliers together with non-linear normalization methods.

Example 1. The three pre-processing steps can be employed one after the other by the function `expresso`. To combine the background correction RMA with constant normalization and to use average differences for the computation of gene expression values, we may use the following.

```
eset <- expresso(MLL.B,bgcorrect.method="rma",
  normalize.method="constant",pmcorrect.method="pmonly",
  summary.method="avgdiff")
```

□

Example 2. Another frequently applied preprocessing method is RMA. It combines convolution background correction, quantile normalization, and summarization based on multi-array model fit in a robust manner by a so-called median polish algorithm.

```
> library(affy)
> data(MLL.B, package = "ALLMLL")
> eset3 <- rma(MLL.B)
Background correcting
Normalizing
Calculating Expression
> boxplot(data.frame(exprs(eset3)))
```

The three stages of preprocessing by `rma` are part of the output. Before a box-and-whiskers plot can be constructed the expression values need to be extracted from the object `eset3`. □

After the foregoing it is often desirable to further preprocess the data in order to remove patient specific means or medians. When the patient median is zero, for instance, testing for a gene to have mean expression value

different from zero becomes meaningful.

Example 3. In the sequel we shall frequently work with the `ALL` data from the `ALL` package of Bioconductor. Here the data set is briefly introduced (see also Section 1.1) and further processing steps are illustrated. The raw data have been jointly normalized by RMA and are available in the form of an exprSet object. 12625 gene expression values are available from microarrays of 128 different persons suffering from acute lymphoblastic leukemia (ALL). A number of interesting phenotypical co-variates are available. For instance, the `ALL$mol` variable has TRUE/FALSE values for each of the 128 patients depending on whether a reciprocal translocation occurred between the long arms of Chromosome 9 and 22. This is casually related to chronic and acute leukemia. One can also ask for `table(ALL$BT)` to obtain an overview of the numbers of patients which are in certain phases of a disease. See also the general help `?ALL` for further information on the data or the article by Chiaretti et al. (2004).

```
> data(ALL, package = "ALL")
> slotNames(ALL)
[1] "assayData"         "phenoData"         "featureData"
[4] "experimentData"    "annotation"        ".__classVersion__"
> row.names(exprs(ALL))[1:10]
 [1] "1000_at"   "1001_at"   "1002_f_at" "1003_s_at" "1004_at"   "1005_at"
 [7] "1006_at"   "1007_s_at" "1008_f_at" "1009_at"
```

By `feno <- pData(ALL)` phenotypical information from the patients is stored in a data frame, which is useful for further analysis. In case the gene expression values over the patients are non-normally distributed one may want to subtract the median and divide by the MAD. An efficient manner to do so is to use an `apply` function to compute the column mad and median, and `sweep` to subtract the median from each column entry and, next, to divide each column entry by the MAD.

```
ALL1pp <- ALL1 <- ALL[,ALL$mol == "ALL1/AF4"]
mads <- apply(exprs(ALL1), 2, mad)
meds <- apply(exprs(ALL1), 2, median)
dat <- sweep(exprs(ALL1), 2, meds)
exprs(ALL1pp) <- sweep(dat, 2, mads, FUN="/")
```

By this script the patients are selected with assigned molecular biology equal to ALL1/AF4. Then `ALL1` is copied in order to overwrite the expression values in a later stage. The median and the MAD are computed per column by the specification 2 (column index) in the `apply` function. Then the first `sweep` function subtracts the medians from the expression values and second divides these by the corresponding MAD. By comparing the box plots in Figure 6.3 and 6.4 the effect of preprocessing can be observed. The medians of the preprocessed data are equal to zero and the variation is smaller due to the division by their MAD. Note that by box plotting a data frame a fast overview of the distributions of columns in a data frame is obtained. □

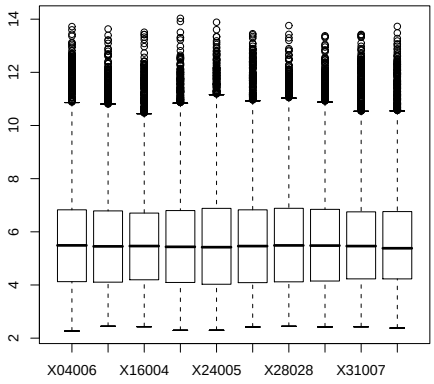


Figure 6.3: Boxplot of the ALL1/AF4 patients.

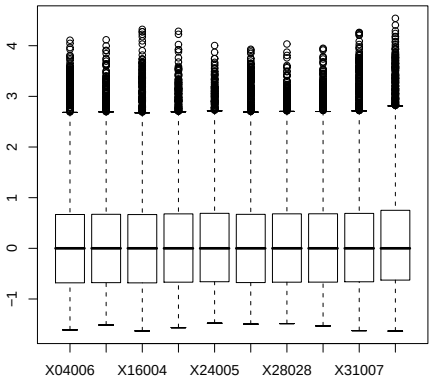


Figure 6.4: Boxplot of the ALL1/AF4 patients after median subtraction and MAD division.

6.3 Gene filtering

A few important methods to filter genes are illustrated here. It is wise to keep in mind that there are statistical as well as and biological criteria for filtering genes and that a combination of these often gives the most satisfactory results. The examples stress the importance of careful thinking.

Example 1. Filtering by the coefficient of variation. A manner to filter genes is by the coefficient of variation, which is defined as the standard deviation divided by the absolute value of the mean: $cv = \sigma/|\mu|$. If $cv = 1$, then the standard deviation equals the mean, so that the experimental effect is small relative to the precision of measurement. If, however, $cv < 0.2$, then the mean is five times larger than the standard deviation, so that both the experimental effect and the measurement precision are large. Let's compute the coefficient of variation per gene for the `ALL1pp` data of the previous section.

```
> cvval <- apply(exprs(ALL1pp),1,function(x){sd(x)/abs(mean(x))})
```

Now using `sum(cvval<0.2)` yields 4751 genes with a coefficient of variation smaller than 0.2. These genes can be selected by `ALL1pp[cvval<0.2,]`. □

Example 2. Combining several filters. It is often desired to combine several filters. Of course it is possible to program filters completely on your own, however, we may conveniently use the function `filterfun` to combine several filters. The script in this example is useful when several functions are to be applied to a single data set.

```
library("genefilter")
f1 <- function(x)(IQR(x)>0.5)
f2 <- pOverA(.25, log2(100))
f3 <- function(x) (median(2^x) > 300)
f4 <- function(x) (shapiro.test(x)$p.value > 0.05)
f5 <- function(x) (sd(x)/abs(mean(x))<0.1)
f6 <- function(x) (sqrt(10)* abs(mean(x))/sd(x) >  qt(0.975,9))
ff <- filterfun(f1,f2,f3,f4,f5,f6)
library("ALL"); data(ALL)
selected <- genefilter(exprs(All[,ALL$BT=="B"]), ff)
```

After running this script and using `sum(selected)` one obtains 317 genes that pass the combined filter. The first function returns TRUE if the interquartile range is larger than 0.5, the second if 25% of the gene expression values is larger than 6.643856, the third if the median of the expression values taken as powers to the base two is larger than 300, the fourth if it passes the Shapiro-Wilk normality test, the fifth if the coefficient of variation is smaller than 0.1, and the sixth if the one-sample t-value is significant. The filter

functions are combined by `filterfun` and the function `genefilter` returns a logical vector indicating whether the gene passed all the filters or failed at least one of them. In order to use these filter steps properly it is well to think them through because several filters focus on similar properties. In particular, since the IQR divided by 1.349 is a robust estimator of the standard deviation, the first filter selects genes with a certain minimal standard deviation. With respect to the third filter note that $2^x > 300$ is equivalent to $x > {}^2\log(300) \approx 8.228819$, which is highly similar to the second filter. Furthermore, $s/|\overline{x}| < 0.1$ is equivalent to $\sqrt{10}|\overline{x}|/s > 1/\sqrt{10}$, so that the last two filters are highly similar. □

Example 3. Filtering by t-test and normality. One may also want to select genes with respect to p-values of a two-sample t-test over B-cell ALL versus T-cell ALL. This can be combined with a normality test in the sense that only those genes are filtered which pass the Shapiro-Wilk normality test. The latter will be applied separately for the B-cell ALL patients and for the T-cell ALL patients. For this we write a function that will be used twice. First, however, we create a logical factor `patientB` indicating patients with B-cell ALL (TRUE) and with T-cell ALL (FALSE). The filter defined selects genes that have their p-value from the Welch two-sample t-test smaller than the significance level 0.05. A logical variable named `selected` is defined which attains TRUE only if `sel1`, `sel2`, as well as `sel3` have the value TRUE.

```
library("genefilter");library("ALL"); data(ALL)
patientB <- factor(ALL$BT %in% c("B","B1","B2","B3","B4"))
f1 <- function(x) (shapiro.test(x)$p.value > 0.05)
f2 <- function(x) (t.test(x ~ patientB)$p.value < 0.05)
sel1 <- genefilter(exprs(ALL[,patientB==TRUE]), filterfun(f1))
sel2 <- genefilter(exprs(ALL[,patientB==FALSE]), filterfun(f1))
sel3 <- genefilter(exprs(ALL), filterfun(f2))
selected <- sel1 & sel2 & sel3
ALLs <- ALL[selected,]
```

This gives 1817 genes which pass the three filters. For these genes it holds that the expression values for B-cell ALL patients as well as for T-cell ALL patients are normally distributed (in the sense of non-rejection). A fundamental manner to visualize how the genes are divided among filters is

by construction of a Venn diagram. This can conveniently be done by using functions from the `limma` package (Smyth, 2005).

```
library(limma)
x <- matrix(as.integer(c(sel1,sel2,sel3)),ncol = 3,byrow=FALSE)
colnames(x) <- c("sel1","sel2","sel3")
vc <- vennCounts(x, include="both")
vennDiagram(vc)
```

From the resulting Venn diagram in Figure 6.5 it can be seen that 1817 genes pass all three filters, 1780 genes pass none, 3406 genes pass the normality tests but not the t-test filter, etc. □

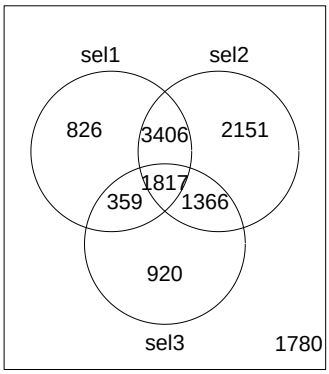


Figure 6.5: Venn diagram of seleced ALL genes.

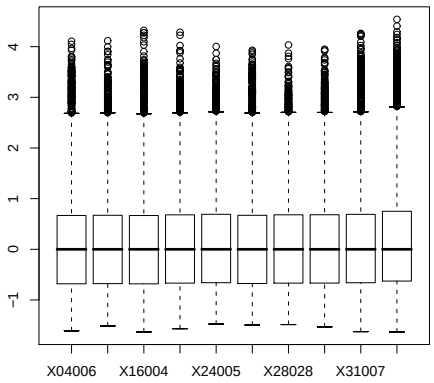


Figure 6.6: Boxplot of the ALL1/AF4 patients after median subtraction and MAD division.

6.4 Applications of linear models

The `limma` package is frequently used for analyzing microarray data by linear models, such as ANOVA.

Example 1. Analysis of variance. We select patients with B-cell leukemia in a beginning stage B and in more progressive stages B1 and B2. The type of analysis is specified by using a factor that defines the model (design) matrix. Then the linear model is fitted to the data and an empirical Bayes procedure is used to adapt the gene specific variances with a global variance estimator (Smyth, 2004)[2].

```
library("ALL"); library("limma");
data(ALL, package = "ALL")
allB <- ALL[,which(ALL$BT %in% c("B","B1","B2"))]
design.ma <- model.matrix(~ 0 + factor(allB$BT))
colnames(design.ma) <- c("B","B1","B2")
fit <- lmFit(allB, design.ma)
fit <- eBayes(fit)
> toptab <- topTable(fit, coef=2,5,adjust.method="fdr")
> print(toptab[,1:5],digits=4)
                    ID logFC AveExpr     t   P.Value
12586 AFFX-hum_alu_at 13.42   13.50 326.0 3.165e-99
2488         32466_at 12.68   12.70 306.3 1.333e-97
2773         32748_at 12.08   12.11 296.3 9.771e-97
5328         35278_at 12.44   12.45 295.5 1.146e-96
4636       34593_g_at 12.64   12.58 278.0 4.431e-95
```

By `topTable` the five genes are selected with the smallest p-values adjusted for the false discovery rate. Let's call the mean of the B patients μ, that of B1 μ_1, and that of B2 μ_2. In the current case we are not so much interested in the hypothesis $H_0 : \mu - \mu_2$, because this is the difference between Stage 0 and Stage 3. Rather, we are interested in the hypothesis $H_0 : \mu - \mu_1$ and $H_0 : \mu_1 - \mu_2$. Such a specific hypothesis can be tested by using a contrast matrix, which can be specified as follows.

```
> cont.ma <- makeContrasts(B-B1,B1-B2, levels=factor(allB$BT))
> cont.ma
      Contrasts
Levels B - B1 B1 - B2
    B       1       0
    B1     -1       1
    B2      0      -1
```

[2]To obtain the appropriate number of levels we make a factor of ALLB$BT.

Observe that the contrast matrix specifies the difference between the levels B and B1 as well as between B1 and B2. It can be implemented as follows.

```
fit1 <- contrasts.fit(fit, cont.ma)
fit1 <- eBayes(fit1)
toptabcon <- topTable(fit, coef=2,5,adjust.method="fdr")
print(toptabcon[,1:5],digits=4)
> toptabcon <- topTable(fit1, coef=2,5,adjust.method="fdr")
> print(toptabcon[,1:5],digits=4)
           ID   logFC AveExpr      t   P.Value
3389 33358_at  1.4890   5.260  7.374 5.737e-10
419   1389_at -1.7852   9.262 -7.081 1.816e-09
1016  1914_at  2.0976   4.939  7.019 2.315e-09
6939 36873_at  1.8646   4.303  6.426 2.361e-08
7542 37471_at  0.8701   6.551  6.106 8.161e-08
```

Here, we have applied a method called "false discovery rate" (fdr) which increases the p-values somewhat in order to reduce the number false positives. The number of genes requested equals 5. □

A very convenient manner to summarize, collect, and communicate various types of results is in the form of an HTML file.

Example 2. Summarizing output in HTML format. It is often desired to combine the typical output from a function like `topTable` with that of an HTML output page containing various types of information. To illustrate this we proceed with the object `toptabcon` of the previous example.

```
library("annaffy");library("hgu95av2.db")
anntable <- aafTableAnn(as.character(toptabcon$ID), "hgu95av2.db",  aaf.handle
saveHTML(anntable, "ALLB123.html", title = "B-cell 012 ALL")
```

By the function `aafTableAnn` various types of information are gathered from the output `topTable` of the estimated linear model, the annotation package, and the `aaf.handler` functionality. The information collected contains the following: Probe, Symbol, Description, Function, Chromosome, Chromosome Location, GenBank, LocusLink, Cytoband, UniGene, PubMed, Gene Ontology, and Pathway. The resulting `anntable` is saved in HTML format in the working directory or the Desktop. It contains a wealth of information

on e.g. Chromosome location, KEGG mappings, summaries from Pubmed articles, etc. □

Example 3. Using basic R functions. It is also possible to summarize results in an HTML table on the basis of p-values from e.g. analysis of variance (ANOVA). That is, the selected genes can directly be used as input for `aafTableAnn`.

```
library("multtest"); library("annaffy"); library("hgu95av2.db")
library("ALL"); data(ALL, package = "ALL")
ALLB <- ALL[,which(ALL$BT %in% c("B","B1","B2"))]
panova <- apply(exprs(ALLB), 1, function(x) anova(lm(x ~ ALLB$BT))$Pr[1])
genenames <- featureNames(ALLB)[panova<0.000001]
atab <- aafTableAnn(genenames, "hgu95av2.db", aaf.handler()[c(1:3,8:9,11:13)])
saveHTML(atab, file="ANOVAonB-cellGroups.html")
```

`hgu95av2.db` is a meta data annotation package connecting the requested information by the call to `aaf.handler`. The meaning of the columns can be obtained from the help page of the function. The resulting `table` is saved as an HTML file in the working directory (`getwd()`) or desktop. In a similar manner the p-values from the Kruskal-Wallis test can be used to select genes. □

Bioconductor has a useful facility to download publicly available microarray data sets from NCBI.

Example 4. Analyzing public available data. The GDS1365 data contain primed macrophage response to IFN-gamma restimulation after different time periods. The purpose is to gain insight into the influence of IFN-gamma priming on IFN-gamma induced transcriptional responses. Among the phenotypical covariates of the data there is a factor time with levels 0, 3 and 24 hours and a factor protocol with the levels "IFN-gamma primed" and "unprimed", which can be extracted by the function `pData`. Since researchers are often interested in the interaction between factors, we shall select genes with a significant interaction effect.

```
library(GEOquery); library(limma); library(hgu95av2.db);library(annaffy)
gds <- getGEO("GDS1365")
eset <- GDS2eSet(gds,do.log2=T)
```

```
prot <- pData(eset)$protocol
time <- pData(eset)$time
pval <- apply(exprs(eset)[1:12625,], 1,
   function(x) anova(lm(x ~ prot * time))$Pr[1:3])
pvalt <- data.frame(t(pval))
colnames(pvalt) <- c("meffprot","mefftime","interaction")
genenames <- featureNames(eset)[pvalt$meffprot< 0.01 &
   pvalt$mefftime < 0.01 & pvalt$interaction < 0.01]
atab <- aafTableAnn(genenames,"hgu95av2.db",aaf.handler()[c(1:3,8:9,11:13)])
saveHTML(atab, file="Two-way ANOVA protocol by time.html")
```

By `getGEO` the data are downloaded to the disk and next these can be loaded into the R system. By `GDS2eSet` these are transformed to an expression set so that these can be analyzed statistically. The function `pData` extracts the factors from the expression set `eset`. The function `anova` extracts the p-value of the interaction effect from the estimated linear model. We restrict the analysis to the first 12625 rows because the additional ones contain not available values. The resulting html file seems to contain may interesting genes. □

6.5 Searching an annotation package

Detailed information on microarray experiments is stored in an annotation package.

```
> library("ALL"); data(ALL)
> annotation(ALL)
[1] "hgu95av2"
```

Hence, the annotation package we need is `hgu95av2.db`. Let's load it and obtain an overview of its functionality.

```
> library(hgu95av2.db)
> ls("package:hgu95av2.db")
 [1] "hgu95av2"              "hgu95av2_dbconn"       "hgu95av2_dbfile"
 [4] "hgu95av2_dbInfo"       "hgu95av2_dbschema"     "hgu95av2ACCNUM"
 [7] "hgu95av2ALIAS2PROBE"   "hgu95av2CHR"           "hgu95av2CHRLENGTHS"
[10] "hgu95av2CHRLOC"        "hgu95av2CHRLOCEND"     "hgu95av2ENSEMBL"
```

```
[13] "hgu95av2ENSEMBL2PROBE" "hgu95av2ENTREZID"      "hgu95av2ENZYME"
[16] "hgu95av2ENZYME2PROBE"  "hgu95av2GENENAME"      "hgu95av2GO"
[19] "hgu95av2GO2ALLPROBES"  "hgu95av2GO2PROBE"      "hgu95av2MAP"
[22] "hgu95av2MAPCOUNTS"     "hgu95av2OMIM"          "hgu95av2ORGANISM"
[25] "hgu95av2PATH"          "hgu95av2PATH2PROBE"    "hgu95av2PFAM"
[28] "hgu95av2PMID"          "hgu95av2PMID2PROBE"    "hgu95av2PROSITE"
[31] "hgu95av2REFSEQ"        "hgu95av2SYMBOL"        "hgu95av2UNIGENE"
[34] "hgu95av2UNIPROT"
```

The annotation package contains environments with different types of information. An easy manner to make the content of an environment available is by converting it into a list and to print part of it to the screen.

```
> ChrNrOfProbe <- as.list(hgu95av2CHR)
> ChrNrOfProbe[1]
$`1000_at`
[1] "16"
```

We recognize the manufacturers identifier of genes and the corresponding chromosome. Asking information by `?hgu95av2CHR` reveals that it is an environment (hash table) which provides mappings between identifiers and chromosomes. From these we obtain various types of information on the basis of the manufacturer's identifier such as `"1389_at"`. Below we obtain, respectively, the GenBank accession number, the Entrez Gene identifier, the gene abbreviation, gene name, brief summaries of functions of the gene products, and the UniGene identifier. For this we use the `get` function in order to search an environment for a name.

```
> get("1389_at", env = hgu95av2ACCNUM)
[1] "J03779"
> get("1389_at", env = hgu95av2ENTREZID)
[1] 4311
> get("1389_at", env = hgu95av2SYMBOL)
[1] "MME"
> get("1389_at", env = hgu95av2GENENAME)
[1] "membrane metallo-endopeptidase (neutral endopeptidase,
   enkephalinase, CALLA, CD10)"
> get("1389_at", env = hgu95av2SUMFUNC)
[1] NA
```

```
> get("1389_at", env = hgu95av2UNIGENE)
[1] "Hs.307734"
```

Let's use the GenBank accession number to search its nucleotide data base.

```
> library(annotate)
> genbank("J03779",disp="browser")
```

From this we obtain the corresponding GI:179833 number, which can be used to obtain a complete XML document.

```
> genbank(1430782,disp="data",type="uid")
```

Obviously, probes correspond to genes and frequently we are interested in their chromosome location, and, specifically, in starting position(s).

```
> get("1389_at", env = hgu95av2CHRLOC)
        3         3         3
156280152 156280327 156280748
```

Its cytoband location can also be obtained.

```
> get("1389_at", env = hgu95av2MAP)
[1] "3q25.1-q25.2"
```

Hence, we see that the gene is on Chromosome 3 at q arm band 25 subband 1 and 2. In case we have a LocusLink ID, e.g. 4121, available the corresponding GO terms can be obtained and stored in a list.

```
ll1<-GOENTREZID2GO[["4121"]]
```

6.6 Using annotation to search literature

Given the manufactures probe identifier it is possible to search literature by collecting Pubmed ID's and to use these to collect relevant articles.

```
> library(hgu95av2.db);library(annotate); library(ALL); data(ALL)
> pmid <- get("1389_at",env=hgu95av2PMID)
> pubmed(pmid,disp="browser")
```

Another possibility is to collect a list containing PubMed ID, authors, abstract text, title, journal, and publication date.

```
> absts <- pm.getabst("1389_at", "hgu95av2")
> pm.titles(absts)
```

The list can obviously be searched for regular expressions.

```
ne <- pm.abstGrep("neutral endopeptidase",absts[[1]])
```

Another possibility is to construct an HTML table with the titles.

```
> pmAbst2HTML(absts[[1]],filename="pmon1389_at.html")
```

6.7 Searching GO numbers and evidence

By the phrase "ontology" we mean a structured language about some conceptual domain. The gene ontology consortium defines three ontologies: A Molecular Function (MF) describes a phenomenon at the biochemical level such as "enzyme", "transporter", or "ligand". A Biological Process (BP) may coordinate various related molecular functions such as "DNA replication" or "signal transduction". A Cellular Component (CC) is a unit within a part of the cell such as "chromosome", "nucleus", or "ribosome".

Each term is identified by a unique GO number. To find GO numbers and their dependencies we use `get` to extract a list from the annotation files `hgu95av2GO` for example. From the latter we extract a list and use an `apply` type of function to extract another list containing GO identification numbers.

```
> go1389 <- get("1389_at", env = hgu95av2GO)
> idl <- lapply(go1389,function(x) x$GOID)
> idl[[1]]
[1] "GO:0006508"
```

The list `idl` contains 8 members of which only the first is printed to the screen. By changing `GOID` into `Ontology` more specific information pertaining to ontology is extracted. From the `annotate` package we may now select the GO numbers which are related to a biological process.

```
> library(annotate)
> getOntology(go1389,"BP")
[1] "GO:0006508" "GO:0007267"
```

There are various types of evidence such as: inferred from genetic interaction (IGI), inferred from electronic annotation (IEA), traceable author statement (TAS), etc. Per GO identifier the type of evidence can be obtained.

```
> getEvidence(go1389)
GO:0004245 GO:0005886 GO:0005887 GO:0006508 GO:0007267 GO:0008237 GO:0008270
     "IEA"      "TAS"      "TAS"      "TAS"      "TAS"      "TAS"      "IEA"
GO:0046872
     "IEA"
```

When we now want to select the GO numbers with evidence of a traceable author statement we can use the `subset` function to create a list.

```
go1389TAS <- subset(go1389,getEvidence(go1389)=="TAS")
```

A manner to extract information from this list is by using an `apply` type of function.

```
> sapply(go1389TAS,function(x) x$GOID)
> sapply(go1389TAS,function(x) x$Evidence)
> sapply(go1389TAS,function(x) x$Ontology)
```

We shall use this list in the below.

6.8 GO parents and children

The term “transmembrane receptor protein-tyrosine kinase” is more specific and therefore a ’child’ of the more general term parent term “transmembrane receptor” (Gentleman, et. al, 2005).

Example 1. Collecting GO information. There are functions to obtain parents and children from a GO identifier.

```
> GOMFPARENTS$"GO:0003700"
         isa          isa
"GO:0003677" "GO:0030528"
> GOMFCHILDREN$"GO:0003700"
         isa
"GO:0003705"
```

In case of a list of GO identifiers you may want to collect the ontology, parents, and children identifiers in a vector.

```
go1389 <- get("1389_at", env = hgu95av2GO)
gonr <- getOntology(go1389, "BP")
gP <- getGOParents(gonr)
gC <- getGOChildren(gonr)
gPC <- c(gonr,gP,gC)
pa <- sapply(gP,function(x) x$Parents)
ch <- sapply(gC,function(x) x$Children)
gonrc <- c(gonr,unlist(pa),unlist(ch))
```

□

Example 2. Probe selection by GO. A research strategy may be to start with a probe number, to find the GO identifiers of the biological process, to obtains its parents, and next to transform these to probes.

```
library(GO); library(annotate); library("ALL"); data(ALL)
go1389 <- get("1389_at", env = hgu95av2GO)
gonr <- getOntology(go1389, "BP")
gP <- getGOParents(gonr)
pa <- sapply(gP,function(x) x$Parents)
probes <- mget(pa,hgu95av2GO2ALLPROBES)
probeNames <- unlist(probes)
ALLpr <- ALL[probeNames,]
> dim(exprs(ALLpr))
[1] 7745  128
```

Indeed, you may end up with many genes, useful for further analysis. □

6.9 Gene filtering by a biological term

An application of working with GO numbers is to filter for genes which are related to a biological term.

Example 1. Filter gene by a term. From a biological point of view it is most interesting to select genes which are related to a certain biological process to be specified by a term such as ”transcriptional repression”.

We combine this with the previous filter. For this we need the annotation package used in the stage of data collection. This can be obtained by `annotation(ALL)`. First we define a function (Gentleman, et al., 2005, p. 123) to collect appropriate GO numbers from the environment `GOTERM`.

```
library("GO"); library("annotate"); library("hgu95av2.db")
GOTerm2Tag <- function(term) {
 GTL <- eapply(GOTERM, function(x) {grep(term, x@Term, value=TRUE)})
 Gl <- sapply(GTL, length)
 names(GTL[Gl>0])
}
>  GOTerm2Tag("transcriptional repressor")
[1] "GO:0016564" "GO:0016565" "GO:0016566" "GO:0017053"
```

The functions `eapply` and `sapply` search an *e*nvironment like `GOTERM` by `grep` for matches of a specified term. A precaution is taken to select only those names which are not empty. This gives the GO terms which can now be translated to probe of the `ALLs` data.

```
tran1 <- hgu95av2GO2ALLPROBES$"GO:0016564"
tran2 <- hgu95av2GO2ALLPROBES$"GO:0016566"
tran3 <- hgu95av2GO2ALLPROBES$"GO:0017053"
tran <- c(tran1,tran2,tran3)
inboth <- tran %in% row.names(exprs(ALLs))
ALLtran <- ALLs[tran[inboth],]
```

The GO translated probe names are intersected with the row names of the data giving the logical variable `inboth`. The variable `tran[inboth]` gives the ids by which genes can be selected. Next, gene ids for which `inboth` equals `TRUE` are selected and the corresponding data are collected in the data frame `ALLtran`. More information can be obtained by `GOTERM$"GO:0016564`. By `dim(exprs(ALLtran))` it can be observed that 26 genes which passed the normality filter are related to "transcriptional repression". □

6.10 Significance per chromosome

After a statistical analysis to filter and order genes it is often quite useful to do post analysis on the results. In particular, after collecting p-values from

a t-test one may wonder whether genes with significant p-values occur more often within a certain chromosome. To test for such over or under representation the Fisher test is very useful (see Section 4.1.7).

Example 1. On the expression values of the ALL data we perform a two sample t-test using the patient group for which remission was achieved and for which it was not achieved. Per chromosome it can be tested whether the odds ratio differs from 1 or, equivalently, whether there is independence. The data for the test consist of the number of significant probes on Chromosome 19, the number of non-significant probes on Chromosome 19, the number of remaining significant probes, and the number of remaining non-significant probes.

```
> library("ALL"); data(ALL); library("hgu95av2.db")
> rawp <- apply(exprs(ALL), 1, function(x) t.test(x ~ ALL$remission)$p.value)
> xx <- as.list(hgu95av2CHR)
> AffimIDChr <- names(xx[xx=="19"])
> names(rawp) <-  featureNames(ALL)
> f <- matrix(NA,2,2)
> f[1,1] <- sum(rawp[AffimIDChr]<0.05); f[1,2] <- sum(rawp[AffimIDChr]>0.05)
> f[2,1] <- sum(rawp<0.05) - f[1,1]   ; f[2,2] <- sum(rawp>0.05) - f[1,2]
> print(f)
     [,1]  [,2]
[1,]  106   638
[2,]  832 11049
> fisher.test(f)

        Fisher's Exact Test for Count Data

data:  f
p-value = 4.332e-11
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 1.757949 2.748559
sample estimates:
odds ratio
  2.206211
```

```
> chisq.test(f)

        Pearson's Chi-squared test with Yates' continuity correction

data:  f
X-squared = 52.3803, df = 1, p-value = 4.573e-13
```

The number of significant probes is larger for Chromosome 19 resulting in an odds ratio of 2.2. The hypothesis of independence is rejected by both tests. □

6.11 Overview and concluding remarks

Many examples are given on using analysis of variance or T-tests for selecting genes with large experimental effects on different patient groups. The above statistical methods seem to cover the majority of problems occurring in practice.

6.12 Exercises

1. Gene filtering on normality per group of B-cell ALL patients.
   (a) Use `genefilter` to program the Shapiro normality test separately for each gene of the groups "B1","B2","B3","B4".
   (b) How many pass the filter?
   (c) Compute a Venn diagram for group "B2", "B3", and "B4", plot it, and give a correct interpretation for each number.
2. Analysis of gene expressions of B-cell ALL patients using Limma.
   (a) Construct a data frame containing the expression values for the B-cell ALL patients in stage B, B1, B2, B3, B4 from the `ALL` data.
   (b) Construct the design matrix and an appropriate contrast matrix.
   (c) Compute the twenty best genes by topTable.
   (d) Collect information on the twenty best gene s in an HTML page.

3. Finding a row number. Use grep to find the row number of gene `1389_at`. Hint: Use `row.names` or `featureNames`.

4. Remission (genezing) from acute lymphocytic leukemia (ALL). With respect to the `ALL` data from the `ALL` library there is a phenotypical variable called `remission` indicating complete remission `CR` or refractory `REF` meaning improvement from disease and less or no improvement, respectively.

   (a) How many persons are classified as CR and REF, respectively? Hint: Use `pData` to extract a data frame with phenotypical data.

   (b) Program the two-sample t-test not assuming equal variances to select genes with p-values smaller than 0.001. Hint: You may have to select the persons with values on remission, excluding not available data.

   (c) Collect and give the manufactures probe names of the genes with p-values smaller than 0.001.

   (d) Use the probe names to find the corresponding gene names. Give the code.

   (e) Is the famous protein p53 is among them?

   (f) How many unique gene names are there?

5. Remission achieved. For the ALL data from its `ALL` library the patients are checked for achieving remission. The variable `ALL$CR` has values CR (became healthy) and REF (did not respond to therapy; remain ill).

   (a) Construct a separate data frame consisting of only those gene expression values from patients that have values CR or REF.

   (b) How many genes have a p-value smaller than 0.0001 from the two-sample T-test not assuming equal variances? Hint: Use the `apply` functionality to program the test.

   (c) Give the affymetrix names (symbols) of the genes the pass the selection criterion of p-value smaller than 0.0001.

   (d) Use the latter to find the biological names.

   (e) How many oncogenes are there is total?

(f) Do the Fisher test on the number of oncogenes out of the total versus the number of significant oncogenes out of the selected.

6. Gene filtering of ALL data. The data are in the library called "ALL". The persons with T-cell leukemia which are in stage T2 and T3 can be selected by the variable `ALL$BT`. You may use the function "table" to find the frequencies of the patient types and leukemia stages. To answer the questions below functions from the library "genefilter" are helpful.

   (a) Program a gene filter step separately for T2 and T3 patients such that only those genes pass which are normally distributed.

   (b) Program a second filter step which passes only those genes with a significant p-value from the two sample T-test.

   (c) How many genes pass all filter steps?

   (d) How many genes pass normality?

7. Stages of B-cell ALL in the ALL data. Use the limma package to answer the questions below.

   (a) Select the persons with T-cell leukemia which are in stage B1, B2, B3, and B4.

   (b) What type of contrast matrix would you like to suggest in this situation? Give its code.

   (c) Perform analysis of variance to test the hypothesis of equal population means. Use the Benjamini & Hochberg (1995) ("BH") adjustment method for the false discovery rate and topTable to report the five best genes.

   (d) For how many genes is the null-hypothesis to be rejected?

8. Analysis of public micro array data on rheumatoid arthritis.

   (a) Download GDS486 and transform it into eset form. Here we meet a missing data problem. A manner to solve it is as follows. Use the function `function(x) sum(is.na(x))` in `apply` on the rows to count the number of missing values per row. Select the rows without missing value to perform a two-sample t-test with the

groups in `cell.line`. Overwrite the vector with the number of missing values with the p-values in a suitable manner.

(b) Download GDS711 and repeat the above using ANOVA p-values with the covariate `disease.state` to indicate the groups.

(c) Download GDS2126 and repeat the above using ANOVA p-values with the covariate `disease.state` to indicate the groups.

(d) Compute the symbols of the twenty best genes in the sense of having smallest summed p-values.

(e) Summarize information of the twenty best genes in an HTML table. Does p53 play a role in the path way of the best gene?

9. Analysis of genes from a GO search.

(a) Select the patients on the covariate `mol.biol` with values `ALL1/AF4`, `BCR/ABL`, and `NEG`.

(b) Collect the ANOVA p-values with contrast between `NEG` and `ALL1/AF4`, and between `NEG` and `BCR/ABL`. Report the number of significant affy ID's and the total. Hint: Re-order the columns into "NEG", "ALL1/AF4", and "BCR/ABL".

(c) Find the GO ID's refereing to the term "protein-tyrosine kinase" since it mediates many steps due to BCR/ABL translocation.

(d) Select the affy ID's corresponding to the GO ID's and report its number and the number of significant genes.

(e) Perform Fisher exact to test the odds ratio equal to one hypothesis.

Chapter 7

Cluster Analysis and Trees

Given the expression values of several genes, a problem which often arises is to find genes which are similar or close. Genes with expressions in small distance may have similar functions and may be potentially interesting for further research. In order to discover genes which form a group there are several methods developed called cluster analysis. These methods are based on a *distance function* and an algorithm to join data points to clusters. The so-called single linkage cluster analysis is intuitively appealing and often applied in bioinformatics. By this method several clusters of genes can be discovered without specifying the number of clusters on beforehand. The latter is necessary for another method called k-means cluster analysis. Each analysis produces a tree which represents similar genes as close leaves and dissimilar ones on different edges.

An other measure to investigate similarity or dependency of pairs of gene expressions is the *correlation coefficient*. Various examples of applications will be given. It prepares the way to searching a data set for directions of large variance. That is, since gene expression data sets tend to be large, it is of importance to have a method available which discovers important "directions" in the data. A frequently used method to find such directions is that by *principal components analysis*. Its basic properties will be explained as well as how it can be applied in combination with cluster analysis.

In applications where it is difficult to formulate distributional assumptions of the statistic it may still be of importance to construct a confidence interval. It will be illustrated by several examples how the *bootstrap* can be applied to construct 95% confidence intervals. Many examples are given to clarify the application of cluster analysis and principal components analysis.

7.1 Distance

The concept of distance plays a crucial role in all types of cluster analysis. For real numbers a and b a *distance* function d is defined as the absolute value of their difference

$$d(a,b) = |a-b| = \sqrt{(a-b)^2}.$$

The properties of a distance function should be in line with our intuition. That is, if $a = b$, then $d(a,a) = 0$ and if $a \neq b$, then $d(a,b) > 0$. Hence, the distance measure should be *definitive* in the sense that $d(a,b) = 0$ if and only if $a = b$. Since the square is *symmetric*, it follows that

$$d(a,b) = |a-b| = \sqrt{(a-b)^2} = \sqrt{(b-a)^2} = |b-a| = d(b,a).$$

In other words, $d(a,b) = d(b,a)$, the distance between a and b equals that between b and a. Furthermore, it holds for all points c between a and b that $d(a,b) = d(a,c) + d(c,b)$. For all points c not between a and b, it follows that $d(a,b) < d(a,c) + d(c,b)$. The latter two notions can be summarized by the so-called triangle inequality. That is, for all real c it holds that

$$d(a,b) \leq d(a,c) + d(c,b).$$

Directly going from a to b is shorter than via c. Finally, the distance between two points a and b should increase as these move further apart.

Example 1. Let $a = 1$ and $b = 3$. Then, obviously, the distance $d(1,3) = 2$. The number $c = 2$ is between a and b, so that $d(1,3) = 2 = 1 + 1 = d(1,2) + d(2,3)$ and the triangle inequality becomes an equality. □

For the situation where gene expression values for several patients are available, it is of importance to define a distance for vectors of gene expressions such as $\boldsymbol{a} = (a_1, \cdots, a_n)$ and $\boldsymbol{b} = (b_1, \cdots, b_n)$. We shall concentrate mainly on the Euclidian distance, which is defined as the root of the sum of the squared differences

$$d(\boldsymbol{a}, \boldsymbol{b}) = \sqrt{\sum_{i=1}^{n} (a_i - b_i)^2}.$$

The distance measure satisfies the above properties of definiteness, symmetry, and triangle inequality. Although many other, but often highly similar, distance functions are available we shall mainly concentrate on Euclidian distance because it is applied most frequently in bioinformatics.

Example 2. Suppose that $\boldsymbol{a} = (a_1, a_2) = (1, 1)$ and $\boldsymbol{b} = (b_1, b_2) = (4, 5)$. Then

$$d(\boldsymbol{a}, \boldsymbol{b}) = \sqrt{(a_1 - b_1)^2 + (a_2 - b_2)^2} = \sqrt{(1-4)^2 + (1-5)^2} = \sqrt{9 + 16} = 5.$$

Since the differences are squared it is immediate that $d(\boldsymbol{a}, \boldsymbol{b}) = d(\boldsymbol{b}, \boldsymbol{a})$, the distance from $\boldsymbol{a}$ to $\boldsymbol{b}$ equals that from $\boldsymbol{b}$ to $\boldsymbol{a}$. For $\boldsymbol{c} = (c_1, c_2) = (2, 2)$ we have that $d(\boldsymbol{a}, \boldsymbol{c}) = \sqrt{2}$, $d(\boldsymbol{b}, \boldsymbol{c}) = \sqrt{2^2 + 3^2} = \sqrt{13}$. Hence,

$$d(\boldsymbol{a}, \boldsymbol{b}) = 5 < \sqrt{2} + \sqrt{13} = d(\boldsymbol{a}, \boldsymbol{c}) + d(\boldsymbol{b}, \boldsymbol{c}),$$

so that the triangle inequality is strict. This is in line with our intuitive idea that the road directly from $\boldsymbol{a}$ to $\boldsymbol{b}$ is shorter than from $\boldsymbol{a}$ to $\boldsymbol{b}$ via $\boldsymbol{c}$. □

Example 3. To compute the Euclidian distance between two vectors one may use the following.

```
> a <- c(1,1); b <- c(4,5)
> sqrt(sum((a-b)^2))
[1] 5
```

□

Example 4. Distances between Cyclin gene expressions. By the build-in-function `dist` the Euclidian distance between two vectors of gene expression values can be computed. To select genes related to the biological term "Cyclin" and to compute the Euclidian distance between the gene expression values of the Golub et al. (1999) data, we may use the following.

```
> library(multtest); data(golub)
> index <- grep("Cyclin",golub.gnames[,2])
> golub.gnames[index,2]
 [1] "CCND2 Cyclin D2"
 [2] "CDK2 Cyclin-dependent kinase 2"
 [3] "CCND3 Cyclin D3"
 [4] "CDKN1A Cyclin-dependent kinase inhibitor 1A (p21, Cip1)"
 [5] "CCNH Cyclin H"
```

```
 [6] "Cyclin-dependent kinase 4 (CDK4) gene"
 [7] "Cyclin G2 mRNA"
 [8] "Cyclin A1 mRNA"
 [9] "Cyclin-selective ubiquitin carrier protein mRNA"
[10] "CDK6 Cyclin-dependent kinase 6"
[11] "Cyclin G1 mRNA"
[12] "CCNF Cyclin F"
> dist.cyclin <- dist(golub[index,],method="euclidian")
> diam <- as.matrix(dist.cyclin)
> rownames(diam) <- colnames(diam) <- golub.gnames[index,3]
> diam[1:5,1:5]
          D13639_at M68520_at M92287_at U09579_at U11791_at
D13639_at  0.000000  8.821806  11.55349 10.056814  8.669112
M68520_at  8.821806  0.000000  11.70156  5.931260  2.934802
M92287_at 11.553494 11.701562   0.00000 11.991333 11.900558
U09579_at 10.056814  5.931260  11.99133  0.000000  5.698232
U11791_at  8.669112  2.934802  11.90056  5.698232  0.000000
```

By the `grep` function the order numbers of the genes with the phrase "Cyclin" in their names are assigned to the vector called `index`. The euclidian distances are assigned to the matrix called `diam`. Its diagonal has distances between identical genes which are, of course, zero. The distance between the first (CCND2 Cyclin D2) and the third (CCND3 Cyclin D3) is relatively small, which is in line with the fact the these genes have related functions. Note, however, that there are genes with in smaller distance. □

Example 5. Finding the ten closest genes to a given one. After selecting certain genes it often happens that one wants to find genes which are close to the selected ones. This can be done with the `genefinder` functionality by specifying either an index or a name (consistent with the geneNames of the exprSet). To find genes from the ALL data (Chiaretti et al., 2004) close to the MME expression values of the probe with identifier `1389_at`, we may use the following.

```
library("genefilter"); library("ALL"); data(ALL)
closeto1389_at <- genefinder(ALL, "1389_at", 10, method = "euc")
closeto1389_at[[1]]$indices
round(closeto1389_at[[1]]$dists,1)
featureNames(ALL)[closeto1389_at[[1]]$indices]
```

The function `genefilter` produces a list from which the selected row numbers can be extracted as well as the probe names can be found.[1] If desired, these can be used for further analysis. From the output it can be observed that the gene expressions of row 2653 with probe identifier `32629_f_at` has the smallest distance (12.6) to those of `1389_at`. □

7.2 Two types of Cluster Analysis

Some important types of cluster analysis are defined and illustrated here.

7.2.1 Single Linkage

A cluster I is simply a set of data points $I = \{\boldsymbol{x}_i\}$, where $\boldsymbol{x}_i$ is the i-th vector with gene expressions. In single linkage cluster analysis the distance between clusters I and J is defined as the smallest distance over all pairs of points of the two clusters:

$$d(I, J) = \min_{i,j} \{d(\boldsymbol{x}_i, \boldsymbol{x}_j,) : \boldsymbol{x}_i \text{ in } I \quad \text{and} \quad \boldsymbol{x}_j \text{ in } J\} .$$

Hence, the distance between the two clusters is the same as that of the *nearest neighbors*. The algorithm of single linkage cluster analysis starts with creating as many clusters as data points. Next, the nearest two are determined and these are merged into one cluster. Then the next two nearest clusters are determined and merged into one cluster. This process continuous until all points belong to one cluster.

Example 1. An explanatory example. To illustrate single linkage cluster analysis suppose we the following five gene expressions $\boldsymbol{g}_1 = (1, 1)$, $\boldsymbol{g}_2 = (1, 1.2)$, $\boldsymbol{g}_3 = (3, 2)$, $\boldsymbol{g}_4 = (3, 2.2)$, and $\boldsymbol{g}_5 = (5, 5)$, from two persons. The expressions for each gene can be seen as coordinates on two perpendicular axis $\boldsymbol{p}_1$ and $\boldsymbol{p}_2$. The script below produces Figure 7.1 which illustrates the idea. It computes the the distances between the genes and performs a single linkage cluster analysis.

```
names <- list(c("g1","g2","g3","g4","g5"),c("p1","p2"))
```

[1] For information on lists, see Chapter 6 of the manual "An Introduction to R".

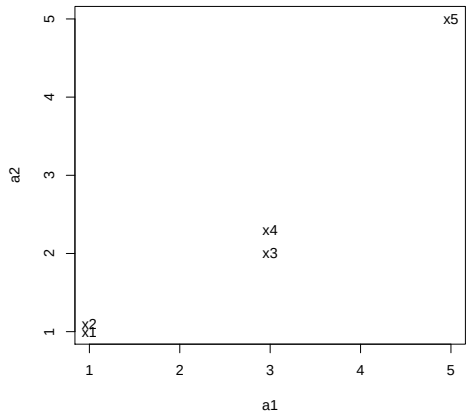


Figure 7.1: Plot of five points to be clustered.

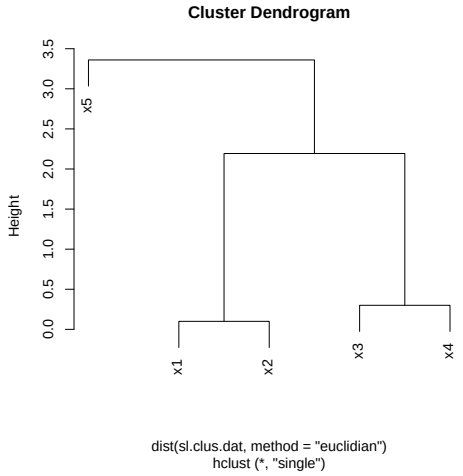


Figure 7.2: Tree of single linkage cluster analysis.

```
sl.clus.dat <- matrix(c(1,1,1,1.1,3,2,3,2.3,5,5),ncol = 2,
  byrow = TRUE,dimnames = names)
plot(sl.clus.dat,type="n", xlim=c(0,6), ylim=c(0,6))
text(sl.clus.dat,labels=row.names(sl.clus.dat))
> print(dist(sl.clus.dat,method="euclidian"),digits=3)
     x1   x2   x3   x4
x2 0.10
x3 2.24 2.19
x4 2.39 2.33 0.30
x5 5.66 5.59 3.61 3.36
> sl.out<-hclust(dist(sl.clus.dat,method="euclidian"),method="single")
> plot(sl.out)
```

At the start each data point is seen as a separate cluster. Then the nearest two points (genes) from the Euclidian distance matrix are $\boldsymbol{g}_1$ and $\boldsymbol{g}_2$, having $d(\boldsymbol{g}_1, \boldsymbol{g}_2) = 0.10$. These two data points are merged into one cluster, say $I = \{\boldsymbol{g}_1, \boldsymbol{g}_2\}$. In Figure 7.2 this is illustrated by the horizontal line at height 0.10 in the tree. The other three data points $\boldsymbol{g}_3, \boldsymbol{g}_4, \boldsymbol{g}_5$ are seen as three different clusters. Next, the minimal distance between clusters can be read from the Euclidian distance matrix. Since the smallest is $d(\boldsymbol{g}_3, \boldsymbol{g}_4) = 0.30$, the new cluster $J = \{\boldsymbol{g}_3, \boldsymbol{g}_4\}$, corresponding to the horizontal line at height

0.30. Now there are three clusters, I, J, and $K = \{\boldsymbol{x}_5\}$. From the Euclidian distance matrix, it can be observed that the distance between cluster I and J is 2.19, see the corresponding horizontal line at this height. Hence, the cluster I and J are merged into one. Finally, the distance between cluster $\{\boldsymbol{g}_1, \boldsymbol{g}_2, \boldsymbol{g}_3, \boldsymbol{g}_4\}$, and the data point $\boldsymbol{g}_5$ equals $d(\boldsymbol{g}_4, \boldsymbol{g}_5) = 3.36$, see the corresponding horizontal line at this height. □

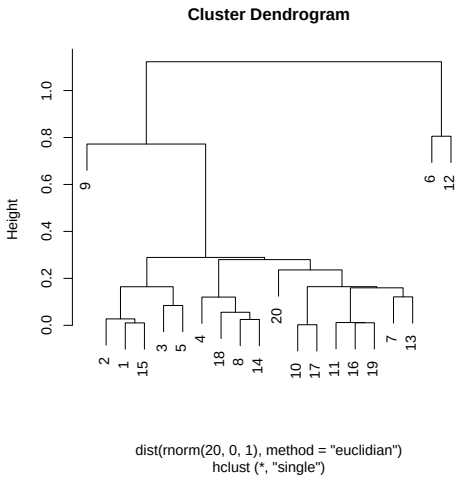


Figure 7.3: Example of three without clusters.

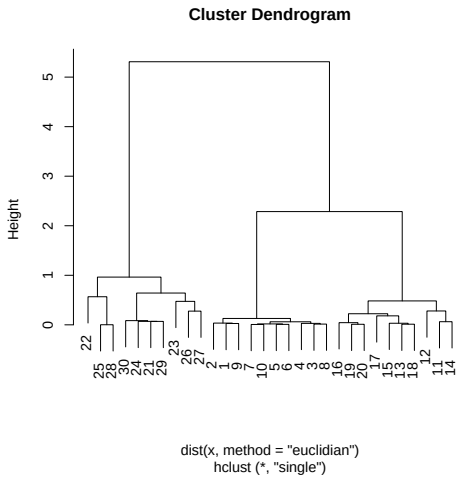


Figure 7.4: Three clusters with different standard deviations.

Example 2. Relating data generation processes to cluster trees. It is of importance to have some experience with data that does and does not contain clusters. To illustrate this we perform single linkage cluster analysis on twenty data points from the standard normal population.

```
sl.out<-hclust(dist(rnorm(20,0,1),method="euclidian"),method="single")
plot(sl.out)
```

From the resulting tree in Figure 7.3 one might get the impression that there are five separate clusters in the data. Note, however, that there is no underlying data generation process which produces separate clusters from different populations.

If, however, the data are generated by different normal distributions, then there are different processes producing separate clusters. To illustrate

this, ten data points were sampled from the $N(0, 0.1)$ population, ten from $N(3, 0.5)$, and ten from $N(10, 1)$.

```
x <- c(rnorm(10,0,0.1),rnorm(10,3,0.5),rnorm(10,10,1.0))
plot(hclust(dist(x,method="euclidian"),method="single"))
plot(sl.out)
```

From the tree in Figure 7.4, it can be observed that there clearly exist three clusters. □

These examples illustrate that results from cluster analysis may very well reveal population properties, but that some caution is indeed in order.

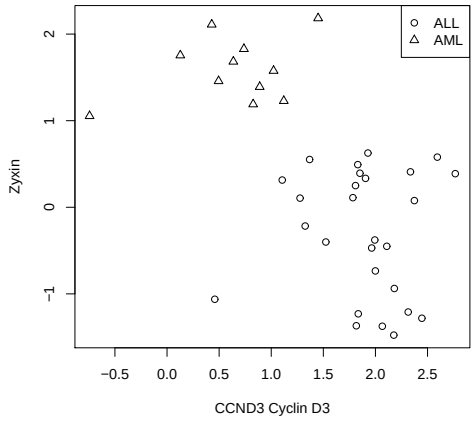


Figure 7.5: Plot of gene "CCND3 Cyclin D3" and "Zyxin" expressions for ALL and AML patients.

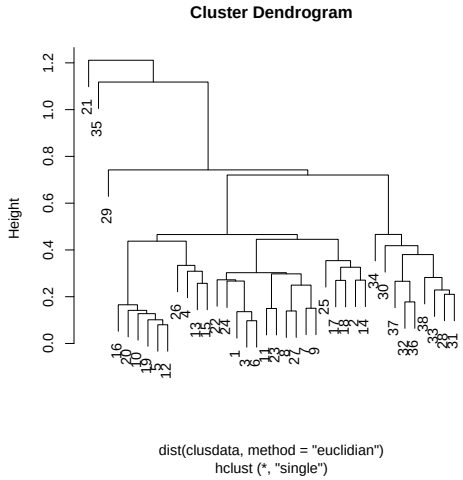


Figure 7.6: Single linkage cluster diagram from gene "CCND3 Cyclin D3" and "Zyxin" expressions values.

Example 3. Application to the Golub (1999) data. Recall that the first twenty seven patients belong to ALL and the remaining eleven to AML and that we found earlier that the expression values of the genes "CCND3 Cyclin D3" and "Zyxin" differ between the patient groups ALL and AML. Figure 7.5 illustrates that the patient groups differ with respect to gene expression values. How to produce this plot and a single linkage cluster analysis is shown by the script below.

```
data(golub, package="multtest")
clusdata <- data.frame(golub[1042,],golub[2124,])
colnames(clusdata)<-c("CCND3 Cyclin D3","Zyxin")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
plot(clusdata, pch=as.numeric(gol.fac))
legend("topright",legend=c("ALL","AML"),pch=1:2)
plot(hclust(dist(clusdata,method="euclidian"),method="single"))
```

Figure 7.6 gives the tree from single linkage cluster analysis. Apart from three expressions the tree shows two clusters corresponding to the two patient groups. □

7.2.2 k-means

K-means cluster analysis is a popular method in bioinfomatics. It is defined by minimizing the within cluster sum of squares over K clusters. That is, given the data points $\boldsymbol{x}_1, \cdots, \boldsymbol{x}_n$ the method seeks to minimize the function

$$\sum_{k=1}^{K} \sum_{i \in I_k} d^2(\boldsymbol{x}_i, \boldsymbol{a}_k)$$

over all possible points $\boldsymbol{a}_1, \cdots \boldsymbol{a}_K$. This is accomplished by an algorithm (Hartigan & Wong, 1979) which starts by partitioning the data points into K initial clusters, either at random or using some heuristic device. It then computes the cluster means (step 1) and constructs a new partition by associating each point with the closest cluster mean (step 2). The latter yields new clusters of which the means are calculated (step 1). Then it constructs a new partition by associating each point with the closest cluster mean (step 2). These two steps are repeated until convergence. The latter occurs when the data points no longer change clusters. The iterative algorithm is fast in the sense that it often converges in less iterations than the number of points n, but it need not to attain the global minimum. For the optimal points $\boldsymbol{a}_1, \cdots \boldsymbol{a}_K$, it holds that these are equal to the mean per cluster, that is $\boldsymbol{a}_k = \overline{\boldsymbol{x}}_k$ for each cluster k. When the data points are independent and identically distributed, then the cluster means converge in probability to the corresponding population means (Pollard, 1981).

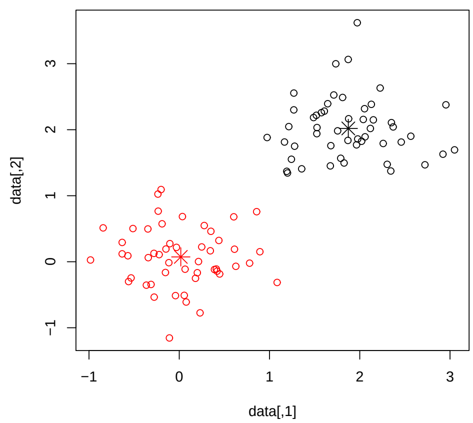


Figure 7.7: K-means cluster analysis.

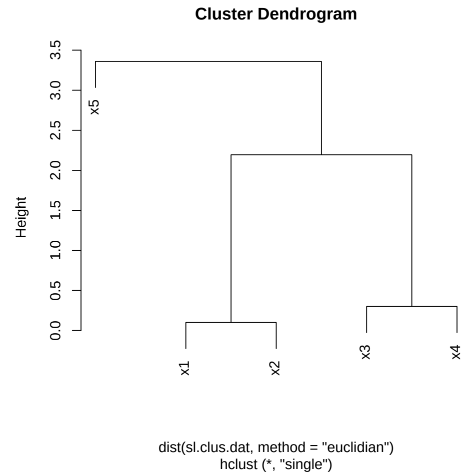


Figure 7.8: Tree of single linkage cluster analysis.

Example 1. Relating a data generation process to k-means cluster analysis. To illustrate k-means cluster analysis we shall simulate gene expressions from two different normal populations. That is, we randomly take fifty gene expressions for two persons from the $N(0, 0.5)$ population and fifty expressions for two persons from the $N(2, 0.5)$ population. The data points are collected in two matrices of order fifty by two which are placed one above the other. On the total of one hundred data points a $(k =)$2-means cluster analysis is performed.

```
> data <- rbind(matrix(rnorm(100,0,0.5), ncol = 2),
  + matrix(rnorm(100,2,0.5), ncol = 2))
> cl <- kmeans(data, 2)
K-means clustering with 2 clusters of sizes 50, 50

Cluster means:
        [,1]       [,2]
1 1.87304978 2.01940342
2 0.01720177 0.07320413

Clustering vector:
  [1] 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
```

```
 [38] 2 2 2 2 2 2 2 2 2 2 2 2 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
 [75] 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1

Within cluster sum of squares by cluster:
[1] 22.60733 20.54411

Available components:
[1] "cluster"  "centers"  "withinss" "size"
```

The output of k-means cluster analysis is assigned to a list called `cl`. Observe that the cluster means are fairly close to the population means $(0, 0)$ and $(2, 2)$. The `Clustering vector` indicates to which cluster each data point (gene) belongs and that these correspond exactly to the two populations from which the data are sampled. The variable `cl$cluster` contains cluster membership and can be used to specify the color of each data point a plot, as follows.

```
> plot(data, col = cl$cluster)
> points(cl$centers, col = 1:2, pch = 8, cex=2)
```

The data points are plotted in red and black circles and the cluster means by a star, see Figure 7.7. The sum of the within cluster sum of squares equals the minimal function value obtained by the algorithm.

Before performing a k-means cluster analysis a plot from a single linkage cluster analysis may reveal the number of clusters. If the number of clusters is not at all clear, then it becomes questionable whether k-means is appropriate. For cases where the number of clusters is only moderately clear, the algorithm is more sensible to get stuck into a solution which is only locally optimal. Such solutions are of limited scientific value. To cope with the danger of suboptimal solutions one may simply run the algorithm repeatedly by using the `nstart` option. Another possibility is to use rational initial starting values for the cluster means. In particular, the sample means of potential clusters or the hypothesized population means can be used.

```
> initial <- matrix(c(0,0,2,2), nrow = 2, ncol=2, byrow=TRUE)
> cl<- kmeans(data, initial, nstart = 10)
```

The so-called bootstrap (Efron, 1979) can be used to estimate 95% confidence intervals around cluster means. The idea is to re-sample with replacement from the given sample one thousand times with replacement and to compute quantiles for the corresponding confidence intervals.

```
n <- 100; nboot<-1000
boot.cl <- matrix(0,nrow=nboot,ncol = 4)
for (i in 1:nboot){
  dat.star <- data[sample(1:n,replace=TRUE),]
  cl <- kmeans(dat.star, initial, nstart = 10)
  boot.cl[i,] <- c(cl$centers[1,],cl$centers[2,])
}
> quantile(boot.cl[,1],c(0.025,0.975))
      2.5%      97.5%
-0.1098886  0.1627979
> quantile(boot.cl[,2],c(0.025,0.975))
       2.5%       97.5%
-0.04830563  0.19721732
> quantile(boot.cl[,3],c(0.025,0.975))
    2.5%    97.5%
1.730495 2.009014
> quantile(boot.cl[,4],c(0.025,0.975))
    2.5%    97.5%
1.898407 2.162019
```

From the bootstrap confidence intervals the null hypothesis that the cluster population means are equal to $(0, 0)$ and $(2, 2)$ are accepted. □

Example 2. Application to the Golub (1999) data. In the above we found that the expression values of the genes "CCND3 Cyclin D3" and "Zyxin" are closely related to the distinction between ALL and AML. Hence, a 2-means cluster analysis of these gene expression values is appropriate here.

```
> data <- data.frame(golub[1042,],golub[2124,])
> colnames(data)<-c("CCND3 Cyclin D3","Zyxin")
> cl <- kmeans(data, 2,nstart = 10)
> cl
K-means clustering with 2 clusters of sizes 11, 27

Cluster means:
  CCND3 Cyclin D3      Zyxin
1       0.6355909  1.5866682
2       1.8938826 -0.2947926
```

```
Clustering vector:
 1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
 2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2  2
27 28 29 30 31 32 33 34 35 36 37 38
 2  1  1  1  1  1  1  1  1  1  1  1

Within cluster sum of squares by cluster:
[1]  4.733248 19.842225
```

The two clusters discriminate exactly the ALL patients from the AML patients. This can also be seen from Figure 7.9, where expression values of CCND3 Cyclin D3 are depicted on the horizontal axis and those of Zyxin on the vertical, and the ALL patients are in red and the AML patients in black. By the bootstrap the cluster means and their confidence intervals can be estimated.

```
> mean(data.frame(boot.cl))
        X1         X2         X3         X4
 0.6381860  1.5707477  1.8945878 -0.2989426
> quantile(boot.cl[,1],c(0.025,0.975))
     2.5%     97.5%
0.2548907 0.9835898
> quantile(boot.cl[,2],c(0.025,0.975))
    2.5%    97.5%
1.259608 1.800581
> quantile(boot.cl[,3],c(0.025,0.975))
    2.5%    97.5%
1.692813 2.092361
> quantile(boot.cl[,4],c(0.025,0.975))
       2.5%       97.5%
-0.60802142 -0.02420802
```

The difference between the bootstrap means and the k-means from the original data gives an estimate of the estimation bias. It can be observed that the bias is small. The estimation is quite precise because the 95% bootstrap confidence intervals are fairly small. □

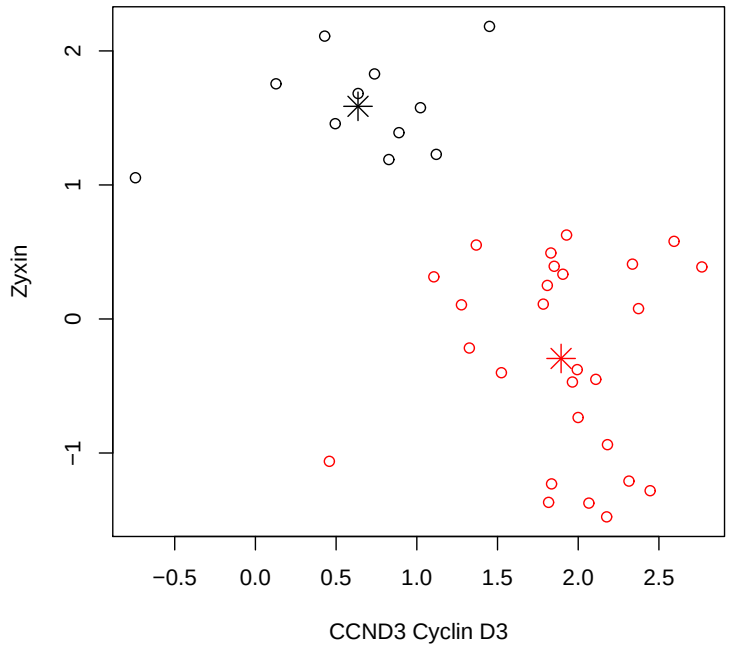


Figure 7.9: Plot of kmeans (stars) cluster analysis on CCND3 Cyclin D3 and Zyxin discriminating between ALL (red) and AML (black) patients.

7.3 The correlation coefficient

A frequently used coefficient to express the degree of linear relationship between two sets of gene expression values is the correlation coefficient ρ. For two sequences of gene expressions such as $\boldsymbol{x} = (x_1, \cdots, x_n)$ and $\boldsymbol{y} = (y_1, \cdots, y_n)$, the correlation coefficient ρ is estimated by

$$\widehat{\rho} = \frac{\sum_{i=1}^{n}(x_i - \overline{x}_i)(y_j - \overline{y}_j)}{\sqrt{\sum_{i=1}^{n}(x_i - \overline{x}_i)^2 \sum_{i=1}^{n}(y_j - \overline{y}_j)^2}}.$$

The value of the correlation coefficient is always between minus one and plus one. If the value is close to either of these values, then the variables are

linearly related in the sense that the first is a linear transformation of the second. That is, there are constants a and b such that $ax_i + b = y_i$ for all i. By the function `cor.test`, the null hypothesis $H_0 : \rho = 0$ can be tested against the alternative $H_0 : \rho \neq 0$.

Example 1. Teaching demonstration. To develop intuition with respect to the correlation coefficient the function `run.cor.examp(1000)` of the `TeachingDemos` package is quite useful. It launches an interactive plot with 1000 data points on two random variables X and Y. When the correlation is near zero, then the data points are distributed along contours of circles. By moving the slider slowly from the left to the right it can be observed that all points are approximately on a straight line. If the sign of the correlation coefficient is positive, then small/large values of X tend to go together with small/large values of Y. □

Example 2. Another teaching demonstration. By the function `put.points.demo()` it is possible to add and delete points to a plot which interactively recomputes the value for the correlation coefficient. By first creating a few points that lie together on a circle the corresponding correlation coefficient will be near zero. By next adding one outlier, it can be observed that the correlation coefficient changes to nearly ± 1. This illustrates that the correlation coefficient is not robust against outliers. □

Example 3. Application to the Golub (1999) data. We shall illustrate the correlation coefficient by two sets of expression values of the MCM3 gene of the Golub et al. (1999) data. This gene encodes for highly conserved mini-chromosome maintenance proteins (MCM) which are involved in the initiation of eukaryotic genome replication. Here, we find its row numbers, collect the gene expression value in vectors $\boldsymbol{x}$ and $\boldsymbol{y}$, and compute the value of the correlation coefficient by the function `cor(x,y)`.

```
> library(multtest); data(golub)
> x <- golub[2289,]; y <- golub[2430,]
> cor(x,y)
[1] 0.6376217
```

The value is positive which means that larger values of $\boldsymbol{x}$ occur together with larger values of $\boldsymbol{y}$ and vice versa. This can also be observed by `plot(x,y)`. By

the function `cor.test`, the null hypothesis $H_0 : \rho = 0$ can be tested against the alternative $H_0 : \rho \neq 0$. It also estimates a 95% confidence interval for ρ.

```
> cor.test(x,y)

        Pearson's product-moment correlation

data:  x and y
t = 4.9662, df = 36, p-value = 1.666e-05
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 0.3993383 0.7952115
sample estimates:
      cor
0.6376217
```

The test is based on the normality assumption and prints therefore a t-value. Since the corresponding p-value is very small, we reject the null hypothesis of zero correlation. The left bound of the confidence interval falls far to the right hand side of zero. □

Example 4. Confidence interval by the bootstrap. Another method to construct a 95% confidence interval is by the bootstrap. The idea (Efron, 1979) is to obtain thousand samples from the original sample with replacement and to compute the correlation coefficient for each of these. This yields thousand coefficients from which the quantiles for the 95% confidence interval can be computed.

```
> nboot <- 1000; boot.cor <- matrix(0,nrow=nboot,ncol = 1)
> data <- matrix(c(x,y),ncol=2,byrow=FALSE)
> for (i in 1:nboot){
+    dat.star <- data[sample(1:nrow(data),replace=TRUE),]
+    boot.cor[i,] <- cor(dat.star)[2,1]}
> mean(boot.cor)
[1] 0.6534167
> quantile(boot.cor[,1],c(0.025,0.975))
     2.5%     97.5%
0.2207915 0.9204865
```

Observe that the 95% confidence interval is larger than that found by `cor.test`. This indicates that the assumption of normality may not be completely valid here. Since the confidence interval does not contain zero, we reject the null-hypothesis of zero correlation. □

Example 5. Application to the Golub (1999) data. The ALL and AML patients of the Golub et al. (1999) data are indicated by zero and ones of the binary vector `golub.cl`. A manner to select genes it by the correlation of the expression values with this binary vector. Such can be computed by using the `apply` functionality.

```
> library(multtest); data(golub)
> corgol<- apply(golub, 1, function(x) cor(x,golub.cl))
> o <- order(corgol)
```

By `golub.gnames[o[3041:3051],2]` it can be seen that various of these genes seem indeed to have important cell functions referred to by Golub et al. (1999). In particular, Interleukin 8 is recently related to inflammatory cytokine production in myeloid cells (Tessarz et al., 2007). □

7.4 Principal Components Analysis

To make the basic ideas behind principal components analysis explicit, it is wise to start with a small artificial example. Suppose that for six genes the standardized expression values on two patients (variables) became available as these are given in Table 7.1. The data are collected in a 6 by 2 data matrix $\boldsymbol{Z}$, where e.g. element z_{21} is expression value -0.40 which belongs to the second gene of the first patient.

The whole idea of principal components analysis is to find new directions in the data along which there is maximal variation. A direction is defined as a linear combination $\boldsymbol{Zk}$ of the data $\boldsymbol{Z}$ by a vector $\boldsymbol{k}$ with weights. The i-th element of the linear combination is the weighted sum $\sum_{j=1}^{2} z_{ij}k_j$. The direction of maximal variation is defined as the linear combination with maximal variance. To find this direction the correlation matrix plays an important role. The latter contains the correlations between each pair of patients (variables). In our case correlations between the columns (patients) in Table 7.1

Table 7.1: Data set for principal components analysis.

	Var 1	Var 2
gene 1	1.63	1.22
gene 2	−0.40	0.79
gene 3	0.93	0.97
gene 4	−1.38	−1.08
gene 5	−0.17	−0.96
gene 6	−0.61	−0.93

can be placed in a matrix $\boldsymbol{R}$, which has ones on the diagonal and the value 0.8 elsewhere.

To illustrate a direction let's try the linear combination $\boldsymbol{k} = (2, 1)$[2] of the sample correlation matrix $\boldsymbol{R}$. This gives

$$\boldsymbol{Rk} = \begin{bmatrix} 1 & 0.8 \\ 0.8 & 1 \end{bmatrix} \begin{bmatrix} 2 \\ 1 \end{bmatrix} = \begin{bmatrix} 2.8 \\ 2.6 \end{bmatrix}.$$

Both vectors $\boldsymbol{k}$ and $\boldsymbol{Rk}$ can be plotted in the xy-plane. The vector $(2, 1)$ is plotted by drawing an arrow from (0,0) to the point with $x = 2$ and $y = 1$. This is done completely similar for $(2.8, 2.6)$ in Figure 7.10. It can be observed that the two vectors (arrows) do not fall on the same line and therefore have different directions. The crux of principal components analysis is that a linear combination with the same direction as the weights represent the direction of maximum variation. Such is the case if $\boldsymbol{Rk}$ differs from $\boldsymbol{k}$ only by a constant of multiplication, that is there exists a constant d such that $\boldsymbol{Rk} = d\boldsymbol{k}$. We shall determine such a constant by finding the weights vector first. To do so observe from our correlations matrix that the sum of both rows equals 1.8. Taking $\boldsymbol{k} = (1, 1)$ yields

$$\boldsymbol{Rk} = \begin{bmatrix} 1 & 0.8 \\ 0.8 & 1 \end{bmatrix} \begin{bmatrix} 1 \\ 1 \end{bmatrix} = \begin{bmatrix} 1.8 \\ 1.8 \end{bmatrix} = 1.8 \begin{bmatrix} 1 \\ 1 \end{bmatrix} = 1.8\boldsymbol{k}.$$

So that we obtain $d = 1.8$. A similar result follows by observing that the differences per row are equal in absolute value. That is, taking $\boldsymbol{k} = (1, -1)$

[2]For the sake of simple notation we shall not use the transposition operator ^{T} to indicate rows.

yields

$$\boldsymbol{Rk} = \begin{bmatrix} 1 & 0.8 \\ 0.8 & 1 \end{bmatrix} \begin{bmatrix} 1 \\ -1 \end{bmatrix} = \begin{bmatrix} 0.2 \\ -0.2 \end{bmatrix} = 0.2 \begin{bmatrix} 1 \\ -1 \end{bmatrix} = 0.2\boldsymbol{k}.$$

A vector $\boldsymbol{k}$ for which $\boldsymbol{Rk} = d\boldsymbol{k}$ holds is called an *eigenvector* corresponding to the *eigenvalue* d. Eigenvectors are often re-scaled by dividing by their Euclidian length. Since the Euclidian length of $(1, 1)$ is $\sqrt{1^2 + 1^2} = \sqrt{2}$, we obtain the new eigenvector $\boldsymbol{k}_1 = (1/\sqrt{2}, 1/\sqrt{2}) \approx (0.71, 0.71)$. Since the length of eigenvector $(1, -1)$ also equals $\sqrt{2}$ the re-scaled second eigenvector equals $\boldsymbol{k}_2 = (1/\sqrt{2}, -1/\sqrt{2}) \approx (0.71, -0.71)$. Now the first principal component is defined as $\boldsymbol{Zk}_1$ and the second as $\boldsymbol{Zk}_2$. In practical applications the actual computation of eigenvectors and eigenvalues is performed by well-designed numerical methods (Golub & Van Loan, 1983).

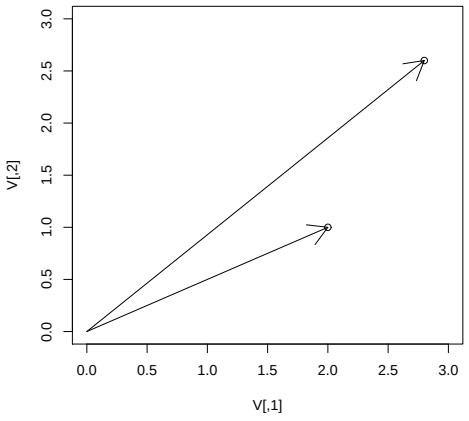


Figure 7.10: Vectors of linear combinations.

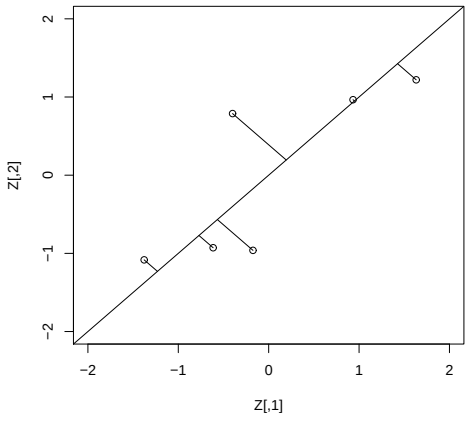


Figure 7.11: First principal component with projections of data.

Example 1. Using R on the above data. It is convenient to store the data of the first two columns of Table 7.1 as a matrix object called `Z`. The correlations matrix can be computed by the built-in-function `cor` and the eigenvectors and eigenvalues by the built-in-function `eigen`, as follows.

```
Z <- matrix(c( 1.63, 1.22, -0.40, 0.79, 0.93, 0.97, -1.38,
```

```
  -1.08, -0.17, -0.96, -0.61, -0.93), nrow=6, byrow=TRUE)
K <- eigen(cor(Z))
```

The output is stored as an object called `K` which can be printed to the screen in two digits.

```
> print(K,digits=2)
$values
[1] 1.8 0.2

$vectors
     [,1]  [,2]
[1,] 0.71  0.71
[2,] 0.71 -0.71
```

The eigenvalues are assigned to `K$values` and the eigenvectors are the columns of `K$vectors`. To compute the principal components we use the matrix multiplication operator `%*%`. Then the first principal component is defined as the linear combination of the data with the first eigenvector, `Z%*%K$vec[,1]`. To print the scores on the first and the second principal component one can use the following.

```
> print(Z %*% K$vec, digits=2)
      [,1]   [,2]
[1,]  2.02  0.290
[2,]  0.28 -0.841
[3,]  1.34 -0.028
[4,] -1.74 -0.212
[5,] -0.80  0.559
[6,] -1.09  0.226
```

To illustrate the first principal component the six data points from the `Z` matrix are plotted as small circles in Figure 7.11. Gene 1, for instance, has x coordinate 1.63 and y coordinate 1.22 and appears therefore in the right upper corner.

A convenient manner to perform principal components analysis is by using the built-in-function `princomp`, as follows.

```
pca <- princomp(Z, center = TRUE, cor=TRUE, scores=TRUE)
pca$scores
```

The `scores` are the component scores and the `loadings` from `princomp` are the eigenvectors. □

The eigenvalues represent an amount of variance related to the component. In the previous example the first component has variance 1.8 and the second 0.2, so that the first represents $1.8/2 = 0.9$ or 90% of the variance. On the basis of the eigenvalues the number of interesting directions in the data can be evaluated by two rules of thumb. The first is that each eigenvalue should represent more variance than that of any of the observed variables. The second is the so-called elbow rule saying that when the first few eigenvalues are large and the remaining considerably smaller, then the first few are the most interesting.

Principal components analysis is a descriptive method to analyze dependencies (correlations) between variables. If there are a few large eigenvalues, then there are equally many directions in the data which summarize the most important variation among the gene expressions. Then it may be useful to explore simultaneously a two dimensional visualization of the genes and the patients. Furthermore, it can be rewarding to study the weights of the eigenvectors because these may reveal a structure in the data otherwise gone unnoticed. Finally, the principal components contain less (measurement) error than the individual variables. For this reason, cluster analysis on the values on the principal components may be useful.

Example 2. Application to the Golub (1999) data. The first five eigenvalues from the correlation matrix of `golub` can be printed by the following.

```
> eigen(cor(golub))$values[1:5]
[1] 25.4382629  2.0757158  1.2484411  1.0713373  0.7365232
```

Because the eigenvalues are arranged in decreasing order the sixth to the 38th are smaller than one. Reason for which these will be neglected. The first eigenvalue is by far the largest, indicating that the persons are dependent to a large extent. Applying the previous bootstrap methods to estimate 95% confidence intervals for the eigenvalues we obtain the following intervals.

```
data <- golub; p <- ncol(data); n <- nrow(data) ; nboot<-1000
eigenvalues <- array(dim=c(nboot,p))
for (i in 1:nboot){dat.star <- data[sample(1:n,replace=TRUE),]
  eigenvalues[i,] <- eigen(cor(dat.star))$values}
```

```
> for (j in 1:p) print(quantile(eigenvalues[,j],c(0.025,0.975)))
    2.5%    97.5%
for (j in 1:5) cat(j,as.numeric(quantile(eigenvalues[,j],
 + c(0.025,0.975))),"\n" )
1 24.83581 26.00646
2 1.920871 2.258030
3 1.145990 1.386252
4 0.9917813 1.154291
5 0.6853702 0.7995948
```

The `cat` function allows for much control in printing. The null hypothesis of eigenvalue being equal to one is accepted for the fourth component and rejected for the first three and the fifth. Thus the fourth represents less variance than an individual variable, reason for which it is neglected.

The percentages of variance explained by the first two components can be computed by `sum(eigen(cor(golub))$values[1:2])/38*100`, which yields the amount 72.4052%. Thus the first two components represent more than 72% of the variance in the data. Hence, the data allow for a reduction in dimensions from thirthy eight to two.

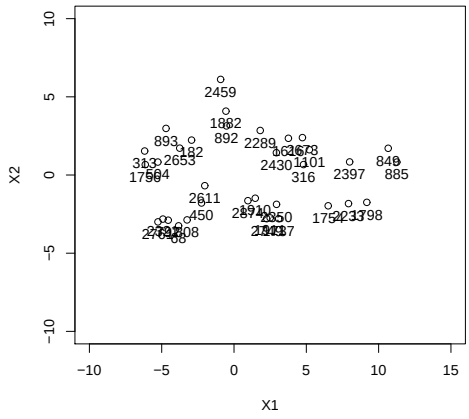


Figure 7.12: Scatter plot of selected genes with row labels on the first two principal components.

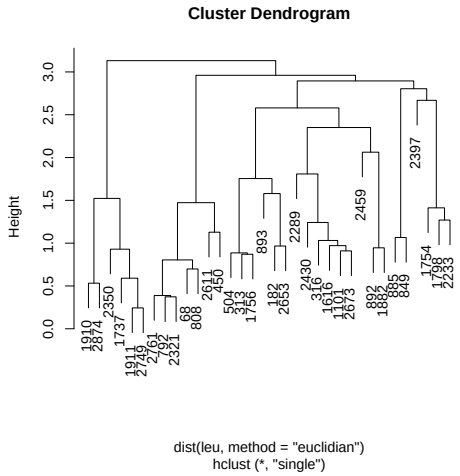


Figure 7.13: Single linkage cluster diagram of selected gene expression values.

It can be checked that all correlations between the patients are positive.

This implies that large expression values on gene i co-vary positively with large deviations of gene j. The positivity of the correlations also implies that the weights of the first eigenvector have the same sign, so that these can be taken to be positive for all patients (Horn & Johnson, 1985). Unfortunately, this is not automatic in R so that caution is in order with respect to interpretation of the components. By using `-eigen(cor(golub))$vec[,1:2]` to print the weights to the screen it can be observed that those that correspond to the first component are positive. All weights of the first eigenvector are positive and have very similar size (all are between 0.13 and 0.17). Thus the first component is almost equal to the sum of the variables (the correlation equals 0.9999). The weights of the second component have a very interesting pattern. Namely, almost all of the first 27 weights are positive and the last 11 weights are negative. Thus the second component contrasts the ALL patients with the AML patients. By contrasting ALL patients with AML patients a second to the largest amount of variance is explained in the data. Hence, the AML-ALL distinction is discovered by the second component, which is in line with findings of Golub et al. (1999).

Obviously the genes with the largest expression values from the first component can be printed. We shall, however, concentrate on the second component because it appears to be more directly related to the research intentions of Golub et. al. (1999). The first and the last ten gene names with respect to the values on the second component can be printed by the following.

```
> pca <- princomp(golub, center = TRUE, cor=TRUE, scores=TRUE)
> o <- order(pca$scores[,2])
> golub.gnames[o[1:10],2]
> golub.gnames[o[3041:3051],2]
```

Many of these genes are related to leukemia (Golub, et al., 1999). □

Example 3. Biplot. A useful manner to plot both genes (cases) and patients (variables) is the biplot, which is based on a two-dimensional approximation of the data very similar to principal components analysis. Here, we illustrate how it can be combined with principal components analysis.

```
> biplot(princomp(data,cor=TRUE),pc.biplot=TRUE,cex=0.5,expand=0.8)
```

The resulting plot is given by Figure 7.14. The left and bottom axis refer to the component scores and the top and right to the patient scores, which

are scaled to unit length by the specification `cor`. It can be seen that the patients are clearly divided in two groups corresponding to ALL and AML.

Example 4. Critical for S-phase. Golub et al. (1999) mention that among genes which are useful for tumor class prediction there are genes that encode for proteins critical for S-phase cell cycle progression such as Cyclin D3, Op18, and MCM3. We select genes which carry "CD", "Op", or "MCM" in their names and collect the corresponding row numbers.

```
data(golub, package = "multtest")
factor <- factor(golub.cl)
o1 <- grep("CD",golub.gnames[,2])
o2 <- grep("Op",golub.gnames[,2])
o3 <- grep("MCM",golub.gnames[,2])
o <- c(o1,o2,o3)
```

This yields 110 genes. In order to select those that do have an experimental effect, we use a two-sample t-test.

```
pt <- apply(golub, 1, function(x) t.test(x ~  gol.fac)$p.value)
oo <- o[pt[o]<0.01]
```

This yields 34 genes, of which the row numbers are selected in the vector `oo`. In order to identify genes in directions of large variation we use the scores on the first two principal components.

```
Z <- as.matrix(scale(golub, center = TRUE, scale = TRUE))
K <- eigen(cor(Z))
P <- Z %*% -K$vec[,1:2]
leu <- data.frame(P[oo,], row.names= oo)
attach(leu)
```

The scores on the first two principal components of the selected genes are stored in the data frame `leu`. From the plotted component scores in Figure 7.12, it seems that there are several sub-clusters of genes. The genes that belong to these clusters can be identified by hiearchical cluster analysis.

```
cl <- hclust(dist(leu,method="euclidian"),method="single")
plot(cl)
```

From the tree (dendrogram) in Figure 7.13 various clusters of genes are apparent that also appear in Figure 7.12. [3] The ordered genes can be obtained from the object `cl` as follows.

```
> a <- as.integer(rownames(leu)[cl$order])
> for (i in 1:length(a)) cat(a[i],golub.gnames[a[i],2],"\n")
1910 FCGR2B Fc fragment of IgG, low affinity IIb, receptor for (CD32)
2874 GB DEF = Fas (Apo-1, CD95)
```

The cluster with rows 504, 313, 1756, and 893 consists of antigenes. The genes MCM3 Minichromosome maintenance deficient (S. cerevisiae) 3 with row numbers 2289 and 2430 appear adjacent to each other. This illustrates that genes with similar functions may indeed be close with respect to their gene expression values. □

7.5 Overview and concluding remarks

Single linkage cluster analysis can be applied to explore for groups in a set of gene expressions. When groups are present a k-means cluster analysis can be applied in combination with the bootstrap to estimate confidence intervals for the cluster means.

The correlation coefficient measures the degree of dependency between pairs of gene expression values. It can also be used to find gene expressions which are highly dependent with a phenotypical variable. It is reassuring to find in applications that the confidence interval for a correlation coefficient is small.

Principal components analysis is very useful for finding directions in the data where the gene expression values vary maximally, see Jolliffe (2002) for a complete treatment of the principal component analysis. When these directions can be represented well by the first two components a biplot helps to simultaneously visualize genes and patients. Principal components analysis can be useful in identifying clusters of genes in a lower dimensional space.

[3] Unfortunately, some row numbers of genes are less readable because the points are very close.

7.6 Exercises

1. Cluster analysis on the "Zyxin" expression values of the Golub et al. (1999) data.

   (a) Produce a chatter plot of the gene expression values using showing different symbols for the two groups.

   (b) Use single linkage cluster analysis to see whether the three indicates two different groups.

   (c) Use k-means cluster analysis. Are the two clusters according to the diagnosis of the patient groups?

   (d) Perform a bootstrap on the cluster means. You will have to modify the code here and there. Do the confidence intervals for the cluster means overlap?

2. Close to CCND3 Cyclin D3. Recall that we did various analysis on the expression data of the CCND3 Cyclin D3 gene of the Golub (1999) data.

   (a) Use `genefilter` to find the ten closed genes to the expression values of CCND3 Cyclin D3. Give their probe as well as their biological names.

   (b) Produce of combined boxplot separately for the ALL and the AML expression values. Compare it with that on the basis of CCND3 Cyclin D3 and comment of the similarities.

   (c) Compare the smallest distances with those among the Cyclin genes computed above. What is your conclusion?

3. MCM3. In the example on MCM3 a plot shows that there is an outlier.

   (a) Plot the data and invent a manner to find the row number of the outlier.

   (b) Remove the outlier, test the correlation coefficient. Compare the results to those above.

   (c) Perform the bootstrap to construct a confidence interval.

4. Cluster analysis on part of Golub data.

(a) Select the oncogenes from the Golub data and plot the tree from a single linkage cluster analysis.

(b) Do you observe meaningful clusters.

(c) Select the antigenes and answer the same questions.

(d) select the receptor genes and answer the same questions.

5. Principal Components Analysis on part of the `ALL` data.

(a) Construct an expression set with the patients with B-cell in stage B1, B2, and B3. Compute the corresponding ANOVA p-values of all gene expressions. Construct the expression set with the p-values smaller than 0.001. Report the dimensionality of the data matrix with gene expressions.

(b) Are the correlations between the patients positive?

(c) Compute the eigenvalues of the correlation matrix. Report the largest five. Are the first three larger than one?

(d) Program a bootstrap of the largest five eigenvalues. Report the bootstrap 95% confidence intervals and draw relevant conclusions.

(e) Plot the genes in a plot of the first two principal components.

6. Some correlation matrices.

$$\begin{bmatrix} 1 & -0.8 \\ -0.8 & 1 \end{bmatrix}, \begin{bmatrix} 1 & 0.8 & 0.8 \\ 0.8 & 1 & 0.8 \\ 0.8 & 0.8 & 1 \end{bmatrix}, \begin{bmatrix} 1 & -0.5 & -0.5 \\ -0.5 & 1 & -0.5 \\ -0.5 & -0.5 & 1 \end{bmatrix},$$

(a) Verify that the eigenvalues of the matrices are `1.8, 0.2`, `2.6, 0.2, 0.2`, and `1.500000e+00, 1.500000e+00, -7.644529e-17`.

(b) How much variance represents the first component corresponding to the second matrix?

(c) Verify that the first eigen vector of the second correlation matrix has identical signs.

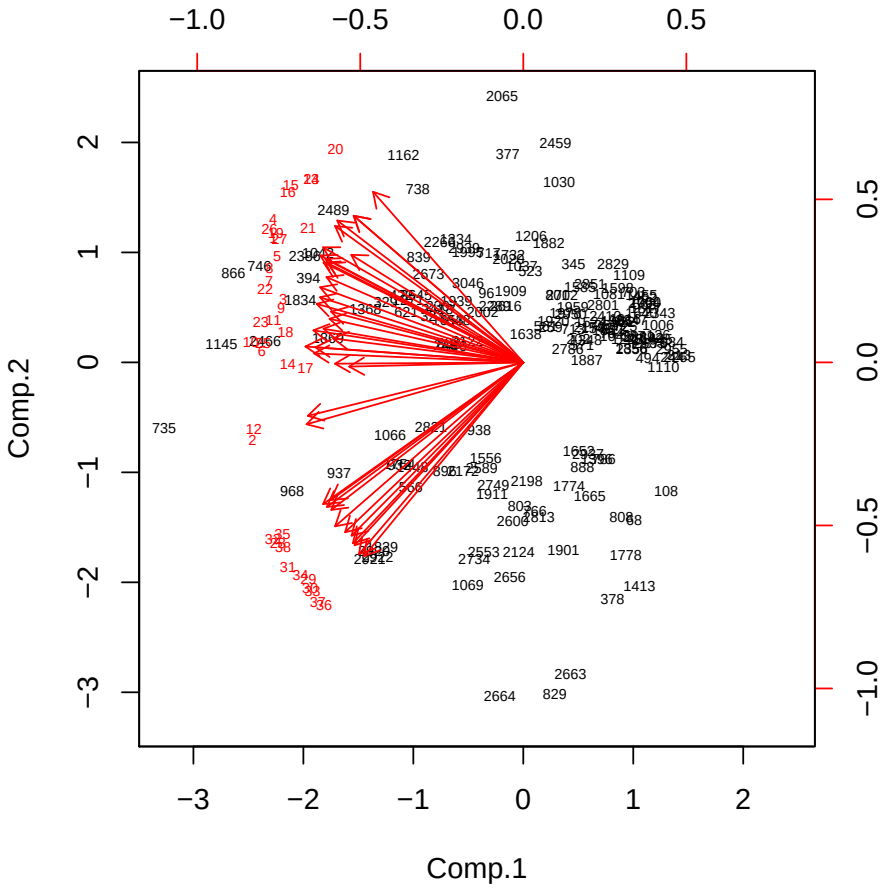


Figure 7.14: Biplot of selected genes from the golub data.

Chapter 8

Classification Methods

In medical settings groups of patients are often diagnosed into classes corresponding to types of diseases. In bioinformatics the question arises whether the diagnosis of a patient can be predicted by gene expression values? Related is the question which genes play an important role in the prediction of class membership. A similar question is the prediction of micro RNA's from values of folding energy. More generally, for objects like proteins, mRNA's, or microRNA's it may be of importance to classify these on the basis of certain measurements.

Many classification methods have been developed for various scientific purposes. In bioinformatics, methods such as recursive partitioning, support vector machine and neural network are frequently applied to solve classification problems.

In this chapter you learn what recursive partitioning is and how to use it. To evaluate the quality of prediction the fundamental concepts of sensitivity and specificity are frequently used. The specificity can be summarized in a single number by the area under the curve of a receiver operator curve. This will be explained and illustrated. Two other methods to predict disease class from gene expression data are the support vector machine and the neural network. It will briefly be explained what these methods are about and how these can be applied. A validation set will be used to evaluate the predictive accuracy.

8.1 Classification of microRNA

The subject of making a correct medical diagnosis is highly similar to that of correctly classifying microRNA.

Example 1. Classification of Micro RNA. MicroRNA are small RNA molecules with important functions in cell growth and disease development. In order to identify microRNA's from arbitrary sequences its characterizing properties are used to distinguish non-microRNA from microRNA molecules. One of these properties is that microRNA's have the capacity to fold in a certain hairpin type of structure. Such a structure typically exhibits a small minimum folding energy (Zuker, 2003; Zuker & Stiegler, 1981). This property can be used as a test to discriminate microRNA's from non-microRNA's (Bonnet, et al., 2004), as follows. Given a set of 3424 different microRNA's the minimum folding energy was computed for each of these. Next, for each microRNA the order of the nucleotides was shuffled with replacement 1000 times. This yielded per microRNA 1000 differently shuffled sequences of nucleotides for which the minimum folding energy is computed.[1] Per microRNA the 1001 energy values were arranged to have increasing order, similar as for empirical distributions in the previous chapter. Then the number of minimum folding energies below that of the original microRNA is counted and divided by 1001 as the p-value. If the minimum folding energie of the original microRNA is the smallest, then the empirical p-value is zero. This procedure yielded a total of 3424 p-values. The number of sequences with p-values below the threshold value 0.01 is given in Table 8.1. The same procedure is conducted for non-microRNA molecules which were taken as sequences with similar length and nucleotide percentages.

Table 8.1: Frequencies empirical p-values lower than or equal to 0.01.

	test positive $p \leq 0.01$	test negative $p > 0.01$	total
microRNA	2973	451	3424
non microRNA	33	3391	3424
total	3006	3842	6848

[1] I am obliged to Sven Warris for computing the minimum energy values.

From the frequency Table 8.1, the sensitivity, the specificity, and the predictive power can be computed in order to evaluate the quality of the test. The sensitivity is the probability that the test is positive given that the sequence is a microRNA (true positive). Thus

$$\text{sensitivity} = P(\text{true positive}) = P(\text{test positive}|\text{microRNA}) = \frac{2973}{3424} = 0.8682.$$

The specificity is the probability that the test is negative given that the sequence is not a microRNA (true negative). Thus

$$\text{specificity} = P(\text{true negative}) = P(\text{test negative}|\text{no microRNA}) = \frac{3391}{3424} = 0.9903.$$

For practical applications of a test the predictive power is of crucial importance. In particular, the predictive value positive is the probability that the sequence is a microRNA given that the test is positive. That is,

$$\text{Predictive value positive} = PV^+ = P(\text{microRNA}|\text{test positive}) = \frac{2973}{3006} = 0.9890$$

Thus when the test is positive we are 98.90% certain that the sequence is indeed a microRNA. The predictive value negative is the probability that the sequence is not a microRNA given that the test is negative.

$$\text{Predictive value negative} = PV^- = P(\text{no microRNA}|\text{test negative}) = \frac{3391}{3842} = 0.8826.$$

Thus when the test is negative we are 88.26% certain that the sequence is not a microRNA. From the estimated conditional probabilities it can be concluded that the test performs quite well in discriminating between microRNA's from non-microRNA's. □

8.2 ROC types of curves

In Chapter 2 we have observed with respect to the Golub et al. (1999) data that the expression values of gene CCND3 Cyclin D3 tend to be greater for ALL patients. We may therefore use these as a test for predicting ALL using a certain cutoff value. In particular, for gene expression values larger than a cutoff we declare the test "positive" in the sense of indicating ALL. By doing

so the corresponding true and false positives can be computed for each cutoff value. To briefly indicate the origin of the terminology, imagine that the test results are a characteristic received by an operator. The receiver operator characteristic (ROC) is a curve where the false positive rates are depicted horizontally and the true positive rates vertically. The larger the area under the ROC curve, the better the test is because then low false positive rates go together with large true positive rates.[2] These ideas are illustrated by several examples.

Example 1. For the sake of illustration we consider the prediction of ALL from the expression values for gene CCND3 Cyclin D3 from Golub et al. (1999) in row 1042 of the matrix `golub`. Now consider cutoff point 1.27. For such a cutoff point we can produce a table with TRUE/FALSE frequencies of predicting ALL/not ALL.

```
> data(golub, package = "multtest")
> gol.true <- factor(golub.cl,levels=0:1,labels= c(" ALL","not ALL"))
> gol.pred <- factor(golub[1042,]>1.27,levels=c("TRUE","FALSE"),
   labels=c("ALL","notALL"))
> table(gol.pred,gol.true)
            gol.true
gol.pred  ALL not ALL
  ALL      25       1
  notALL    2      10
```

There are 25 ALL patients with expression values greater than or equal to 1.27, so that the true positive rate is 25/27=0.93. For this cutoff value there is one false positive because one patient without ALL has a score larger than 1.27. Hence, the false positive rate is $1/11 = 0.09$. □

Example 2. The expression values for gene CCND3 Cyclin D3 from the Golub et al. (1999) data are sorted in decreasing order, see Table 8.2. The procedure to draw the ROC curve starts with cutoff point infinity. Obviously, there are no expression values equal to infinity, so there is no patient tested positive. Next, the cut off point 2.77 is taken and values greater than or equal to 2.77 are tested as positive. This yields one true positive implying a

[2] More detailed information can be obtained from a wikipedia search using "ROC curve".

true positive rate of 1/27, see second row of Table 8.2. For this cutoff value there are no negatives so that the false positive rate is zero.

Now consider cutoff point 1.52. There are 22 ALL patients with expression values greater than or equal to 1.52, so that the true positive rate is 22/27=0.81. For this cutoff value there are no false positives because all patients without ALL have expression values lower than 1.51. Hence, the false positive rate is 0 and the true positive rate is 0.81. To indicate this there is a vertical line drawn in the ROC curve from point $(0, 0)$ to point $(0, 0.81)$ in Figure 8.1. Now consider the next cutoff point 1.45. There are 22 ALL patients with expression values greater than or equal to 1.45, so that the true positive rate is again 22/27=0.81. However, there is one patient without ALL having expression value 1.45, whom receives therefore a positive test. Hence, the number of false positives increases from zero to one, which implies a false positive rate of 1/11=0.09. In the ROC curve this is indicated by point $(0.09, 0.81)$ and the horizontal line from $(0, 0.81)$ to $(0.09, 0.81)$, see Figure 8.1.

This process goes on (see Table 8.2) until the smallest data point -0.74 is taken as cutoff point. For this point all patients are tested positive, so that the false positive rate is 11/11 and the true positive rate is 27/27. This is indicated by the end point $(1, 1)$ in the plot at the top on the right hand side.

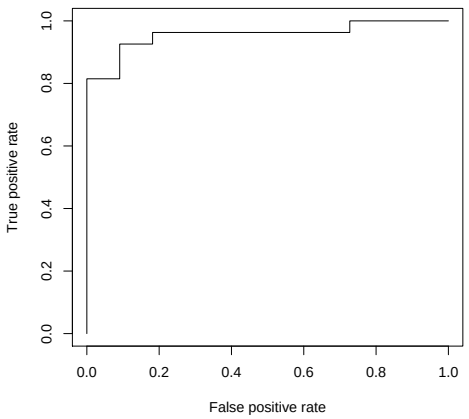


Figure 8.1: ROC plot for expression values of CCND3 Cyclin D3.

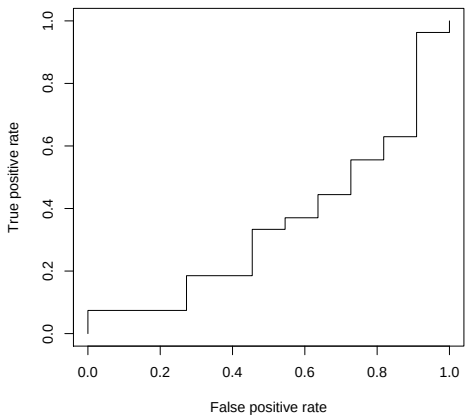


Figure 8.2: ROC plot for expression values of gene Gdf5.

It is obviously helpful to use a computer for producing an ROC such as in Figure 8.1. To do so we construct an appropriate factor with the value TRUE for ALL and FALSE for not ALL `gol.true` and use functions from the `ROCR` package.

```
library(ROCR)
gol.true <- factor(golub.cl,levels=0:1,labels= c("TRUE","FALSE"))
pred <- prediction(golub[1042,], gol.true)
perf <- performance(pred, "tpr", "fpr" )
plot(perf)
```

It seems clear that the expression values are better in testing for ALL when the curve is very steep in the beginning and attains its maximum value soon. In such a case the true positive rate is large for a small false positive rate. A manner to express the predictive accuracy of a test in a single number is by the area under the curve. Using the function `performance(pred,"auc")` we obtain that the area under the curve is 0.96, which is large. Hence, the expression values of CCND3 Cyclin D3 are suitable for discrimination between ALL and not ALL (AML). The ROC curve for the expression values of gene Gdf5 is given by Figure 8.2. It can be observed that the true positive rate is much lower as one moves on the horizontal axis from the left to the right. This corresponds to the area under the curve of 0.35, which is small. This illustrates that genes may express large differences with respect to prediction of the disease status of patients. □

In practical applications one is often interested in a single optimal cut-off value and in combining several predictors in a decision scheme.

8.3 Classification trees

The purpose of classification is to allocate organisms into classes on the basis of measurements on attributes. For instance, in case of the Golub et al. (1999) data the organisms are 38 patients which have measurements on 3051 genes. The classes consist of diagnosis of patients into the ALL class (27 patients) and the AML class (11 patients). A tree model resembles that of a linear model, where the criterion is the factor indicating class membership and the predictor variables are the gene expression values. In case of, for instance, the Golub et al. (1999) data the gene expression values $\{x_1, \cdots, x_{38}\}$

can serve as predictors to form a decision tree. For instance, if $x_j < t$, then patient j is AML, and otherwise if $x_j \geq t$, then patient j is ALL. Obviously, the threshold value t on which the decision is based should be optimal given the predictor. Such can be estimated by a regression tree (Breiman et al., 1984; Chambers & Hastie, 1992; Venables, & Ripley, 2000), which is implemented in the `rpart` package (Therneau & Atkinson, 1997).

A training set is used to estimate the threshold values that construct the tree. When many predictor variables are involved, 3051 for instance, then we have a tremendous gene (variable) selection problem. The `rpart` package automatically selects genes which are important for classification and neglects others. A further problem is that of overfitting where additional nodes of a tree are added to increase prediction accuracy. When such nodes are specific for the training sample set, these can not be generalized to other samples so that these are of limited scientific value. Prevention of such overfitting is called pruning and is automatically done by the `rpart` function. Many basic ideas are illustrated by an elementary example.

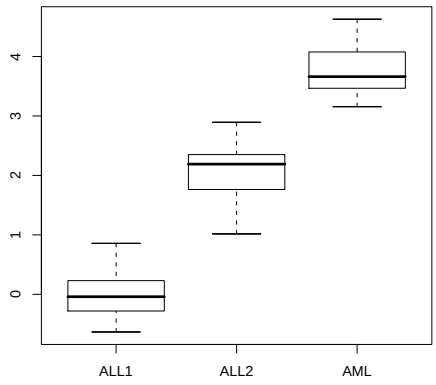


Figure 8.3: Boxplot of expression values of gene a for each leukemia class.

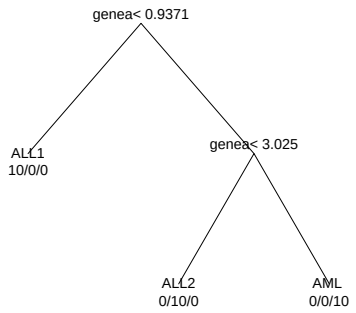


Figure 8.4: Classification tree for gene for three classes of leukemia.

Example 1. Optimal gene expressions. Suppose microarray expression data are available with respect to patients suffering from three types of

leukemia abbreviated as ALL1, ALL2, and AML. Gene A has expression values from the populations (patient groups) $N(0, 0.5^2)$ for ALL1, $N(2, 0.5^2)$ for ALL2, and $N(4, 0.5^2)$ for AML. The script below generates thirty expression values for gene A, the patients of the three disease classes, and the estimates of the classification tree.

```
set.seed(123); n<-10 ; sigma <- 0.5
fac <- factor(c(rep(1,n),rep(2,n),rep(3,n)))
levels(fac) <- c("ALL1","ALL2","AML")
geneA <- c(rnorm(10,0,sigma),rnorm(10,2,sigma),rnorm(10,4,sigma))
dat <- data.frame(fac,geneA)
library(rpart)
rp  <-  rpart(fac ~ geneA, method="class",data=dat)
plot(rp, branch=0,margin=0.1);  text(rp, digits=3, use.n=TRUE)
```

From the boxplot in Figure 8.3 it can be observed that there is no overlap of gene expressions between classes. This makes gene A an ideal predictor for separating patients into classes. By the construction of the gene expression values $x_1, \cdots, x_{30}$ we expect the following partition. If $x_i < 1$, then ALL1, if x_i is in interval $[1, 3]$, then ALL2, and if $x_i > 3$, then AML. From the estimated tree in Figure 8.4 it can be observed that the estimated splits are close to our expectations: If $x_i < 0.971$, then ALL1, if x_i is in $[0.9371, 3.025]$, then ALL2, and if $x_i > 3.025$, then AML. The tree consists of three leaves (nodes) and two splits. The prediction of patients into the three classes perfectly matches the true disease status. □

Obviously, such an ideal gene need not exist because the expression values overlap between the disease classes. In such a case more genes may be used to build the classification tree.

Example 2. Gene selection. Another situation is where Gene A discriminates between ALL and AML and Gene B between ALL1 patients and ALL2 or AML patients and Gene C does not discriminate at all. To simulate this setting we generate expression values for Gene A from $N(0, 0.5^2)$ for both ALL1 and ALL2, and from $N(2, 0.5^2)$ for AML patients. Next, we generate expression values for Gene B from $N(0, 0.5^2)$ for ALL1 and from $N(2, 0.5^2)$ for ALL2 and AML. Finally, we generate for Gene C from $N(1, 0.5^2)$ for ALL1, ALL2, and AML. For this and for estimating the tree, we use the following script.

```
set.seed(123)
n<-10 ; sigma <- 0.5
fac <- factor(c(rep(1,n),rep(2,n),rep(3,n)))
levels(fac) <- c("ALL1","ALL2","AML")
geneA <- c(rnorm(20,0,sigma),rnorm(10,2,sigma))
geneB <- c(rnorm(10,0,sigma),rnorm(20,2,sigma))
geneC <- c(rnorm(30,1,sigma))
dat <- data.frame(fac,geneA,geneB,geneC)
library(rpart)
rp  <-  rpart(fac ~ geneA  + geneB + geneC, method="class",data=dat)
```

Note the addition in the model notation for the `rpart` function.[3] It is convenient to collect the data in the form of a data frame.[4]

From the boxplot in Figure 8.5 it can be seen that Gene A discriminates well between ALL and AML, but not between ALL1 and ALL2. The expression values for Gene B discriminate well between ALL1 and ALL2, whereas those of Gene C do not discriminate at all. The latter can also be seen from the estimated tree in Figure 8.6, where Gene C plays no role at all. This illustrates that `rpart` automatically selects the genes (variables) which play a role in the classification tree. Expression values on Gene A larger than 1.025 are predicted as AML and smaller ones as ALL. Expression values on Gene B smaller than 0.9074 are predicted as ALL1 and larger as ALL2. Hence, Gene A separates well within the ALL class. □

Example 3. Prediction by CCND3 Cyclin D3 gene expression values. From various visualizations and statistical testing in the previous chapters, it can be conjectured that CCND3 Cyclin D3 gene expression values form a suitable predictor for discriminating between ALL and AML patients. Note, however, from Figures 2.2 and 8.7 that there is some overlap between the expression values from the ALL and the AML patients, so that a perfect classification is not possible. By the function `rpart` the regression partitioning can be computed as follows.

```
> library(rpart);data(golub); library(multtest)
> gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
> gol.rp  <-  rpart(gol.fac ~ golub[1042,] , method="class")
```

[3] See Chapter 11 of the manual "An Introduction to R" for more on model notation.
[4] See Chapter 6 of the manual "An Introduction to R" for more on data frames.

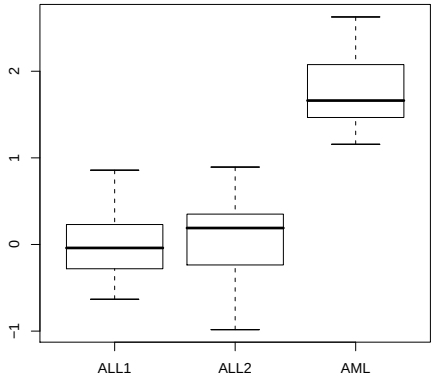


Figure 8.5: Boxplot of expression values of gene a for each leukemia class.

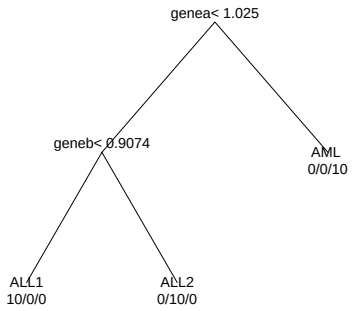


Figure 8.6: Classification tree of expression values from gene A, B, and C for the classification of ALL1, ALL2, and AML patients.

```
> predictedclass <- predict(gol.rp, type="class")
> table(predictedclass, gol.fac)
              gol.fac
predictedclass ALL AML
           ALL  25   1
           AML   2  10
```

Note that $(25 + 10)/38 \cdot 100\% = 92.10\%$ of the ALL/AML patients are correctly classified by gene CCND3 Cyclin D3. By the function `predict(gol.rp,type="class")` the predictions from the regression tree of the patients in the two classes can be obtained. The factor `gol.fac` contains the levels `ALL` and `AML` corresponding to the diagnosis to be predicted. The predictor variable consists of the expression values of gene CCND3 Cyclin D3. The output of recursive partitioning is assigned to an object called `gol.rp`, a list from which further information can be extracted by suitable functions. A summary can be obtained as follows.

```
> summary(gol.rp)
Call:
```

```
rpart(formula = gol.fac ~ golub[1042, ], method = "class")
  n= 38

         CP nsplit rel error    xerror      xstd
1 0.7272727      0 1.0000000 1.0000000 0.2541521
2 0.0100000      1 0.2727273 0.5454545 0.2043460

Node number 1: 38 observations,    complexity param=0.7272727
  predicted class=ALL  expected loss=0.2894737
    class counts:    27    11
   probabilities: 0.711 0.289
  left son=2 (26 obs) right son=3 (12 obs)
  Primary splits:
      golub[1042, ] < 1.198515 to the right, improve=10.37517, (0 missing)

Node number 2: 26 observations
  predicted class=ALL  expected loss=0.03846154
    class counts:    25     1
   probabilities: 0.962 0.038

Node number 3: 12 observations
  predicted class=AML  expected loss=0.1666667
    class counts:     2    10
   probabilities: 0.167 0.833

26
[1] 0.03846154
```

The expected loss in prediction accuracy of Node number 2 is 1/26 and that of Node number 3 is 2/12. This equals the probabilities from the class counts. The primary splits gives the estimated threshold value. To predict the class of the individual patients one may use the function `predict`, as follows.

```
> predict(gol.rp,type="class")
  1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18  19  20
ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL ALL AML ALL ALL ALL
 21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38
AML ALL ALL ALL ALL ALL ALL AML ALL AML AML AML AML AML AML AML AML AML
Levels: ALL AML
```

Hence, Patient 17 and 21 are erroneously predicted as AML and Patient 29 is erroneously predicted in the ALL class. A more precise output is obtained by asking for the probability of class membership.

```
> predict(gol.rp, type="prob")
         ALL        AML
1  0.9615385 0.03846154
2  0.9615385 0.03846154
etc.
```

Based on this the probability of patient 21 to have ALL is 0.16 and that to have AML is 0.83. □

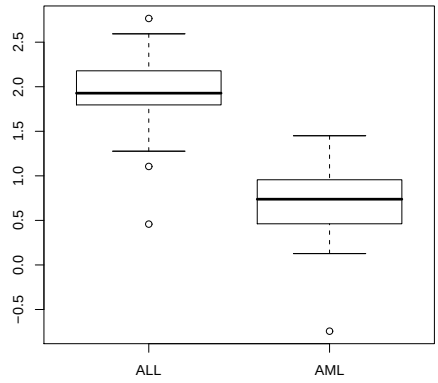


Figure 8.7: Boxplot of expression values from gene CCND3 Cyclin D3 for ALL and AML patients

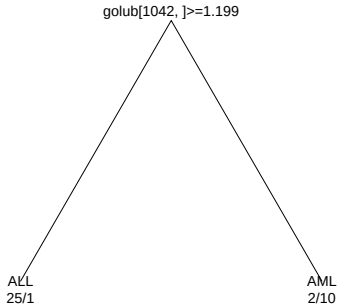


Figure 8.8: Classification tree of expression values from gene CCND3 Cyclin D3 for classification of ALL and AML patients.

Example 4. Gene selection of the Golub (1999) data. By recursive partitioning it is possible to select among the genes of Golub et al. (1999) those which give the best partitioning. For the latter to work we have to specify the gene expressions as the variables (columns). For this we use the transposition operator `t`. To facilitate reading the output we add `gene 1` to `gene 3051` as column names.

```
library(rpart);data(golub); library(multtest)
row.names(golub)<- paste("gene", 1:3051, sep = "")
goldata <- data.frame(t(golub[1:3051,]))
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
gol.rp  <-  rpart(gol.fac~., data=goldata, method="class", cp=0.001)
plot(gol.rp, branch=0,margin=0.1);  text(gol.rp, digits=3, use.n=TRUE)
golub.gnames[896,]
```

Inspection of the plot yields gene "FAH Fumarylacetoacetate" as the predictor by which the two classes of patients can be predicted perfectly. □

In order to further illustrate possibilities of classification methods we use the ALL data collected by Chiaretti, et al. (2004), see also Chapter 6.

Example 5. Application to the Chiaretti (2004) data. With respect to the ALL data we want to predict from the gene expressions the diagnosis of B-cell State B1, B2, and B3. Since the complete set of 12625 gene expressions is too large, we select the genes with different means over the patients groups. It is obvious that only these gene can contribute to the prediction of the disease states. In particular we select the gene with ANOVA p-value is smaller than 0.000001.

```
library("hgu95av2.db");library(ALL);data(ALL)
ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
pano <- apply(exprs(ALLB123), 1, function(x) anova(lm(x ~ ALLB123$BT))$Pr[1])
names <- featureNames(ALL)[pano<0.000001]
symb <- mget(names, env = hgu95av2SYMBOL)
ALLBTnames <- ALLB123[names, ]
probedat <- as.matrix(exprs(ALLBTnames))
row.names(probedat)<-unlist(symb)
```

The probe symbols are extracted from the `hgu95av2SYMBOL` environment and used as row names to facilitate readability of the resulting tree. There are 78 patients selected and 29 probes. The recursive partitioning to find the tree can be performed by the following script.

```
> diagnosed <- factor(ALLBTnames$BT)
> tr <- rpart(factor(ALLBTnames$BT) ~ ., data = data.frame(t(probedat)))
> plot(tr, branch=0,margin=0.1);  text(tr, digits=3, use.n=TRUE)
```

```
> rpartpred <- predict(tr, type="class")
> table(rpartpred,diagnosed)
         diagnosed
rpartpred B1 B2 B3
       B1 17  2  0
       B2  1 33  5
       B3  1  1 18
```

The rows to the left of the table give the frequencies of the predicted B cell stages and the columns on top the diagnosed B cell stages from the factor. The matrix with frequencies of the predicted and true patient status is often called a "confusion table". The resulting tree in Figure 8.9 should be read as follows. If gene expression MME is strictly smaller than the cutoff value 8.395, then the patient is predicted to be in state (class) B1. If the expression of LSM6 smaller than 4.192, then the predicted state is B2, and if it is larger than the predicted state it is B3.

The misclassification rate is 10/78=0.1282051, which is low, but not zero. It may happen that the probability of the predicted class is close to that of the diagnosed. An overview of the latter can be obtained as follows.

```
predicted.class <- predict(tr, type="class")
predicted.probabilities <- predict(tr, type="prob")
out <- data.frame(predicted.probabilities,predicted.class,
  diagnosis=factor(ALLBTnames$BT))
> print(out,digits=2)
         B1   B2   B3 predicted.class diagnosis
01005 0.026 0.85 0.13              B2        B2
01010 0.026 0.85 0.13              B2        B2
04006 0.895 0.11 0.00              B1        B1
04007 0.026 0.85 0.13              B2        B2
04008 0.895 0.11 0.00              B1        B1
04010 0.050 0.05 0.90              B3        B1
04016 0.895 0.11 0.00              B1        B1
06002 0.026 0.85 0.13              B2        B2
08001 0.026 0.85 0.13              B2        B2
08011 0.026 0.85 0.13              B2        B3
08012 0.026 0.85 0.13              B2        B3
08018 0.050 0.05 0.90              B3        B3
08024 0.895 0.11 0.00              B1        B2
```

```
09008 0.026 0.85 0.13              B2         B3
...
```

For instance, the sixth patient is with probability .90 in class B3 and with probability .05 in class B1, which is the diagnosed disease state. □

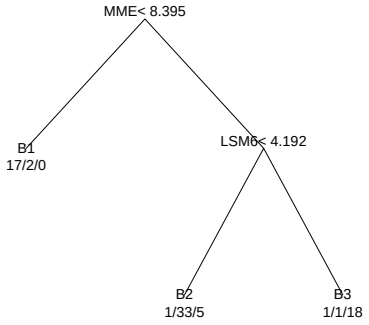


Figure 8.9: rpart on ALL B-cel 123 data.

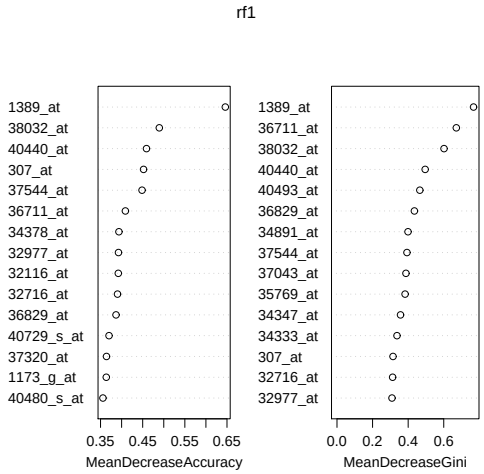


Figure 8.10: Variable importance plot on ALL B-cell 123 data.

Note the reduction in variables from twenty nine to two in the actual construction of the tree. In a construction like this the gene expressions (variables) are linearly dependent in the sense that once the first gene is selected for the first split, then highly similar ones are not selected anymore. It can be instructive to leave out the variables selected from the data and to redo the analysis.

A generally applied manner to evaluate an estimated model is by its predictive accuracy with respect to a future data set. When such a future data set is not available, it is common practice to split the available data in two parts: A training set and a validation set. Then the model is estimated from the training set and this is used to predict the class of the patients in the validation set. Then a confusion matrix is constructed with the frequencies of true classes against predicted classes. Next, the misclassification rate can be computed to evaluate the predictive accuracy. This can very well be seen

as a method to detect for over fitting where the model estimates are so data specific that generalization to future data sets is in danger.

Example 6. Training and validation. In the setting of B-cell ALL data with State 1, 2, and 3 the manner to split the data centers around randomly splitting the patients in two halves. The 78 patients in State 1, 2 or 3 can be split in two halves, as follows.

```
i <- sample(1:78, 39, replace = FALSE)
noti <- setdiff(1:78,i)
df <- data.frame(Y = factor(ALLBTnames$BT), X =t(probedat))
rpart.est <- rpart(Y ~ ., data = df, subset=i)
rpart.pred.t <- predict(rpart.est, df[i,], type="class")
> table(rpart.pred.t,factor(ALLBTnames$BT[i]))
rpart.pred.t B1 B2 B3
          B1 11  1  0
          B2  0 12  0
          B3  0  1 14
> rpart.pred.v <- predict(rpart.est,df[noti,], type="class")
> table(rpart.pred.v,factor(ALLBTnames$BT[noti]))
rpart.pred.v B1 B2 B3
          B1  6  1  0
          B2  1 19  3
          B3  1  2  6
```

The misclassification rate in the training set is $2/39 = 0.05$ and in the validation set is $7/39 = 0.18$. Note that the differences mainly occur between State 2 and 3. Generally the prediction of disease state from the training set is better because the model is estimated from these data. □

The same split of the data into training and validation set will be used for other methods as well.

8.4 Support Vector Machine

A support vector machine finds separating lines (hyper planes) between groups of points. This works like a classification problem where the classes

of patients are to be predicted from gene expression values. If such separating lines do exist in the data, then a linear support vector machine will find these. This is because the optimization method behind it is based on quadratic programming by iterative algorithms which find the globally optimal solution with certainty. Support vector machines do not automatically select variables and are designed for continuous predictor variables. Since the mathematical details are beyond the current scope, we shall confine with illustrating applications to gene expression data.

Example 1. Application to the Chiaretti (2004) data. The parameters for the support vector machine can be determined by the function `svm` from the `e1071` package, as follows.

```
library(e1071)
df <- data.frame(Y = factor(ALLBTnames$BT), X =t(probedat))
Y <- factor(ALLBTnames$BT);X <- t(probedat)
svmest <- svm(X, Y, data=df, type = "C-classification", kernel = "linear")
svmpred <- predict(svmest, X, probability=TRUE)
> table(svmpred, factor(ALLBTnames$BT))
svmpred B1 B2 B3
     B1 19  0  0
     B2  0 36  1
     B3  0  0 22
```

The confusion matrix shows that the misclassification rate of the three classes of B-cell ALL is 1/78=0.0128 is very small, so that the prediction is almost perfect. Note, however, from `summary(svmest)` that the number of support vectors per class equals 20, 9, and 11, for class B1, B2, and B3, respectively. These have values for all input variables (genes) as can be obtained from `dim(svmest$SV)` and the coefficient vectors `dim(svmest$coefs)`. Hence, the excellent prediction properties are obtained by a very large number of estimated parameters. □

Example 2. Training and validation. A generally applied manner to evaluate the predictive quality of an estimated model is by splitting the data into a training and a validation set. The model is estimated by the training set and then the class of the patients in the validation set is predicted. We shall use the same split as in Example 6 of the previous section.

```
> Yt <- factor(ALLBTnames$BT)[i]; Yv <- factor(ALLBTnames$BT)[noti]
> X <- t(probedat); Xt <- X[i,]; Xv <- X[noti,]
> svmest <- svm(Xt, Yt, type = "C-classification", kernel = "linear")
> svmpredt <- predict(svmest, Xt, probability=TRUE)
> table(svmpredt, Yt)
        Yt
svmpredt B1 B2 B3
      B1 11  0  0
      B2  0 14  0
      B3  0  0 14
> svmpredv <- predict(svmest, Xv, probability=TRUE)
> table(svmpredv, Yv)
        Yv
svmpredv B1 B2 B3
      B1  5  0  0
      B2  1 19  4
      B3  2  3  5
```

The predictions of the disease states of the patients from the training set perfectly match the diagnosed states. The predictions, however, of the classes of the patients from the validation set have misclassification rate 10/39=0.25 and are therefore less accurate. Hence, the parameter estimates from the training set are sample specific and do not generalize with the same accuracy to the validation set. □

8.5 Neural Networks

Neural networks are nonlinear models consisting of nonlinear hyperplanes around classes of objects given a set of prediction variables (Ripley, 1996). We confine with illustrating the method by two examples.

Example 1. Application to the Chiaretti (2004) data. The models can be estimated by the function `nnet` from the package that goes under the same name. To avoid having to many variables we randomly select a subset of 20 genes.

```
> Y <- factor(ALLBTnames$BT);X <- t(probedat)
```

```
> library(nnet)
> df <- data.frame(Y = Y, X = X[, sample(ncol(X), 20)])
> nnest <- nnet(Y ~ .,data = df, size = 5, maxit = 500, decay = 0.01,
  + MaxNWts = 5000)
> pred <- predict(nnest, type = "class")
> table(pred, Y) # prints confusion ma
    Y
pred B1 B2 B3
  B1 19  0  0
  B2  0 36  0
  B3  0  0 23
```

The confusion matrix shows that zero out of 78 patients are mis-classified.□

Example 2. Training and validation. The results from cross validation on the neural networks are as follows.

```
> nnest.t <- nnet(Y ~ ., data = df,subset=i, size = 5,decay = 0.01,
 + maxit=500)
> prednnt <- predict(nnest.t, df[i,], type = "class")
> table(prednnt,Ytrain=Y[i])
       Ytrain
prednnt B1 B2 B3
     B1 11  0  0
     B2  0 14  0
     B3  0  0 14
> prednnv <- predict(nnest.t, df[noti,], type = "class")
> table(prednnv, Yval= Y[noti])
       Yval
prednnv B1 B2 B3
     B1  4  1  0
     B2  4 17  4
     B3  0  4  5
```

The predictions on the training set have misclassification rate zero and that on the validation set 13/39=0.33. □

8.6 Generalized Linear Models

Within the framework of generalized linear models the diagnosis of a patient is seen as a response. In case the response has the values healthy or disease for which it may be assumed that the binomial distribution holds with a succes probability p_i. Recall from Chapter 3 that for a binomially distributed variable with y_i successes out of n_i it holds that the probability of y_i successes out of ni equals

$$P(Y_i = y_i) = \frac{n_i!}{y_i!(n_i - y_i)!} p_i^{y_i} (1 - p_i)^{n_i - y_i}, \qquad \text{for} \quad k = 0, \cdots, n_i.$$

The value of p_i is closely related to one or more predictor variables x_1 and x_2 via a linear combination. That is, the linear model holds such that $\eta_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2}$. The predictors are linked to the succes probability via the so-called logit link

$$p_i = \frac{e^{\eta_i}}{e^{\eta_i} + 1} = \frac{\exp(\beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2})}{1 + \exp(\beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2})}.$$

Rather than going deeper into the details, the usefulness of generalized linear models will be illustrated with two examples.

Example 1. CCND3 Cyclin D3. In the Golub et al. (1999) data we may model $Y_i = 1$ if the patient is diagnosed as ALL and $Y_i = 0$ if (s)he is diagnosed as AML. We use the CCND3 Cyclin D3 gene expression values as predictor. To will be convenient to compute the response by `-golub.cl + 1`. This yield 1 for ALL and 0 for not ALL[5].

```
library(faraway)
logitmod <- glm((-golub.cl + 1) ~ golub[1042,],
  family=binomial(link = "logit"))
pchisq(deviance(logitmod),df.residual(logitmod),lower=FALSE)
plot((-golub.cl + 1) ~ golub[1042,], xlim=c(-2,5), ylim = c(0,1),
  xlab="CCND3 expression values ", ylab="Probability of ALL")
x <- seq(-2,5,.1)
lines(x,ilogit(-4.844124  + 4.439953*x))
pchisq(deviance(logitmod),df.residual(logitmod),lower=FALSE)
```

[5] One may also conveniently use a factor as response variable

```
> summary(logitmod)

Call:
glm(formula = (-golub.cl + 1) ~ golub[1042, ], family = binomial(link = "logit"))

Coefficients:
              Estimate Std. Error z value Pr(>|z|)
(Intercept)     -4.844      1.849  -2.620  0.00880 **
golub[1042, ]    4.440      1.488   2.984  0.00284 **

    Null deviance: 45.728  on 37  degrees of freedom
Residual deviance: 18.270  on 36  degrees of freedom
AIC: 22.270
```

From Figure 8.11 it can be seen that the logit curve fits the data fairly well. From the summary of the output it can be seen that the estimated intercept is -4.844 and the estimated slope is 4.440. Both coefficients are significantly different from zero. The goodness-of-fit value of the model is computed from the chi-square distribution and equals .99. The model fits the data well. The predictive accuracy of the model may be obtained as follows.

```
> pred <- predict(logitmod,type="response") > 0.5
> pred.fac <- factor(pred,levels=c(TRUE,FALSE),labels=c("ALL","not ALL"))
> table(pred.fac,gol.fac)
         gol.fac
pred.fac  ALL AML
  ALL      26   2
  not ALL   1   9
```

The diagnosis of the majority of patients is predicted correctly. □

Example 2. Application to the Chiaretti (2004) data. With respect to the ALL data we want model the diagnosis of B-cell State B1, B2, and B3 as a response. The factor representing these levels can be used as input for the response. Here we use the gene expressions with greatest importance from the classification tree in Section 8.3. We assign the biological name to the predictor variables and estimate the generalized linear model.

```
library(nnet);library("hgu95av2.db");library(ALL);data(ALL)
```

```
probe.names <- c("1389_at","35991_at","40440_at")
ALLB123 <- ALL[,ALL$BT %in% c("B1","B2","B3")]
probedat <- exprs(ALLB123)[probe.names,]
row.names(probedat) <- unlist(mget(probe.names, env = hgu95av2SYMBOL))
fac <- factor(ALLB123$BT,levels=c("B1","B2","B3"))
dat <- data.frame(fac,t(probedat))
mnmod <- multinom(fac ~ ., family=binomial(link = "logit"),data=dat)
> summary(mnmod)
Call:
multinom(formula = fac ~ ., data = dat, family = binomial(link = "logit"))

Coefficients:
   (Intercept)     MME       LSM6    SERBP1
B2    14.36158 4.14002 -0.8494635 -5.104337
B3   -12.90584 4.94908  4.7415802 -5.655420

Std. Errors:
   (Intercept)      MME     LSM6   SERBP1
B2    16.36959 1.367058 1.716513 2.222486
B3    17.97896 1.424526 1.744425 2.313700

Residual Deviance: 59.88298
AIC: 75.88298
```

Apart from the intercepts, the estimated coefficients are significantly different from zero.

```
> predmn <- predict(mnmod,type="class")
> table(predmn,fac)
      fac
predmn B1 B2 B3
    B1 17  2  1
    B2  1 31  5
    B3  1  3 17
```

The model predict the diagnosed classes quite well. □

Generalized linear models are statistical models which have to be estimated by an iterative process which may need some computation time. It

has the advantage that confidence intervals or the significance of the parameters can be estimated. On the other hand, using statistical model building procedures may not designed to handle large amounts of predictor variables. Hence, the researcher does need to have some idea on which gene expressions (s)he want to use as predictors. Indeed, better models than those estimated in the above example may certainly exist.

8.7 Overview and concluding remarks

Central themes in prediction methods are the face validity (clarity) of the model, the size of the model, and predictive accuracy on a validation set. For many researchers it is of crucial importance to have a clear idea on what a method is essentially doing. Some models and their estimation procedures are mathematically intricate and seem to be recollected in the mind of many researchers as black boxes. Even from a more pragmatic point of view such need not be devastating if the predictive accuracy is excellent. However, support vector machines and neural networks typically use a large number of parameters to predict well on a test set, but less well on validation sets. It is, furthermore, questionable whether a zero misclassification rate is rational since patients may be misclassified by the diagnosis or very close to transferring from one state to the other.

Recursive partitioning to estimate a classification tree performs very well on variable selection and pruning in order to discover as few variables (gene expressions) as possible for maximum predictive accuracy. In addition, it seems obvious that classification trees have great clarity, see e.g. the CART package (Breiman et al., 1984) for further types of recursive trees. Note that several methods have different misclassification rates with respect to the whole sample, but comparable rates on the validation sets. It should, however, be clear that when there are non-linear relationships between predictor variables and classes, then nonlinear models should outperform linear ones[6].

8.8 Exercises

1. Classification tree of Golub data. Use recursive partitioning in `rpart`

[6]Some people may want to use the `ade4TkGUI()`

(a) Find a manner to identify an optimal gene with respect the Golub data to prediction of the ALL AML patients.

(b) Explain what the code does.

(c) Use `rpart` to construct the classification tree with the genes that you found. Does it have perfect predictions?

(d) Find the row number of gene Gdf5, which is supposed not to have any relationship with leukemia. Estimate a classification tree and report the probability of misclassification. Give explanations of the results.

2. Sensitivity versus specificity.

(a) Produce a sensitivity versus specificity plot for the gene expression values of CCND3 Cyclin D3.

(b) In what sense does it resemble Figure 8.2.

(c) Compute the area under the curve for sensitivity versus specificity curve.

3. Comparing Classification Methods. To obtain an idea on the misclassification rate when there is no relation between the predictors and the factor indicating groups, we perform a small simulation study.

(a) Construct a factor with 100 values one and two and a matrix with predictor variables of 500 by 4 with values from the normal distribution. Use the first four letters of the alphabet for the column names.

(b) Use `rpart` to construct a recursive tree and report the misclassification rate. Comment on the results.

(c) Do the same for support vector machines.

(d) Do the same for neural networks.

(e) Think through your results and comment on these.

4. Prediction of achieved remission. For the ALL data from its `ALL` library the patients are checked for achieving remission. The variable `ALL$CR` has values CR (became healthy) and REF (did not respond to therapy; remain ill).

(a) Construct an expression set containing the patients with values on the phenotypical variable `remission` and the gene expressions with a significant p-value on the t-test with the patient groups CR or REF.

(b) Use recursive partitioning to predict the remission. Report the misclassification rate and the names of the genes that play a role in the tree.

5. Gene selection by area under the curve. A strategy of selecting genes is to compute the auc for each gene and to use the best 10 for further investigation. Compute the auc for each row with gene expressions of the Golub at al. (1999) data. Collect these in a vector and select the ten best. Is "CCND3 Cyclin D3" among these?

6. Classification Tree for Ecoli. The ecoli data can be download by the following: (Hint: Copy two separated lines into one before running it.)

```
ecoli <- read.table(
  "http://www.grappa.univ-lille3.fr/~torre/Recherche/Datasets/
 downloads/ecoli/ecoli.data",sep=",",header = TRUE)
colnames(ecoli) <- c("SequenceName","mcg","gvh","lip","chg",
  "aac","alm1","alm2","ecclass")
```

(a) Use `ecclass` to construct a factor containing the "cp","im",and "pp".

(b) Construct a classification tree using the variables "mcg","gvh","lip","aac","alm1","alm2". Give the code. Hint: Use the addition notation.

(c) Plot the tree and report the variables that play a role in the constructed tree.

(d) Predict the class by the tree. Report the code and the miss-classification rate.

(e) Leaf out the upper variable in the classification tree and re-estimate the tree. Report the miss-classification rate. Is it much worse?

Table 8.2: Ordered expression values of gene CCND3 Cyclin D3, index 2 indicates ALL, 1 indicates AML, cutoff points, number of false positives, false positive rate, number of true positives, true positive rate.

	data	index	cutoff	fp	fpr	tp	tpr
1			Inf	0	0.00	0	0.00
2	2.77	2	2.77	0	0.00	1	0.04
3	2.59	2	2.59	0	0.00	2	0.07
4	2.45	2	2.45	0	0.00	3	0.11
			⋮				
22	1.78	2	1.78	0	0.00	21	0.78
23	1.52	2	1.52	0	0.00	22	0.81
24	1.37	2	1.45	1	0.09	22	0.81
25	1.33	2	1.37	1	0.09	23	0.85
26	1.28	2	1.33	1	0.09	24	0.89
27	1.11	2	1.28	1	0.09	25	0.93
28	0.46	2	1.12	2	0.18	25	0.93
29	1.45	1	1.11	2	0.18	26	0.96
30	1.12	1	1.02	3	0.27	26	0.96
31	1.02	1	0.89	4	0.36	26	0.96
32	0.89	1	0.83	5	0.45	26	0.96
33	0.83	1	0.74	6	0.55	26	0.96
34	0.74	1	0.64	7	0.64	26	0.96
35	0.64	1	0.49	8	0.73	26	0.96
36	0.49	1	0.46	8	0.73	27	1.00
37	0.43	1	0.43	9	0.82	27	1.00
38	0.13	1	0.13	10	0.91	27	1.00
39	−0.74	1	−0.74	11	1.00	27	1.00

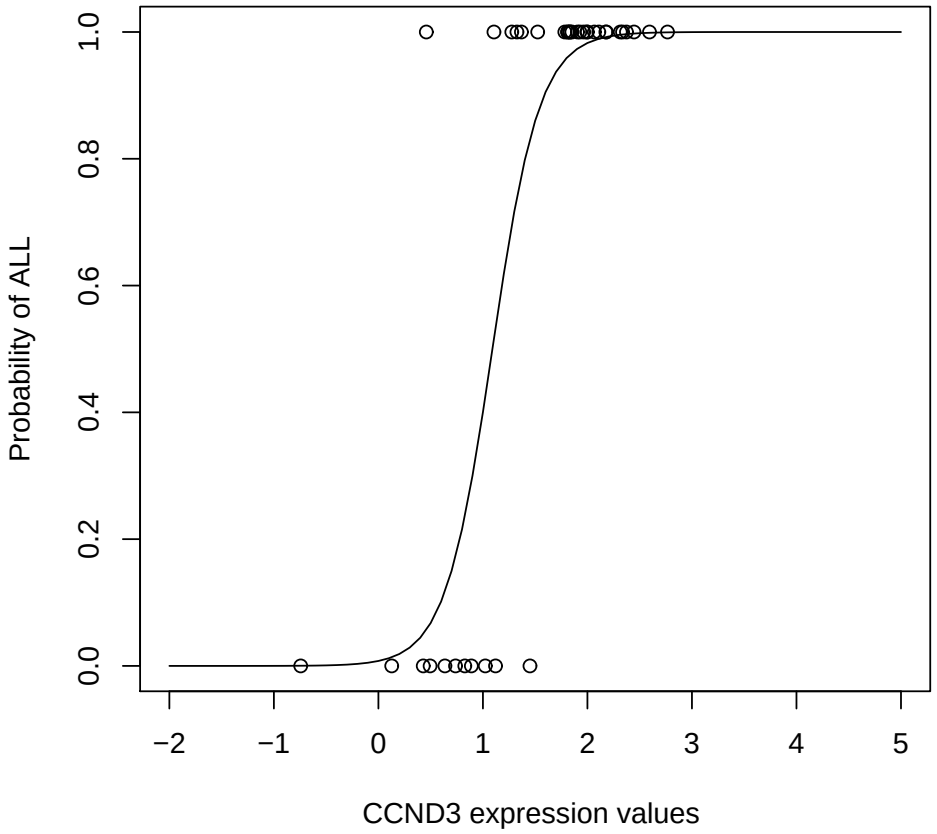


Figure 8.11: Logit fit to the CCND3 Cyclin D3 expression values.

Chapter 9

Analyzing Sequences

For many purposes in bioinformatics nucleotide or amino acid sequences are analyzed. The idea is that highly similar sequences may have identical biological functions. For expressing the similarity of sequences it is necessary to compute first their optimal alignment. It will be explained and illustrated how optimal pairwise alignment can be obtained. Furthermore, it is of importance to compute quantities for DNA sequences such as the CG fraction, or, for amino acid sequences, the isoelectric point or the hydropathy score. It will be explained and illustrated how such quantities can be computed. In this chapter you learn how to query online data bases, to translate RNA into protein sequences, to match patterns, and to program pairwise alignments. We will start, however, with a query language in order to download sequences.

9.1 Using a query language

It will be illustrated how the query language from the `seqinr` package can be used for various types of searches. However, before we download anything, it is important to know which banks can be chosen.

```
> library(seqinr)
> choosebank()
 [1] "genbank"     "embl"        "emblwgs"     "swissprot"   "ensembl"
 [6] "refseq"      "nrsub"       "hobacnucl"   "hobacprot"   "hovergendna"
[11] "hovergen"    "hogenom"     "hogenomdna"  "hogennucl"   "hogenprot"
[16] "hoverclnu"   "hoverclpr"   "homolens"    "homolensdna" "greview"
```

```
[21] "polymorphix" "emglib"      "HAMAPnucl"   "HAMAPprot"   "hoppsigen"
[26] "nurebnucl"   "nurebprot"   "taxobacgen"
```

There are many possibilities to use the `query` language e.g. for answering questions about sequences from online data bases (Gouy, et al. 1984). We give a few examples to illustrate some of its possibilities. For this we shall temporary use the option `virtual=TRUE` to save time by preventing actual downloading.[1] We may ask: How many ccnd sequences has genbank?

```
> choosebank("genbank")
> query("ccnd","k=ccnd",virtual=TRUE)$nelem
[1] 147
```

More specific: How many sequences ccnd sequences has genbank for the species homo sapiens.

```
> query("ccnd3hs","sp=homo sapiens AND k=ccnd3",virtual=TRUE)$nelem
[1] 9
```

For many other combinations of search options we refer to the manual of the `seqinr` package and for a book length treatment with many examples to Charif et al. (2008).

9.2 Getting information on downloaded sequences

After sequences are downloaded in binary format it is essential to obtain information with respect to their accession number, length, actual elements, translation to amino acids, and annotation. How to do this will briefly be illustrated by an example.

Example 1. Let's download sequences related to the species homo sapiens and a gene name like "CCND3".

```
> choosebank("genbank")
> query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
> ccnd3hs$nelem
[1] 9
```

[1] The results below are obviously time dependent.

The sequences are downloaded in binary format. The symbol `@` acts as a wildcard for any zero or other characters. There are a number of useful functions available to obtain further information. Some of these are `getName`, `getLength`, `getSequence`, `getTrans`, and `getAnnot`. To use these on a list containing sets of sequences the functionality `sapply` is very convenient. This is illustrated by extracting the NCBI accession numbers.

```
> sapply(ccnd3hs$req, getName)
 [1] "AF517525.CCND3"   "AL160163.CCND3"   "AL160163.PE5"     "AL161651"
 [5] "BC011616.CCND3"   "CR542246"         "HUMCCND3A.CCND3"  "HUMCCND3PS.PE1"
 [9] "HUMCCNDB04.CCND3" "HUMCYCD3A.CCND3"
```

The length of the sequences can be obtained by the `getLength` function.

```
> sapply(ccnd3hs$req, getLength)
 [1] "879" "879" "729" "211627" "879" "879" "879" "537" "559" "879"
```

Let's obtain the first sequence and print its first fifteen nucleotides to the screen. [2]

```
> getSequence(ccnd3hs$req[[1]])[1:15]
 [1] "a" "t" "g" "g" "a" "g" "c" "t" "g" "c" "t" "g" "t" "g" "t"
```

Its translation into amino acids can be obtained

```
> getTrans(ccnd3hs$req[[1]])[1:15]
 [1] "M" "E" "L" "L" "C" "C" "E" "G" "T" "R" "H" "A" "P" "R" "A"
```

as well as its annotation from the corresponding web page:

```
> getAnnot(ccnd3hs$req[[1]])
 [1] "  CDS join(1051..1248,2115..2330,5306..5465,6005..6141,"
 [2] "  6593..6760)"
 [3] "  /gene=\"CCND3\""
 [4] "  /codon_start=1"
 [5] "  /product=\"cyclin D3\""
 [6] "  /protein_id=\"AAM51826.1\""
 [7] "  /db_xref=\"GI:21397158\""
 [8] "  /translation=\"MELLCCEGTRHAPRAGPDPRLLGDQRVLQSLLRLEERYVPRASY"
```

[2] Use double brackets to extract a sequence from a list.

```
 [9] "  FQCVQREIKPHMRKMLAYWMLEVCEEQRCEEEVFPLAMNYLDRYLSCVPTRKAQLQLL"
[10] "  GAVCMLLASKLRETTPLTIEKLCIYTDHAVSPRQLRDWEVLVLGKLKWDLAAVIAHDF"
[11] "  LAFILHRLSLPRDRQALVKKHAQTFLALCATDYTFAMYPPSMIATGSIGAAVQGLGAC"
[12] "  SMSGDELTELLAGITGTEVDCLRACQEQIEAALRESLREAAQTSSSPAPKAPRGSSSQ"
[13] "  GPSQTSTPTDVTAIHL\""
```

□

9.3 Computations on sequences

A basic quantity to compute are the nucleotide and the dinucleotide frequencies.

Example 1. Frequencies of (di)nucleotides. We shall continue with the first result from the CCND3 (Cyclin D3) search with accession number "AF517525.CCND3". To compute the frequencies we may extract the sequence from a list in order to use the basic function `table`, as follows.

```
> table(getSequence(ccnd3hs$req[[1]]))

  a   c   g   t
162 288 267 162
```

This table can also be computed by the `seqinr` function `count`, which is more general in the sense that frequencies of dinucleotides can be computed.

```
> count(getSequence(ccnd3hs$req[[1]]),2)

 aa  ac  ag  at  ca  cc  cg  ct  ga  gc  gg  gt  ta  tc  tg  tt
 25  44  64  29  68  97  45  78  52 104  76  34  16  43  82  21
```

This will be quite useful in the next chapter. Indeed, changing 2 into 3 makes it possible to count trinucleotides. □

Example 2. G + C percentage. We are often interested in the fraction G plus C in general (`GC`), or starting from the first position of the codon bases (`GC1`), the second (`GC2`), or third (`GC3`).

```
> GC(getSequence(ccnd3hs$req[[1]]))
[1] 0.6313993
```

```
> GC1(getSequence(ccnd3hs$req[[1]]))
[1] 0.6484642
> GC2(getSequence(ccnd3hs$req[[1]]))
[1] 0.4641638
> GC3(getSequence(ccnd3hs$req[[1]]))
[1] 0.78157
```

Hence, the G + C percentage is largest when started at position three. It is also possible to compute the G + C fraction in a window of length 50 nt, say, and to plot it along the sequence.

```
GCperc <- double()
n <- length(ccnd3[[1]])
for (i in 1:(n - 50)) GCperc[i] <-  GC(ccnd3[[1]][i:(i+50)])
plot(GCperc,type="l")
```

By `double()` we first create a vector. From Figure 9.1 it can be seen that the G + C fraction changes drastically along a window of 50 nucleotides. □

With respect to over or under representation of dinucleotides there is a function ρ (rho) available, which is defined as

$$\rho(xy) = \frac{f_{xy}}{f_x \cdot f_y},$$

where f_{xy}, f_x, and f_y are the frequencies of the (di)nucleotide xy, x, and y, respectively. The z-score is computed by subtracting the mean and dividing by the standard deviation (Palmeira, et al., 2006). The latter is somewhat more sensitive for over and under representation.

Example 3. Rho and z-scores. The coefficient rho and the corresponding z-scores will be computed from the sequence with NCBI accession number "AF517525.CCND3".

```
> round(rho(getSequence(ccnd3hs$req[[1]])),2)

  aa   ac   ag   at   ca   cc   cg   ct   ga   gc   gg   gt   ta   tc   tg   tt
0.84 0.83 1.30 0.97 1.28 1.03 0.51 1.47 1.06 1.19 0.94 0.69 0.54 0.81 1.67 0.70

> round(zscore(getSequence(ccnd3hs$req[[1]]),modele='base'),2)
```

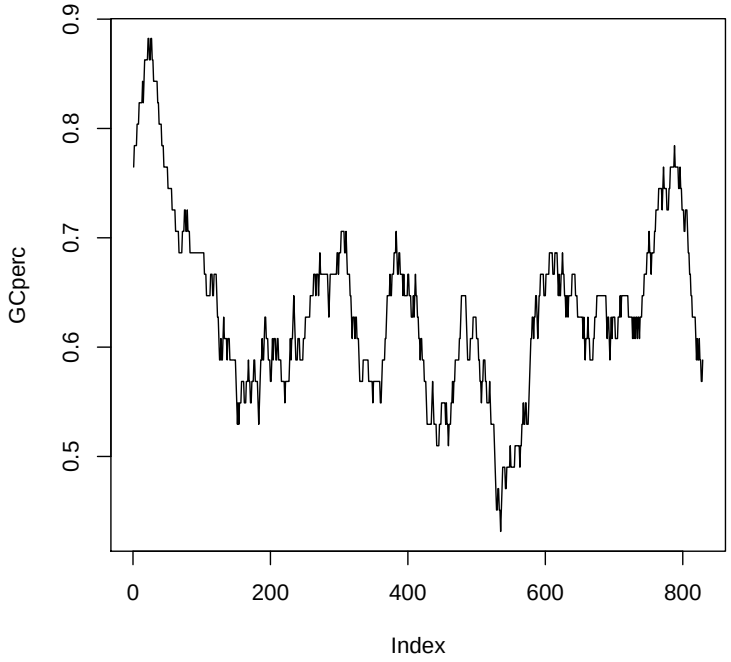


Figure 9.1: G + C fraction of sequence "AF517525.CCND3" along a window of length 50 nt.

```
   aa    ac    ag    at    ca    cc    cg    ct    ga    gc    gg    gt    ta
-1.08 -1.67  2.81 -0.18  2.78  0.42 -6.63  4.64  0.54  2.60 -0.80 -2.87 -3.10
   tc    tg    tt
-1.86  6.22 -1.98
```

The rho value for CG is not extreme, but its z-score certainly is. □

In case we have an amino acid sequence it may be useful to obtain a plot of the amino acid frequencies. When we have translated the nucleotide sequence into an amino acid sequence, it may be interesting to construct a

plot expressing their frequencies. Such can be useful for a first impression on sequence similarity.

Example 4. Comparing Amino acid frequencies. We continue with the first result from the CCND3 (Cyclin D3) search, translate and order it, and, next, produce a `dotchart` with amino acid frequencies.

```
tab <- table(getTrans(ccnd3hs$req[[1]]))
taborder <- tab[order(tab)]
names(taborder) <- aaa(names(taborder))
dotchart(taborder,pch=19,xlab="Stop and amino-acid-counts")
abline(v=1,lty=2)
```

The script was run on both sequences AF517525.CCND3 and AL160163.CCND3 resulting in Figure 9.2 and 9.3, respectively. The two sequences are highly similar with respect to amino acid frequencies. □

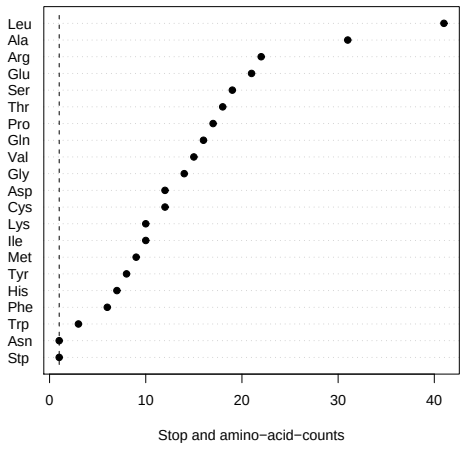


Figure 9.2: Frequency plot of amino acids from accession number AF517525.CCND3.

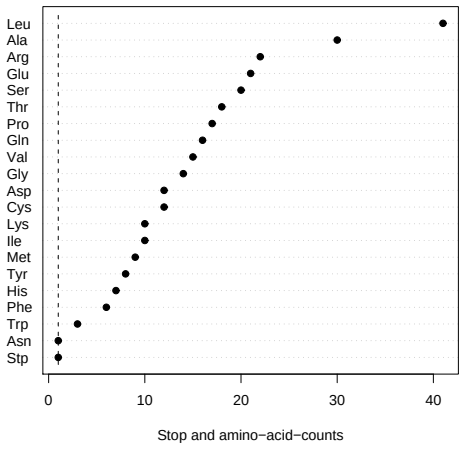


Figure 9.3: Frequency plot of amino acids from accession number AL160163.CCND3.

For amino acid sequences it may be of importance to compute the theoretical isoelectric point or the molecular weight of the corresponding protein.

Example 5. Isoelectric point. The function `computePI` computes the theoretical isoelectric point of a protein, which is the pH at which the protein has a neutral charge (Gasteiger, et al. 2005).

```
> computePI(getTrans(ccnd3hs$req[[1]]))
[1] 6.657579
```

The protein molecular weight can be computed as follows.

```
> pmw(getTrans(getSequence(ccnd3hs$req[[1]])))
[1] 32503.38
```

Note that it is easy to compute these for all downloaded proteins and to compare these. □

Another important quantity is hydropathy score (Kyte & Doolittle, 1982) of proteins, which is defined as a weighted sum $\sum_{i=1}^{20} \alpha_i f_i$ of amino acid coefficients α_i and the relative frequencies f_i. An example will illustrate how it can be computed.

Example 6. Hydropathy score. The coefficients $\alpha_1, \cdots, \alpha_{20}$ are available as `KD` data from the `EXP` list of the `seqinr` package. The unique names are lexicographically ordered and stored in the object `kdc`. The scale is changed by the minus sign below so that hydrophilic proteins are positive, but smaller than one. A function is defined to compute the hydropathy score for a set of amino acid sequences.

```
ccnd3 <- sapply(ccnd3hs$req, getSequence)
ccnd3transl <- sapply(ccnd3, getTrans)
data(EXP)
names(EXP$KD) <- sapply(words(), function(x) translate(s2c(x)))
kdc <- EXP$KD[unique(names(EXP$KD))]
kdc <- -kdc[order(names(kdc))]

linform <- function(data, coef) {   #data are sequences
 f <- function(x) {
     freq <- table(factor(x, levels = names(coef)))/length(x)
     return(coef %*% freq) }
 res <- sapply(data, f)
 names(res) <- NULL
```

```
 return(res)
}
kdath <- linform(ccnd3transl, kdc)
> print(kdath,digits=3)
 [1] 0.0874 0.0962 0.0189 0.1496 0.0962 0.0874 0.0874 0.2659 0.2220
```

Indeed, the largest score is still much smaller than one, so the conclusion is that there are no hydrophilic proteins among our sequences. □

The data set `aaindex` of the `seqinr` library contains more than five hundred sets of coefficients for computing specific quantities with respect to proteins.

9.4 Matching patterns

A manner to investigate a long sequence is to search for identical patterns, eventually allowing for a specified number of mismatches. There are many relevant examples such as seeking for one of the stop codons `UAG`, `UGA` `UAA` in RNA, or recognition sequences of enzymes (e.g. Roberts, et al., 2007). We sustain with a brief example.

Example 1. Pattern match. In the sequence with NCBI accession number "AF517525.CCND3", we seek the pattern "cccggg" with zero mismatches as well as those with a single mismatch. By the function `c2s` a sequence of characters is converted into a single string.

```
library(seqinr)
choosebank("genbank")
query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
ccnd3 <- sapply(ccnd3hs$req, getSequence)
ccnd3nr1 <- c2s(ccnd3[[1]])
> ccnd3nr1
[1] "atggagctgctgtgttgcgaaggcacccggcacgcgccccgggccgggccggacccgcgg"...
> subseq <- "cccggg"
> countPattern(subseq, ccnd3nr1, mismatch = 0)
[1] 2
> matchPattern(subseq, ccnd3nr1, mismatch = 0)
  Views on a 879-letter BString subject
```

```
Subject: atggagctgctgtgttgcgaaggcacccggcacg...actcctacagatgtcacag
Views:
    start end width
[1]    38  43     6 [cccggg]
[2]   809 814     6 [cccggg]
> matchPattern(subseq, ccnd3nr1, mismatch = 1)
  Views on a 879-letter BString subject
Subject: atggagctgctgtgttgcgaaggcacccggcacg...actcctacagatgtcacag
Views:
     start end width
 [1]    26  31     6 [cccggc]
 [2]    37  42     6 [ccccgg]
 [3]    38  43     6 [cccggg]
 [4]    43  48     6 [gccggg]
 [5]    54  59     6 [cccgcg]
 [6]   119 124     6 [cccgcg]
 [7]   236 241     6 [ccctgg]
 [8]   303 308     6 [cctggg]
 [9]   512 517     6 [cccgtg]
[10]   612 617     6 [cacggg]
[11]   642 647     6 [cctggg]
[12]   661 666     6 [tccggg]
[13]   662 667     6 [ccgggg]
[14]   808 813     6 [ccccgg]
[15]   809 814     6 [cccggg]
[16]   810 815     6 [ccgggg]
```

The number of counted patterns allowing two mismatches is much larger. □

9.5 Pairwise alignments

Among the basic questions about genes or proteins is to what extent a pair of sequences are similar. To find this out these are aligned in a certain manner after which a similarity score can be computed. In order to understand sequence alignment it is fundamental to have some idea about recursion.

Example 1. Basic recursion. The idea of recursion is to generate a sequence by defining the current value as a function of the previous. Suppose that the first element is one, $x_1 = 1$, and that the sequence is defined by

$$x_i = x_{i-1} + 1.$$

Then we obtain $x_1 = 1$, $x_2 = 2$, $x_3 = 3$, etc, so that the sequence becomes $1, 2, 3, \cdots$. Indeed, this is as fundamental as counting.

Another manner to define a sequence is by multiplying the previous value by a constant. For example, let $x_i = 2x_{i-1}$ with $x_1 = 1$. Then the values of the sequence are $x_1 = 1$, $x_2 = 2$, $x_3 = 4$, $x_3 = 8$, etc. Also we see that in fact $x_n = 2^n$, so that a value of the sequence can be computed without actually computing all previous elements.

Another example would be $x_i = 2x_{i-1} - 10$, with $x_1 = 1$. In order to compute the value x_{10} we may use R, as follows.

```
> x<-double();x[1]<-1
> for (i in 2:10) {x[i]<- 2*x[i-1]-10}
> x[10]
[1] -4598
```

This illustrates basic ideas about recursively defined sequences. □

Suppose we want to compute an alignment score for two small DNA sequences GAATTC and GATTA (Durbin et. al., 1998, p.18). We agree that a match between two letters should have the score +2 and a mismatch the score -1. A gap at a certain position of the sequences should be punished by subtracting a score by $d = 2$. A possible alignment is $\begin{smallmatrix} G & A & A & T & T & C \\ G & A & T & T & - & A \end{smallmatrix}$, where the minus sign indicates a gap. Then the alignment consists of a match, match, mismatch, match, gap, mismatch, respectively, so that the score is $2 + 2 - 1 + 2 - 2 - 1 = 2$. Now the question is whether this alignment is optimal in the sense that the score is maximal? The answer is: No! To see this, consider the alignment $\begin{smallmatrix} G & A & A & T & T & C \\ G & A & - & T & T & A \end{smallmatrix}$. Then we have a match, match, gap, match, match, mismatch, respectively, so that the score is $2+2-2+2+2-1 = 5$. This is better, but still we do not know whether this alignment is optimal.

In order to ascertain that the alignment is optimal we have to build an alignment score matrix $F(i, j)$. To do so it is convenient to start with building the (mis)match score matrix $s(i, j)$. Its (i, j)th element $s(i, j)$ has the value 2 in case of a match and the value -1 is case of a mismatch. Note that for

each step we can choose between a gap, a match, or a mismatch. Building up the matrix $F(i,j)$ recursively, means that we define its elements on the basis of the values of its preceding elements. That is, given the values of the previous elements $F(i-1,j-1)$, $F(i-1,j)$, and $F(i,j-1)$, we will be able to find the best consecutive value for $F(i,j)$. In particular, in case of a match or a mismatch, we take $F(i,j) = F(i-1,j-1) + s(x_i, y_j)$ and in case of a gap we take $F(i,j) = F(i-1,j) - d$ or $F(i,j) = F(i,j-1) - d$. The famous Needleman-Wunsch alignment algorithm consists of taking the maximum out of these possibilities at each step (e.g, Durbin et. al., 1998, p.21). Their algorithm can be summarized, as follows.

$$F(i,j) = \max \begin{cases} F(i-1,j-1) + s(i,j) \\ F(i-1,j) - d \\ F(i,j-1) - d \end{cases}$$

Note, however, that this will not yet work because we have not defined any initial values. In fact we will agree to start with $F(0,0) = 0$ and due to the gap penalties we take $F(i,0) = -id$ for the first column and $F(0,j) = -jd$ for the first row. Then, the final score $F(n,m)$ is the optimal score and the values of the matrix $F(i,j)$ indicates the optimal path. By informaticians this recursive scheme is often called a "dynamic programming algorithm".

Example 2. Dynamic programming of DNA sequences. Consider again the DNA sequences GAATTC, GATTA, the score +2 for a match, -1 for a mismatch, and the gap penalty $d = 2$. It is clarifying to first construct the score matrix $s(i,j)$. For this we use the string-to-character function `s2c`, a for loop, and an `if else` statement.

```
library(seqinr)
x <- s2c("GAATTC"); y <- s2c("GATTA"); d <- 2
s <- matrix(data=NA,nrow=length(y),ncol=length(x))
for (i in 1:(nrow(s))) for (j in 1:(ncol(s)))
   {if (y[i]==x[j]) s[i,j]<- 2 else  s[i,j]<- -1 }
rownames(s) <- c(y); colnames(s) <- c(x)
> s
   G  A  A  T  T  C
G  2 -1 -1 -1 -1 -1
A -1  2  2 -1 -1 -1
T -1 -1 -1  2  2 -1
```

```
T -1 -1 -1  2  2 -1
A -1  2  2 -1 -1 -1
```

To initialize the first row and column of the matrix $F(i,j)$, it is convenient to use the function `seq`. The purpose of the `max` function seems obvious.

```
F <- matrix(data=NA,nrow=(length(y)+1),ncol=(length(x)+1))
rownames(F) <- c("",y); colnames(F) <- c("",x)
F[,1] <- -seq(0,length(y)*d,d); F[1,] <- -seq(0,length(x)*d,d)
for (i in 2:(nrow(F)))
  for (j in 2:(ncol(F)))
  {F[i,j] <- max(c(F[i-1,j-1]+s[i-1,j-1],F[i-1,j]-d,F[i,j-1]-d))}
>  F
      G  A  A  T   T   C
    0 -2 -4 -6 -8 -10 -12
G  -2  2  0 -2 -4  -6  -8
A  -4  0  4  2  0  -2  -4
T  -6 -2  2  3  4   2   0
T  -8 -4  0  1  5   6   4
A -10 -6 -2  2  3   4   5
```

From the lower corner to the right hand side we see that the optimal score is indeed 5. □

Optimal alignment for pairs of amino acid sequences are often considered to be more relevant because these are more closely related to biological functions. For this purpose we may modify the previous scheme by changing the gap penalty d and the (mis)match scores $s(i,j)$. In particular, we shall use the gap penalty $d = 8$ and for the (mis)match the scores from the so-called BLOSUM50 matrix.

Example 3. Programming Needleman-Wunsch. For the two sequences "PAWHEAE" and "HEAGAWGHEE" (see, Durbin et. al., 1998, p.21) we seek the Needleman-Wunsch optimal alignment score, using the BLOSUM50 (mis)match score matrix and gap penalty $d = 8$. You can either directly read a BLOSUM matrix from NCBI

```
>  file <- "ftp://ftp.ncbi.nih.gov/blast/matrices/BLOSUM50"
>  BLOSUM50 <- as.matrix(read.table(file, check.names=FALSE))
```

Table 9.1: BLOSUM50 matrix.

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	5	-2	-1	-2	-1	-1	-1	0	-2	-1	-2	-1	-1	-3	-1	1	0	-3	-2	0
R	-2	7	-1	-2	-4	1	0	-3	0	-4	-3	3	-2	-3	-3	-1	-1	-3	-1	-3
N	-1	-1	7	2	-2	0	0	0	1	-3	-4	0	-2	-4	-2	1	0	-4	-2	-3
D	-2	-2	2	8	-4	0	2	-1	-1	-4	-4	-1	-4	-5	-1	0	-1	-5	-3	-4
C	-1	-4	-2	-4	13	-3	-3	-3	-3	-2	-2	-3	-2	-2	-4	-1	-1	-5	-3	-1
Q	-1	1	0	0	-3	7	2	-2	1	-3	-2	2	0	-4	-1	0	-1	-1	-1	-3
E	-1	0	0	2	-3	2	6	-3	0	-4	-3	1	-2	-3	-1	-1	-1	-3	-2	-3
G	0	-3	0	-1	-3	-2	-3	8	-2	-4	-4	-2	-3	-4	-2	0	-2	-3	-3	-4
H	-2	0	1	-1	-3	1	0	-2	10	-4	-3	0	-1	-1	-2	-1	-2	-3	2	-4
I	-1	-4	-3	-4	-2	-3	-4	-4	-4	5	2	-3	2	0	-3	-3	-1	-3	-1	4
L	-2	-3	-4	-4	-2	-2	-3	-4	-3	2	5	-3	3	1	-4	-3	-1	-2	-1	1
K	-1	3	0	-1	-3	2	1	-2	0	-3	-3	6	-2	-4	-1	0	-1	-3	-2	-3
M	-1	-2	-2	-4	-2	0	-2	-3	-1	2	3	-2	7	0	-3	-2	-1	-1	0	1
F	-3	-3	-4	-5	-2	-4	-3	-4	-1	0	1	-4	0	8	-4	-3	-2	1	4	-1
P	-1	-3	-2	-1	-4	-1	-1	-2	-2	-3	-4	-1	-3	-4	10	-1	-1	-4	-3	-3
S	1	-1	1	0	-1	0	-1	0	-1	-3	-3	0	-2	-3	-1	5	2	-4	-2	-2
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	2	5	-3	-2	0
W	-3	-3	-4	-5	-5	-1	-3	-3	-3	-3	-2	-3	-1	1	-4	-4	-3	15	2	-3
Y	-2	-1	-2	-3	-3	-1	-2	-3	2	-1	-1	-2	0	4	-3	-2	-2	2	8	-1
V	0	-3	-3	-4	-1	-3	-3	-4	-4	4	1	-3	1	-1	-3	-2	0	-3	-1	5

or load a BLOSUM matrix from the `Biostrings` package. For the sake of clarity we shall conveniently construct the matrix $s(i, j)$ without any concern about computer memory.

```
library(seqinr);library(Biostrings);data(BLOSUM50)
x <- s2c("HEAGAWGHEE"); y <- s2c("PAWHEAE"); s <- BLOSUM50[y,x]; d <- 8
F <- matrix(data=NA,nrow=(length(y)+1),ncol=(length(x)+1))
F[1,] <- -seq(0,80,8); F[,1] <- -seq(0,56,8)
rownames(F) <- c("",y); colnames(F) <- c("",x)
for (i in 2:(nrow(F)))
  for (j in 2:(ncol(F)))
  {F[i,j] <- max(c(F[i-1,j-1]+s[i-1,j-1],F[i-1,j]-d,F[i,j-1]-d))}
```

```
> F
        H   E   A   G   A   W   G   H   E   E
    0  -8 -16 -24 -32 -40 -48 -56 -64 -72 -80
P  -8  -2  -9 -17 -25 -33 -41 -49 -57 -65 -73
A -16 -10  -3  -4 -12 -20 -28 -36 -44 -52 -60
W -24 -18 -11  -6  -7 -15  -5 -13 -21 -29 -37
H -32 -14 -18 -13  -8  -9 -13  -7  -3 -11 -19
E -40 -22  -8 -16 -16  -9 -12 -15  -7   3  -5
A -48 -30 -16  -3 -11 -11 -12 -12 -15  -5   2
E -56 -38 -24 -11  -6 -12 -14 -15 -12  -9   1
```

Hence, from the lower-right corner we observe that the optimal score equals one. □

Example 4. Needleman-Wunsch. We may also conveniently use the `pairwiseAlignment` function from the `Biostrings` package to find the optimal Needleman-Wunsch aligment score for the sequences PAWHEAE" and "HEAGAWGHEE" (see, Durbin et. al., 1998, p.21).

```
library(Biostrings);data(BLOSUM50)
> pairwiseAlignment(AAString("PAWHEAE"), AAString("HEAGAWGHEE"),
  + substitutionMatrix = "BLOSUM50",gapOpening = 0, gapExtension = -8,
  + scoreOnly = FALSE)
Global Pairwise Alignment
1:  --P-AW-HEAE
2:  HEAGAWGHE-E
Score:  1
```

Hence, we obtain the optimal score 1 as well as a representation of the optimal alignment. □

An obvious question is whether in the previous example the obtained score 1 is to be evaluated as being "large" or not. A manner to answer this question is by comparing it with the alignment score of random sequences. That is, we may compute the probability of alignment scores larger than 1.

Example 5. Comparing with random sequences. To illustrate how the probability of alignment scores larger than 1 can be computed we sample randomly from the names of the amino acids, seven for y and 10 for x and

compute the maximum alignment score. This is repeated 1000 times and the probability of optimal alignment scores greater than 1 is estimated by the corresponding proportion.

```
library(seqinr);library(Biostrings);data(BLOSUM50)
randallscore <- double()
for (i in 1:1000) {
 x <- c2s(sample(rownames(BLOSUM50),7, replace=TRUE))
 y <- c2s(sample(rownames(BLOSUM50),10, replace=TRUE))
 randallscore[i] <- pairwiseAlignment(AAString(x), AAString(y),
 substitutionMatrix = "BLOSUM50",gapOpening = 0, gapExtension = -8,
 scoreOnly = TRUE)
}
> sum(randallscore>1)/1000
[1] 0.003
```

By the option `scoreOnly = TRUE` the optimal score is written to the vector `randallscore`. The probability of scores larger than 1 equals 0.003 and is therefore small and the alignment is stronger than expected from randomly constructed sequences. □

Example 6. Sliding window on Needleman-Wunsch scores. We may also program a sliding window such that for each the Needleman-Wunsch alignment score is computed. Then the maximum can be found and localized.

```
choosebank("genbank"); library(seqinr)
query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
ccnd3 <- sapply(ccnd3hs$req, getSequence)
ccnd3transl <- sapply(ccnd3, getTrans)
x <- c2s(ccnd3transl[[1]])
y <- c2s(ccnd3transl[[1]][50:70])
nwscore <- double() ; n <- length(ccnd3transl[[1]])
for (i in 1:(n-21))
  nwscore[i] <-
    pairwiseAlignment(AAString(c2s(ccnd3transl[[1]][i:(i+20)])),
    AAString(y),substitutionMatrix = "BLOSUM50",gapOpening = 0,
    gapExtension = -8, scoreOnly = TRUE)
> pairwiseAlignment(AAString(y), AAString(y),
     substitutionMatrix = "BLOSUM50", gapOpening = 0, gapExtension = -8,
```

```
  +   scoreOnly = TRUE)
[1] 152
> max(nwscore)
[1] 152
> which.max(nwscore)
[1] 50
```

Note that the maximum occurs when the subsequences are identical. The value of the maximum is 152 which occurs at position 50. □

9.6 Overview and concluding remarks

It was illustrated how the query language of the `seqinr` library can be used to download sequences, to translate these and to compute relevant quantities such as the isoelectric point or the hydropathy score. Furthermore, it was illustated how patterns can be matched and how algorithms for optimal pairwise alignment can be programmed. Further applications are given by the exercises below.

The package `Biostrings` contains the various PAM matrices for optimal alignment, as well as facilities to find palindromes, and to read and write data in FASTA format (`readFASTA`).

9.7 Exercises

1. Writing to a FASTA file. Read, similar to the above, the ccnd3 sequences using the query language and write the first sequence to a files in FASTA format. Also try to write them all to FASTA format.

2. Dotplot of sequences. Use the function `dotPlot` of the `seqinr` package and `par(mfrow=c(1,2))` to produce two adjacent plots.

   (a) Construct two random sequence of size 100 and plot the first against second and the first against the first.

   (b) Construct a plot of the first against the first and the first against the first in reverse order.

(c) Download the sequences related to the species homo sapiens and a gene name like "CCND3 Cyclin D3". Construct a dotplot of the most similar and the least similar sequences. Report your observations.

3. Local alignment. The Smith-Waterman algorithm seeks maximum local alignment between *sub*sequences of sequences. Their algorithm can be summarized (Durbin et al., 2005, p.22), as follows.

$$F(i,j) = \max \begin{cases} F(i-1,j-1) + s(i,j) \\ F(i-1,j) - d \\ F(i,j-1) - d \end{cases}$$

The algorithm allows the score zero if the others have negative values. The idea is that the maximum alignment can occur anywhere in the matrix, optimal alignment is defines as the maximum over the whole matrix. Program the Smith-Waterman algorithm and find the optimal local alignment of the sequences PAWHEAE" and "HEAGAWGHEE".

4. Probability of more extreme alignment score. Sample x and y randomly from the names of the amino acids, seven for y and 10 for x. repeat this 1000 times and compute the optimal alignment score and use it to evaluate the significance of the previously obtained score.

5. Prochlorococcus marinus. Each of three strains of P. marinus is exposed to different intensities of UV radiation because these live in different depths in water. The MIT 9313 strain lives at depth 135 m, SS120 at 120 m, and MED4 at 5 m. The latter strain is considered to be high-light-adapted. The residual intensities of 260-nm UVb irradiation corresponding to the given depths is 0.00007%, 0.0002% and 70%, respectively. It is hypothesized that the G + C content depends on the amount of radiation. The accession numbers of Gen bank are AE017126, BX548174, and BX548175, respectively.

   (a) Use the operator OR together with the accession numbers to download the sequences of the bacteria strains.

   (b) Compte the GC fraction of each of the sequences.

   (c) Is there a relation between UVb radiation and GC fraction?

   (d) Formulate a relevant hypothesis and test it.

6. Sequence equality. Download the sequences "AF517525.CCND3" and "AL160163.CCND3". Hint: These are the first two from the query "ccnd3" within homo sapiens.

   (a) Compute the length of the sequences.

   (b) Translate the sequences into amino acids and compare their frequencies.

   (c) Are they equal or, if not, in what position do they differ?

7. Conserved region. At `http://blocks.fhcrc.org` there are blocks of highly conserved regions for proteins in PROSITE. Find `PR00851A` which contains blocks of protein related to a human gene responsible for DNA-repair defect xeroderma pigmentosum (sensitivity to ultraviolet light) Perform a pairwise alignment with these subsequences and report the ones most and least similar. Use BLOSUM50.

8. Plot of CG proportion from Celegans.

   (a) Produce a plot of the CG proportion of the chromosome I of Celegans (Celegans.UCSC.ce2) along a window of 100 nucleotides. Take the first 10,000 nucleotides.

   (b) A binding sequence of the enzyme EcoRV is the subsequence GATATC. How many exact matches has Chromosome I of Celegans. How many do you expect by chance?

9. Plot of codon usage. Go to the `seqinr` help page on `dotchart.uco`.

   (a) Redo the example and briefly describe its usage.

   (b) Use the query language to find

Chapter 10

Markov Models

The idea of a Markov process forms the basis of many important models in bioinformatics such as (Hidden) Markov Models, models for sequence alignment, and models for phylogenetic trees. By the latter it is possible to estimate distances between several sequences and to visualize these in a tree. Classical matrices for sequence alignment such as BLOSUM and PAM are constructed on the basis of a Markov process. By (Hidden) Markov Models the specific repetitive order of DNA sequences can be modeled so that predictions of families becomes possible.

In this chapter you learn what a probability transition matrix is and which role it plays in a Markov process to construct specific sequences. Various models for phylogenetic trees are explained in terms of the rate matrix as well as the probability transition matrix. The basic ideas of the Hidden Markov Model are briefly explained and illustrated by an example[1].

10.1 Random sampling

Models to predict and classify DNA type of sequences make it possible to draw a sample from a population. The latter is the same as a distribution with certain properties. Recall from Chapter 3 that a discrete distribution is a set of values with certain probabilities that add up to one. Two basic examples illustrate this point.

[1]This chapter is somewhat more technical in its notation with respect to e.g. conditional probability. This is, however, inevitable for the understanding of Markov processes.

Example 1. Throwing a coin. A fair coin X attains Head and Tail with probability 1/2. Thus we may write $P(X = H) = 0.5$ and $P(X = T) = 0.5$. With such a random variable there always correspond a population as well as a sampling scheme which can be simulated on a computer (e.g. Press, et al., 1992).

```
> sample(c("H","T"),30,rep=TRUE,prob=c(0.5,0.5))
 [1] "H" "H" "T" "T" "T" "H" "H" "T" "T" "H" "H" "H" "T" "T" "H" "T"
[20] "H" "T" "T" "T" "H" "T" "H" "T" "T" "T" "T"
```

Thus the sampled values Head and Tail correspond to the process of actually throwing with a fair coin. The function `sample` randomly draws thirty times one of the values `c("H","T")` with replacement (`rep=TRUE`) and equal probabilities (`prob=c(0.5,0.5)`). □

Example 2. Generating a sequence of nucleotides. Another example is that of a random variable X which has the letters of the nucleotides as its values. So the events are $X = A$, $X = C$, $X = G$, and $X = T$. These events may occur in a certain DNA sequence with probabilities $P(X = A) = 0.1$, $P(X = G) = 0.4$, $P(X = C) = 0.4$, and $P(X = T) = 0.1$, respectively. Then the actual placement of the nucleotides along a sequence can be simulated.

```
> sample(c("A","G","C","T"),30,rep=TRUE,prob=c(0.1,0.4,0.4,0.1))
 [1] "G" "C" "T" "G" "C" "G" "G" "G" "T" "C" "T" "T" "C" "C" "C"
[20] "G" "G" "C" "G" "G" "G" "C" "C" "C" "G" "C"
```

Of course, if you do this again, then the resulting sequence will differ due to the random nature of its generation. □

For these sampling schemes it holds that the events occur independently from the previous.

10.2 Probability transition matrix

In order to build a model that produces specific sequences we will consider a certain type of random variable. In particular, we will consider a sequence $\{X_1, X_2, \cdots\}$ with values from a certain *state space* E. The latter is simply a set containing the possible values or states of a process. If, for instance, $X_n = i$, then the process is in state i at time n. Similarly, the expression

$P(X_1 = i)$ denotes the probability that the process is in state i at time point 1. The event that the process changes its state from i to j (transition) between time point one and two corresponds to the event $(X_2 = j|X_1 = i)$, where the bar means "given that". The probability for this event to happen is denoted by $P(X_2 = j|X_1 = i)$. In general, the probability of the transition from i to j between time point n and $n+1$ is given by $P(X_{n+1} = j|X_n = i)$. These probabilities can be collected in a *probability transition matrix* $\boldsymbol{P}$ with elements

$$p_{ij} = P(X_{n+1} = j|X_n = i).$$

We will assume that the transition probabilities are the same for all time points so that there is no time index needed on the left hand side. Given that the process X_n is in a certain state, the corresponding row of the transition matrix contains the distribution of X_{n+1}, implying that the sum of the probabilities over all possible states equals one. The probability transition matrix contains a (conditional) discrete probability distribution on each of its rows. For a Markov process it holds that the state at time point $n+1$ depends upon the state at time point n, but not on states at earlier time points.

Example 1. Using the probability transition matrix to generate a Markov sequence. Suppose X_n has two states: 1 for a pyrimidine and 2 for a purine. A sequence can now be generated, as follows. If $X_n = 1$, then we throw with a fair die: If the outcome is lower than or equal to 5, then $X_{n+1} = 1$ and, otherwise, (outcome equals 6) $X_{n+1} = 2$. If $X_n = 2$, then we throw with a fair coin: If the outcome equals Tail, then $X_{n+1} = 1$, and otherwise $X_{n+1} = 2$. For this process the two by two probability transition matrix equals

from \ to	1	2
1	p_{11}	p_{12}
2	p_{21}	p_{22}

where p_{21} is the probability that the process changes from 2 to 1. This transition matrix can also be written as

$$\boldsymbol{P} = \begin{pmatrix} p_{11} & p_{12} \\ p_{21} & p_{22} \end{pmatrix} = \begin{pmatrix} P(X_1 = 1|X_0 = 1) & P(X_1 = 2|X_0 = 1) \\ P(X_1 = 1|X_0 = 2) & P(X_1 = 2|X_0 = 2) \end{pmatrix} = \begin{pmatrix} \frac{5}{6} & \frac{1}{6} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix}.$$

Any matrix probability transition matrix $\boldsymbol{P}$ can be visualized by a *transition graph*, where the transition probabilities are visualized by an arrow from

state i to state j and the value of p_{ij}. For the current example the transition graph is given by Figure 10.1[2]. The values 1 and 2 of the process are written within the circles and the transition probabilities are written near the arrows. To actually generate a sequences with values equal to 1 and 2 according the

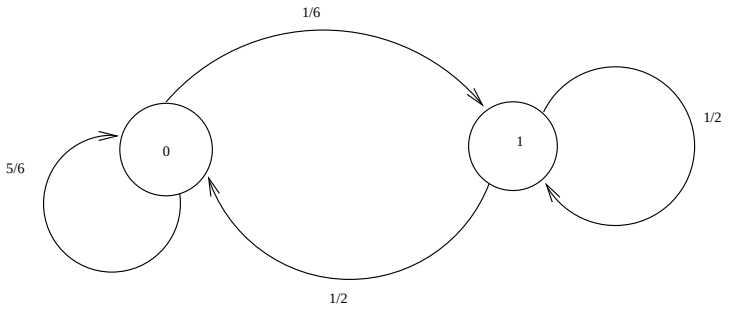


Figure 10.1: Graph of probability transition matrix

transition matrix we may use the following.

```
markov1 <- function(x,P,n){ seq <- x
 for(k in 1:(n-1)){
 seq[k+1] <- sample(x, 1, replace=TRUE, P[seq[k],])}
 return(seq)
 }
P <- matrix(c(1/6,5/6,0.5,0.5), 2, 2, byrow=TRUE)
rownames(P) <- colnames(P) <- StateSpace <- x <- c(1,2)
> markov1(x,P,30)
 [1] 1 2 1 2 1 2 2 1 2 1 2 2 1 2 1 2 2 1 2 1 2 1 2 1 2 2 2 2 2 2
```

In the function `markov1` the actual sampling is conducted by `sample`. We sample one element from the set containing 1 and 2 according to the probabilities in row `seq[k]` of the matrix `P`. This makes the probabilities of the states dependent on the corresponding row of the transition matrix. We conveniently use the fact that R adds an element to the sequence; we do not have to declare its length on beforehand (although we could!). The sequence has a fixed start at State 1 and thereafter the first row in the probability transition matrix. Note that without the return command the function does

[2]The values 1 and 2 are erroneously depicted as 0 and 1, respectively

not give any output. □

Example 2. A sequence with a large frequency of C and G. To illustrate that certain probability transition matrices imply a large frequency of C and G residues, we use the following.

```
markov2 <- function(StateSpace,P,pi0,n){
 seq <- character(n)
 seq[1] <- sample(StateSpace, 1, replace=TRUE, pi0)
 for(k in 1:(n-1)){
 seq[k+1] <- sample(StateSpace, 1, replace=TRUE, P[seq[k],])}
 return(seq)
}
P <- matrix(c(
  1/6,5/6,0,0,
  1/8,2/4,1/4,1/8,
  0,2/6,3/6,1/6,
  0,1/6,3/6,2/6),4,4,byrow=TRUE)
rownames(P) <- colnames(P) <- StateSpace <- c("a","c","g","t")
pi0 <- c(1/4,1/4,1/4,1/4)
x <- markov2(StateSpace,P,pi0,1000)
> table(x)
x
  a   c   g   t
 72 409 378 141
```

The function starts with sampling just once from the distribution with equal probabilities `pi0`. It conveniently uses the the column and row names of the probability transition matrix for the sampling. The probabilities to go from "a" or "t" to "c" or "g" are large and as well as that to stay within "c" or "g". From the frequency table it can be observed that the majority of residues are "c" or "g". □

Example 3. A sequence with high phenylalanine frequency. Now it is possible to construct a sequence which produces the amino acid phenylalanine (F) with high probability. Recall that it is coded by the triple TTT or TTC. We use the function `getTrans` of the `seqinr` package to translate nucleotide triplets into amino acids.

```
pi0 <- c(1/4,1/4,1/4,1/4)
P <- matrix(c(.01,.01,.01,.97,
              .01,.01,.01,.97,
              .01,.01,.01,.97,
              .01,.28,.01,0.70),4,4,byrow=T)
rownames(P) <- colnames(P) <- StateSpace <- c("a","c","g","t")
x <- markov2(StateSpace,P,pi0,30000)
> table(getTrans(x))

   *    A    C    D    F    H    I    L    M    N    P    R    S    T    V
   2    1   75    2 5205   24   76 2260    1    2   19   26 2154    1   91
   Y
  60
```

From the table it is clear that the F frequency is the largest among the generated amino acids. □

Example 4. To illustrate estimation of the probability transition matrix we proceed with the sequence produced by the previous example.

```
nr <- count(x,2)
names(nr)
A <- matrix(NA,4,4)
A[1,1]<-nr["aa"]; A[1,2]<-nr["ag"]; A[1,3]<-nr["ac"]; A[1,4]<-nr["at"]
A[2,1]<-nr["ga"]; A[2,2]<-nr["gg"]; A[2,3]<-nr["gc"]; A[2,4]<-nr["gt"]
A[3,1]<-nr["ca"]; A[3,2]<-nr["cg"]; A[3,3]<-nr["cc"]; A[3,4]<-nr["ct"]
A[4,1]<-nr["ta"]; A[4,2]<-nr["tg"]; A[4,3]<-nr["tc"]; A[4,4]<-nr["tt"]
rowsumA <- apply(A, 1, sum)
Phat <- sweep(A, 1, rowsumA, FUN="/")
rownames(Phat) <- colnames(Phat) <- c("a","g","c","t")
> round(Phat,3)
      a     g     c     t
a 0.011 0.000 0.007 0.982
g 0.017 0.003 0.010 0.969
c 0.010 0.011 0.012 0.967
t 0.009 0.009 0.279 0.703
```

The number of transitions are counted and divided by the row totals. The estimated transition probabilities are quite close to the true transition probabilities. The zero transition probabilities are exactly equal to the true because

these do not occur. This estimation procedure can easily be applied to DNA sequences. □

10.3 Properties of the transition matrix

In the above, the sequence was started at a certain state. Often, however, the probabilities of the initial states are available. That is, we have a vector $\boldsymbol{\pi}_0$ with initial probabilities $\pi_{10} = P(X_0 = 1)$ and $\pi_{20} = P(X_0 = 2)$. Furthermore, if the transition matrix

$$\boldsymbol{P} = \begin{pmatrix} p_{11} & p_{12} \\ p_{21} & p_{22} \end{pmatrix} = \begin{pmatrix} P(X_1 = 1|X_0 = 1) & P(X_1 = 2|X_0 = 1) \\ P(X_1 = 1|X_0 = 2) & P(X_1 = 2|X_0 = 2) \end{pmatrix},$$

then the probability that the process is in State 1 at time point 1 can be written as

$$P(X_1 = 1) = \pi_{10}p_{11} + \pi_{20}p_{21} = \boldsymbol{\pi}_0^T\boldsymbol{p}_1, \tag{10.1}$$

where $\boldsymbol{p}_1$ is the first column of $\boldsymbol{P}$, see Section 10.7. Note that the last equality holds by definition of matrix multiplication. In a similar manner, it can be shown that $P(X_1 = 2) = \boldsymbol{\pi}_0^T\boldsymbol{p}_2$, where $\boldsymbol{p}_2$ is column 2 of the transition matrix $\boldsymbol{P} = (\boldsymbol{p}_1, \boldsymbol{p}_2)$. It can be concluded that $\boldsymbol{\pi}_0^T\boldsymbol{P} = \boldsymbol{\pi}_1^T$, where $\boldsymbol{\pi}_1^T = (P(X_1 = 1), P(X_1 = 2))$; the probability at time point 1 that the process is in State 1, State 2, respectively. This holds in general for all time points n, that is

$$\boldsymbol{\pi}_n^T\boldsymbol{P} = \boldsymbol{\pi}_{n+1}^T. \tag{10.2}$$

Thus to obtain the probabilities of the states at time point $n + 1$, we can simply use matrix multiplication [3].

Example 1. Matrix multiplication to compute probabilities. Suppose the following initial distribution and probability matrix

$$\boldsymbol{\pi}_0 = \begin{pmatrix} \frac{2}{3} & \frac{1}{3} \end{pmatrix}, \boldsymbol{P} = \begin{pmatrix} \frac{5}{6} & \frac{1}{6} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix},$$

for State 1 and 2, respectively. Then $P(X_1 = 1)$ and $P(X_1 = 2)$ collected in $\boldsymbol{\pi}_1^T = (P(X_1 = 1), P(X_1 = 2))$ can be computed as follows.

$$\boldsymbol{\pi}_1^T = \boldsymbol{\pi}_0^T\boldsymbol{P} = \begin{pmatrix} \frac{2}{3} & \frac{1}{3} \end{pmatrix} \begin{pmatrix} \frac{5}{6} & \frac{1}{6} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix} = \begin{pmatrix} \frac{2}{3} \cdot \frac{5}{6} + \frac{1}{3} \cdot \frac{1}{2} & \frac{2}{3} \cdot \frac{1}{6} + \frac{1}{3} \cdot \frac{1}{2} \end{pmatrix} = \begin{pmatrix} \frac{13}{18} & \frac{5}{18} \end{pmatrix}$$

[3] The transposition sign ^{T} simply transforms a column into a row.

Using R its operator `%*%` for matrix multiplication, the product $\boldsymbol{\pi}_0^T \boldsymbol{P}$ can be computed as follows.

```
> P <- matrix(c(5/6,1/6,0.5,0.5),2,2,byrow=T)
> pi0 <- c(2/3,1/3)
> pi0 %*% P
          [,1]      [,2]
[1,] 0.7222222 0.2777778
```

□

Yet, another important property of the probability transition matrix deals with the probability of being in state 1 given that the process is in state 1 two time points before. In particular, it holds (see Section 10.7) that

$$P(X_2 = 1 | X_0 = 1) = p_{11}^2, \tag{10.3}$$

where the latter is element $(1, 1)$ of the matrix[4] $\boldsymbol{P}^2$. In general, we have that

$$P(X_n = j | X_0 = i) = p_{ij}^n,$$

which is element i, j of $\boldsymbol{P}^n$.

Example 3. Given the probability matrix of the previous example, the values $P(X_2 = j | X_0 = i)$ for all of i, j can be computed by matrix multiplication.

$$\boldsymbol{P}^2 = \begin{pmatrix} \frac{5}{6} & \frac{1}{6} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix} \cdot \begin{pmatrix} \frac{5}{6} & \frac{1}{6} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix} = \begin{pmatrix} (\frac{5}{6})^2 + \frac{1}{6}\frac{1}{2} & \frac{5}{6}\frac{1}{6} + \frac{1}{6}\frac{1}{2} \\ \frac{1}{2}\frac{5}{6} + (\frac{1}{2})^2 & \frac{1}{2}\frac{1}{6} + (\frac{1}{2})^2 \end{pmatrix} = \begin{pmatrix} \frac{28}{36} & \frac{8}{36} \\ \frac{24}{36} & \frac{12}{36} \end{pmatrix}.$$

Obviously, such matrix multiplications can be accomplished much more convenient on a personal computer.

```
> P %*% P
          [,1]      [,2]
[1,] 0.7777778 0.2222222
[2,] 0.6666667 0.3333333
```

Larger powers of $\boldsymbol{P}$ can be computed more efficiently by methods given below. □

[4] For a brief definition of matrix multiplication, see Pevsner (2003, p.56) or wikipedia using the search string "wiki matrix multiplication".

10.4 Stationary distribution

A probability distribution $\boldsymbol{\pi}$ satisfying

$$\boldsymbol{\pi}^T = \boldsymbol{\pi}^T \boldsymbol{P}$$

is *stationary* because the transition matrix does not change the probabilities of the states of the process. Such a distribution usually exists, is unique, and plays an essential role in the long term behavior of the process. It sheds light on the question: What is the probability $P(X_n = 1|X_0 = 1) = p_{11}^n$, as n increases without bound. That is: What is the probability that the process is in State 1, given that it started in State 1, as time increases without bound? To answer such a question we need large powers of the probability transition matrix. To compute these we need the eigen-decomposition of the probability transition matrix

$$\boldsymbol{P} = \boldsymbol{V}\boldsymbol{\Lambda}\boldsymbol{V}^{-1},$$

where $\boldsymbol{V}$ is the eigenvector matrix and $\boldsymbol{\Lambda}$ the diagonal matrix with eigenvalues. The latter are usually sorted in decreasing order so that the first (left upper) is the largest. Now the third power of the probability transition matrix can be computed, as follows

$$\boldsymbol{P}^3 = \boldsymbol{V}\boldsymbol{\Lambda}\boldsymbol{V}^{-1}\boldsymbol{V}\boldsymbol{\Lambda}\boldsymbol{V}^{-1}\boldsymbol{V}\boldsymbol{\Lambda}\boldsymbol{V}^{-1} = \boldsymbol{V}\boldsymbol{\Lambda}\boldsymbol{\Lambda}\boldsymbol{\Lambda}\boldsymbol{V}^{-1} = \boldsymbol{V}\boldsymbol{\Lambda}^3\boldsymbol{V}^{-1}.$$

So that, indeed, in general

$$\boldsymbol{P}^n = \boldsymbol{V}\boldsymbol{\Lambda}^n\boldsymbol{V}^{-1}.$$

The latter is a computationally convenient expression because we only have to take the power of the eigenvalues in $\boldsymbol{\Lambda}$ and to multiply by the left and right eigenvector matrices. This will be illustrated below.

In the long term the Markov process tends to a certain value (Brémaud, 1999, p.197) because a probability transition matrix has a unique largest eigenvalue equal to 1 with corresponding eigenvectors $\mathbf{1}$ and $\boldsymbol{\pi}$ (or rather normalized versions of these). It follows that, as n increases without bound, then $\boldsymbol{P}^n$ tends to $\mathbf{1}\boldsymbol{\pi}^T$. In other words, $P(X_n = j|X_0 = i) = p_{ij}^n$ tends to element (i, j) of $\mathbf{1}\boldsymbol{\pi}^T$, which is equal to element j of $\boldsymbol{\pi}$. For any initial distribution $\boldsymbol{\pi}_0$, it follows that $\boldsymbol{\pi}_0'\boldsymbol{P}^n$ tends to $\boldsymbol{\pi}^T$.

Example 1. Stationary distribution. To compute the eigen-decomposition of the probability transition matrix $\boldsymbol{P}$ as well as powers of it, we may use the function `eigen`.

```
> P <- matrix(c(1/6,5/6,0.5,0.5),2,2,byrow=T)
> V <- eigen(P,symmetric = FALSE)
> V$values
[1]  1.0000000 -0.3333333
> V$vectors
           [,1]       [,2]
[1,] -0.7071068 -0.8574929
[2,] -0.7071068  0.5144958
```

The output of the function `eigen` is assigned to the list `V` from which the eigenvalues and eigenvectors can be extracted and printed to the screen.

Now we can compute $\boldsymbol{P}^{16}$; the probability transition matrix raised to the power sixteen.

```
> V$vec %*% diag(V$va)^(16) %*% solve(V$vec)
      [,1]  [,2]
[1,] 0.375 0.625
[2,] 0.375 0.625
```

So that the stationary distribution $\boldsymbol{\pi}^T$ equals $(0.375, 0.625)$. □

Example 2. Diploid. Suppose A is a dominant gene, a a recessive and that we start with a heterozygote aA. From the latter we obtain the initial state probability $\boldsymbol{\pi}^T = (0, 1, 0)$ for the events (AA, aA, aa). When we consider pure self-fertilization, then the offspring from AA is AA with probability $(1, 0, 0)$, that of aa is aa with probability $(0, 0, 1)$, and that of aA is (AA, aA, aa) with probability $1/4, 1/2, 1/4$, respectively. Hence, the probability transition matrix becomes

$$\boldsymbol{P} = \begin{pmatrix} 1 & 0 & 0 \\ 1/4 & 1/2 & 1/4 \\ 0 & 0 & 1 \end{pmatrix}$$

We can now compute the transition probability matrix after five generations.

```
P <- matrix(c(1,0,0, 1/4,1/2,1/4,0,0,1),3,3,byrow=T)
V <- eigen(P,symmetric = FALSE)
> V$vec %*% diag(V$va)^(5) %*% solve(V$vec)
         [,1]    [,2]     [,3]
[1,] 1.000000 0.00000 0.000000
```

```
[2,] 0.484375 0.03125 0.484375
[3,] 0.000000 0.00000 1.000000
```

Hence, the distribution we obtain can be read from the second row which is highly homozygotic. A little more precise, using Equation 10.2, it can be shown that

$$\boldsymbol{\pi}_{n+1}^T = \left(\frac{1}{2} - \left(\frac{1}{2}\right)^n, \left(\frac{1}{2}\right)^n, \frac{1}{2} - \left(\frac{1}{2}\right)^n\right),$$

so that the distribution converges to $(1/2, 0, 1/2)$. □

Note that this method of raising the transition probability matrix to a large power can easily be applied to determine the stationary distribution. The idea of taking a transition matrix to a certain power is also used to construct the PAM250 matrix given the PAM1 matrix (Pevsner, 2003, p.53) and for the construction of various BLOSUM matrices (Pevsner, 2003, p.50-59; Deonier, et al. 2005, 187-190).

10.5 Phylogenetic distance

Phylogenetic trees are constructed on the basis of distances between DNA sequences. These distances are computed from substitution models which are defined by a matrix representing the rate of substitutions of one state to the other. The latter is usually expressed as a matrix $\boldsymbol{Q}$. The rates of staying in a state are given by a negative number on the diagonal of the substitution matrix. The probability transition matrix $\boldsymbol{P}$ can be computed by matrix exponentiation $\boldsymbol{P} = \exp(\boldsymbol{Q})$. How to do this in practice will be illustrated by an example.

Example 1. From a rate matrix to a probability transition matrix. Suppose the rate matrix

$$\boldsymbol{Q} = \begin{array}{c} \\ A \\ G \\ C \\ T \end{array} \begin{array}{c} \begin{array}{cccc} A & G & C & T \end{array} \\ \begin{bmatrix} -0.60 & 0.20 & 0.20 & 0.20 \\ 0.20 & -0.60 & 0.20 & 0.20 \\ 0.20 & 0.20 & -0.60 & 0.20 \\ 0.20 & 0.20 & 0.20 & -0.60 \end{bmatrix} \end{array}.$$

Thus within a certain time period a proportion of 0.20 A changes into G, 0.20 A into C, and 0.20 A into T. Consequently, a proportion of 0.60 of the

residues goes back to A. Given this rate matrix, we can find the probability transition matrix $\boldsymbol{P} = \exp(\boldsymbol{Q})$ by using the function `expm(Q)` from the package `Matrix`.

```
library(Matrix)
Q <- 0.2 * Matrix(c(-3,1,1,1,1,-3,1,1,1,1,-3,1,1,1,1,-3),4)
rownames(Q) <- colnames(Q) <- c("A","G","C","T")
P <- as.matrix(expm(Q))
> round(P,2)
     A    G    C    T
A 0.59 0.14 0.14 0.14
G 0.14 0.59 0.14 0.14
C 0.14 0.14 0.59 0.14
T 0.14 0.14 0.14 0.59
```

Thus the probability that the state changes from A to A is 0.59, from A to G is 0.14, etc. □

Because all phylogenetic models are defined in terms of rate matrices, we shall concentrate on these. For instance, the rate matrix for the Jukes and Cantor (1969) (JC69) model can be written as

$$\boldsymbol{Q}_{JC69} = \begin{array}{c} \\ A \\ G \\ C \\ T \end{array} \begin{array}{c} \begin{array}{cccc} A & G & C & T \end{array} \\ \begin{bmatrix} \cdot & \alpha & \alpha & \alpha \\ \alpha & \cdot & \alpha & \alpha \\ \alpha & \alpha & \cdot & \alpha \\ \alpha & \alpha & \alpha & \cdot \end{bmatrix} \end{array}.$$

The sum of each row of a rate matrix equals zero, so that from this requirement the diagonal elements of the JC69 model are equal to -3α. Furthermore, the non-diagonal substitution rates of the JC69 model all have the same value α. That is, the change from i to j equals that from j to i, so that the rate matrix is symmetric. Also the probability that the sequence equals one of the nucleotides is 1/4. This assumption, however, is unrealistic is many cases.

Transitions are substitutions of nucleotides within types of nucleotides, thus purine to purine or pyrmidine to pyrmidine ($A \leftrightarrow G$ or $C \leftrightarrow T$). Transversions are substitutions between nucleotide type ($A \leftrightarrow T$, $G \leftrightarrow T$,$A \leftrightarrow C$, and $C \leftrightarrow G$). In the JC69 model a transition is assumed to

happen with equal probability as a transversion. That is, it does not account for the fact that transitions are more common that transversions. To cover this for more general type of models are proposed by Kimura (1980, 1981), which are commonly abbreviated by K80 and K81. In terms of the rate matrix these models can we written as

$$\boldsymbol{Q}_{K80} = \begin{bmatrix} \cdot & \alpha & \beta & \beta \\ \alpha & \cdot & \beta & \beta \\ \beta & \beta & \cdot & \alpha \\ \beta & \beta & \alpha & \cdot \end{bmatrix}, \qquad \boldsymbol{Q}_{K81} = \begin{bmatrix} \cdot & \alpha & \beta & \gamma \\ \alpha & \cdot & \gamma & \beta \\ \beta & \gamma & \cdot & \alpha \\ \gamma & \beta & \alpha & \cdot \end{bmatrix}.$$

In the K80 model a change within type (transition) occurs at rate α and between type (transversion) at rate β. In the K81 model all changes occur at a different though symmetric rate; the rate of change $A \to G$ is α and equals that of $A \leftarrow G$. If α is large, then the amount of transitions is large; if both β and γ are very small, then the number of transversions is small.

A model is called "nested" if it is a special case of a more general model. For instance, the K80 model is nested in the K81 model because when we take $\gamma = \beta$, then we obtain the K80 model. Similarly, the JC69 model is nested in the K80 model because if we take $\beta = \alpha$, then we obtain the JC69 model.

Some examples of models with even more parameters are the Hasegawa, Kishino, and Yano (1985) (HKY85) model and the General Time-Reversable Model (GTR) model

$$\boldsymbol{Q}_{HKY85} = \begin{bmatrix} \cdot & \alpha\pi_G & \beta\pi_C & \beta\pi_T \\ \alpha\pi_A & \cdot & \beta\pi_C & \beta\pi_T \\ \beta\pi_A & \beta\pi_G & \cdot & \alpha\pi_T \\ \beta\pi_A & \beta\pi_G & \alpha\pi_C & \cdot \end{bmatrix}, \qquad \boldsymbol{Q}_{GTR} = \begin{bmatrix} \cdot & \alpha\pi_G & \beta\pi_C & \gamma\pi_T \\ \alpha\pi_A & \cdot & \delta\pi_C & \epsilon\pi_T \\ \beta\pi_A & \delta\pi_G & \cdot & \zeta\pi_T \\ \gamma\pi_A & \epsilon\pi_G & \zeta\pi_C & \cdot \end{bmatrix}.$$

The distance between DNA sequences is defined on the basis of these models. From these distances the phylogenetic tree is computed by a neighbor-joining algorithm such that it has the smallest total branch length.

Example 2. The K81 model. To compute the rate matrix of the K81 model with $\alpha = 3/6$, $\beta = 2/6$, $\gamma = 1/6$ we may use the following.

```
alpha <- 3/6; beta <- 2/6; gamma<- 1/6; Q <- matrix(data=NA,4,4)
Q[1,2] <- Q[2,1] <- Q[3,4] <- Q[4,3] <- alpha
```

```
Q[1,3] <- Q[3,1] <- Q[2,4] <- Q[4,2] <- beta
Q[1,4] <- Q[4,1] <- Q[2,3] <- Q[3,2] <- gamma
> diag(Q) <- -(alpha + beta + gamma)
> Q
           [,1]       [,2]       [,3]       [,4]
[1,] -1.0000000  0.5000000  0.3333333  0.1666667
[2,]  0.5000000 -1.0000000  0.1666667  0.3333333
[3,]  0.3333333  0.1666667 -1.0000000  0.5000000
[4,]  0.1666667  0.3333333  0.5000000 -1.0000000
> Q <- Matrix(Q)
> P <- as.matrix(expm(Q))
> P
          [,1]      [,2]      [,3]      [,4]
[1,] 0.4550880 0.2288517 0.1767105 0.1393498
[2,] 0.2288517 0.4550880 0.1393498 0.1767105
[3,] 0.1767105 0.1393498 0.4550880 0.2288517
[4,] 0.1393498 0.1767105 0.2288517 0.4550880
```

By raising the power of the probability transition matrix to a sufficiently large number, it can be observed that the stationary distribution $\boldsymbol{\pi}^T = (0.25, 0.25, 0.25, 0.25)$. □

Example 3. Stationarity for the JC69 model. Let's take $\alpha = 1/5$ as in Example 1 and compute the rate matrix $\boldsymbol{Q}$ of the JC69 model, the corresponding probability transitionmatrix $\boldsymbol{P}$, and raise it to the power 50.

```
library(Matrix)
alpha <- 1/5;  Q <- matrix(rep(alpha,16),4,4)
diag(Q) <- -3 * alpha
Q <- Matrix(Q)
P <- as.matrix(expm(Q))
V <- eigen(P,symmetric = FALSE)
> V$vec %*% diag(V$va)^(50) %*% solve(V$vec)
     [,1] [,2] [,3] [,4]
[1,] 0.25 0.25 0.25 0.25
[2,] 0.25 0.25 0.25 0.25
[3,] 0.25 0.25 0.25 0.25
[4,] 0.25 0.25 0.25 0.25
```

Hence, the stationary distribution is $\boldsymbol{\pi}^T = (0.25, 0.25, 0.25, 0.25)$ (cf. Ewens & Grant, 2005, p. 477). □

Example 4. Distance between two sequences according to the JC69 model. In case of the JC69 model, the distance between sequences is a function of the proportion of different nucleotides. Namely,

$$d = -\frac{3}{4}\log(1 - 4p/3),$$

where p is the proportion of different nucleotides of the two sequences. The pairwise distances between DNA sequences can be computed by the function `dist.dna` from the `ape` package.

```
> library(ape);library(seqinr)
> accnr <- paste("AJ5345",26:27,sep="")
> seqbin <- read.GenBank(accnr, species.names = TRUE, as.character = FALSE)
> dist.dna(seqbin, model = "JC69")
          AJ534526
AJ534527 0.1326839
```

Hence, the distance is 0.133. Over a total of 1143 nucleotides there are 139 differences, som that the proportion of different nucleotides $139/1143 = p$. Inserting this into the previous distance formula gives the distance. This can be verified as follows.

```
> seq <- read.GenBank(accnr, species.names = TRUE, as.character = TRUE)
> p <- sum(seq$AJ534526==seq$AJ534527)/1143
> d <- -log(1-4*p/3)*3/4
> d
[1] 0.1326839
```

□

Example 5. Phylogenetic tree of a series of downloaded sequences. To further illustrate distances between DNA sequences we shall download the Chamaea fasciata mitochondrial cytb gene for cytochrome b for 10 species of warblers of the genus sylvia (Paradis, 2006). The function `paste` is used to quickly define the accession numbers and `read.GenBank` to actually download the sequences. The species names are extracted and attached to the sequences. We shall use the `dist.dna` function with the `K80` model.

```
library(ape);library(seqinr)
accnr <- paste("AJ5345",26:35,sep="")
seq <- read.GenBank(accnr)
names(seq) <- attr(seq, "species")
dist <- dist.dna(seq, model = "K80")
plot(nj(dist))
```

Obviously, in this manner various trees can be computed and their plots compared. □

When various different models are defined the question becomes apparent which of these fits best to the data relative to the number of parameters (symbols) of the model. When the models are estimated by maximum likelihood, then the Akaike information criterion (AIC = -2 · loglik + 2 · number of free parameters) can be used to select models. The best model is the one with the smallest AIC value.

Example 6. A program called PHYML (Guindon & Gascuel, 2003) can be downloaded from `http://atgc.lirmm.fr/phyml/` and run by the R function `phymltest`, if the executable is available at the same directory. We first write the sequences to the appropriate directory. The output from the program is written to the object called `out` for which the functions `plot(out)` and `summary(out)` can be used to extract more detailed information.

```
> setwd("/share/home/wim/bin")
> write.dna(seq,"seq.txt", format ="interleaved")
> out <-phymltest("seq.txt",format = "interleaved", execname ="phyml_linux")
> print(out)
          nb.free.para    loglik      AIC
JC69                 1 -4605.966 9213.931
JC69+I               2 -4425.602 8855.203
JC69+G               2 -4421.304 8846.608
JC69+I+G             3 -4421.000 8848.001
K80                  2 -4423.727 8851.455
K80+I                3 -4230.539 8467.079
K80+G                3 -4224.457 8454.915
K80+I+G              4 -4223.136 8454.272
F81                  4 -4514.331 9036.662
```

```
F81+I                5 -4309.600 8629.199
F81+G                5 -4304.530 8619.060
F81+I+G              6 -4303.760 8619.519
F84                  5 -4351.164 8712.328
F84+I                6 -4112.006 8236.012
F84+G                6 -4106.568 8225.135
F84+I+G              7 -4105.500 8225.001
HKY85                5 -4333.086 8676.171
HKY85+I              6 -4102.262 8216.524
HKY85+G              6 -4097.401 8206.802
HKY85+I+G            7 -4096.624 8207.248
TN93                 6 -4323.291 8658.581
TN93+I               7 -4097.099 8208.198
TN93+G               7 -4091.461 8196.922
TN93+I+G             8 -4090.790 8197.580
GTR                  9 -4293.398 8604.795
GTR+I               10 -4084.522 8189.043
GTR+G               10 -4079.010 8178.020
GTR+I+G             11 -4078.149 8178.299
```

The notation "+I" and "+G" indicates the presence of invariant sites and/or a gamma distribution of substitution rates. It can be seen that the smallest AIC corresponds to model 27 called `GTR+G`. To plot it, we have to read the trees, and, next, to extract the 27th, see Figure 10.3.

```
tr <- read.tree("seq.txt_phyml_tree.txt")
plot(tr[[27]])
add.scale.bar(length=0.01)
```

In case similar sequences have slightly different lengths, these have to be aligned by programs such as `clustalx` or `clustalw` before these can be used. □

10.6 Hidden Markov Models

In a Hidden Markov Model (HMM) there are two probability transition matrices. There is an emission matrix $\boldsymbol{E}$ and a transition matrix $\boldsymbol{A}$. The

generation of an observable sequence goes in two steps. First, there is a transition from a Markov process of a hidden state and given this value there is an emission of an observable value. We shall illustrate this by the classical example of the occasionally dishonest casino (Durbin et. al., 1998, p.18).

Example 1. Occasionally dishonest casino. A casino uses a fair die most of the time, however, occasionally it switches to an unfair die. The state with respect to fairness is hidden for the observer. The observer can only observe the values of the die and not its hidden state with respect to its fairness. It is convenient to denote fair by 1 and unfair by 2. The transition probabilities of the hidden states are by the emission matrix

$$\boldsymbol{E} = \begin{bmatrix} P(D_i = 1|D_{i-1} = 1) & P(D_i = 2|D_{i-1} = 1) \\ P(D_i = 1|D_{i-1} = 2) & P(D_i = 2|D_{i-1} = 2) \end{bmatrix} = \begin{bmatrix} 0.95 & 0.05 \\ 0.10 & 0.90 \end{bmatrix}.$$

Thus the probability is 0.95 that the die is fair at time point i, given that it is fair at time point $i-1$. The probability that it will switch from fair to unfair is 0.05. The probability that it will switch from loaded to fair is 0.10 and that it stays loaded is 0.90. With this emission matrix we can generate a sequence of hidden states, where the values 1 and 2 indicate whether the die is fair (1) or loaded (2). Given the fairness of the die we define the probability transition matrix.

$$\begin{aligned} \boldsymbol{A} &= \begin{bmatrix} P(O_i = 1|D_i = 1) & P(O_i = 2|D_i = 1) & P(O_i = 3|D_i = 1) & \cdots \\ P(O_i = 1|D_i = 2) & P(O_i = 2|D_i = 2) & P(O_i = 3|D_i = 2) & \cdots \end{bmatrix} \\ &= \begin{bmatrix} 1/6 & 1/6 & 1/6 & 1/6 & 1/6 & 1/6 \\ 1/10 & 1/10 & 1/10 & 1/10 & 1/10 & 1/2 \end{bmatrix}. \end{aligned} \tag{10.4}$$

Thus given that the die is fair, the probability of any outcome equals 1/6. However, given that the die is unfair (loaded), the probability of outcome 6 equals 1/2 and that of any other outcome equals 1/10.

The HMM with this transition and emission matrix can be programmed. After sampling the hidden states from a Markov chain and the outcomes of the die are sampled according to the value of the hidden state (die type).

```
hmmdat <- function(A,E,n){
 observationset <- c(1:6)
 hiddenset <- c(1,2)
 x <- h <- matrix(NA,nr=n,nc=1)
```

```
 h[1]<-1; x[1]<-sample(observationset,1,replace=T,E[h[1],])
 h <- markov(hiddenset,A,n)
 for(k in 1:(n-1)){x[k+1] <- sample(observationset,1,replace=T,E[h[k],])}
 out <- matrix(c(x,h),nrow=n,ncol=2,byrow=FALSE)
  return(out)
  }
E <- matrix(c(rep(1/6,6),rep(1/10,5),1/2),2,6,byrow=T) #emission matrix
A <- matrix(c(0.95,0.05,0.1,0.9),2,2,byrow=TRUE)       #transition matrix
dat <- hmmdat(A,E,100)
colnames(dat) <- c("observation","hidden_state")
rownames(dat)  <- 1:100
> t(dat)
              1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
observations  5 2 3 1 6 1 3 1 1  5  6  6  2  2  3  5  4  6  1  2  4  4  3  2  3
hidden_states 1 1 1 1 1 1 1 1 1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
              26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47
observations   4  3  2  4  1  6  6  6  6  6  5  5  3  6  1  6  5  2  4  1  4  2
hidden_states  1  1  1  2  2  2  2  2  2  1  1  1  1  1  1  1  1  1  1  1  1  1
              48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69
observations   5  6  5  2  3  3  1  3  3  5  6  6  2  4  5  4  6  1  6  5  2  6
hidden_states  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
              70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91
observations   1  1  4  4  1  5  6  4  3  5  4  2  6  1  3  6  5  2  2  6  6  1
hidden_states  1  1  1  1  1  1  1  1  1  2  2  2  2  2  2  2  2  2  2  2  1  1
              92 93 94 95 96 97 98 99 100
observations   4  1  6  5  5  6  5  3   4
hidden_states  1  1  1  1  1  1  1  1   1
```

□

In certain applications to bioinformatics, it is of most importance to estimate the value of the hidden state given the data. The Viterbi algorithm is developed to predict the hidden state given the data and the (estimated) transition and emission matrix. The algorithm builds up a matrix $v(i,l)$, where i runs from one to the number of observations and l from one to the number of states. The initial values are $v(1,1) = 1$, and $v(1,l) = 0$ for all l. Then the values for $v(i,l)$ are recursively defined by

$$v(i,l) = e(l,x(i)) \cdot \max_k \{v(i-1,k)a(k,l)\}\,.$$

For each row of the matrix the maximum is taken as the best predictor of the hidden state.

Example 2. The viterbi algorithm can be programmed and applied to the hidden states of the data generated with respect to the occasionally dishonest casino.

```
viterbi <- function(A,E,x) {
  v <- matrix(NA, nr=length(x), nc=dim(A)[1])
  v[1,] <- 0; v[1,1] <- 1
  for(i in 2:length(x)) {
    for (l in 1:dim(A)[1]) {v[i,l] <- E[l,x[i]] * max(v[(i-1),] * A[l,])}
   }
  return(v)
}
vit <- viterbi(A,E,dat[,1])
vitrowmax <- apply(vit, 1, function(x) which.max(x))
hiddenstate <- dat[,2]
> table(hiddenstate, vitrowmax)
           vitrowmax
hiddenstate  1  2
          1 72 11
          2 15  2

datt <- cbind(dat,vitrowmax)
colnames(datt) <- c("observation","hidden_state","predicted state")
> t(datt)
                1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
observation     5 2 3 1 6 1 3 1 1  5  6  6  2  2  3  5  4  6  1  2  4  4  3  2
hidden_state    1 1 1 1 1 1 1 1 1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
predicted state 1 1 1 1 1 1 1 1 1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
                25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45
observation      3  4  3  2  4  1  6  6  6  6  6  5  5  3  6  1  6  5  2  4  1
hidden_state     1  1  1  1  2  2  2  2  2  2  1  1  1  1  1  1  1  1  1  1  1
predicted state  1  1  1  1  1  1  1  1  2  2  2  2  2  2  2  2  2  2  2  2  2
                46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66
observation      4  2  5  6  5  2  3  3  1  3  3  5  6  6  2  4  5  4  6  1  6
hidden_state     1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
```

```
predicted state  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
                67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87
observation      5  2  6  1  1  4  4  1  5  6  4  3  5  4  2  6  1  3  6  5  2
hidden_state     1  1  1  1  1  1  1  1  1  1  1  1  2  2  2  2  2  2  2  2  2
predicted state  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1  1
                88 89 90 91 92 93 94 95 96 97 98 99 100
observation      2  6  6  1  4  1  6  5  5  6  5  3   4
hidden_state     2  2  1  1  1  1  1  1  1  1  1  1   1
predicted state  1  1  1  1  1  1  1  1  1  1  1  1   1
```

The misclassification rate is 0.27 which is quite large given the fact that we used the true transition and emission matrix. An important observation is that after a transition of a hidden state, it takes a few values for the prediction to change. This is caused by the recursive nature of the algorithm. □

10.7 Appendix

The probability that the process is in State 1 at time point 1 can be computed as follows.

$$\begin{aligned} P(X_1 = 1) &= P(X_1 = 1, X_0 = 1) + P(X_1 = 1, X_0 = 2) \\ &= P(X_1 = 1|X_0 = 1) \cdot P(X_0 = 1) + P(X_1 = 1|X_0 = 2) \cdot P(X_0 = 2) \\ &= \pi_{10} p_{11} + \pi_{20} p_{21} \\ &= \boldsymbol{\pi}_0^T \boldsymbol{p}_1, \end{aligned}$$

where $\boldsymbol{p}_1$ is the first column of $\boldsymbol{P}$.

In particular, it holds that

$$\begin{aligned} P(X_2 = 1|X_0 = 1) &= P(X_2 = 1, X_1 = 1|X_0 = 1) + P(X_2 = 1, X_1 = 2|X_0 = 1) \\ &= \sum_{k=1}^{2} P(X_2 = 1, X_1 = k|X_0 = 1) \\ &= \sum_{k=1}^{2} P(X_2 = 1|X_1 = k, X_0 = 1) \cdot P(X_1 = k|X_0 = 1) \\ &= \sum_{k=1}^{2} P(X_2 = 1|X_1 = k) \cdot P(X_1 = k|X_0 = 1) \\ &= p_{11}p_{11} + p_{21}p_{12} \\ &= \text{row 1 of } \boldsymbol{P} \text{ times column 1 of } \boldsymbol{P} = \boldsymbol{P}^2_{11}, \end{aligned}$$

where the latter is element $(1, 1)$ of the matrix $\boldsymbol{P}^2 = \boldsymbol{P} \cdot \boldsymbol{P}$.

10.8 Overview and concluding remarks

The probability transition matrix is extensively explained and illustrated because it is a cornerstone to many ideas in bioinformatics. A thorough treatment of phylogenetics is given by Paradis (2006) and of Hidden Markov Models by Durbin et. al (2005).

10.9 Exercises

1. Visualize by a transition graph the following transition matrices. For the process with four states take the names of the nucleotides in the order A, G, T, and C.

$$\begin{pmatrix} \frac{1}{3} & \frac{2}{3} \\ \frac{3}{4} & \frac{1}{4} \end{pmatrix}, \begin{pmatrix} 1 & 0 \\ 0 & 1 \end{pmatrix}, \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix}, \begin{pmatrix} \frac{1}{4} & \frac{2}{4} & 0 & \frac{1}{4} \\ \frac{1}{6} & \frac{2}{6} & \frac{2}{6} & \frac{1}{6} \\ 0 & \frac{2}{7} & \frac{5}{7} & 0 \\ \frac{1}{8} & \frac{1}{8} & \frac{2}{8} & \frac{4}{8} \end{pmatrix}, \begin{pmatrix} \frac{1}{4} & \frac{3}{4} & 0 & 0 \\ \frac{1}{6} & \frac{5}{6} & 0 & 0 \\ 0 & 0 & \frac{5}{7} & \frac{2}{7} \\ 0 & 0 & \frac{3}{8} & \frac{5}{8} \end{pmatrix}.$$

2. Computing probabilities. Given the states 0 and 1 and the following initial distribution and probability matrix

$$\boldsymbol{\pi}_0 = \begin{pmatrix} \frac{1}{2} & \frac{1}{2} \end{pmatrix}, \boldsymbol{P} = \begin{pmatrix} \frac{3}{4} & \frac{1}{4} \\ \frac{1}{2} & \frac{1}{2} \end{pmatrix}.$$

(a) Compute $P(X_1 = 0)$.

(b) Compute $P(X_1 = 1)$.

(c) Compute $P(X_2 = 0|X_0 = 0)$.

(d) Compute $P(X_2 = 1|X_0 = 0)$.

3. Programming GTR. Use $\pi_A = 0.15$, $\pi_G = 0.35$, $\pi_C = 0.35$, $\pi_T = 0.15$, $\alpha = 4$, $\beta = 0.5$, $\gamma = 0.4$, $\delta = 0.3$, $\epsilon = 0.2$, and $\zeta = 4$.

   (a) Program the rate matrix in such a manner that it is simple to adapt for other values of the parameters.

   (b) Is the transversion rate larger or smaller then the transition rate?

   (c) Compute the corresponding the probability transition matrix.

   (d) Try to argue whether you expect a large frequency of transversions or translations.

   (e) Generate a sequence of 99 nucleotide residues according to the markov model.

4. Distance according to JC69.

   (a) Down load the sequences `AJ534526` and `AJ534527`. Hint: Use `as.character = TRUE` in the `read.GenBank` function.

   (b) Compute the proportion of different nucleotides.

   (c) Use this proportion to verify the distances between these sequences according to the JC69 model.

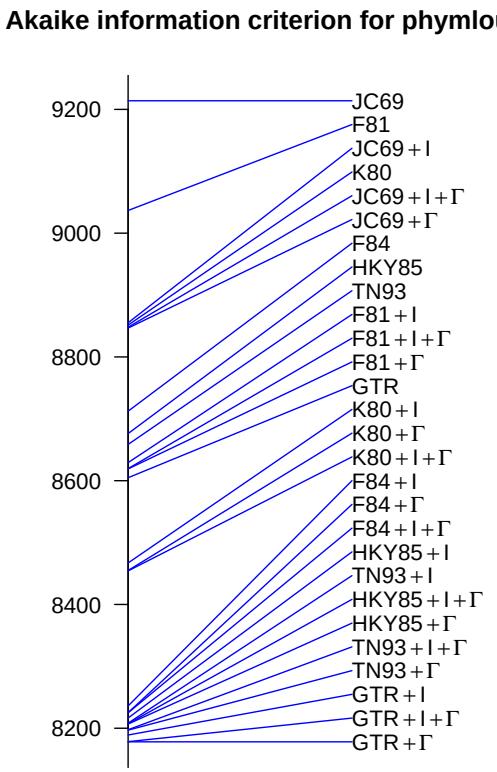


Figure 10.2: Evaluation of models by AIC .

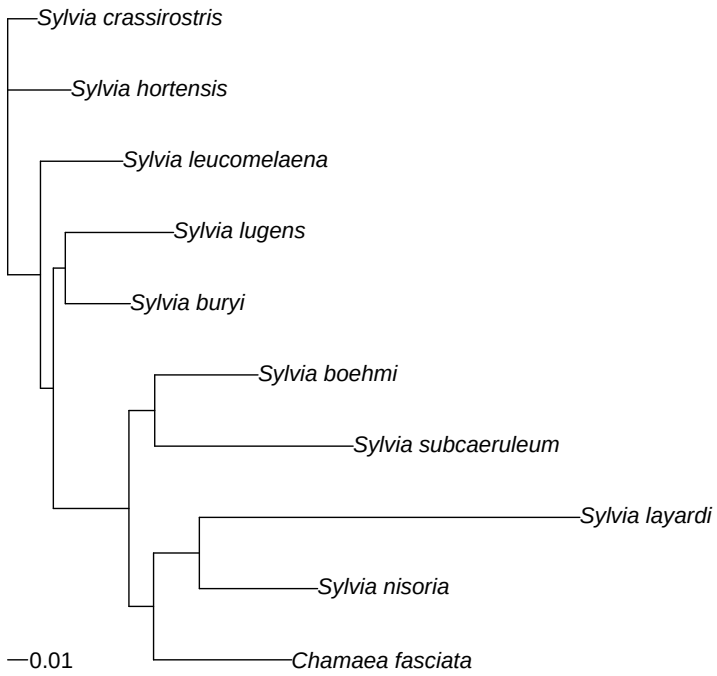


Figure 10.3: Tree according to GTR model.

Appendix A

Answers to exercises

Answers to exercises of Chapter 1: Brief Introduction to R

1. Some questions to orientate yourself.

   (a) matrix, numeric, numeric, matrix, function, function, factor, standardGeneric, ExpressionSet.

   (b) remove, summation, product, sequence, standard deviation, number of rows,

   (c) Use R its help or use the internet search key "r wiki grep" to find the following answers: searching regular expressions, return a vector from a function on the rows or columns of a matrix, generate a factor by specifying the pattern of levels, load add-on packages, make R reading input from a file or URL, set the working directory to a certain map, print the last · commands given from the command line, give the structure of an object.

2. `gendat`

   (a) `apply(gendat,2,sd)`.

   (b) `apply(gendat,1,sd)`.

   (c) To order the data frame according to the gene standard deviations.

   ```
   sdexprsval <- apply(gendat,1,sd)
   o <- order(sdexprsval,decreasing=TRUE)
   gendat[o,]
   ```

(d) `gene1`

3. Computations on gene means of the Golub data.

   (a) Computation of mean gene expression values.

   ```
   data(golub, package = "multtest")
   meangol <- apply(golub,1,mean)
   ```

   (b) To order the data frame use `o <- order(meangol,decreasing=TRUE)` and `golub[o,]`

   (c) Give the names of the three genes with the largest mean expression value.

   ```
   > golub.gnames[o[1:3],3]
   [1] "U43901_rna1_s_at" "M13934_cds2_at"   "X01677_f_at"
   ```

   (d) Give their biological names.

   ```
   > golub.gnames[o[1:3],2]
   [1] "37 kD laminin receptor precursor/p40 ribosome associated protein
   [2] "RPS14 gene (ribosomal protein S14) extracted from Human ribosoma
   [3] "GAPD Glyceraldehyde-3-phosphate dehydrogenase"
   ```

4. Computations on gene standard deviations of the Golub data.

   (a) The standard deviation per gene can be computed by `sdgol <- apply(golub,1,sd)`.

   (b) The gene with standard deviation larger than 0.5 can be selected by `golubsd <- golub[sdgol>0.5,]`.

   (c) `sum(sdgol>0.5)` gives that the number of genes having sd larger than 0.5 is 1498.

5. Oncogenes in Golub data.

   (a) `length(agrep("^oncogene",golub.gnames[,2]))` gives 42.

   (b) By the script below the `"Cellular oncogene c-fos` is found.

   ```
   data(golub, package="multtest")
   rowindex <- agrep("^oncogene",golub.gnames[,2])
   oncogol <- golub[rowindex,]
   ```

```
oncogolub.gnames <- golub.gnames[rowindex,]
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
meangol <- apply(oncogol[,gol.fac=="ALL"],1,mean)
o <- order(meangol,decreasing=TRUE)
> oncogolub.gnames[o[1:3],2]
[1] "PIM1 Pim-1 oncogene"       "JUNB Jun B proto-oncogene"
[3] "Proto-oncogene BCL3 gene"
```

(c)
```
meangol <- apply(oncogol[,gol.fac=="AML"],1,mean)
o <- order(meangol,decreasing=TRUE)
> oncogolub.gnames[o[1:3],2]
[1] "PIM1 Pim-1 oncogene"       "JUNB Jun B proto-oncogene"
[3] "Proto-oncogene BCL3 gene"
```

(d) Writing results to a `csv` file. Be aware of the correct column separation.

```
x <- oncogolub.gnames[o[1:10],c(3,2)]
colnames(x) <- c("probe ID","gene name")
write.csv(x,file="goluboutcsv")
write.table(x,file="goluboutnorowname",row.names=FALSE)
```

6. Constructing a factor.

   (a) `gl(2,4)`.

   (b) `gl(5,3)`.

   (c) `gl(3,5)`.

7. Gene means for B1 patients.

```
library(ALL); data(ALL)
meanB1 <- apply(exprs(ALL[,ALL$BT=="B1"]),1, mean)
o <- order(meanB1,decreasing=TRUE)
> meanB1[o[1:3]]
AFFX-hum_alu_at        31962_at      31957_r_at
       13.41648        13.16671        13.15995
```

Answers to exercises of Chapter 2: Descriptive Statistics and Data Display

1. Illustration of mean and standard deviation.

   (a) Use `x<- c(1,1.5,2,2.5,3)` and `mean(x)` and `sd(x)` to obtain the mean is 2 and the standard deviation is 0.7905694.

   (b) Now the mean is 7.4 and dramatically increased the standard deviation 12.64615.

   (c) The outlier increased the mean as well as the standard deviation.

2. Comparing two genes. Take `i <- 66` or `i <- 790`.

   (a) Use `boxplot(golub[i,]~gol.fac)` to observe that 790 has three outliers and 66 has no.

   (b) Use `qqnorm(golub[i,gol.fac=="ALL")` and `qqline(golub[i,gol.fac=="ALL"])` to observe that nearly all values of 66 are on the line, where as for 790 the three outliers are way of the normality line. Hypothesis: The expression values of 66 are normally distributed, but those of row 790 are not.

   (c) Use `mean(golub[i,gol.fac=="ALL"])` and `median(golub[i,gol.fac=="ALL"])`. The mean (-1.174024) is larger than the median (-1.28137) due to outliers on the right hand side. For the gen in row 66 the mean is 1.182503 and the median 1.23023. The differences are smaller.

3. Effect size.

   (a) The size 11 is large, because the mean is eleven times larger than the standard deviation.

```
data(golub, package="multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
efs <- apply(golub[,gol.fac=="ALL"],1,function(x) mean(x)/sd(x))
o <- order(efs,decreasing=TRUE)
> efs[o[1:5]]
[1] 11.138128 10.638308  9.155108  8.954115  8.695353
> golub.gnames[o[1:5],2]
[1] "YWHAZ Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activa
[2] "ZNF91 Zinc finger protein 91 (HPF7, HTF10)"
[3] "HnRNP-E2 mRNA"
[4] "54 kDa protein mRNA"
[5] "Immunophilin homolog ARA9 mRNA"
```

(b) The robust variant can be defined by dividing the median by the MAD. An alternative would be to divide the median by the IQR. This gives other best genes indicating that the some genes may have outliers that influence the outcome.

```
refs <- apply(golub[,gol.fac=="ALL"],1,function(x) median(x)/mad(x))
o <- order(refs,decreasing=TRUE)
> refs[o[1:5]]
[1] 14.51217 13.57425 13.27698 13.14419 12.91608
> golub.gnames[o[1:5],2]
[1] "COX6B gene (COXG) extracted from Human DNA from overlapping chromosome
   F25451, and R31076 containing COX6B and UPKA, genomic sequence"
[2] "AFFX-HSAC07/X00351_M_at (endogenous control)"
[3] "ATP5A1 ATP synthase, H+ transporting, mitochondrial F1 complex, alpha s
    isoform 1, cardiac muscle"
[4] "ATP SYNTHASE GAMMA CHAIN, MITOCHONDRIAL PRECURSOR"
[5] "YWHAZ Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation pr
   zeta polypeptide"
```

4. Plotting gene expressions "CCND3 Cyclin D3". The answers in the script below.

```
data(golub, package = "multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
stripchart(golub[1042,] ~ gol.fac,method="jitter")
stripchart(golub[1042,] ~ gol.fac,method="jitter",vertical = TRUE)
stripchart(golub[1042,] ~ gol.fac,method="jitter",col=c("red", "blue"),
  vertical = TRUE)
stripchart(golub[1042,] ~ gol.fac,method="jitter",col=c("red", "blue"),
  pch="*",vertical = TRUE)
title("CCND3 Cyclin D3 expression value for ALL and AMl patients")
```

5. Box-and-Whiskers plot of "CCND3 Cyclin D3"..

```
locator()
x11()
x <- data(golub, package = "multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
boxplot(x,xlim=c(0,4))
```

```
arrows(2.0,1.93,1.24,1.93);text(2.5,1.93,"Median")
arrows(2.0,1.1,1.24,1.1)  ;text(2.5,1.1,"Outlier")
arrows(2.0,1.79,1.24,1.79);text(2.5,1.79,"first quartile")
arrows(2.0,2.17,1.24,2.17);text(2.5,2.17,"third quartile")
arrows(2.0,1.27,1.24,1.27);text(2.5,1.27,"lower wisker")
arrows(2.0,2.59,1.24,2.59);text(2.5,2.59,"upper wisker")
dev.copy2eps(device=x11,file="BoxplotWithExplanation.eps")
boxplot.stats(x, coef = 1.5, do.conf = TRUE, do.out = TRUE)  #finds value
```

6. Box-and-wiskers plot of persons of Golub et al. (1999) data..

   (a) The medians are all around zero, the inter quartile range differ only slightly, the minimal values are all around minus 1.5. All persons have outliers near three.

   (b) The means are very close to zero. The medians are all between $(-0.15383, 0.06922)$, so these are also close to zero.

   ```
   personmean <- apply(golub,2,mean)
   personmedian <- apply(golub,2,median)
   ```

   (c) The data seem preprocessed to have standard deviation equal to one. The re-scaled IQR and MAD have slightly larger range.

   ```
   > range(apply(golub,2,sd))
   [1] 0.9999988 1.0000011
   > range(apply(golub,2,function(x) IQR(x)/1.349))
   [1] 0.9599036 1.3361527
   > range(apply(golub,2,mad))
   [1] 0.9590346 1.2420185
   ```

7. Oncogenes of Golub et al. (1999) data.

   (a) Note that we need the transpose operator `t` to change rows into columns. The script below will do.

   ```
   data(golub, package="multtest")
   gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
   rowindex <- agrep("^oncogene",golub.gnames[,2])
   oncogol <- golub[rowindex,]
   oncogolub.gnames <- golub.gnames[rowindex,]
   ```

```
row.names(oncogol) <- oncogolub.gnames[,3]
boxplot(data.frame(t(oncogol[,gol.fac=="ALL"])))
```

(b) The plot gives a nice overview of the distributions of the gene expressions values of the onco gene separately for the ALL and the AML patients. Several genes behave similarly for ALL and AML. Some are clearly distributed around zero, but others not. Also, some have a small inter quartile ranges, while for others this is large. A similar statement holds for outliers, some do not have outliers, but others certainly have. Some gene show distinct distributions between patient groups. For instance, the sixth has ALL expressions around zero, but those for AML are larger than zero.

```
par(mfrow=c(2,1))
boxplot(data.frame(t(oncogol[,gol.fac=="ALL"])))
title("Box and wiskers plot for oncogenes of ALL patients ")
boxplot(data.frame(t(oncogol[,gol.fac=="AML"])))
title("Box and wiskers plot for oncogenes of AML patients ")
par(mfrow=c(1,1))
```

8. Descriptive statistics for the ALL gene expression values of the Golub et al. (1999) data.

(a) The ranges indicate strong difference in means. The range of the mean and of the median are similar. The bulk of the data seems symmetric.

```
> range(apply(golub[,gol.fac=="ALL"],1,mean))
[1] -1.330984  3.278551
> range(apply(golub[,gol.fac=="ALL"],1,median))
[1] -1.36832  3.35455
```

(b) The range of the standard deviation is somewhat smaller than of the re-scaled IQR and MAD.

```
> range(apply(golub[,gol.fac=="ALL"],1,sd))
[1] 0.1336206 2.0381309
> range(apply(golub[,gol.fac=="ALL"],1,function(x) IQR(x)/1.349))
[1] 0.1153113 2.7769867
> range(apply(golub[,gol.fac=="ALL"],1,mad))
```

```
[1] 0.1056649 2.9656744
```

Answers to exercises of Chapter 3: Important Distributions

1. Binomial
   (a) $P(X = 24) = 0.1046692$, $P(X \leq 24) = 0.5557756$, and $P(X \geq 30) = 0.0746237$.
   (b) $P(20 \leq X \leq 30) = 0.83856$, $P(20 \leq X) = 0.8830403$.
   (c) $P(20 \leq XorX \geq 40) = 0.8830403$, and $P(20 \leq XandX \geq 10) = 0.999975$.
   (d) $E(X) = 24$, $\text{var}(X) = 3.794733$ Use: `sqrt(60 * 0.4 *0.6)`
   (e) $x_{0.025} = 17$, $x_{0.5} = 24$, and $x_{0.975} = 32$.
2. Standard Normal.
   (a) $P(1.6 < Z < 2.3) = 0.04408$.
   (b) $P(Z < 1.64) = 0.9495$.
   (c) $P(-1.64 < Z < -1.02) = 0.1034$.
   (d) $P(0 < Z < 1.96) = 0.4750$.
   (e) $P(-1.96 < Z < 1.96) = 0.9500$.
   (f) $z_{0.025} = -1.959964$, $z_{0.05} = -1.644854$, $z_{0.5} = 0$, $z_{0.95} = 1.644854$, and $z_{0.975} = 1.959964$.
3. Normal.
   (a) $P(X < 12) = 0.8413$.
   (b) $P(X > 8) = 0.8413$.
   (c) $P(9 < X < 10,5) = 0.2917$.
   (d) The quantiles $x_{0.025} = 6.080072$, $x_{0.5} = 10$, and $x_{0.975} = 13.91993$.
4. T-distribution.
   (a) $P(T_6 < 1) = 0.8220412$.
   (b) $P(T_6 > 2) = 0.04621316$.
   (c) $P(-1 < T_6 < 1) = 0.6440823$.

(d) $P(-2 < T_6 < -2) = 0.9075737$.

(e) $t_{0.025} = -2.446912$, $t_{0.5} = 0$, and $t_{0.975} = 2.446912$.

5. F distribution.

(a) $P(F_{8,5} < 3) = 0.8792198$.

(b) $P(F_{8,5} > 4) = 0.07169537$.

(c) $P(1 < F_{8,5} < 6) = 0.4931282$.

(d) The quantiles $f_{0.025} = 0.2075862$, $f_{0.5} = 1.054510$, and $f_{0.975} = 6.757172$.

6. Chi-squared distribution.

(a) $P(\chi^2_{10} < 3) = 0.01857594$.

(b) $P(\chi^2_{10} > 4) = 0.947347$.

(c) $P(1 < \chi^2_{10} < 6) = 0.1845646$.

(d) The quantiles $g_{0.025} = 3.246973$, $g_{0.5} = 9.341818$, and $g_{0.975} = 20.48318$.

7. MicroRNA.

(a) $P(X = 14) = \texttt{dbinom}(14, 20, 0.7) = 0.191639$.

(b) $P(X \leq 14) = \texttt{pbinom}(14, 20, 0.7) = 0.5836292$.

(c) $P(X > 10) = 1 - P(X \leq 10) = 1 - \texttt{pbinom}(10, 20, 0.7) = 0.9520381$.

(d) $P(10 \leq X \leq 15) = P(X \leq 15) - P(X \leq 9) = \texttt{pbinom}(15, 20, 0.7) - \texttt{pbinom}(9, 20, 0.7) = 0.7453474$.

(e) $20 \cdot 0.7 = 14$.

(f) `sqrt(20* 0.7 * 0.3)`=2.04939.

8. Zyxin.

(a) $P(X \leq 1.2)$ =`pnorm(1.2,1.6,0.4)`=0.1586553.

(b) $P(1.2 \leq X \leq 2.0)$ =`pnorm(2.0,1.6,0.4) - pnorm(1.2,1.6,0.4)`=0.6826895.

(c) $P(2.4 \leq X \leq 0.8)$ =`pnorm(2.4,1.6,0.4) - pnorm(0.8,1.6,0.4)`=0.9544997.

(d) $x_{0.025}$ =`qnorm(0.025,1.6,0.4)`=0.8160144. Similarly, $x_{0.975}$ = 2.383986.

(e) `x <- rnorm(1000,1.6,0.4)` gives `mean(x)` = 1.608401 and `sd(x)`=0.4022082. Both are close to the values in the population.

9. Some computations on Golub et al. (1999) data.

(a) The tree larges t-value 57.8, 55.2, and 47.5 are extremely large.

```
data(golub, package="multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
tval <- apply(golub[,gol.fac=="ALL"],1,function(x) sqrt(27) * mean(x)
o <- order(tval,decreasing=TRUE)
tval[o[1:3]]
golub.gnames[o[1:3],2]
```

(b) The scrip below gives 2185 ratios between 0.5 and 1.5.

```
sdall <- apply(golub[,gol.fac=="ALL"],1, sd)
sdaml <- apply(golub[,gol.fac=="AML"],1, sd)
sdratio <- sdall/sdaml
sum( sdratio > 0.5 & sdratio < 1.5)
```

10. Extreme value investigation. The blue line (extreme value) fits to the black line (density of generated extreme data) much better than the red line (normal distribution).

```
an <- sqrt(2*log(n)) - 0.5*(log(log(n))+log(4*pi))*(2*log(n))^(-1/2)
bn <- (2*log(n))^(-1/2)
e <- double(); n <- 10000 # Serfling p.90
for (i in 1:1000) e[i] <- (max(rnorm(n))-an)/bn
plot(density(e),ylim=c(0,0.5))
f<-function(x){exp(-x)*exp(-exp(-x))}
curve(f,range(density(e)$x),add=TRUE,col = "blue")
curve(dnorm,add=TRUE,col = "red")
```

Answers exercise chapter 4: Estimation and Inference

1. Gene CD33. Use `agrep("^CD33",golub.gnames[,2])` to find 808.

(a) The code

```
library(multtest);data(golub)
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
shapiro.test(golub[i,gol.fac=="ALL"])
```

gives p-value = 0.592 and changing ALL into AML gives p-value = 0.2583. Hence, for normality is accepted.

(b) `var.test(golub[i,] ~ gol.fac)` gives p-value = 0.1095 so equality of variances is accepted.

(c) `t.test(golub[i,] ~ gol.fac, var.equal = TRUE)` gives `p-value = 1.773e-09`, so equality of means is rejected.

(d) Yes, `t = -7.9813` is quite extreme.

2. Gene MYBL2 V-myb avian myeloblastosis viral oncogene homolog-like 2. Take `i <- 1788`.

(a) Use `boxplot(golub[i,] ~ gol.fac)` to observe from the boxplot that one is quite certain that the null-hypothesis of no experimental effect holds.

(b) `t.test(golub[i,] ~ gol.fac, var.equal = TRUE)` gives `p-value = 0.8597`, so that the null hypothesis of equal means is accepted.

3. HOXA9. Use `i <- 1391`.

(a) `shapiro.test(golub[i,gol.fac=="ALL"])` gives `p-value = 1.318e-07`, so that normality is rejected.

(b) `wilcox.test(golub[i,] ~ gol.fac)` gives `p-value = 7.923e-05`, so that equality of means is rejected. Note that the p-value from Grubbs test of the ALL expression values is 0.00519, so the null hypothesis of no outliers is rejected. Nevertheless the Welch two-sample T-test is also rejects the null-hypothesis of equal means. Its t-value equals -4.3026 and is quite large.

4. Zyxin.

(a) Searching NCBI UniGene on zyxin gives BC002323.2.

(b) Use `chisq.test(as.data.frame(table(read.GenBank(c("BC002323.2"))))$Freq)` to find `p-value < 2.2e-16`, so that the null-hypothesis of equal frequencies is rejected.

(c) We download and store the frequencies of the sequences in `x` and `y`. Next the empirical probabilities from `y` are use to predict the frequencies from `y`.

```
x <- as.data.frame(table(read.GenBank(c("X94991.1"))))$Freq
y <- as.data.frame(table(read.GenBank(c("BC002323.2"))))$Freq
>chisq.test(x, p=y/sum(y))
        Chi-squared test for given probabilities
data:  x
X-squared = 0.0277, df = 3, p-value = 0.9988
```

5. Gene selection.

```
ptg <- apply(golub, 1, function(x) t.test(x ~  gol.fac,
   alternative = c("greater"))$p.value)
golub.gnames[order(ptg)[1:10],2]
```

6. Antigenes.

```
library(multtest); data(golub)
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
pt <- apply(golub, 1, function(x) t.test(x ~  gol.fac)$p.value)
index <-agrep("^antigen",golub.gnames[,2])
golub.index<-golub[index,]
pt.index<-pt[index]
golub.gnames.index<-golub.gnames[index,]
golub.gnames.index[order(pt.index)[1:length(index)],2]
```

7. Genetic Model. From the output below the null hypothesis that the probabilities are as specified is accepted.

```
> chisq.test(x=c(930,330,290,90),p=c(9/16,3/16,3/16,1/16))

        Chi-squared test for given probabilities

data:  c(930, 330, 290, 90)
X-squared = 4.2276, df = 3, p-value = 0.2379
```

8. Comparing two genes.

```
all66 <- golub[66,gol.fac=="ALL"]
all790 <- golub[790,gol.fac=="ALL"]
boxplot(all66,all790)
mean(all66);mean(all790)
median(all66);median(all790)
sd(all66);sd(all790)
IQR(all66)/1.349 ;IQR(all790)/1.349
mean(all66);mean(all790)
mad(all66);mad(all790)
shapiro.test(all66);shapiro.test(all790)
```

9. Normality tests.

```
library(multtest);data(golub)
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
allsh <- apply(golub[,gol.fac=="ALL"], 1, function(x) shapiro.test(x)$p.value)
amlsh <- apply(golub[,gol.fac=="AML"], 1, function(x) shapiro.test(x)$p.value)
> 100 * sum(allsh>0.05)/length(allsh)
[1] 58.27598
> 100 * sum(amlsh>0.05)/length(amlsh)
[1] 78.5644
> 100 * sum(allsh>0.05 & allsh>0.05)/length(allsh)
[1] 58.27598
```

10. Two-sample tests on gene expression values of the Golub et al. (1999) data.

    (a)
```
data(golub, package = "multtest");
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
pt <- apply(golub, 1, function(x) t.test(x ~  gol.fac)$p.value)
pw <- apply(golub, 1, function(x) wilcox.test(x ~  gol.fac)$p.value)
o <- order(pt,decreasing=FALSE)
> golub.gnames[o[1:10],2]
 [1] "Zyxin"
 [2] "FAH Fumarylacetoacetate"
 [3] "APLP2 Amyloid beta (A4) precursor-like protein 2"
 [4] "LYN V-yes-1 Yamaguchi sarcoma viral related oncogene homolog"
 [5] "CST3 Cystatin C (amyloid angiopathy and cerebral hemorrhage)"
```

```
 [6] "X-LINKED HELICASE II"
 [7] "RB1 Retinoblastoma 1 (including osteosarcoma)"
 [8] "TOP2B Topoisomerase (DNA) II beta (180kD)"
 [9] "TCRA T cell receptor alpha-chain"
[10] "T-COMPLEX PROTEIN 1, GAMMA SUBUNIT"
```

(b)
```
> o <- order(pw,decreasing=FALSE)
> golub.gnames[o[1:10],2]
 [1] "FAH Fumarylacetoacetate"
 [2] "Zyxin"
 [3] "CST3 Cystatin C (amyloid angiopathy and cerebral hemorrhage)"
 [4] "ELA2 Elastatse 2, neutrophil"
 [5] "TCF3 Transcription factor 3 (E2A immunoglobulin enhancer bindin
 [6] "Macmarcks"
 [7] "LYN V-yes-1 Yamaguchi sarcoma viral related oncogene homolog"
 [8] "CD33 CD33 antigen (differentiation antigen)"
 [9] "VIL2 Villin 2 (ezrin)"
[10] "APLP2 Amyloid beta (A4) precursor-like protein 2"
```

11. Biological hypotheses.

    (a) $n = 1000$, $p = 0.05$ so $np = 50$

    (b) `pbinom(9,1000,.05)`$=5.24 \cdot 10^{-13}$.

    (c) `sum(dbinom(6:1000,1000,.05))`$=1$.

    (d) `sum(dbinom(2:8,1000,.05))`$= 8.8 \cdot 10^{-14}$.

12. Programming some tests.

    (a)
```
data(golub,package="multtest")
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
x <- golub[1042,gol.fac=="ALL"]
n <- length(x)
y <- golub[1042,gol.fac=="AML"]
m <- length(y)
t <- (mean(x)-mean(y))/sqrt(var(x)/n + var(y)/m)
v <- (var(x)/n + var(y)/m)^2/( (var(x)/n)^2/(n-1) + (var(y)/m)^2/(m-1
2*pt(-abs(t),v)
mean(x) - mean(y) + qt(0.025,v)* sqrt(var(x)/n + var(y)/m)
mean(x) - mean(y) + qt(0.975,v)* sqrt(var(x)/n + var(y)/m)
```

(b)
```
z <- golub[1042,]
> sum(rank(z)[1:27]) - 0.5*27*(27+1)
[1] 284
```

(c)
```
x <- golub[1042,gol.fac=="ALL"]
y <- golub[1042,gol.fac=="AML"]
w <- 0
for (i in 1:27) w <- w + sum(x[i]>y)
> w
[1] 284
```

Answers to exercises of Chapter 5 Linear Models

1. Analysis of gene expressions of B-cell ALL patients.

```
library(ALL); data(ALL)
ALLB <- ALL[,ALL$BT %in% c("B","B1","B2","B3","B4")]
> table(ALLB$BT)

 B B1 B2 B3 B4  T T1 T2 T3 T4
 5 19 36 23 12  0  0  0  0  0

psw <- apply(exprs(ALLB), 1, function(x) shapiro.test(residuals(lm(x ~ ALLB$BT))))
library(lmtest)
pbp <-apply(exprs(ALLB), 1, function(x)
   as.numeric(bptest(lm(x ~ ALLB$BT),studentize = FALSE)$p.value))
> sum(psw > 0.05)
[1] 6847
> sum(pbp > 0.05)
[1] 10057
> sum(psw > 0.05 & pbp > 0.05)
[1] 6262
```

2. Further analysis of gene expressions of B-cell ALL patients.

```
> panova <- apply(exprs(ALLB), 1, function(x) anova(lm(x ~ ALLB$BT))$Pr[1])
> featureNames(ALLB)[panova<0.000001]
 [1] "1125_s_at"  "1126_s_at"  "1134_at"    "1389_at"    "1500_at"
```

```
 [6] "1866_g_at"  "1914_at"    "205_g_at"   "31472_s_at" "31615_i_at"
[11] "31616_r_at" "33358_at"   "35614_at"   "35991_at"   "36873_at"
[16] "37809_at"   "37902_at"   "38032_at"   "38555_at"   "39716_at"
[21] "40155_at"   "40268_at"   "40493_at"   "40661_at"   "40763_at"
[26] "41071_at"   "41139_at"   "41448_at"   "873_at"
> pkw <- apply(exprs(ALLB), 1, function(x) kruskal.test(x ~ ALLB$BT)$p.va
> featureNames(ALLB)[pkw<0.000001]
[1] "1389_at"   "1866_g_at" "38555_at"  "40155_at"  "40268_at"

> panovasmall <- panova < 0.001
> pkwsmall <- pkw < 0.001
> table(panovasmall,pkwsmall)
           pkwsmall
panovasmall FALSE  TRUE
      FALSE 12172    38
      TRUE    124   291
```

There are 124 significant gene expressions from ANOVA which are not significant on Kruskal-Wallis. There are only 38 significant gene expressions from Kruskal-Wallis which are non-significant according to ANOVA. The tests agree on the majority of gene expressions.

3. Finding the ten best best genes among gene expressions of B-cell ALL patients.

```
> sort(panova)[1:10]
     1914_at      1389_at     38555_at     33358_at     40268_at     3971
1.466523e-14 5.891702e-14 4.873245e-10 1.117406e-09 1.145502e-09 4.748615
    40763_at     37809_at     36873_at    1866_g_at
5.256410e-09 2.155457e-08 2.402379e-08 3.997065e-08
> sort(pkw)[1:10]
     1389_at     40268_at     38555_at    1866_g_at     40155_at      191
2.348192e-09 7.764046e-08 1.123068e-07 2.335279e-07 6.595926e-07 1.074525
   1125_s_at   40662_g_at     38032_at     40661_at
1.346907e-06 1.384281e-06 1.475170e-06 1.719456e-06

npanova <- names(sort(panova)[1:10])
npkw <- names(sort(pkw)[1:10])
```

```
> intersect(npanova,npkw)
[1] "1914_at"   "1389_at"   "38555_at"  "40268_at"  "1866_g_at"
```

4. A simulation study for ANOVA.

```
> x <- matrix(rnorm(90000),nrow = 10000, ncol = 9)
> a <- gl(3,3)
> panova <- apply(x, 1, function(x) anova(lm(x ~ a))$Pr[1])
> sum(panova<0.05)
[1] 514
```

The number of false positives is 514. The expected number is $\alpha \cdot n = 0.05 \cdot 10,000 = 500$, which is quite close to the observed.

A matrix with differences between three groups of gene expression values.

```
sigma <- 1; n <- 10000
data <- cbind(matrix(rnorm(n*3,0,sigma),ncol=3),
  matrix(rnorm(n*3,1,sigma), ncol = 3),matrix(rnorm(n*3,2,sigma), ncol = 3))
a <- gl(3,3)
panova <- apply(data, 1, function(x) anova(lm(x ~ a))$Pr[1])
> sum(panova<0.05)
[1] 3757
> pkw <- apply(data, 1, function(x) kruskal.test(x ~ a)$p.value)
> sum(pkw<0.05)
[1] 1143
```

Thus the number of true positives from ANOVA is 3757 and the number of false negatives is 6243. For the Kruskal-Wallis test there are 1143 true positives and 8857 false negatives. This can be impoved by increasing the number of gene expressions per group.

Answers to exercises of Chapter 6: Micro Array Analysis.

1. Gene filtering on normality per group of B-cell ALL patients.

```
library("genefilter")
data(ALL, package = "ALL")
ALLB <- ALL[,ALL$BT %in% c("B1","B2","B3","B4")]
f1 <- function(x) (shapiro.test(x)$p.value > 0.05)
sel1 <- genefilter(exprs(ALL[,ALLB$BT=="B1"]), filterfun(f1))
sel2 <- genefilter(exprs(ALL[,ALLB$BT=="B2"]), filterfun(f1))
sel3 <- genefilter(exprs(ALL[,ALLB$BT=="B3"]), filterfun(f1))
sel4 <- genefilter(exprs(ALL[,ALLB$BT=="B4"]), filterfun(f1))
selected <- sel1 & sel2 & sel3 & sel4

library(limma)
x <- matrix(as.integer(c(sel2,sel3,sel4)),ncol = 3,byrow=FALSE)
colnames(x) <- c("sel2","sel3","sel4")
vc <- vennCounts(x, include="both")
vennDiagram(vc)
137 pass filter 2 but not the other
510 pass filter 2 and 3 but not 4
1019 pas filter 2 and 4 but not 3
5598 pass filter 2, 3 and 4. etc.
```

2. Analysis of gene expressions of B-cell ALL patients using Limma.

```
library("ALL"); library("limma");library("annaffy");library(hgu95av2.db)
data(ALL)
ALLB <- ALL[,ALL$BT %in% c("B1","B2","B3","B4")]
design.ma <- model.matrix(~0 + factor(ALLB$BT))
colnames(design.ma) <- c("B1","B2","B3","B4")
cont.ma <- makeContrasts(B2-B1,B3-B2,B4-B3,levels=factor(ALLB$BT))
fit <- lmFit(ALLB, design.ma)
fit1 <- contrasts.fit(fit, cont.ma)
fit1 <- eBayes(fit1)
topTable(fit1, coef=2,5,adjust.method="fdr")
tab <- topTable(fit1, coef=2, number=20, adjust.method="fdr")
anntable <- aafTableAnn(as.character(tab$ID), "hgu95av2",  aaf.handler())
saveHTML(anntable, "ALLB1234.html", title = "B-cell ALL of stage 1,2,3,4"
```

3. Finding a row number: `grep("1389_at",row.names(exprs(ALL)))`.

4. Remission (genezing) from acute lymphocytic leukemia (ALL).

```
library(ALL); data(ALL)
table(pData(ALL)$remission)
remis <-  which(pData(ALL)$remission %in% c("CR","REF"))
ALLrem <- ALL[,remis]
remfac <-factor(pData(ALLrem)$remission)
pano <- apply(exprs(ALLrem),1,function(x) t.test(x ~ remfac)$p.value)
sum(pano<0.001)
> sum(pano<0.001)
[1] 45

library(hgu95av2.db)
names <- featureNames(ALLrem)[pano<.001]
ALLremsel<- ALLrem[names,]

symb <- mget(names, env = hgu95av2SYMBOL)
genenames <-  mget(names,hgu95av2GENENAME)
listofgenenames <- as.list(hgu95av2GENENAME)
unlistednames <- unlist(listofgenenames[names],use.names=F)

> grep("p53",unlistednames)
[1] 12 21

> length(unique(unlistednames))
[1] 36
```

5. Remission achieved.

```
library(ALL); data(ALL)
ALLCRREF <- ALL[,which(ALL$CR %in% c("CR","REF"))]
pano <- apply(exprs(ALLCRREF),1,function(x) t.test(x ~ ALLCRREF$CR)$p.value)
> sum(pano<0.0001)
[1] 11
> featureNames(ALLCRREF)[pano<.0001]
 [1] "1472_g_at"  "1473_s_at"  "1475_s_at"  "1863_s_at"  "34098_f_at" "36574_at"

library("hgu95av2.db")
affynames <- featureNames(ALLCRREF)[pano<.0001]
genenames <- mget(affynames, env = hgu95av2GENENAME)
```

```
> grep("oncogene",genenames)
[1] 1 2 3

affytot <- unique(featureNames(ALLCRREF))
genenamestot <- mget(affytot, env = hgu95av2GENENAME)
> length(grep("oncogene",genenamestot))
[1] 239
> length(genenamestot)
[1] 12625

> dat <- matrix(c(12625,239,11,3),2,byrow=TRUE)
> fisher.test(dat)

        Fisher's Exact Test for Count Data

data:  dat
p-value = 0.002047
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
  2.562237 54.915642
sample estimates:
odds ratio
  14.39959
```

6. Gene filtering of ALL data.

```
library("ALL")
data("ALL")
table(ALL$BT)
ALLT23 <- ALL[,which(ALL$BT %in% c("T2","T3"))]
library(genefilter)
f1 <- function(x) (shapiro.test(x)$p.value > 0.05)
f2 <- function(x) (t.test(x ~ ALLT23$BT)$p.value < 0.05)
sel1 <- genefilter(exprs(ALLT23[,ALLT23$BT=="T2"]), filterfun(f1))
sel2 <- genefilter(exprs(ALLT23[,ALLT23$BT=="T3"]), filterfun(f1))
sel3 <- genefilter(exprs(ALLT23), filterfun(f2))
> sum(sel1 & sel2 & sel3)
```

```
[1] 905
> sum(sel1 & sel2)
[1] 9388
> sum(sel3)
[1] 1204
```

7. Stages of B-cell ALL in the ALL data.

```
library("ALL")
library("limma");
allB <- ALL[,which(ALL$BT %in% c("B1","B2","B3","B4"))]
facB123 <- factor(allB$BT)
cont.ma <- makeContrasts(B2-B1,B3-B2,B4-B3, levels=facB123)
design.ma <- model.matrix(~ 0 + facB123)
colnames(design.ma) <- c("B1","B2","B3","B4")
fit <- lmFit(allB, design.ma)
fit1 <- contrasts.fit(fit, cont.ma)
fit1 <- eBayes(fit1)
> topTable(fit1, coef=2,5,adjust.method="BH")
           ID      logFC  AveExpr         t      P.Value    adj.P.Val         B
6048 35991_at  0.5964481 4.144598  6.624128 2.578836e-09 0.0000325578 10.842989
3909 33873_at  0.5707770 7.217570  6.083524 2.891823e-08 0.0001825464  8.625253
5668 35614_at  1.7248509 5.663477  5.961231 4.946078e-08 0.0002081474  8.132884
6776 36711_at -2.3664712 7.576108 -5.759565 1.187487e-07 0.0003054110  7.329631
7978 37902_at  0.8470235 4.258491  5.742783 1.276579e-07 0.0003054110  7.263298
> sum(fit1$p.value<0.05)
[1] 4328
```

8. Analysis of public micro array data.

```
library(GEOquery); library(limma); library(hgu95av2.db);library(annaffy)
gds486 <- getGEO("GDS486"); eset486 <- GDS2eSet(gds486,do.log2=T)

nrmissing <- apply(exprs(eset486), 1, function(x) sum(is.na(x)) )
eset486sel <- eset486[nrmissing<1,]
pval486sel <- apply(exprs(eset486sel), 1, function(x) t.test(x ~  eset486sel$cell
pval486 <- nrmissing
pval486[pval486==0]<-pval486sel
```

```
pval486[pval486>1]<-1

gds711 <- getGEO("GDS711"); eset711 <- GDS2eSet(gds711,do.log2=T)
nrmissing <- apply(exprs(eset711), 1, function(x) sum(is.na(x)) )
eset711sel <- eset711[nrmissing<1,]
panova711sel <- apply(exprs(eset711sel), 1, function(x) anova(lm(x ~ eset
pval711sel <- panova711sel
pval711 <- nrmissing
pval711[pval711==0]<-pval711sel
pval711[pval711>1]<-1

gds2126 <- getGEO("GDS2126"); eset2126 <- GDS2eSet(gds2126,do.log2=T)
nrmissing <- apply(exprs(eset2126), 1, function(x) sum(is.na(x)) )
eset2126sel <- eset2126[nrmissing<1,]
pval2126sel <- apply(exprs(eset2126sel), 1, function(x) anova(lm(x ~ eset
pval2126 <- nrmissing
pval2126[pval2126==0]<-pval2126sel
pval2126[pval2126>1]<-1

sumpval <- pval486 + pval711 + pval2126
o <- order(sumpval,decreasing=FALSE)
genenames <- names(sumpval[o[1:20]])
symb <- "aap"
for (i in 1:20) symb[i] <- get(genenames[i], env = hgu95av2SYMBOL)

> symb
 [1] "GADD45A" "DUSP4"   "OAS1"    "STAT1"   "STAT1"   "AKR1C3"  "PSMB9"
[16] "TKT"     "NFKB1"   "SLC7A5"  "CXCL2"   "DLG5"

library("KEGG");library("GO");library("annaffy")
atab <- aafTableAnn(genenames, "hgu95av2", aaf.handler() )
saveHTML(atab, file="ThreeExperiments.html")
# p53 plays a role.
```

9. Analysis of genes from a GO search.

```
library(ALL)
data(ALL,package="ALL")
```

```
ALLP <- ALL[,ALL$mol.biol  %in% c("ALL1/AF4","BCR/ABL","NEG")]

neg <- which(ALLP$mol.biol=="NEG")
aal1 <- which(ALLP$mol.biol=="ALL1/AF4")
bcr <- which(ALLP$mol.biol=="BCR/ABL")
orderpat <- c(neg,aal1,bcr)

ALLP <- ALL[,ALL$mol.biol  %in% c("ALL1/AF4","BCR/ABL","NEG")]
ALLPo <- ALLP[,c(neg,aal1,bcr)]

facnr <- c(rep(1,74),rep(2,10),rep(3,37))
nab.fac <- factor(facnr,levels=1:3, labels= c("NEG","ALL1/AF4","BCR/ABL"))
panova <- apply(exprs(ALLPo), 1, function(x) anova(lm(x ~ nab.fac))$Pr[1])

library("GO"); library("annotate"); library("hgu95av2")
GOTerm2Tag <- function(term) {
 GTL <- eapply(GOTERM, function(x) {grep(term, x@Term, value=TRUE)})
 Gl <- sapply(GTL, length)
 names(GTL[Gl>0])
}
> GOTerm2Tag("protein-tyrosine kinase")
[1] "GO:0004713"

probes <- hgu95av2GO2ALLPROBES$"GO:0004713"

> sum(panova[probes]<0.05)
[1] 86
> sum(panova[probes]<1)
[1] 320
> sum(panova<0.05)
[1] 2581
> sum(panova<1)
[1] 12625

> fisher.test(matrix(c(12625, 2581,320,86),2,byrow=TRUE))

        Fisher's Exact Test for Count Data
```

```
data:  matrix(c(12625, 2581, 320, 86), 2, byrow = TRUE)
p-value = 0.03222
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 1.019848 1.679625
sample estimates:
odds ratio
  1.314569

the odds ratio differs significantly from zero; there are more significan
```

Answers to exercises of Chapter 7: Cluster Analysis and Trees.

1. Cluster analysis on the "Zyxin" expression values of the Golub et al. (1999) data.

```
data(golub, package="multtest")
data <- data.frame(golub[2124,])
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))

stripchart(golub[2124,]~gol.fac, pch=as.numeric(gol.fac))
plot(hclust(dist(clusdata,method="euclidian"),method="single"))

initial <- as.matrix(tapply(golub[2124,],gol.fac,mean), nrow = 2, ncol=1,
cl<- kmeans(data, initial, nstart = 10)

table(cl$cluster,gol.fac)
n <- length(data); nboot<-1000
boot.cl <- matrix(0,nrow=nboot,ncol = 2)
for (i in 1:nboot){
  dat.star <- data[sample(1:n,replace=TRUE)]
  cl <- kmeans(dat.star, initial, nstart = 10)
  boot.cl[i,] <- c(cl$centers[1,],cl$centers[2,])
}

> quantile(boot.cl[,1],c(0.025,0.975))
      2.5%       97.5%
-1.07569310 -0.03344292
```

```
> quantile(boot.cl[,2],c(0.025,0.975))
    2.5%    97.5%
0.731493 1.784468
```

2. Close to CCND3 Cyclin D3.

```
library("genefilter"); data(golub, package = "multtest")
closeg <- genefinder(golub, 1042, 10, method = "euc", scale = "none")
golub.gnames[closeg[[1]][[1]],2]
boxplot(golub[394,] ~gol.fac)
```

3. MCM3.

```
data(golub, package = "multtest")
x <- golub[2289,]; y <- golub[2430,]
plot(x,y)
which.min(y)  # the plot suggests the smallest y as the outlier
> cor.test(x[-21],y[-21])

        Pearson's product-moment correlation

data:  x[-21] and y[-21]
t = 10.6949, df = 35, p-value = 1.42e-12
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 0.7690824 0.9341905   # much smaller
sample estimates:
     cor
0.875043  # much larger than 0.6376217
nboot <- 1000; boot.cor <- matrix(0,nrow=nboot,ncol = 1)
data <- matrix(c(x[-21],y[-21]),ncol=2,byrow=FALSE)
for (i in 1:nboot){
    dat.star <- data[sample(1:nrow(data),replace=TRUE),]
    boot.cor[i,] <- cor(dat.star)[2,1]}
>  mean(boot.cor)
[1] 0.8725835   # very similar to cor.test
> quantile(boot.cor[,1],c(0.025,0.975))
     2.5%     97.5%
0.7755743 0.9324625   # very similar to cor.test
```

4. Cluster analysis on part of Golub data.

```
library(multtest);data(golub);
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
o1 <- grep("oncogene",golub.gnames[,2])
plot(hclust(dist(golub[o1,],method="euclidian"),method="single"))

o2 <- grep("antigene",golub.gnames[,2])
plot(hclust(dist(golub[o2,],method="euclidian"),method="single"))

o3 <- grep("receptor",golub.gnames[,2])
plot(hclust(dist(golub[o3,],method="euclidian"),method="single"))
```

5. Principal Components Analysis on part of the `ALL` data.

```
library(ALL); data(ALL)
ALLB <- ALL[,ALL$BT %in% c("B1","B2","B3")]
panova <- apply(exprs(ALLB), 1, function(x) anova(lm(x ~ ALLB$BT))$Pr[1])
ALLBsp <- ALLB[panova<0.001,]
> dim(exprs(ALLBsp))
[1] 499  78
> min(cor(exprs(ALLBsp)))
[1] 0.5805595
> eigen(cor(exprs(ALLBsp)))$values[1:5]
[1] 65.2016203  2.9652965  2.4781567  0.7556439  0.6040647

data <- exprs(ALLBsp); p <- ncol(data); n <- nrow(data) ; nboot<-1000
eigenvalues <- array(dim=c(nboot,p))
for (i in 1:nboot){dat.star <- data[sample(1:n,replace=TRUE),]
  eigenvalues[i,] <- eigen(cor(dat.star))$values}
> for (j in 1:p) print(quantile(eigenvalues[,j],c(0.025,0.975)))
    2.5%    97.5%
63.43550 66.77785
    2.5%    97.5%
2.575413 3.530350
    2.5%    97.5%
2.081573 2.889933
     2.5%     97.5%
```

```
0.6475809 0.9942871  #Hence, the first three are significant!
     2.5%     97.5%
0.5067404 0.7482680
     2.5%     97.5%

biplot(princomp(data,cor=TRUE),pc.biplot=T,cex=0.5,expand=0.8)
```

6. Some correlation matrices.

```
eigen(matrix(c(1,-0.8,-0.8,1),nrow=2))
eigen(matrix(c(1,0.8,0.8,0.8,1,0.8,0.8,0.8,1),nrow=3))
eigen(matrix(c(1,-0.5,-0.5,-0.5,1,-0.5,-0.5,-0.5,1),nrow=3))
> 2.6/3 * 100
[1] 86.66667
> eigen(matrix(c(1,0.8,0.8,0.8,1,0.8,0.8,0.8,1),nrow=3))$vectors
           [,1]       [,2]       [,3]
[1,] -0.5773503  0.8164966  0.0000000
[2,] -0.5773503 -0.4082483 -0.7071068
[3,] -0.5773503 -0.4082483  0.7071068
```

Answers to exercises of Chapter 8: Classification Methods.

1. Classification tree of Golub data. Use recursive partitioning in `rpart`

```
library(multtest);data(golub);
gol.fac <- factor(golub.cl,levels=0:1, labels= c("ALL","AML"))
maxgol <- apply(golub[,gol.fac=="ALL"], 1, function(x) max(x))
mingol <- apply(golub[,gol.fac=="AML"], 1, function(x) min(x))
sum(maxgol < mingol)
> which.min(maxgol - mingol)
[1] 2124
> golub.gnames[2124,]
[1] "4847"      "Zyxin"     "X95735_at"
> boxplot(golub[2124,] ~gol.fac)

gol.rp  <-  rpart(gol.fac ~ golub[2124,], method="class", cp=0.001)
plot(gol.rp, branch=0,margin=0.1);  text(gol.rp, digits=3, use.n=TRUE)
```

```
grep("Gdf5",golub.gnames[,2])

> grep("Gdf5",golub.gnames[,2])
[1] 2058

gol.rp  <-  rpart(gol.fac ~ golub[2058,], method="class", cp=0.001)
plot(gol.rp, branch=0,margin=0.1);  text(gol.rp, digits=3, use.n=TRUE)


gol.rp  <-  rpart(gol.fac ~., data.frame(t(golub)), method="class", cp=0.
plot(gol.rp, branch=0,margin=0.1);  text(gol.rp, digits=3, use.n=TRUE)
```

2. Sensitivity versus specificity.

   (a)
   ```
   library(multtest);library(ROCR);data(golub)
   golub.clchanged <-  -golub.cl +1
   pred <- prediction(golub[1042,], golub.clchanged)
   perf <- performance(pred, "sens", "spec")
   plot(perf)
   ```

   (b) The function is essentially the same.

   (c) Use auc as before.

3. Comparing Classification Methods.

```
library(rpart)
predictors <- matrix(rnorm(100*4,0,1),100,4)
colnames(predictors) <- letters[1:4]
groups <-  gl(2,50)
simdata <- data.frame(groups,predictors)
rp<-rpart(groups ~  a + b + c + d,method="class",data=simdata)
predicted <- predict(rp,type="class")
table(predicted,groups)
plot(rp, branch=0,margin=0.1);  text(rp, digits=3, use.n=TRUE)

> table(predicted,groups)
         groups
predicted  1  2
        1 41 12
```

```
        2  9 38

library(e1071)
svmest <- svm(predictors, groups, data=df, type = "C-classification", kernel = "l
svmpred <- predict(svmest, predictors, probability=TRUE)
> table(svmpred, groups)
       groups
svmpred  1  2
      1 31 25
      2 19 25

library(nnet)
nnest <- nnet(groups ~ ., data = simdata, size = 5,maxit = 500, decay = 0.01, Max
pred <- predict(nnest, type = "class")
> table(pred, groups) # prints confusion ma
    groups
pred  1  2
   1 45 10
   2  5 40
```

The misclassification rate of rpart, svm, and nnet is, respectively, 21/100, 44/100, and 15/100. If we increase the number of predictors, then the misclassification rate decreases.

4. Prediction of achieved remission.

```
library(ALL); library(hgu95av2.db); library(rpart); data(ALL)
ALLrem <- ALL[,which(pData(ALL)$remission %in% c("CR","REF"))]
remfac <-factor(pData(ALLrem)$remission)
pano <- apply(exprs(ALLrem),1,function(x) t.test(x ~ remfac)$p.value)
names <- featureNames(ALLrem)[pano<.001]
ALLremsel<- ALLrem[names,]
data <- data.frame(t(exprs(ALLremsel)))
all.rp  <-  rpart(remfac ~., data, method="class", cp=0.001)
plot(all.rp, branch=0,margin=0.1);  text(all.rp, digits=3, use.n=TRUE)
rpart.pred <- predict(all.rp, type="class")
> table(rpart.pred,remfac)
          remfac
```

```
rpart.pred CR REF
       CR  93   1
       REF  6  14
> 7/(93+1+6+14)
[1] 0.06140351
> mget(c("1840_g_at","36769_at","1472_g_at","854_at"), env = hgu95av2GENE
$`1840_g_at`
[1] NA

$`36769_at`
[1] "retinoblastoma binding protein 5"

$`1472_g_at`
[1] "v-myb myeloblastosis viral oncogene homolog (avian)"

$`854_at`
[1] "B lymphoid tyrosine kinase"
```

5. Gene selection by area under the curve.

```
library(ROCR); data(golub, package = "multtest")
gol.true <- factor(golub.cl,levels=0:1,labels= c("TRUE","FALSE"))
auc.values <- apply(golub,1,
  function(x) performance(prediction(x, gol.true),"auc")@y.values[[1]])
o <- order(auc.values,decreasing=TRUE)
golub.gnames[o[1:25],2]
```

6. Classification Tree for Ecoli.

```
ecoli <- read.table("http://www.grappa.univ-lille3.fr/~torre/Recherche/Da
  downloads/ecoli/ecoli.data",sep=",",header = TRUE)
colnames(ecoli) <- c("SequenceName","mcg","gvh","lip","chg","aac","alm1",
ecolisel<- ecoli[which(ecoli$ecclass %in% c("cp","im","pp")),]
ecolisel$ecclass <- factor(ecolisel$ecclass, levels=c("cp","im","pp"))
library(rpart)
rpfit <- rpart(ecolisel$ecclass ~ mcg + gvh + lip + aac + alm1 + alm2,dat
plot(rpfit, branch=1,margin=0.1); text(rpfit, digits=3, use.n=TRUE)
title(main = "rpartfit ecoli classes cp im and pp")
```

```
predictedclass <- predict(rpfit, type="class")
table(predictedclass,ecolisel$ecclass) #predictors are alm1, gvh and im
> (1+2+7+4)/length(ecolisel$ecclass)
[1] 0.05166052
```

Answers to exercises of Chapter 9: Analyzing Sequences

1. Writing to a FASTA file.

```
choosebank("genbank"); library(seqinr)
query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
ccnd3 <- sapply(ccnd3hs$req, getSequence)
x1 <- DNAStringSet(c2s(ccnd3[[1]]))
write.XStringSet(x1, file="ccnd3.fa", format="fasta", width=80)

ccnd3c2sn <- sapply(ccnd3, c2s)
x1 <- DNAStringSet(ccnd3c2sn)
write.XStringSet(x1, file="ccnd3n.fa", format="fasta", width=80)
```

   An alternative would be to use the `write.dna` function of the `ape` package.

2. Dotplot of sequences.

```
seq1 <- sample(c("A","G","C","T"),100,rep=TRUE,prob=c(0.1,0.4,0.4,0.1))
seq2 <- sample(c("A","G","C","T"),100,rep=TRUE,prob=c(0.1,0.4,0.4,0.1))

par(mfrow=c(1,2))
dotPlot(seq1, seq2, main = "Dot plot of different random sequences\nwsize = 1, ws
dotPlot(seq1, seq1, main = "Dot plot of equal random sequnces\nwsize = 1, wstep =
par(mfrow=c(1,1))

par(mfrow=c(1,2))
dotPlot(seq1, seq2, main = "Dot plot of different random sequences\nwsize = 3, ws
dotPlot(seq1, seq1, main = "Dot plot of equal random sequnces\nwsize = 3, wstep =
par(mfrow=c(1,1))

par(mfrow=c(1,2))
dotPlot(seq1, seq1, main = "Dot plot of different random sequences\nwsize = 3, ws
```

```
dotPlot(seq1, seq1[100:1], main = "Dot plot of equal random sequnces\nwsi
par(mfrow=c(1,1))

x <- c("RPLWVAPDGHIFLEAFSPVYK")
y <- c("RPLWVAPDGHIFLEAFSPVYK")
z <- c("PLWISPSDGRIILESFSPLAE")

choosebank("genbank"); library(seqinr)
query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
ccnd3 <- sapply(ccnd3hs$req, getSequence)
sapply(ccnd3hs$req, getName)
ccnd3prot <- sapply(ccnd3hs$req, getTrans)
dotPlot(ccnd3prot[[1]], s2c("EEEVFPLAMN"), main = "Dot plot of two protei
dotPlot(ccnd3prot[[7]], ccnd3prot[[8]], main = "Dot plot of two protein\n
dotPlot(s2c(x), s2c(z), main = "Dot plot of two protein\nwsize = 1, wstep
```

3. Local alignment.

```
library(seqinr);library(Biostrings);data(BLOSUM50)
x <- s2c("HEAGAWGHEE"); y <- s2c("PAWHEAE")
s <- BLOSUM50[y,x]; d <- 8
F <- matrix(data=NA,nrow=(length(y)+1),ncol=(length(x)+1))
F[1,] <- 0 ; F[,1] <- 0
rownames(F) <- c("",y); colnames(F) <- c("",x)
for (i in 2:(nrow(F)))
  for (j in 2:(ncol(F)))
  {F[i,j] <- max(c(0,F[i-1,j-1]+s[i-1,j-1],F[i-1,j]-d,F[i,j-1]-d))}
> max(F)
[1] 28
```

4. Probability of more extreme alignment score.

```
library(seqinr);library(Biostrings);data(BLOSUM50)
randallscore <- c(1,1)
for (i in 1:1000) {
 x <- c2s(sample(rownames(BLOSUM50),7, replace=TRUE))
 y <- c2s(sample(rownames(BLOSUM50),10, replace=TRUE))
 randallscore[i] <- pairwiseAlignment(AAString(x), AAString(y), substitut
```

```
    gapOpening = 0, gapExtension = -8, scoreOnly = TRUE)
}
> sum(randallscore>1)/1000
[1] 0.003
> plot(density(randallscore))
```

5. Prochlorococcus marinus.

```
library(seqinr)
choosebank("genbank")
query("ccmp","AC=AE017126 OR AC=BX548174 OR AC=BX548175")
ccmpseq <- sapply(ccmp$req,getSequence)
gc <- sapply(ccmpseq, GC)
> wilcox.test(gc[1:2],gc[3:9])

        Wilcoxon rank sum test

data:  gc[1:2] and gc[3:9] W = 0, p-value = 0.05556 alternative
hypothesis: true location shift is not equal to 0

> t.test(gc[1:2],gc[3:9])

        Welch Two Sample t-test

data:  gc[1:2] and gc[3:9] t = -5.8793, df = 1.138, p-value =
0.08649 alternative hypothesis: true difference in means is not
equal to 0 95 percent confidence interval:
 -0.4507417  0.1079848
sample estimates: mean of x mean of y 0.3362065 0.5075849

gc in the left group is lower, the tests are not significant.
```

6. Sequence equality.

```
\begin{verbatim}
library(seqinr)
choosebank("genbank")
query("ccnd3hs","sp=homo sapiens AND k=ccnd3@")
```

```
sapply(ccnd3hs$req,getLength)
> ccnd3prot <- sapply(ccnd3hs$req, getTrans)
> table(ccnd3prot[[1]])

 *  A  C  D  E  F  G  H  I  K  L  M  N  P  Q  R  S  T  V  W  Y
 1 31 12 12 21  6 14  7 10 10 41  9  1 17 16 22 19 18 15  3  8
> table(ccnd3prot[[2]])

 *  A  C  D  E  F  G  H  I  K  L  M  N  P  Q  R  S  T  V  W  Y
 1 30 12 12 21  6 14  7 10 10 41  9  1 17 16 22 20 18 15  3  8
 # Hence, there is only one difference!
> which(!ccnd3prot[[1]]==ccnd3prot[[2]])
[1] 259
```

7. Conserved region.

```
ID   XRODRMPGMNTB; BLOCK
AC   PR00851A; distance from previous block=(52,131)
DE   Xeroderma pigmentosum group B protein signature
BL   adapted;   width=21; seqs=8; 99.5%=985; strength=1287
XPB_HUMAN|P19447   (  74) RPLWVAPDGHIFLEAFSPVYK  54
XPB_MOUSE|P49135   (  74) RPLWVAPDGHIFLEAFSPVYK  54
P91579             (  80) RPLYLAPDGHIFLESFSPVYK  67
XPB_DROME|Q02870   (  84) RPLWVAPNGHVFLESFSPVYK  79
RA25_YEAST|Q00578  ( 131) PLWISPSDGRIILESFSPLAE 100
Q38861             (  52) RPLWACADGRIFLETFSPLYK  71
O13768             (  90) PLWINPIDGRIILEAFSPLAE 100
O00835             (  79) RPIWVCPDGHIFLETFSAIYK  86

library(Biostrings);data(BLOSUM50)
x <- c("RPLWVAPDGHIFLEAFSPVYK")
y <- c("RPLWVAPDGHIFLEAFSPVYK")
z <- c("PLWISPSDGRIILESFSPLAE")

x == y
pairwiseAlignment(AAString(x), AAString(z), substitutionMatrix = "BLOSUM5
> pairwiseAlignment(AAString(x), AAString(y), substitutionMatrix = "BLOSU
Global Pairwise Alignment
```

```
1:  RPLWVAPDGHIFLEAFSPVYK
2:  RPLWVAPDGHIFLEAFSPVYK
Score:  154
>
> z <- c("PLWISPSDGRIILESFSPLAE")
>
> x == y
[1] TRUE
> pairwiseAlignment(AAString(x), AAString(z), substitutionMatrix = "BLOSUM50",gap
Global Pairwise Alignment
1:  RPLWVAP-DGHIFLEAFSPVYK
2:  -PLWISPSDGRIILESFSPLAE
Score:  85
```

8. Plot of CG proportion from Celegans.

   (a) Produce a plot of the CG proportion of the chromosome I of Celegans (Celegans.UCSC.ce2) along a window of 100 nucleotides. Take the first 10,000 nucleotides.

   ```
   library(seqinr)
   source("http://bioconductor.org/biocLite.R")
   biocLite("BSgenome.Celegans.UCSC.ce2")
   library(BSgenome.Celegans.UCSC.ce2)
   GCperc <- double()
   for (i in 1:10000) GCperc[i] <- GC(s2c(as.character(Celegans$chrI[i:(i+100)]
   plot(GCperc,type="l")
   ```

   (b) A binding sequence of the enzyme EcoRV is the subsequence GATATC. How many exact matches has Chromosome I of Celegans.

   ```
   > subseq <- "gatatc"
   > countPattern(subseq, Celegans$chrI, max.mismatch = 0)
   [1] 3276
   > length(s2c(as.character(Celegans$chrI))) * (1/4)^6
   [1] 3681.759
   ```

9. Plot of codon usage.

```
data(ec999)
ec999.uco <- lapply(ec999, uco, index="eff")
df <- as.data.frame(lapply(ec999.uco, as.vector))
row.names(df) <- names(ec999.uco[[1]])
global <- rowSums(df)
title <- "Codon usage in 999 E. coli coding sequences"
dotchart.uco(global, main = title)

choosebank("genbank"); library(seqinr)
query("ccndhs","sp=homo sapiens AND k=ccnd@")
ccnd <- sapply(ccndhs$req, getSequence)
ccnd.uco <- lapply(ccnd3, uco, index="eff")
df <- as.data.frame(lapply(ccnd.uco, as.vector))
row.names(df) <- names(ccnd.uco[[1]])
global <- rowSums(df)
title <- "Codon usage in ccnd3 homo sapiens coding sequences"
dotchart.uco(global, main = title)
```

Answers to exercises of Chapter 10: Markov Models.

1. Visualize by a transition graph the following transition matrices. Consult your teacher.

2. Computing probabilities. The answers are provided by the following.

```
> P <- matrix(c(3/4,1/4,1/2,1/2),2,2,byrow=T)
> pi0 <- c(1/2,1/2)
> pi0 %*% P
      [,1]  [,2]
[1,] 0.625 0.375
> P %*% P
       [,1]   [,2]
[1,] 0.6875 0.3125
[2,] 0.6250 0.3750
> P
     [,1] [,2]
[1,] 0.75 0.25
[2,] 0.50 0.50
```

3. Programming GTR. Use $\pi_A = 0.15$, $\pi_G = 0.35$, $\pi_C = 0.35$, $\pi_T = 0.15$, $\alpha = 4$, $\beta = 0.5$, $\gamma = 0.4$, $\delta = 0.3$, $\epsilon = 0.2$, and $\zeta = 4$.

   (a) Program the rate matrix in such a manner that it is simple to adapt for other values of the parameters.

```
library(Matrix)
piA <- 0.15; piG <- 0.35; piC <- 0.35; piT <- 0.15
alpha <- 4; beta <- 0.5; gamma <- 0.4; delta <- 0.3
epsilon <- 0.2; zeta <- 4
Q <- matrix(data=NA,4,4)

                      Q[1,2] <- alpha * piG;  Q[1,3] <- beta * piC;
                                              Q[1,4] <- gamma * piT
Q[2,1] <- alpha * piA;                        Q[2,3] <- delta * piC;
                                              Q[2,4] <- epsilon * piT
Q[3,1] <- beta * piA;  Q[3,2] <- delta * piG;
                                                        Q[3,4] <- delta* p
Q[4,1] <- gamma * piA; Q[4,2] <- epsilon* piG; Q[4,3] <- zeta * piC
diag(Q) <- 0
diag(Q) <- -apply(Q,1,sum)
Q <- Matrix(Q)
> Q
4 x 4 Matrix of class "dgeMatrix"
       [,1]   [,2]   [,3]   [,4]
[1,] -1.635  1.400  0.175  0.060
[2,]  0.600 -0.735  0.105  0.030
[3,]  0.075  0.105 -0.225  0.045
[4,]  0.060  0.070  1.400 -1.530
```

   (b) The transversion rate is larger then the transition rate because the blocks outside the main diagonal have lower values.

   (c) The probability transition matrix is

```
> P <- as.matrix(expm(Q))
> P
           [,1]       [,2]      [,3]       [,4]
[1,] 0.32199057 0.51569256 0.1392058 0.02311107
[2,] 0.22097363 0.64908639 0.1115233 0.01841667
```

```
[3,] 0.05203969 0.09913633 0.8263804 0.02244359
[4,] 0.04621015 0.08457814 0.6397090 0.22950271

rownames(P) <- colnames(P) <- StateSpace <- c("a","g","c","t")
pi0 <- c(1/4,1/4,1/4,1/4)
markov2 <- function(StateSpace,P,n){
 seq <- matrix(0,nr=n,nc=1)
 seq[1] <- sample(StateSpace,1,replace=T,pi0)
 for(k in 1:(n-1)){ seq[k+1] <- sample(StateSpace,1,replace=T,P[seq[k
 return(seq) }
seq <- markov2(StateSpace,P,99)
```

4. Distance according to JC69.

   (a) `accnr <- paste("AJ5345",26:27,sep="")`
   `seqbin <- read.GenBank(accnr, species.names = TRUE, as.character = FA`
   Down load the sequences `AJ534526` and `AJ534527`. Hint: Use `as.character = TRUE` in the `read.GenBank` function.

   (b) Two solution of computing the proportion of different nucleotides are

   ```
   dist.dna(seqbin, model = "raw")
   p <- sum(seq$AJ534526 != seq$AJ534527)/1143
   ```

   (c) Simply insert the obtained `p` in the formula `d <- -log(1-4*p/3)*3/4`.

Appendix B

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