

# Online Reproducible Research: An Application to Multivariate Analysis of Bacterial DNA Fingerprint Data

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**Abstract** This paper presents an example of online reproducible multivariate data analysis. This example is based on a web page providing an online computing facility on a server. HTML forms contain editable R code snippets that can be executed in any web browser thanks to the Rweb software. The example is based on the multivariate analysis of DNA fingerprints of the internal bacterial flora of the poultry red mite *Dermanyssus gallinae*. Several multivariate data analysis methods from the `ade4` package are used to compare the fingerprints of mite pools coming from various poultry farms. All the computations and graphical displays can be redone interactively and further explored online, using only a web browser. Statistical methods are detailed in the duality diagram framework, and a discussion about online reproducibility is initiated.

## Introduction

Reproducible research has gained much attention recently (see particularly <http://reproducibleresearch.net/> and the references therein). In the area of Statistics, the availability of Sweave (Leisch (2002), <http://www.stat.uni-muenchen.de/~leisch/Sweave/>) has proved extremely useful and Sweave documents are now widely used. Sweave offers the possibility to have text, R code, and outputs of this code in the same document. This makes reproducibility of a scientific paper written in Sweave very straightforward.

However, using Sweave documents implies a good knowledge of R, of Sweave and of  $\LaTeX$ . It also requires having R installed on one's computer. The installed version of R must be compatible with the R code in the Sweave document, and all the needed packages must also be installed. This may be a problem for some scientists, for example for many biologists who are not familiar with R and  $\LaTeX$ , or who do not use them on a regular basis.

In this paper, we demonstrate an online reproducibility system that helps circumvent these problems. It is based on a web page that can be used with any web browser. It does not require R to be installed locally, nor does it need a thorough knowledge of R and  $\LaTeX$ . Nevertheless it allows the user to present

and comment R code snippets, to redo all the computations and to draw all the graphical displays of the original paper.

The example presented here relates to multivariate data analysis. Reproducibility of multivariate data analyses is particularly important because there is a large number of different methods, with poorly defined names, making it often difficult to know what has been done exactly. Many methods have several different names, according to the community where they were invented (or re-invented), and where they are used. Translation of technical terms into languages other than English is also difficult, as many are ambiguous and have "false friends". Moreover, data analysis methods make intensive use of graphical displays, with a large number of graphical parameters which can be changed to produce a wide variety of displays.

This situation is similar to what was described by Buckheit and Donoho (1995) in the area of wavelet research. Their solution was to publish the Matlab code used to produce the figures in their research articles. Rather than do this we decided to set up a simple computer environment using R to offer online reproducibility. In 2002, we installed an updated version of the Rweb system (Banfield, 1999) on our department server (see <http://pbil.univ-lyon1.fr/Rweb/Rweb.general.html>), and we implemented several computational web services in the field of comparative genomics (Perrière et al. (2003), see for example <http://pbil.univ-lyon1.fr/mva/coa.php>).

This server now combines the computational power of R with simple HTML forms (as suggested by de Leeuw (2001) for Xlisp-Stat) and the ability to search online molecular databases with the `seqinr` package (Charif and Lobry, 2007). It is also used by several researchers to provide an online reproducibility service for scientific papers (see for example <http://pbil.univ-lyon1.fr/members/lobry/>).

The present paper provides an example of an application of this server to the analysis of DNA fingerprints by multivariate analysis methods, using the `ade4` package (Chessel et al., 2004; Dray and Dufour, 2007). We have shown recently (Valiente-Moro et al., 2009) that multivariate analysis techniques can be used to analyse bacterial DNA fingerprints and that they make it possible to draw useful conclusions about the composition of the bacterial communities from which they originate. We also demonstrate the effectiveness of principal component anal-

ysis, between-group analysis and within-group analysis [PCA, BGA and WGA, Benzécri (1983); Dolédec and Chessel (1987)] to show differences in diversity between bacterial communities of various origins.

In summary, we show here that it is easy to set up a software environment offering full online reproducibility of computations and graphical displays of multivariate data analysis methods, even for users who are not familiar with R, Sweave or L<sup>A</sup>T<sub>E</sub>X.

## Data and methods

In this section, we first describe the biological material used in the example data set. Then we present the statistical methods (PCA, BGA and WGA), in the framework of the duality diagram (Escoufier, 1987; Holmes, 2006). We also detail the software environment that was used.

### Biological material

The poultry red mite, *Dermanyssus gallinae* is an haematophagous mite frequently present in breeding facilities and especially in laying hen facilities (Chauve, 1998). This arthropod can be responsible for anemia, dermatitis, weight loss and a decrease in egg production (Kirkwood, 1967). It has also been involved in the transmission of many pathogenic agents responsible for serious diseases in both animals and humans (Valiente Moro et al., 2005; Valiente-Moro et al., 2007). The poultry red mite is therefore an emerging problem that must be studied to maintain good conditions in commercial egg production facilities. Nothing is known about its associated non-pathogenic bacterial community and how the diversity of the microflora within mites may influence the transmission of pathogens.

Most studies on insect microflora are based on isolation and culture of the constituent microorganisms. However, comparison of culture-based and molecular methods reveals that only 20-50% of gut microbes can be detected by cultivation (Suau et al., 1999). Molecular methods have been developed to analyse the bacterial community in complex environments. Among these methods, Denaturing Gradient and Temporal Temperature Gel Electrophoresis (DGGE and TTGE) (Muyzer, 1998) have already been used successfully.

Full details of the example data set used in this paper (mites sampling, DNA extraction, PCR amplification of 16S rDNA fragments and TTGE banding pattern achieving) are given in Valiente-Moro et al. (2009). Briefly, 13 poultry farms were selected in the Bretagne region in France, and in each farm 15 single mites, five pools of 10 mites and one pool of 50 mites were collected. The results for single mites and mite pools were analysed separately, but only the results of mite pools are presented in this study, as they

had the most illustrative analysis. Banding patterns can be analysed as quantitative variables (intensity of bands), or as binary indicators (presence/absence of bands), but for the same reason, only the presence/absence data were used in this paper.

TTGE banding patterns were collected in a data table, with bands in columns (55 columns) and mite pools in rows (73 rows). This table was first subjected to a principal component analysis (PCA) to get an overall idea of the structure of the banding patterns. Between-group analysis (BGA) was then applied to study the differences between poultry farms, and finally within-group analysis (WGA) was used to eliminate the farm effect and obtain the main characteristics of the common bacterial flora associated with *D. gallinae* in standard breeding conditions. A good knowledge of these characteristics would allow comparisons of the standard bacterial flora among various situations, such as geographic regions, type and location of breeding facilities (particularly organic farms), or developmental stages of *D. gallinae*.

### Duality diagram of principal component analysis

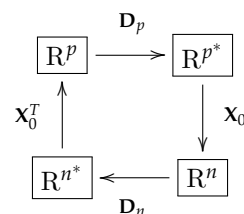
Let  $\mathbf{X} = [x_{ij}]_{(n,p)}$  be the TTGE data table with  $n$  rows (individuals = mite pools) and  $p$  columns (variables = TTGE bands). Variables have mean  $\bar{x}_j = \frac{1}{n} \sum_i x_{ij}$  and variance  $\sigma_j^2 = \frac{1}{n} \sum_i (x_{ij} - \bar{x}_j)^2$ . Individuals belong to  $g$  groups (or classes), namely  $G_1, \dots, G_g$ , with group counts  $n_1, \dots, n_g$ , and  $\sum n_k = n$ .

Using duality diagram theory and triplet notation, the PCA of  $\mathbf{X}$  is the analysis of a triplet  $(\mathbf{X}_0, \mathbf{D}_p, \mathbf{D}_n)$ .  $\mathbf{X}_0$  is the table of standardized values:

$$\mathbf{X}_0 = [\tilde{x}_{ij}]_{(n,p)}$$

with  $\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_j}{\sigma_j}$ , and  $\mathbf{D}_n$  and  $\mathbf{D}_p$  are the diagonal matrices of row and column weights:  $\mathbf{D}_p = \mathbf{I}_p$  and  $\mathbf{D}_n = \frac{1}{n} \mathbf{I}_n$ .

This information is summarized in the following mnemonic diagram, called the "duality diagram" because  $\mathbf{R}^{p*}$  is the dual of  $\mathbf{R}^p$  and  $\mathbf{R}^{n*}$  is the dual of  $\mathbf{R}^n$ :



$\mathbf{X}_0^T$  is the transpose of  $\mathbf{X}_0$ . The analysis of this triplet leads to the diagonalization of matrix:

$$\mathbf{X}_0^T \mathbf{D}_n \mathbf{X}_0 \mathbf{D}_p$$

i.e., the matrix obtained by proceeding counter-clockwise around the diagram, starting from  $\mathbf{R}^p$ .

Principal components, (variable loadings and row scores), are computed using the eigenvalues and eigenvectors of this matrix.

### Between-group analysis

The between-group analysis (BGA) is the analysis of triplet  $(\mathbf{X}_B, \mathbf{D}_p, \mathbf{D}_{n_k})$ , where  $\mathbf{X}_B$  is the  $(g, p)$  matrix of group means:

$$\mathbf{X}_B = [\bar{x}_j^k]_{(g,p)}.$$

The term  $\bar{x}_j^k = \frac{1}{n_k} \sum_{i \in G_k} \tilde{x}_{ij}$  is the mean of variable  $j$  in group  $k$ . In matrix notation, if  $\mathbf{B}$  is the matrix of class indicators:  $\mathbf{B} = [b_i^k]_{(n,g)}$ , with  $b_i^k = 1$  if  $i \in G_k$  and  $b_i^k = 0$  if  $i \notin G_k$ , then we have:

$$\mathbf{X}_B = \mathbf{D}_{n_k} \mathbf{B}^T \mathbf{X}_0.$$

Matrix  $\mathbf{D}_{n_k} = \text{Diag}(\frac{1}{n_k})$  is the diagonal matrix of (possibly non uniform) group weights, and  $\mathbf{B}^T$  is the transpose of  $\mathbf{B}$ .

BGA is therefore the analysis of the table of group means, leading to the diagonalization of matrix  $\mathbf{X}_B^T \mathbf{D}_{n_k} \mathbf{X}_B \mathbf{D}_p$ . Its aim is to reveal any evidence of differences between groups. The statistical significance of these differences can be tested with a permutation test. Row scores of the initial data table can be computed by projecting the rows of the standardized table  $\mathbf{X}_0$  onto the principal components subspaces.

### Within-group analysis

The within-group analysis (WGA) is the analysis of triplet  $(\mathbf{X}_W, \mathbf{D}_p, \mathbf{D}_n)$ , where  $\mathbf{X}_W$  is the  $(n, p)$  matrix of the differences between the standardized values and the group means:

$$\mathbf{X}_W = [\tilde{x}_{ij} - \bar{x}_{ij}^k]_{(n,p)}$$

with  $\tilde{x}_{ij}^k = \tilde{x}_{ij}^k, \forall i \in G_k$ . In matrix notation

$$\mathbf{X}_W = \mathbf{X}_0 - \mathbf{X}_B$$

where  $\mathbf{X}_B$  is the matrix of groups means, repeated inside each group.

WGA is therefore the analysis of the matrix of the residuals obtained by eliminating the between-group effect. It leads to the diagonalization of matrix  $\mathbf{X}_W^T \mathbf{D}_n \mathbf{X}_W \mathbf{D}_p$ . It is useful when looking for the main features of a data table after removing an unwanted characteristic.

### Software

We used R version 2.11.0 (R Development Core Team, 2009) for all computations, and the **ade4** package (version 1.4-14) for multivariate analyses (Chessel et al., 2004; Dray and Dufour, 2007; Thioulouse and Dray, 2007). PCA, BGA and WGA were computed with the `dudi.pca`, `between` and `within` functions of **ade4**. BGA permutation tests were done with the `randtest` function.

## Online reproducibility

The statistical analyses presented in Valiente-Moro et al. (2009) are reproducible (Gentleman and Lang, 2004) via the web page <http://pbil.univ-lyon1.fr/TTGE/>.

This page presents the problem, gives access to the data set, and allows the user to execute R code snippets that reproduce all the computations and graphical displays of the original article (Valiente-Moro et al., 2009). The R code is explained, with a description of important variables and function calls, and the R outputs are documented. Links to the information pages of the main **ade4** functions are also provided.

This page is therefore an example of *online literate programming* (Knuth, 1992), and also of an *online dynamic document* (Gentleman and Lang, 2004). This is made possible using HTML forms containing R code stored in editable text fields. The code itself can be modified by the user and executed on demand. Code execution is achieved by sending it to the Rweb software (Banfield, 1999) running on our department server: <http://pbil.univ-lyon1.fr/Rweb/Rweb.general.html>.

Links to the complete R code and data sets are provided on the web page. They can be used to download these files and use them locally if desired. The full R code (the collection of all code snippets) is available directly from <http://pbil.univ-lyon1.fr/TTGE/allCode.R>.

### PCA, BGA and permutation test

The first code snippet in the reproducibility page reads the TTGE data table, builds the factor describing the poultry farms, and computes the three analyses (PCA, BGA, and WGA). The PCA is first done using the `dudi.pca` function of the **ade4** package, and the resulting object is passed to the `between` and `within` functions to compute BGA and WGA respectively. The BGA permutation test is then computed and the test output is plotted.

On the reproducibility web page, these successive steps are explained: links to the data files are presented, the R code is displayed and commented, and the "Do it again!" button can be used to redo the computations. The code can be freely modified by the user and executed again. For example, it is possible to change the scaling of the PCA, or the number of random permutations in the Monte Carlo test to check the influence of these parameters on analysis outputs.

Figure 1 shows the result of the permutation test of the BGA. The null hypothesis is that there is no difference between farms. The test checks that the

observed value of the ratio of between-group to total inertia (0.67) is much higher than expected under the null hypothesis. Under the null hypothesis, mite pools can be permuted randomly among farms without changing significantly the ratio of between to total inertia. To compute the test, the rows of the dataframe are randomly permuted, and the ratio is computed again. This is done many times, to get an idea of the distribution of the between to total inertia ratio. Figure 1 shows that the observed value (black diamond, far on the right) is much higher than all the values obtained after permuting the pools. The  $p$ -value is 0.001, implying that the hypothesis of no differences between the farms can confidently be rejected.

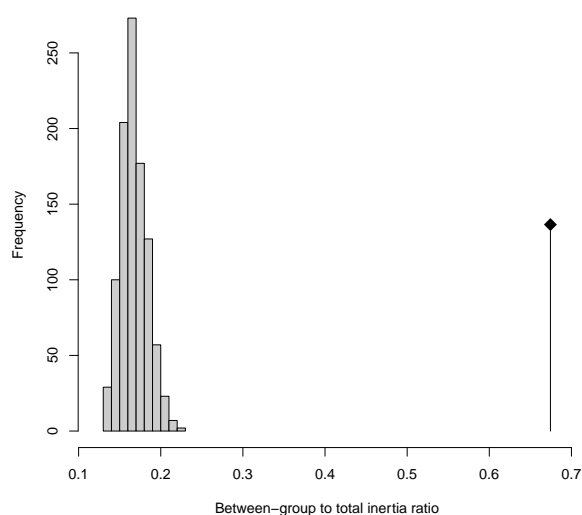


Figure 1: Permutation test of the BGA. The observed value of the between-group to total inertia ratio is equal to 0.67 (black diamond on the right). The histogram on the left shows the distribution of 1000 values of this ratio obtained after permuting the rows of the data table.

## BGA plots

The second code snippet draws Figure 2, showing the factor maps of the BGA.

The loadings of TTGE bands are in the `bga1$co` dataframe, and they are plotted using the `s.label` function (first panel). To get an idea of the dispersion of the six mite pools in each farm, we can plot the projection of each pool on the factor map (second panel). The row scores of the pools are in the `bga1$li` dataframe, and two graphs are superimposed: the graph of pool stars (with `s.class`), and the graph of convex hulls surrounding the pools belonging to the same farm (with `s.chull`). We can see that, as the permutation test had just evidenced, the farms are indeed very different.

Here also, the user can very easily change the R code to draw alternative graphs. It is possible, for example, to explore the effect of the arguments to the `s.label` and `s.class` functions.

Interpreting the differences between farms evidenced in Figure 2 was not easy. All 13 farms are standard poultry farms, using exactly the same breeding conditions, and the differences between TTGE banding patterns could not be attributed to any other factors.

Since the aim of the study was to find the common bacterial flora associated with *D. gallinae* in standard breeding conditions, we decided to remove this unwanted between-farm effect by doing a within-group analysis.

## WGA and cluster analysis plots

The third code snippet draws Figure 3, showing the factor maps of WGA and the dendrogram of the cluster analysis on TTGE band loadings.

The loadings of the 55 TTGE bands are in the `wga1$co` dataframe, and they are plotted using the `s.label` function (top-left panel). The scores of the 73 mite pools are in the `wga1$li` dataframe. They are grouped by convex hulls, according to the poultry farm from which they originate using the `s.class` and `s.chull` functions (top-right panel). The row scores of the WGA are centered by group, so the 13 farms are centered on the origin (this corresponds to the fact that the "farm effect" has been removed in this analysis).

The TTGE bands corresponding to the common dominant bacterial flora associated with *D. gallinae* in standard breeding conditions were selected on Figure 3 (top-left panel), using cluster analysis (lower panel). This was done using the complete linkage algorithm, with euclidean distances computed on WGA variable loadings on the first three axes (`wga1$co`) and not on raw data.

The reproducibility page can be used to compare these results and the ones obtained with other clustering algorithms or other distance measures. Another possibility is to check the effect of the number of axes on which variable loadings are computed (three axes in the R code proposed by default).

We included the two leftmost outgroups (bands 0.15, 0.08, 0.11, 0.51, and 0.88) and the right group (0.38, 0.75, 0.03, 0.26, 0.41, 0.59, 0.13, 0.32, 0.23, 0.21, 0.31, 0.28, and 0.3). Band 0.39 was excluded because it was present in only two mite pools belonging to one single farm.

These results show that there is a strong between-farm effect in the TTGE banding patterns corresponding to the bacterial flora associated with *D. gallinae* in standard breeding conditions. However, it was not possible to give a satisfying biological interpretation of this effect: the farms evidenced by the BGA did not show any particular characteristic. The



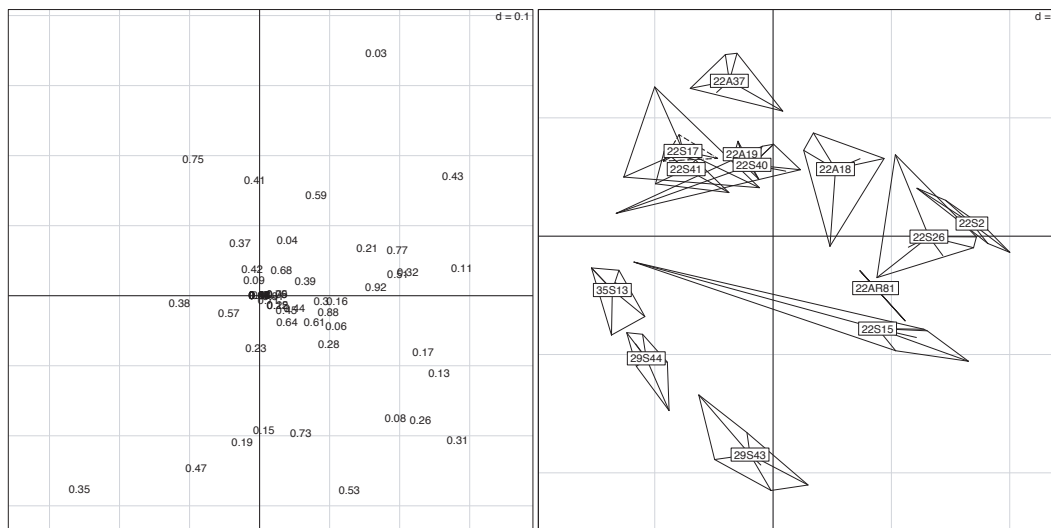


Figure 2: Factor maps of BGA (x-axis=first principal component, y-axis=second principal component, inertia percentages: 20% and 17%). The scale is given by the value  $d$  (top-right corner) that represents the size of the background grid. The first panel shows the map of the 55 TTGE bands (labels correspond to the position of the band on the electrophoresis gel). The second panel shows the 73 mite pools, grouped by convex hulls according to the poultry farm from which they originate (labels correspond to the farms).

WGA could remove this farm effect, and revealed the common dominant bacterial flora (Valiente-Moro et al., 2009). The knowledge of this flora will allow us to compare the variations observed in different conditions, such as different geographic regions, different types of breeding farms (for example organic farms), or different developmental stages of *D. gallinae*. Data from farms in different geographic regions and from organic farms are already available, and a paper presenting the results of their analysis has been submitted for publication.

## Discussion

### Statistical methodology

From the point of view of statistical methodology, BGA appears as a robust method that can be used to reveal any evidence for, and test, a simple effect, namely the effect of a single factor, in a multivariate data table. Many recent examples of its use can be found in the area of Genomics (Culhane et al., 2002, 2005; Baty et al., 2005; Jeffery et al., 2007).

Furthermore WGA can eliminate a simple effect from a multivariate data table. Like BGA, it can be used even when the number of cases is less than the number of variables. A recent example of WGA in the area of Genomics can be found in Suzuki et al. (2008).

BGA can be seen as a particular case of redundancy analysis [RDA, Stewart and Love (1968)] and WGA as a particular case of partial RDA [CANOCO (ter Braak and Šmilauer, 2002)]. Both cases corre-

spond to covariates reduced to a single dummy variable.

This can be checked with **vegan**, another classical R package for multivariate ecological data analysis, using the `rda` function, as explained on the online reproducibility page in code snippet 4. Outputs are not presented here, but they are available on that site, where they may be interactively explored.

### Computer setup

The reproducibility web page presented here is just an example of use of a simple setup that can be implemented easily on any web server. The current configuration of our server is an old Sun Fire 800, with 8 UltraSparc III processors at 900 MHz, 28 GB of memory and 6 x 36 GB disks. From the point of view of software, the server is running Solaris 10, the HTTP server is Apache 2.2.13, and we use Rweb 1.03 and R 2.11.0. Rweb is mainly a set of CGI scripts written in Perl, and the installed Perl version is v5.8.4. The page itself is written in plain HTML code, using standard HTML forms to communicate with Rweb CGI scripts.

Rweb source code is available from the Montana State University (<http://bayes.math.montana.edu/Rweb/Rweb1.03.tar.gz>).

There has been many other attempts at mixing R and HTML on the web. Alternative solutions could for example make use of **CGIwithR** (Firth, 2003) and **R2HTML** (Lecoutre, 2003), or Rscript.

Running a server offering public computing services necessarily raises many security issues. The server that is used for Rweb at the PBIL (<http://>

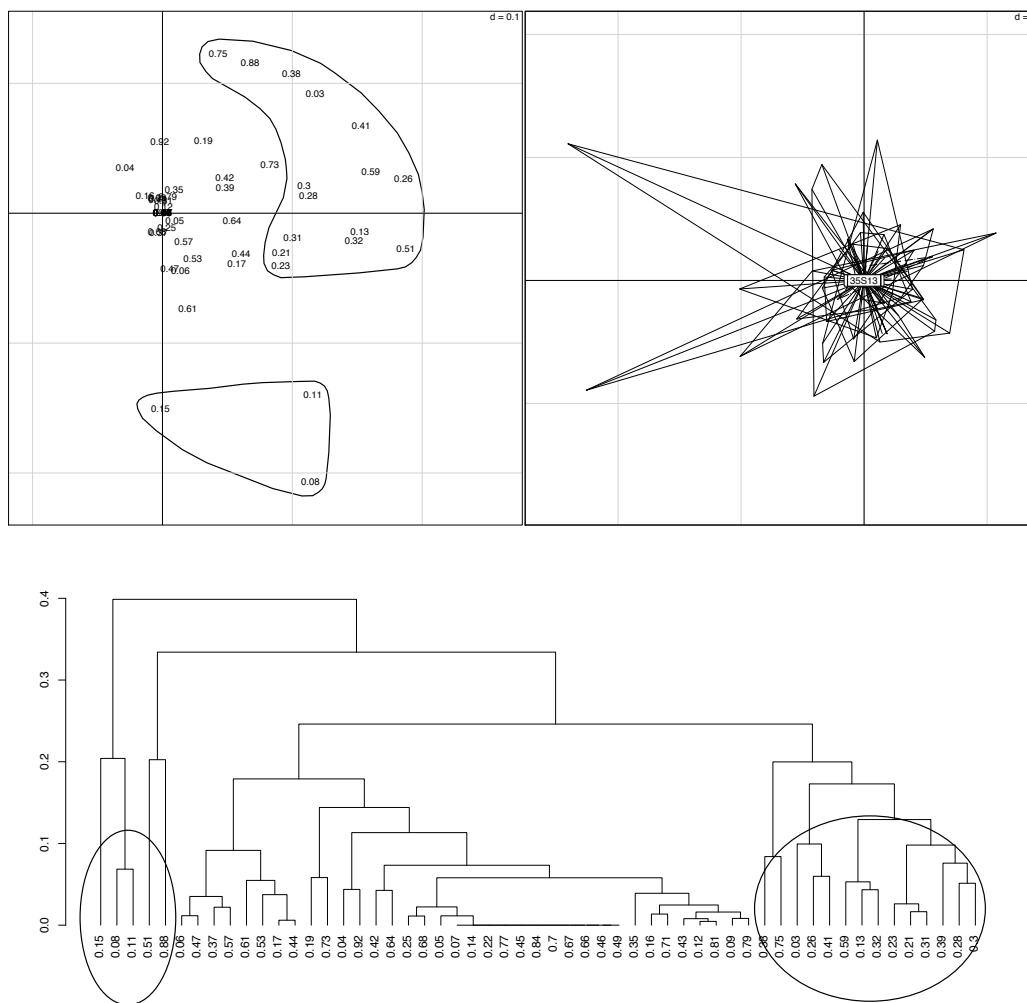


Figure 3: Factor maps of WGA (x-axis=first principal component, y-axis=second principal component, inertia percentages: 14 and 8). The scale is given by the value  $d$  (top-right corner) that represents the size of the background grid. Top-left panel: map of the 55 TTGE bands (see legend to Figure 2). Top-right panel: map of the 73 mite pools, grouped by farms. Bottom panel: dendrogram of the cluster analysis on WGA band loadings.

pbil.univ-lyon1.fr) also offers many other computing services in the area of Genomic research, including complex database exploration and analysis of huge molecular databases (GenBank, EMBL, etc.) This server has been attacked and successfully compromised several times with common rootkits, but in eight years [we started the Rweb service in 2002, Perrière et al. (2003)], the Rweb server has never been used in these attacks. Rweb precludes the use of the most sensitive functions (particularly "system", "eval", "call", "sink", etc.) and an additional security measure taken on our server is that all computations are performed in a temporary directory that is cleaned up automatically after use.

### Online reproducibility

Sweave is a very good solution for the reproducibility of scientific papers' statistical computation and

graphical display. It is more and more frequently used, and not only for reproducible research in the strictest sense. For example, it is used for quality control and for keeping up-to-date the documents used in the statistical teaching modules of the Biometry and Evolutionary Biology department at the University of Lyon, France: <http://pbil.univ-lyon1.fr/R/enseignement.html> (in French).

Using Sweave documents, however, is very demanding for users.  $\LaTeX$  and R must be installed on the local computer, and users must have at least some notions of how to compile these documents. The version of R and the list of packages must be compatible with the R code included in the Sweave document. This can be a serious obstacle for many researchers, for example in Biology and Ecology.

The online reproducibility solution presented in this paper is much easier to use for people who do not know  $\LaTeX$  and who have only vague notions of

R. It can be used with any web browser and it can make the analyses used in an applied statistics paper accessible to anyone. It can even be a good tool to help people get a better knowledge of R and encourage them to install R and use it locally.

## Rweb servers

Many examples of the use of the PBIL Rweb server are available on the home page of J. R. Lobry, <http://pbil.univ-lyon1.fr/members/lobry/>. The articles under the tag “[ONLINE REPRODUCIBILITY]” are reproducible through a reproducibility web page, using HTML forms and Rweb as explained in this paper.

Writing such web pages is very easy, and does not require R, Rweb or any other particular software to be installed on the HTTP server. The HTML code below is a minimal example of such a page. It can be used with any web server using, for example, the apache2 HTTP server, or even locally in any web browser:

```
<html>
<head><title>Rweb</title></head>
<body>
<p style="font-size:30px">
Rweb server at PBIL</p>
<form onSubmit = "return checkData(this)"
action="http://pbil.univ-lyon1.fr/cgi-bin/
Rweb/Rweb.cgi"
enctype="multipart/form-data"
method="post">
<textarea name="Rcode" rows=5 cols=80>
plot(runif(100))
</textarea><br />
<input type="submit" value="Run it!">
</form>
</body>
</html>
```

The main feature of this code is the `form` tag that declares the CGI script of the Rweb server: <http://pbil.univ-lyon1.fr/cgi-bin/Rweb/Rweb.cgi>. The `textarea` tag in this form contains the R code: here by default it is just one plot function call that draws 100 random points. This default code can be changed in the HTML document, or modified interactively by the user when the page is loaded in a web browser. The `input` tag defines the “Run it!” button that can be used to execute the R code. When the user clicks on this button, the code is sent to the Rweb CGI script and executed by R. The browser display is updated, and the execution listing and the graph appear.

Another solution is to install Rweb and run a local (public or private) Rweb server.

## Maintenance and durability

An important problem for reproducible research is durability. How long will a reproducible work stay reproducible?

The answer depends on the availability of the software needed to redo the computations and the graphics. R and contributed packages evolve, and R code can become deprecated if it is not updated on a regular basis. Sweave has been used mainly for just this reason in the documents used in the statistical teaching modules at the University of Lyon. All the documents are recompiled automatically to check their compatibility with the current version of R.

For an online reproducibility system to endure, the same conditions apply. The Biometry and Evolutionary Biology department has been running an Rweb server continuously since 2002, regularly updating the various pieces of software on which it depends (R, contributed packages, Rweb, Perl, Apache, etc.).

Reproducible research needs an ongoing effort; reproducibility ceases when this effort is not sustained.

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