MANUAL

SambaR

Snp datA Management and Basic Analyses in R

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SambaR is a R package for automated SNP dataset management and population genetic analyses. Whilst developing SambaR I was working on datasets generated by the ddRADseq protocol, but this package can also be used to analyse SNP datasets generated with other protocols. Sambar accepts biallelic SNPs only.

Because the datasets I worked on contained information about deer populations, I decided to name the package after a deer species called sambar (*Rusa unicolor*). But maybe an easier way to remember the name is that this package makes analyses of SNP datasets so easy and straightforward that you feel like wanting to dance the samba – or so I hope.

Please don't be scared by the number of pages this manual contains. To run the main analyses with default settings, you only have to go through sections 1 to 7, which will be quick and quite possibly well worth your time. My aim is to help you to get a wide range of standard analyses (e.g. quality control, PCA, CA, DAPC, MDS, Nei's D, Fst, HWE, SFS, LD, theta, Tajima's D, relatedness, inbreeding, admixture and selection analyses) done and your plots made with a few commands, without first having to spend weeks or months to get acquainted with all kinds of software. This way you can focus on what is really important: your study questions.

The functions in this R package depend heavily on other R packages. I make no excuse for that, because SambaR is meant to streamline the workflow. It does mean however that except for citing R and SambaR, you also need to cite many other packages. A Bibtex library of the citations of (most) of these packages is included in the SambaR download. This library can be exported into any referencing software, such as Endnote or Zotero.

SambaR comes with absolutely no warranty. You are free to share and distribute the package and to make changes to the source script as you please.

Run time and data size limitations

SambaR has been tested on datasets of up to hundreds of individuals and millions of SNPs. However, the memory capacity of your computer might pose restrictions and might cause SambaR to run into a memory error. The following table might help you to assess whether SambaR is capable of processing your data on your computer:

Run time of SambaR (excluding installation of dependencies) for various sized SNP datasets

type			**Computer XL** Linux HPC (server)	**Computer M** Windows10 Intel Core™ processer i5-8500 CPU	**Computer S** Windows10 Intel Pentium processer CPU B940 @2 GHz
number of cores			72	6	2
RAM			756 GB	16 GB	4 GB
data set	***n_snps***	***n_inds***			
unpublished data	>1000K	85	2 hours	Memory Error	Memory Error
Bosse et al. 2017	485K	3015	not tested	Memory Error	Memory Error
Bosse et al. 2017 reduced	485K	100	not tested	4 hours	Memory Error
Liu et al. 2014 reduced	1.1M	18	not tested	1 hour	Memory Error
Viengkone et al. 2017	3K	414	not tested	<1 hour	1 hour
De Jong et al. 2020	50K	92	not tested	<1 hour	1 hour

Output directories and files

SambaR will generate within your working directory (the directory which contains your input files) a SambaR_output directory with 7 subdirectories: Demography, Divergence, Diversity, Inputfiles, QC, Selection and Demography. After executing all functions, these subdirectories will contain input files, summary tables and ready to publish plots.
Every time you rerun a function, existing files from previous runs will be overwritten. Make sure that none of the plots are opened in a file viewer when rerunning a function, otherwise you will encounter errors.

SambaR creates most plots in up to 4 formats (i.e. eps, png, pdf, and wmf). Being vector-based, the pdf-files are suitable for stand-alone submission when your paper is accepted. The png and wmf files are of lower quality but are more easily imported into Word files, and therefore suitable to be included in Word files for review rounds. Keep in mind though that imported wmf-plots can cause problems when converting your word to pdf-format.

In principal pdf-files can be imported into Word files as well (see section '9. Manage your plots' on how to do so), but these plots are heavy, and if you want to include many plots (for example supplementary documents), Word can crash. The free software 'Inkscape' can be used to combine pdf-files into vector-based multi-tile figures (see section 9) suitable for publication.

Content of this manual

1. Do all at once

Just 10 R commands suffice to get almost everything done. These wrapper commands, which execute many analyses at once, roughly look like this (but you need to customize them, so keep on reading):

source("SAMBAR_v1xx.txt")	section 2
getpackages()	section 3
setwd("C:/path/to/workdir/")	section 4.1
importdata(inputprefix="yourprefix",sumstatsfile=FALSE,depthfile=FALSE)	section 4.1
filterdata(indmiss=0.25,snpmiss=0.1,min_mac=2,dohefilter=TRUE)	section 6
findstructure(Kmax=6,legend_pos="right",legend_cex=2,symbol_size=2)	section 7.1
calcdistance(legend_cex=2)	section 7.2
calcdiversity(nrsites=NULL)	section 7.3
calckinship()	section 7.4
selectionanalyses(do_meta=TRUE,do_pairwise=FALSE)	section 7.5
backupdata("my_snpdata")	section 8

The getpackages, importdata and filterdata functions should be executed sequentially. Afterwards, you can run any of the remaining functions in the order you prefer. For example, if you are interested in doing selection analyses only, there is no need to execute the findstructure, calcdistance, calcdiversity and calckinship functions.

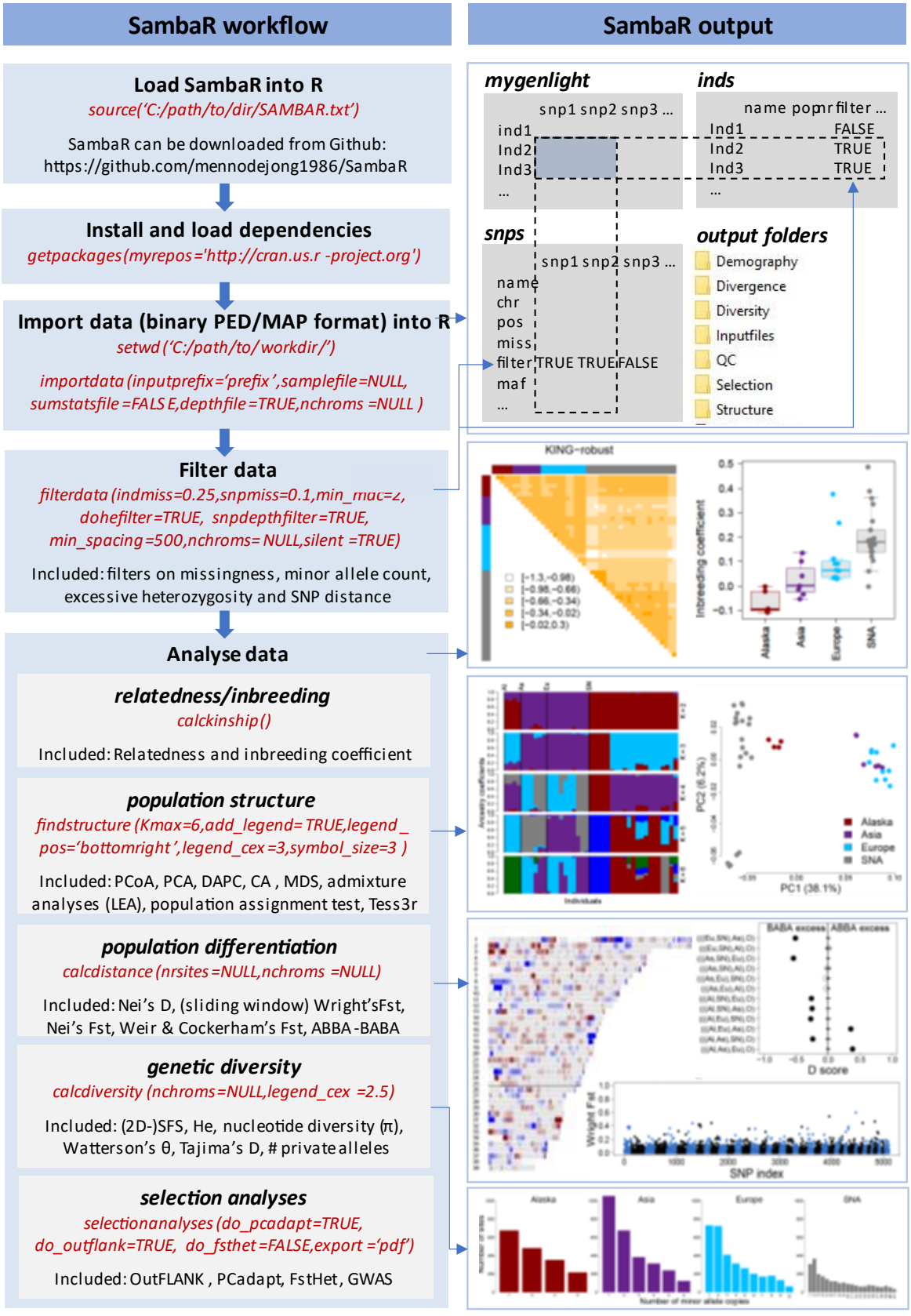
If this is the first time you use SambaR, you will have to go through the manual to understand how to customize the commands, how to prepare the input files, and what to expect in terms of the output. No worries: as stated above, you will get through it quickly.

If you used SambaR before, you know what to do and you can simply fill in the correct paths and desired settings, and next copy paste the commands into R.

Additional analyses

Some additional optional analyses require specific input files, and therefore need to be run independently. In this manual these analyses are labelled as 'ADDITIONAL ANALYSIS'.

Overview of workflow



2. Load SambaR

You are advised to run SambaR on the most recent version of R. This can save you a lot of problems and frustration when SambaR attempts to install all required dependencies (see next section). Preferably also install the latest version of Rtools. This will take a few minutes only. In theory, SambaR might run without problems on fairly recent versions of R and you are welcome to give it a try. There are however no guarantees, as packages are being continuously updated.

Once you opened a new R session, load SambaR simply by typing:
source("C:/ path/to/directory/SAMBAR.txt")

For example, if you have stored the source script on the C drive in the directory 'ProjectA' and the subdirectory 'SNPanalysis', you would type:
source("C:/ ProjectA/SNPanalysis/SAMBAR.txt")
Note: there shouldn't be any white spaces. Also note that R uses forward slashes rather than backward slashes.

Load SambaR directly from Github
You can also source SambaR directly from the Github webpage, without first having to download:
source("https://github.com/mennodejong1986/SambaR/raw/master/SAMBAR_v1.xx.txt")
(Remember to replace xx with the correct version number.)

Trouble shooting
One of the errors you might encounter is this one:
Error: unexpected input in "source(“”
In that case Word (or another text editor) has deformed the double quotes (from "" to “”). If so, either retype the double qoutes and run the command again, or copy paste via Notepad. This applies throughout this manual.

SambaR will tell you if it cannot find the file or path you specified. You can use the command 'list.files()' to see which files are present in any directory. For example, to see which files are present in the directory specified above, you would type:
list.files("C:/ ProjectA/SNPanalysis/")

3. Load dependencies

As mentioned before, SambaR depends heavily on other R packages (>2GB in total). Therefore, you need to install those as well. The good news is that SambaR comes with a function which automatically installs and loads all (or at least most) required packages: the getpackages function. The first time you run this function for a particular R version on your computer, the getpackages function can take several hours to complete. Subsequent executions will run in seconds.

If you are working on a personal computer and you have administrator rights (root permission), follow the instructions in section 3.1.

If you are working remotely on a server and you don't have administrator rights, follow the instructions in section 3.2.

3.1 Load dependencies on a personal computer

If you want to run analyses on your personal computer, you likely have administrator rights, which should make installation of R packages relatively straightforward. Simply run the getpackages() function and hopefully all (or most) packages will be installed automatically.

Usage

getpackages()

Function arguments

flag	default value	description
myrepos	"http://cran.us.r-project.org"	CRAN repository
mylib	NULL	If you do not have root access (administrator rights) on your computer, you need to specify the path to an existing directory where you want install the packages. For example, if you want to install the packages in the directory 'home/userX/Rpackages', you would type: getpackages(myrepos='http://cran.us.r-project.org',mylib='home/userX/Rpackages'). SambaR will automatically add the specified directory to the library paths, so that R knows where to find the installed packages.
noupdates	TRUE	Suppress package updates.
do_halt	TRUE	Abort if an error is encountered; if FALSE, jump to next package.
silent	TRUE	Suppress verbose

Details

If any of the dependencies still need to be downloaded, you will get a lot of rubbish on the screen, which is fine, as long as there are no errors. Warnings are allowed. You might for example get warnings about the R version under which a package has been built, or about objects which have been masked from packages.

If R asks you whether it should create a personal library, answer 'y' for yes (and answer again yes when it proposes a specific directory).
If R asks you whether you want to install from source the packages which needs compilation, answer 'n' for no (because that can take ages).
If R asks you whether it should update packages, answer 'n' for no (because that can take ages).
If R asks you to select a CRAN window, scroll down to 'other windows' and select any http window (rather than a https window).

SambaR will attempt to install of each package the most recent version. The single exception is the package PCadapt. SambaR will install PCadapt version 4.1.0 rather than the most recent version (which will be removed if already present). This is required for correct execution of the selection analyses function.

Trouble shooting

When SambaR does not manage to install and load a certain package, the getpackages function will automatically halt and inform users about the problem. It will also export a control file called 'mypackageslist.txt'. Users can edit this file to prevent SambaR from attempting to install certain packages, for example the ones which are problematic.

The control file classifies packages into three categories: 'essential', 'recommended', and 'optional'. Essential packages are required for SambaR to run without errors. Recommended packages are needed for key analyses. If you run into a problem when trying to install an optional package, the easiest solution would be to simply omit this package. You can do so by setting it's value in the 'do_install' and 'do_load' columns to FALSE.

WARNING: Package not available

If you do not have the most recent version of R, you might encountered the problem:

WARNING: package not available for R version …

If so, this probably means that the package depends on a more recent version of R (assuming that the name has not been misspelled). In that case you could either omit this package (by it's value in the 'do_install' column of the 'mypackageslist.txt' file to FALSE), or, if the package is essential, install the latest R version and then try again.

Problems when installing Bioconductor packages

The getpackages function will try to install Bioconductor packages. It can happen that you run into an error similar to:

ERROR in readRDS(dest): error reading from connection

Or:

Cannot open URL 'https://Bioconductor.org/packages/.rds': HTTP status was '404 Not Found'

I did not manage yet so far to figure out the exact reason, but it appears that SambaR interferes with the Bioconductor installation process. Therefore, follow these instructions:

1. Restart the R session.
2. Do not load SambaR, do not change the working directory, and do not attempt to run the getpackages function.
3. Run the commands (note that you might have to retype the double quotes):
   - if(!requireNamespace("BiocManager", quietly = TRUE)){install.packages("BiocManager")}
   - BiocManager::install(c("gdsfmt","SNPRelate","LEA","qvalue","XML","karyoploteR", "IRanges","Biostrings"),update=FALSE)
4. Next run the SambaR commands as usual.

System Requirements

Some of the packages which SambaR tries to install, most notably 'car' and 'sf', depend on software tools which needs to be installed on your computer. Often these software tools are part of the standard installations, and you will not need to bother about it. But if they are not installed, you will run into an error similar to:

Checking for sqlite… no

no

configure: error: sqlite not found or not executable

ERROR: configuration failed for package 'sf'

To prevent or solve this error, you need to have installed the following software:

sqlite
proj-devel-6.3.2-4.el8
proj-static-6.3.2-4.el8
geos-devel-3.7.2-1.el8
libxml2-devel

Installing is easiest to do if you have root access, using a command like:

yum install sqlite

Note however that, depending on your system, you might have to use an alternative to yum. If you do not have root access yourself, ask your administrator to install the software.

Problems with Rtools and github packages

A few packages are installed from Github and therefore need a software called Rtools. If R can not find it, you will run into an error related to the following warning:

WARNING: Rtools is required to build R packages, but is not currently installed. Please download and install Rtools custom from http://cran.r-project.org/bin/windows/Rtools/

If so, download Rtools and afterwards rerun the getpackages() function.

3.2 Load dependencies on a server

Installing all dependencies on a server can be challenging, because likely you do not have administrator rights. One way to circumvent this problem is to use conda.

First install anaconda or miniconda. Use your internet browser to find the Anaconda (or miniconda) link for download. Then type on Unix command line:
wget https://repo.anaconda.com/archive/Anaconda3-2021.05-Linux-x86_64.sh
bash Anaconda3-2021.05-Linux-x86_64.sh
This will ask you to accept the license agreement, to define a path for installation, and after the installation whether it should also initialize (answer yes). To enable that you can run conda from anywhere without having to specify the path, type: *source ~/.bashrc*

Then create a new environment where to install all R packages (and immediately install some essential packages) by typing:
conda create -n Renv -c conda-forge r-base r-essentials r-devtools r-sf

Optionally update:
conda update –all
conda update -n base -c defaults conda

Activate the 'Renv' environment and launch R:
conda activate Renv
R

In R, load SambaR and run the getpackages() function:
source("C:/ ProjectA/SNPanalysis/SAMBAR.txt")
getpackages()

Hopefully this command will install most packages without any issues. However, if it does encounter a problem and does not manage to install a certain package, it will abort the process and inform you which package is causing a problem. If so, exit R and deactivate the Renv environment:
conda deactivate

Search online (by googling 'conda') and the name of the package, which conda command is needed to install the package. Execute this command, and don't forget to specify to install the package within the 'Renv' environment. For example, if problems are caused by the R packages adegenet and iranges, run the commands:

conda install -n Renv -c conda-forge r-adegenet # for CRAN package

conda install -n Renv -c bioconda-forge bioconductor-iranges # for Bioconductor package

Afterwards, active the 'Renv' environment again, launch R, load SambaR and run the getpackages() function. When you encounter the next problem, install again the problematic packages outside of R. Keep on doing so until the getpackages function finishes without errors.

If some packages keep on causing problems, and if they are not essential packages, you could decide to skip the installation of this particular package (see section 3.1).

4. Load your data

Your data can be loaded and converted into SambaR objects either:
- by importing data into R (section 4.1); or alternatively
- by converting from an existing R genlight object (section 4.2).

Should you filter your data before running SambaR?
If you generated your SNP data with STACKS, there is usually no need for additional filtering, meaning you can proceed directly from STACKS to SambaR. The reason is that STACKS applies filters, and that RADSEQ datasets are relatively small.

If, in contrast, you have whole genome resequencing data, you will need to filter your SNP data, and make sure to remove SNPs with high proportions of missing data. Again, you could use bcftools. Two example commands:
BCFTOOLS filter --exclude-types indels –min.alleles 1 –max-alleles 2 --include " DP>=800 && DP<=2000" input.vcf.gz -O z QC.vcf.gz &
BCFTOOLS view -include "AN>=96" -O z input.vcf.gz -o QC.vcf.gz &

Furthermore, depending on the capacities of the computer you are working on, there might be size limitations, so if you would like to apply SambaR to whole genome sequencing datasets, you might have to thin your data first. On a personal computer you typically should bring the number of SNPs down to less than 200K. For thinning you could use the software vcftools (--thin flag).

4.1 Import your data into R

SambaR uses the 'read.PLINK'-function of the Adegenet package to import biallelic genotype data into R. This function expects your files to be in RAW and BIM format, which are the binary versions of PED and MAP format.

If your files are already in RAW/BIM format, you can proceed to 4.1.3.
If not, follow the instructions in this section (4.1.1-4.1.3) on how to convert your data files into these formats, and how to subsequently import your data into R. These preparatory steps involve using software other than R, namely PLINK2 (or PLINK, although older versions might cause issues) and vcftools and/or PGDspider.

The three steps to load the data are:		Software:
1. Convert your data into a PED/MAP files	4.1.1	plink2 or vcftools or PGDSpider
2. Convert PED/MAP files to RAW/BIM files	4.1.2	plink2
3. Import RAW/BIM into R	4.1.3	R

If you still need to install any of the programs listed above, here are the links:
http://www.cmpg.unibe.ch/software/PGDSpider/
https://vcftools.github.io/downloads.html
https://www.cog-genomics.org/plink2/ (choose the stable beta version)

4.1.1 Convert from any data format to PED/MAP

There are many ways to create a PED/MAP file, depending on your starting point. Here I describe two options: convert from vcf format using vcftools, or convert from genepop format using PGDSpider.

Option 1. Convert from vcf to PED/MAP

Both plink2 and vcftools can quickly convert vcf files into ped/map format. I therefore recommend STACKS users to include the –V flag when running the STACKS populations command. (Another advantage of the vcf format and vcftools is that it can be used to calculate read depth, as explained below.)

To convert vcf to PED and MAP, type on the command line of your computer either:
/path/to/plink –vcf inputfile.vcf –recode --out prefixoutputfile
or:
/path/to/vcftools --vcf inputfile.vcf --plink --out prefixoutputfiles

Vcftools has the disadvantage that it does not like contig or chromosome names other than human chromosome numbers. You are therefore likely to see many warnings on the screen, stating: 'Unrecognized values used for CHROM – Replacing with 0'. As a result, you will find that the first column of the MAP file, which should contain contig names, contains zero's only. However, as vcftools uses the contig names to make SNP names (see column 2), this problem is potentially easy to fix. All you have to do is to insert in the following command the name of your MAP file (as highlighted in grey), and then execute this oneliner on the Linux command line:
cut -f2 yourfile.map | cut -f1 -d ':' > mycontigs.txt && cut -f2,3,4 yourfile.map > mymap.txt && paste mycontigs.txt mymap.txt > yourfile.map && rm mycontigs.txt mymap.txt

Normally, the first column in PED files contains the names of your populations, and the second column the names of your samples. If the population names are not specified in the vcf file in the last row of the vcf header (e.g. pop1_ind1, pop2_ind2, etc), or if using vcftools when converting, you will find that when converting from vcf to PED, both columns in the PED file contain the names of your samples. In that case, you have to provide an additional population file. This should be a tab delimited file with two column called 'name' and 'pop'. The first column contains the sample name and the second column contains the population name, like this:

name	*pop*	*pop2*
sample1	*pop1*	*subpop1*

sample2 pop1 subpop2
sample3 pop2 subpop3
etc.

The third column, 'pop2', is optional, and serves to define subpopulations within your main populations. For example: if you have samples from Europe, Asia and North America, the pop column could serve to assign samples to 'Europe', 'Asia' and 'NA', and the pop2 column could serve to assign samples to specific regions, e,g, MidEast and FarEast for Asia.
WARNING: population names may NOT contain underscores or spaces, because this will interfere with Sambar commands. In addition, because of plot margins, your population names may NOT contain more than 10 characters. Originally, populations needed to contain at least 2 individuals per population, but SambaR versions 1.02 or higher do not longer have this limitation.

You will need this populations file when importing the data into R. But before you can import the data, you need to do one more step: convert from PED/MAP to RAW/BIM format.

Since you are on it, you can also calculate read depths per sample and SNP:
/path/to/vcftools --vcf inputfile.vcf --depth &
/path/to/vcftools --vcf inputfile .vcf --site-mean-depth &

Option 2. Convert from genepop (or other formats) to PED and MAP using vcftools
If your data is not in vcf format, don't worry! You can use PGDspider to convert your data to a PED and a MAP file from almost any format. You can either run PGDspider using the graphical user interface (GUI), or run it from the command line. Note that the GUI of PGDspider has a data size limit, and therefore, depending on the size of your dataset, you might have no other option than to run PGDspider from the Windows command line.

If using the graphical user interface (GUI) of PGDspider, select as data input file 'genepop' and as data output file 'PED'. Click on the button 'create/edit SPID file' , and select 'SNP' as dataset under the tab 'Genepop – Parser Questions'. Under the tab 'PED – Writer Questions', answer 'yes' to the question whether you want to save an additional MAP file, and provide a name. This name should be the same as the name of the PED file. The extension is added automatically.

If running PGDspider from the Windows command line, you will first have to create the appropriate SPID-file (if you prefer, you can do so by using the graphical user interface), and afterwards execute on the command line a command which looks like this:
java -jar PGDSpider2-cli.jar -inputfile infile –outputfile out.ped -spid spidfile.spid

To save the time having to make SPID files yourself, I enclosed two SPID files needed for the conversion from either genepop or vcf format. The actual commands are:
java -jar PGDSpider2-cli.jar -inputfile infile.genepop –outputfile out.ped -spid Genepop2PED.spid
java -jar PGDSpider2-cli.jar -inputfile infile.vcf –outputfile out.ped -spid VCF2PED.spid
Both commands will output two files, called out.ped and out.map, to the working directory.

If you get empty output files (0 or 1 kb), one reason might be that PGDspider doesn't like your population names (if different from pop). So it might help to indicate populations simply with the word 'pop' for now.

As the population names in the PED-file (first column) will be used by Sambar to name the populations, this moment (after the conversion) is a good moment to rethink how you want to name your populations, and possibly rename them. For example: if at the moment your PED-file lists your populations simply as 'pop1' and 'pop2' (etc), you might want to provide more telling names.

It is important to note that population names may NOT contain underscores or spaces, because this will interfere with Sambar commands. In addition, because of plot margins, your population names may NOT contain more than 10 characters. Originally, populations needed to contain at least 2 individuals per population, but SambaR versions 1.02 or higher do not longer have this limitation.

Check your files
Now that you created your PED/MAP files, you might want to do a quick check. Does the number of lines in the PED file correspond with the number of individuals? Does the number of lines in the MAP file correspond with the number of SNPs?
If everything looks right, you are ready to move on to the next step, and convert from PED/MAP format to RAW/BIM (section 4.1.2).

4.1.2 Convert from PED/MAP to RAW/BIM

PED and MAP files of SNP datasets are generally too big to load directly into R. Therefore, you first need to compress your files from PED/MAP formats to RAW/BIM formats.

You can use PLINK to do so. The command, to be executed on either Unix or Windows command line, looks like this:
plink --file prefix –-chr-set 95 –-allow-extra-chr --make-bed --recode A --out prefix

The make-bed flag tells the software to make the bim file. The 'recode A' flag tells the software to create the raw file. In order for the PLINK command to work, the PED and the MAP file should have the same name (prefix), except for the extensions '.ped' and '.map'. Use the –-allow-extra-chr flags to include snps located on contigs rather than on chromosomes. If not, you might end up with empty output files. The –chr-set indicates how many chromosomes are present at max (95 is unrealistically high for most species, but this is to prevent errors).
If you get the error '--file accepts at most 1 parameter', or 'unrecognized flag', or 'doesn't accept parameters', retype all the dashes and try again.

Whilst running the command, PLINK will output some information on the screen. Also here you could check whether the expected number of individuals and SNPs corresponds with the information on the screen. If you made sure your data is biallelic, you can avoid warnings about some SNPs being triallelic – this is probably due to an unexpected character for missing data.

The raw file is binary, but the bim file is not, so just to make sure everything went right you might want to compare it to the map file. (Just note: if you want to observe the files, open these files in a plain text editor, such as Notepad or Notepad++, and not in other programs such as Word, because this might corrupt the file.)

Trouble shooting

If your input file contains indels, PLINK will throw the error:

Possibly irregular .ped line. Restarting scan, assuming multichar alleles.

Rescanning .ped file... 0%

Error: Half-missing call in .ped file at variant …, line 1.

If so, redo the filtering steps (see above).

If your contigs (first column of the MAP-file) start with a number rather than a letter, PLINK assumes they are chromosomes, and might throw the error:

Invalid chromosome '..' on line ... of .map file.

A solution would be to add the word 'contig' in front of the number. You can do so on the Linux command line with the following command:

sed -i -e 's/^/contig/' inputprefix.map

If the data is not properly sorted by chromosome/contig, PLINK might throw the error:

Error: .bim file has a split chromosome. Use –make-bed by itself to remedy this.

This means that your data might have, for example, some chromosome 5 markers, followed by chromosome 6 markers, and then followed again by chromosome 5 markers. Run the following command to sort the data:

plink –file prefix –make-bed –out prefix.sorted

If missing data is encoded by N instead of 0, PLINK might throw the error:

Warning: Variant …. (post-sort) triallelic; setting rarest alleles missing.

Note however that this error can also have other causes.

4.1.3 Import data from RAW/BIM

Once your data is in RAW/BIM format, you are ready to import the data into R. Open the R session in which you loaded Sambar and use the setwd() command to navigate to the directory on your computer where you stored your RAW/BIM files:

setwd("C:/Users/my/path/to/directory/")

To check whether your input files are indeed present, type: *list.files()*

If your input files are present, import your data using the 'importdata' function.

Usage

importdata(inputprefix='yourprefix')

Function arguments

argument	default	description
inputprefix	NULL	Prefix of input RAW and BIM file (i.e. without .raw and .bim extension).
sumstatsfile	FALSE	If TRUE, the function expects to find in the working directory a file called 'sumstats.tsv' generated by the STACKS refmap pipeline. You should include the sumstats.tsv file if you called your SNPs using the STACKS refmap pipeline, regardless of whether you created your PED/MAP files from a genepop file or vcf file. A genepop file does not contain information on the positions of your SNPs, and as result the first two columns of the MAP file wil contain zero's only. A vcf file does contain information about the positions of your SNPs, but the vcf file created by STACKS shows the position of your SNPs in the STACKS loci rather than position in the reference genome
snpinfofile	NULL	Name of tab separated file with additional loci information. Number of lines should equal number of lines of BIM file.
samplefile	NULL	Name of optional input file with population assignment. At minimum the file should contain two tab-separated columns called 'name' and 'pop'. The name column contains the sample name (identical to names specified in PED-file) and the pop column contains the population names (max 10 characters, no underscores or spaces). Population subdivision can be specified in an optional third column called 'pop2'. This sample file is not needed if the populations are defined in the first column of the PED-file.
geofile	NULL	Name of optional input file with geographical coordinates (longitude and latitude) of the samples. Input file should contain three tab-separated columns called 'sample', 'longitude' and 'latitude'. Sample names in the geofile should be identical to sample names in PED-file. The coordinates should be in decimal degrees (e.g. New York: lat = 40.714, lon = -74.006).
depthfile	FALSE	If TRUE, the function expects to find in the working directory the files 'out.idepth' and 'out.ldepth.mean', generated with the software vcftools using the --depth and --site-mean-depth flags. (Note that a vcf file created with 'plink --recode vcf' does not contain read depth information and therefore cannot be to used to calculate mean read depth.)
nchroms	NULL	Number of chromosomes of the reference genome assembly (optional).
colourvector	NULL	Optional vector with colour names, which will be assigned to your populations in alphabetical order. The vector should have the following syntax: c("blue","darkgreen","darkred"), and the length of the vector should be equal or greather than the number of populations defined in the input data.
pop_order	NULL	Optional vector listing your populations in the desired order for output boxplots, structure plots and population distance estimate matrices. If NULL (default), populations will be ordered alphabetically. The population names should be identical to the population names in the input PED-file (or samplefile).
silent	TRUE	Suppress verbose
legend_cex	3	Legend font size of output plots

Example

For example, my inputfiles are called reindeer.raw and reindeer.bim. The data has been generated using STACKS refmap (so I have a sumstatsfile), and I subsequently used vcftools to

calculate read depths stored in files called out.idepth and out.ldepth.mean. I also listed the geographical coordinates of the samples in a file called “geofile.txt”. I type:
importdata(inputprefix=“reindeer”, sumstatsfile=TRUE , depthfile=TRUE,geofile=”geofile.txt”)

Trouble shooting

If you forget to put double quotes around your inputprefix (e.g. inputprefix=reindeer instead of inputprefix=”reindeer”), you will get the error:
Error in paste(inputprefix, "raw", sep = ".", collapse = NULL) :
object 'reindeer' not found

As mentioned elsewhere in this manual, you might encounter the error:
Error: unexpected input in "source(“"
In that case your text editor has deformed the double quotes (from "" to “”). If so, either retype the double qoutes and run the command again, or copy paste via Notepad. This applies throughout this manual.

If by mistake you have set the sumstatsfile flag or depthfile flag to NULL rather than FALSE, you will get the error:
Error in if (sumstatsfile) { : argument is of length zero
Error in if (depthfile) { : argument is of length zero

If you generated the PED and MAP files using vcftools but did not provide input to the flag samplefile whilst running the importdata command, you will get the warning:
WARNING: the number of populations in the 'popnames'-vector does not correspond with the number of populations in raw.file. As a result, could not assign population names.
This will cause errors in subsequent functions.

If there were hidden problems during file conversion (for example the file conversion from vcf to PED/MAP) stopped prematurely, you might encounter an error similar to:
Error in read.PLINK(paste(prefix,"raw",sep=".",collapse=NULL));
some individuals do not have 86754 SNPs
In that case likely something went wrong during the file conversions, resulting in one or more individuals not have the expected number of SNPs (as might be obvious from observing the PLINK). Redo the conversion and afterwards try to import the data again.

4.2 Convert from genlight object (or Genalex, FASTA or DNAbin format)

Rather than importing the data into R, users also have the option to create the SambaR data objects directly from a genlight object (i.e. an existing R object), using the genlight2sambar function.

Usage

genlight2sambar(genlight_object=NULL)

Function arguments

flag	default	description
genlight_object	NULL	Name of input genlight object.
colourvector	NULL	Optional vector with colour names, which will be assigned to your populations in alphabetical order. The vector should have the following syntax: c("blue","darkgreen","darkred"), and the length of the vector should be equal or greater than the number of populations defined in the input data.
popvector	NULL	Optional vector with population names, corresponding to order of individuals in the genlight object.
pop_order	NULL	Optional vector listing your populations in the desired order for output boxplots, structure plots and population distance estimate matrices. If NULL (default), populations will be ordered alphabetically. The population names should be identical to the population names in the input PED-file (or samplefile).
major	NULL	Optional vector which defines allele type of major allele (1,2,3,4, or alternatively "A","C","G","T"). If NULL (default), all major alleles will be set to "A"). This does not affect subsequent analyses, except for calculation of transition-transversion ratios and GC-content.
minor	NULL	Optional vector which defines allele type of minor allele (1,2,3,4, or alternatively "A","C","G","T"). If NULL (default), all minor alleles will be set to "T"). This does not affect subsequent analyses, except for calculation of transition-transversion ratios and GC-content.

Example

If your data is, for example, stored in a genlight object named 'my_gl', all required SambaR objects can be created by running the command:

genlight2sambar(genlight_object="my_gl")

Import data from Genalex format

As long as the data is diploid and bi-allelic, data stored in Genalex format can be converted to genlight and analysed using SambaR.

To import the data in R and subsequently convert to SambaR objects, go through the following steps:

Assuming your Genalex data is currently stored as an Excel file, open the file in Excel, and save the file as a csv file. Next open the file in a plain text editor like Notepad, and use the replace function to replace all semicolons (;) with comma's (,). Check whether they are three header lines, namely:

nrloci,nrsamples,nrpopulations,
optional description of data,
Ind,Pop,Locus1,,Locus2,,Locus3,,etc

Run the following commands in R:

```
install.packages("dartR")
library("dartR")
library("poppr")
mydata          <- poppr::read.genalex("genalexdata.csv",genclone=FALSE)
mygl            <- gi2gl(mydata,parallel=FALSE,verbose = NULL)
source("SAMBAR_vX.XX.txt")
getpackages(myrepos='http://cran.us.r-project.org')
genlight2sambar(genlight_object="mygl")
```

Convert data from DNAbin format (or FASTA format)

As long as the data is diploid and bi-allelic, data stored in DNAbin format (for example imported in R from a FASTA file) can be converted to genlight and analysed using SambaR. Assuming that are working with dnabin object called 'mydata', you can convert the data to SambaR objects using the following commands:

```
source("SAMBAR_vX.XX.txt")
getpackages(myrepos='http://cran.us.r-project.org')
DNAbin2genlight(mydnabin=mydata)
mygl@ind.names      <- gsub(" .*$","",mygl@ind.names)      # optional, shorten names
genlight2sambar(genlight_object="mygl",major=mygl$other[[1]],minor=mygl$other[[2]])
```

5. Observe your data

One of your challenges as a bioinformatician is to manage your input and output files. Because of the big variety of software and of filter settings, the number of files can quickly grow out of control. A main purpose of SambaR is to prevent this from happening, by storing all your data (input and most of the output) conveniently in no more than 3 R objects.

These 3 data objects are:

4. A genlight object called '***mygenlight***', containing the genotype data, with the number of columns equaling the number of loci, and the number of rows equaling the number of samples/individuals. 0 codes for 'homozygous major allele', 1 codes for 'heterozygous' and 2 codes for 'homozygous minor allele'. Missing data is denoted by 'NA'.
5. A dataframe called '***snps***', containing locus specific information, with the number of rows equaling the number of loci.
6. A dataframe called '***inds***', containing sample specific information, with the number of rows equaling the number of individuals.

The 'mygenlight'-object should not be manipulated. The user is free, in contrast, to add as many columns to the snps and inds dataframe as preferred (in addition to the columns generated by SambaR functions). If you are want to know more about the actual meaning of the columns of the snps and inds dataframe, you can find descriptions in section 10.1 and 10.2.

Observe your data

Because many SambaR functions add columns to the snps and/or inds dataframe, you might want to inspect your inds and snps dataframes regularly.

If you type on the R command line 'mygenlight', you will get a summary of the genotype data.
To observe a fraction of the actual data, type:
as.matrix(mygenlight[1:10,1:10])

To observe the first 5 lines of the snps file, type:
head(snps,5)
To observe the last 10 lines of the inds file, type:
tail(inds,10)
On some (not all!) platforms you can observe the snps file in R's built-in data editor by typing:
fix(snps)

Subselect your data

To select a single column within the snps or inds dataframe you have to use the dollar sign. For example, if you want a summary of the identities of the minor alleles, you could type:
table(snps$minor2)

Alternatively, you could also type:
table(snps[,"minor2"])

The square brackets ('[' and ']') are for subselecting a dataset. In the part before the comma, you select rows. (If you don't specify anything, like in in the example above, this is interpreted as: 'select all'.) In the part after the comma, you select columns. If you want to select multiple columns and only the first 10 rows, it would look like this:
snps[1:10,c("chr","name","pos","minor","major")]

The findstructure() will export ordination plots, some of which will show sample numbers. If you want to know the name of a particular sample (for example sample number 51), you could type:
inds[inds$nr==51,]
or:
inds$name[inds$nr==51]

mysambar

The snps, inds and mygenlight objects are the three core objects used by SambaR, but in order to operate correctly it needs more objects. It needs to know for example the paths to the input and the output directories, and the font type to be used for the plots. All this information will be stored in a list object called 'mysambar'. You can observe this list by typing:
summary(mysambar)

For normal use of SambaR, you don't need to know about the existence of the 'mysambar' object. However, it is important to remember that you should NOT name any object 'mysambar', otherwise SambaR will stop working correctly.

6. Filter your data

After having imported the data into R, a mandatory step is to run the filterdata function. This function performs data quality control as well as subsequent data filtering.

Usage

filterdata(indmiss=0.25,snpmiss=0.1,min_mac=2,dohefilter=TRUE,min_spacing=500,nchroms=NULL,TsTvfilter=NULL)

Function arguments

flag	default	description
indmiss	0.25	Maximum allowed proportion of missing data per sample. By default, samples with more than 25% missing data will be excluded from subsequent analyses.
snpmiss	0.1	Maximum allowed proportion of missing data per SNP. By default, SNPs with more than 10% missing data (averaged over samples which passed the indmiss threshold) will be excluded from subsequent analyses.
min_mac	2	Minimum allowed number of minor allele copies per SNP. By default, SNPs with contain only 1 copy of the minor allele (only considering samples which passed the indmiss threshold), will be excluded from subsequent analyses.
dohefilter	TRUE	If TRUE, the function will remove SNPs with heterozygosity levels which are potentially indicative of paralogs (see paralog_threshold1 and paralog_threshold2 flag). These SNPs will be highlighted in red in the 'He_vs_maf' plot which is generated by the filterdata function. Excluded from subsequent analyses are SNPs which meet two criteria: He > 2pq + pT1 and p>T2 (in which p and q denote respectively minor and major allele frequency, and in T1 and T2 denote respectively paralog_threshold1 and paralog_threshold2.
snpdepthfilter	TRUE	If TRUE, the function will remove SNPs with high read depth (if information is available). These SNPs will be highlighed in red in the 'Locusdepth' plot which is generated by the filterdata function.
min_spacing	500	Minimum distance between adjacent SNPs (in bp) when thinning the data. In order to avoid linkage disequillibrium issues, SambaR will use this thinned dataset for structure, genetic distance and diversity analyses.
old_distfilter	FALSE	SambaR version 1.04 and earlier versions used a flawed thinning algorithm, which has been replaced in SambaR version 1.05. For reproducibility, the old thinning algorithm can be invoked with by setting the flag 'old_distfilter' to TRUE.
nchroms	NULL	Number of chromosomes of the reference genome assembly (optional). If a value is provided, SambaR will generate additional plots showing the number of SNPs per chromosome, and try to determine the X-chromosome.
TsTv_filter	NULL	Can be set to either 'Ts' or 'Tv' in order to exclude respectively transversions or transitions.
ychrom	NULL	Name of Y-chromosome (optional).
paralog_threshold1	0.5	Setting to filter out SNPs with high heterozygosity, indicative of paralogs (see dohefilter flag).
paralog_threshold2	0.05	Setting to filter out SNPs with high heterozygosity, indicative of paralogs (see dohefilter flag).
silent	TRUE	Suppress verbose
legend_cex	TRUE	Legend font size of output plots

Details

Despite its name, the function does NOT remove data entries, not from the genlight object, nor from the inds or snps dataframes. In fact, it adds data. First, the function adds columns to the snps and inds dataframes with information about minor allele frequency, observed and expected heterozygosity, inbreeding coefficient, distance between snps, and number of missing datapoints. Based on this information the function will subsequently add columns with boolean

vectors (i.e. FALSE/TRUE) to the snps and inds dataframes, indicating whether loci and individuals pass quality filters.

Function output

Once the execution of the filterdata function is finished, have a look in the Sambar output directory. You should find within the inputfiles directory PED and MAP files of the filtered datasets, both for the entire dataset (metapop) as for each population separately (if applicable), with two filter settings: filter1 and filter2 (more details below), and with genotypes either encoded in letters (A,C,G,T) or numbers (1,2,3,4).

Within the subdirectory 'QC' you will find a number of plots in up to 4 different formats (eps, pdf, png, wmf).

Also, and most importantly, columns have been added to the snps and inds dataframe. The overall filters are inds$filter and snps$filter (thinned dataset) and snps$filter2 (non thinned dataset). These columns determine whether samples and loci will or will not be used during subsequent analyses. If TRUE, the sample or locus will be included in the analysis. If FALSE, the sample or locus won't be considered.

To avoid errors during the execution of subsequent functions, NEVER remove or rename the inds$filter, snps$filter and snps$filter2 columns.

The filter settings you set now will be used for analyses. But if later on you decide you prefer other settings, you can quickly and easily rerun the analyses with different filter settings. For example, you could initially run the filterdata function with certain settings -- e.g *filterdata(indmiss=0.75, snpmiss=0.02)* -- prior to structure analyses, and afterwards rerun the function -- *filterdata(indmiss=0.05, snpmiss=0.15)* -- before doing genetic diversity analyses.

Which filter settings should you choose?

The default values for indmiss and snpmiss (0.25 and 0.10 respectively) are completely arbitrary and can be considered both conservative and liberal. You could argue a sample with 95 percent missing data is of low quality, but if the total dataset contains 50.000 snps, the sample still provides a goodly number of 2.500 non-missing datapoints.

Which values for indmiss and snpmiss should you choose? This depends on your research question. If you are mainly interested in structure analyses, you should probably opt for a strict

snpmiss value (i.e. < 0.05, but preferably as low as possible). A relatively low number of high quality SNPs (with almost no missing data) might give you a more accurate picture of the true population structure than a high number of low quality SNPs. A number of 1000 high quality SNPs is often more than enough to infer population structuring, but this obviously does depend on your study system. Whereas the snpmiss value should be as low as possible, the indmiss value can be relatively high, as long you keep a sufficient number of SNPs. After running the importdata function, you will find in your SambaR directory a pdf-file called 'Data_quality'. Observe this file for guidance.

If you are mainly interested in genetic diversity analyses, you should keep in mind that samples with proportions of missing data likely had low coverage across the genome, which might have complicated the correct calling of heterozygosity. As a result the number of heterozygous sites in these samples might be biased downwards. A relatively small number of high quality individuals might give you therefore a more accurate estimate of genetic diversity within your populations than a high number of low quality samples. Observe the 'He_vs_miss.pop' plot for guidance. You want to set indmiss to a threshold such that for the retained dataset there is no relationship between sample heterozygosity and it's proportion of missing data.

For selection and association analyses, for which your objective is to screen as many SNPs as possibly, you might get away with setting high value for snpmiss and indmiss. The presence of SNPs with high proportions of missing data might not cause major problems because selection analyses often work on a SNP by SNP basis and simply ignore missing data point (this might not be true for PCadapt though). On the other hand you could argue that the distribution of neutral SNPs (from which outlier SNPs need to stand out) might be affected by the presence of missing data, and that therefore some sort of filtering is desirable. To be on the safe side, you should probably run the selection analyses with various filter settings, and observe if the results are generally consistent.

ADDITIONAL ANALYSIS: export files needed for additional analyses

To calculate LD and sample relatedness/kinship with plink, as well as to calculate migration rates with Bayesass and TreeMix (see elsewhere in this manual for more info), you need to generate input files. To do so, execute the following function once the filterdata() function has finished:

exportsambarfiles()

Exclude samples or populations from downstream analyses

If you want to exclude particular samples from downstream analyses (say a sample called 'sample5', simply type:

inds$filter<-inds$filter&as.character(inds$name)!="sample5"

If want to exclude an entire population, say two populations called "pop2" and "pop5", simply type (after the filterdata function finished):

excludepop(do_exclude=c("pop2","pop5"))

Trouble shooting

I mentioned this before, but it might be worth repeating:

If you decide to rerun the filterdata()-function, make sure that none of these plots are opened in a file viewer (like eps viewer or pdf viewer), otherwise this will result in an error similar to:

Error in pdf("He_vs_maf.pdf", family = myfont, width = 10, height = 10) :
cannot open file 'He_vs_maf.pdf'

If for whatever reason SambaR runs into an error whilst exporting a plot, the connection to this plot won't be closed. This will prevent you from opening the plot (you will get an error saying that the file is in use by another device), and might also prevent R from establishing new connections. Therefore, whenever you run into an error, it is good practice to, before running a SambaR function, execute the following command:

dev.off()

Keep on executing this command until you run into the following error message:

Error in dev.off() : cannot shut down device 1 (the null device)

7. Analyse your data

Now you are ready to analyse your data. In this section I will discuss the SambaR functions which will help you to do so. Note that all subsequent functions expect to find:

- a genlight object called 'mygenlight'
- a dataframe called 'inds', containing an inds$filter column
- a dataframe called 'snps', containing a snps$filter' and snps$filter2 column.
- a list object called 'mysambar' in which additional information is stored

You created these objects in the previous steps, when importing and filtering the data.

7.1 Population structure

Likely the first thing you want to do is to find out about the structuring of your samples. In other words: you want to know whether your samples can be divided into distinct populations. This is especially interesting if you don't have a priori information about population structure, but it is also useful to verify a priori expectations.

Usage

findstructure(Kmax=6,add_legend=TRUE,legend_pos="right",legend_cex=2,pop_order=NULL)

Function arguments

flag	default	description
quickrun	TRUE	run a few key analyses only (PCoA, PCA, UPGMA and NJ phylogenetics, admixture analyses) to quickly get an impression of population structure
Kmax	6	Maximum number of clusters to be considered in DAPC and LEA admixture analyses
axis_1	1	PCoA/PCA-component to be displayed on the x-axis
axis_2	2	PCoA/PCA-component to be displayed on the y-axis
domirroraxis	c(FALSE,FALSE)	Logical vector indicating whether to mirror first and second axis of PCoA and PCA plots
dapc_ellipse	FALSE	If true, add ellipses to DAPC scatter plots
dapc_ellipse_level	0.5	Area spanned by ellipse
add_legend	TRUE	If TRUE, add legend to output plots
pop_order	NULL	Vector to specify order of populations in admixture plots (other than the default alphabetical and geographical order). For example: if your populations are named 'America', 'Asia', 'Europe', you can specify an alternative order from left to right by running the command: findstructure(Kmax=6,pop_order=c('Europe','Asia','America').
legend_pos	"bottomright"	String indicating position of legend in ordination plots. Accepted values: bottomright, bottom, bottomleft, right, center, left, topright, top, topleft.
legend_cex	3	Legend size
symbol_size	3	Symbol size
silent	TRUE	Suppress verbose
pop_order	NULL	Order (from left to right) of populations in LEA structure plots
short_pop	NULL	Population labels displayed in LEA structure plots. Length and order should correspond with vector provided to pop_order.
LEAheightfactor	0.75	Setting to change the height of the LEA structure plots
LEAwidthfactor	0.1	Setting to change the width of the LEA structure plots

Details

This function will produce a lot of rubbish on the screen, and in the meantime perform many analyses, including PCoA, PCA, MDS, DAPC, Bayesian Population Assignment tests, and admixture analyses. If the columns longitude and latitude are present in the inds dataframe, it will also produce geographical maps.

Samples will be colored according to the predefined population assignment. If you have defined one population only, all samples will have the same colour. Some of the PCoA, PCA and MDS

plots will show sample numbers. These plots are not meant for publication (i.e small font size), but are for you to figure out which samples clusters together.

Different symbol types based on sample traits

You can optionally create PCoA plots with multiple symbol types based on a certain criterium. For example, to use different symbol types for male and females, first create a txt file with columns named 'name' and 'sex', store this file in the directory with stores your PED and MAP files, and next use the addsample function to insert sex information into the inds dataframe:

addsampleinfo(samplefile="sample_info.txt",filteronly=FALSE)

This will add a column called 'sex' to the inds dataframe.

Next execute:

ape_pcoa(method="hamming",symbolkey="sex",legendpos="topleft",legendcex=2,export="pdf")

Change the size and position of the legend in ordination plots

If the legends are overlapping with the samples, run the findstructure() function again without legends, or with legends positioned at different plot regions:

findstructure(add_legend=FALSE)

findstructure(add_legend=TRUE,legend_pos="topleft")

The legend_pos flag accepts as input 9 different strings: "topleft", "top", "topright", "left", "center", "right", "bottomleft", "bottom", or "bottomright".

You can also edit the size of the legend labels and the the size of the dots:

findstructure(add_legend=TRUE,legend_pos="topleft",legend_cex=1.5,symbol_size=1.5)

Legend_cex and symbol_size are by default set to 2.

Trouble shooting

Depending on the size of your data, the settings of your computer, and on the last time you closed the computer, you might run into the following error:

Creating Nei's genetic distance matrix... Error: cannot allocate vector of size

In that case you could try restarting the computer or omit the nei's D calculations:

findstructure(do_nei=FALSE)

Occasionally, you might run into the following error:

Creating pcoa plot based on nucleotide diversity using Sambar functions and Ape...

Error in array(STATS, dims[perm]) : 'dims' cannot be of length 0

This error occurs when running Adegenet's pcoa function on a matrix on pairwise sequence dissimilarity estimates. I don't know how to solve this error other than to omit this particular analysis by typing:

findstructure(do_pi=FALSE)

Trouble shooting

Occasionally LEA might throw the error:

Error in snmf("LEAinput.geno", K = ndemes, alpha = 100, project = "new") :

internal error in trio library

Simply rerunning it (multiple times) might solve the problem, or else try lower the K-value:

findstructure(onlyLEA=TRUE,Kmax=5)

Reassign samples to populations and rerun analyses

Based on the output of the findstructure()-function, you might want to reassign your individuals to populations, and redo some of the calculations you did before (like minor allele frequency and heterozygosity per population). This is true if you didn't have a priori expectations about population stratification, but it might also be the case if your a priori expectations turned out to be incorrect.

A quick way to reassign samples is to create a (new) samplefile as explained elsewhere in this manual and rerun the importdata() and filterdata() function. Or try function replacepop():
replacepop(samplefile="filename.txt")

You might occasionally find that a few individuals stand out, and that all other individuals are clumped together. If so, you could try to rerun the findstructure() function, this time excluding the individuals which stand out. To do for example the analysis again without individuals numbered 6, 20, and 43, type first:
inds$filter[c(6,20,43),] <- FALSE

Change the size of the piecharts in the geographical maps

If you want to change the size of the piecharts in the geographical maps, you can recreate these plots by running the function:
create_sambarmaps(K_max=6,radius_ratio=50)
The radius_ratio flag determines the size of the piecharts. It defines the radius of the piecharts relative to either the longitude or latitude range (smallest of either). For example: if your longitude values range from 10 to 20, your latitude values from 25 to 30, and if radius_ratio is set to 50, the piecharts will be (30-25)/50 = 0.1 latitudes.

ADDITIONAL ANALYSIS: Plot Admixture output (or any other structure-like output)

On Linux install Admixture in any directory you like:

wget http://dalexander.github.io/admixture/binaries/admixture_linux-1.3.0.tar.gz

tar –zxvf admixture_linux-1.3.0.tar.gz

Run in R the function:

exportsambarfiles()

This will create several files in SambaR's inputfiles directory, including files called 'metapop.retainedinds.filter.number.ped' and 'metapop.retainedinds.filter.number.map'. Open the map file in Excel and replace in the first column the contig names with integers, otherwise Admixture will throw the error: 'Invalid chromosome code! Use integers.'

Use plink to convert the ped file into a bed file:

plink --file metapop.retainedinds.filter.number --allow-extra-chr --make-bed --recode A --out metapop.retainedinds.filter.number

Copy the bed, bim and fam file into the newly created 'admixture-1.3.0' folder, and execute (using 4 threads, as defined by 'j4'):

./admixture –cv metapop.retainedinds.filter.number.bed 2 –j4

You can run the software several times, with different numbers of K. For example, if you want to run to software for K=3 instead of K=2, type:

./admixture –cv metapop.retaindinds.filter.number.ped 3 –j4

All structure like analyses output a matrix with number of columns equalling K and rowsums equalling 1. In the case of Admixture this matrix is stored within a file which has the extension Q. Save the matrices in separates files in SambaR's inputfiles directory, ending on the suffix 'Kn.qmatrix.txt', with *n* indicating the value of K (i.e. number of columns). For example, if you did the analyses for K2, K3 and K4, you should have three files, ending on the names 'K2.qmatrix.txt', 'K3.qmatrix.txt' and 'K4.qmatrix.txt'. Get rid of headers and of the column with sample names, but you need to make sure that the rows are ordered on sample name (as in the inds$name column), which is the case for the 'metapop.retainedinds.filter.number.ped' file.

To plot all matrices together in one plot, execute:

plotstructure(export="pdf",addindnr=TRUE,order_on_longitude=FALSE)

7.2 Population differentiation

Now that you have identified the populations within your dataset, the next step is to calculate the genetic distances between those populations. Which populations are more similar, and which are more divergent?
To find out, you can run the calcdistance function.

Usage

calcdistance(nchroms=NULL)

Function arguments

flag	default	description
nchroms	NULL	Number of chromosomes of the reference genome assembly (optional). If a value is provided, SambaR will generate karyotype plots showing sliding window genetic distance measures.
max_npop_stats	6	Maximum number of populations accepted for ABBA-BABA analyses.
silent	TRUE	Suppress verbose

Function output

This function will calculate several genetic distance measures (i.e. Fst and Nei's genetic D) for all pairwise population comparisons. I will also calculate D-statistics. Note: it only makes sense to run this command if you have defined more than 1 population. If all samples are assigned to the same population, Sambar will not perform any calculations.

7.3 Genetic diversity

You might also want to know how much genetic variation each population contains.

To find out, you can execute the calcdiversity function.

Usage

calcdiversity(nrsites=NULL)

Function arguments

flag	default	description
legend_cex	2.5	Legend size
nrsites	NULL	Combined length (in bp) of the sequences from which your SNP dataset is obtained, including the sequences which did not contain SNPs. If a value is provided to the nrsites flag, the function will calculate estimates of genome wide heterozygosity, nucleotide diversity and Watterson's theta and Tajima's D. For more info, see the section '10.3 SambaR calculations'. How to obtain an estimate of nrsites? If the SNP data has been generated using whole genome resequencing, an estimate of nrsites can be obtained by generating a (thinned and quality filtered) vcf file which contains both polymorphic and monomorphic sites prior to SNP calling (see box 'How to obtain an estimate of nrsites'). If the SNP data has been generated with the software STACKS, an estimate of nrsites can in principle be obtained from the populations.log file. The end of this file contains a line stating: 'Kept ……. loci out,'. (For older versions of STACKS, the line reads: 'Of these, ……. loci/stacks passed the filters.) Multiplying this number with the length (in basepair) of the trimmed reads (e.g. 110 bp), returns a rough estimate of the number of retained sequenced sites (in bp). However, due to the downstream SNP filtering, this estimate will be an overestimate, and I do not know of a suitable way to correct for it. Within mammals, genome wide heterozygosity per individual generally ranges between 0.05 and 0.5% (i.e. 0.0005 < He < 0.005, or 0.5 to 5 heterozygous sites per 1000 bp, see supplementary table in Bruniche-Olsen et al, 2018, *Runs of homozygosity have utility in mammalian conservation and evolutionary studies*). The value that you choose should likely return He-estimates which are within this range. If you are lucky, more precise estimates of genome wide heterozygosity for your particular study species can be obtained from the literature, and used for comparison.
silent	TRUE	Suppress verbose

Function output

This function generates SFS vectors (using both imputed and non-imputed datasets), as well as estimates of heterozygosity, the number of segregating sites, Watterson's theta and counts of private alleles. See section 10.3 for more detailed explanations.

How to obtain an estimate of 'nrsites'

To obtain an estimate of nrsites, there are two things to keep in mind when calling SNPs:

1. Make sure that the output file (e.g. vcf file) contains both polymorphic and monomorphic sites, rather than monomorphic sites only. If you use for example the bcftools mpileup/call pipeline, do NOT include the -v option when running the bcftools call command, because otherwise monomorphic sites are not included in the VCF output file.
2. Wait with extracting (biallelic) SNPs until you finished filtering the vcf file. This way you can easily count the total number of sequenced and retained sites, which is the number of sites prior to extracting biallelic SNPs.

Here are some rough instructions for using the bcftools mpileup/call pipeline:

STEP 1. Calculate the genotype score for each position (monomorphic and polymorphic):
bcftools mpileup -A -ugf reference.fa -b bamfiles.txt > allsites.bcf

bcftools call -m allsites.bcf | bcftools norm --check-ref w --fasta-ref reference.fa -O z > allsites.vcf

Note: don't include the -v option, otherwise monomorphic sites won't be included.

STEP 2. Filter the data:
bcftools view -O z --include 'DP>=8' allsites.vcf > allsites.mindepth10.vcf.gz

vcftools --vcf allsites.mindepth8.vcf.gz --remove-indels --minQ 30 --max-missing 0.95 --recode --recode-INFO-all --out allsites.filtered &

STEP 3. Extract biallelic SNPs:
vcftools --vcf allsites.filtered.recode.vcf --min-alleles 2 --max-alleles 2 --recode --recode-INFO-all --out SNPsonly.filtered &

The estimate of nrsites, the total number of sequenced sites in the retained dataset (from which the SNPs are extracted), is simply:
grep -v '#' allsites.filtered.recode.vcf | wc -l

ADDITIONAL ANALYSIS: Linkage disequilibrium

SambaR contains a function to plot LD output generated by the software PLINK. For that purpose, you will find in the inputfiles directory (if you executed the exportsambarfiles() function), PED and MAP files ending on 'filter2.number.map' and 'filter2.number.ped'. (Note: for this analysis you want to use 'filter2', not 'filter'.

To calculate linkage disequilibrium, navigate on the command line of your computer (so not in R) to the Sambar_output directory, and execute for each population the following command:
plink --noweb --cow --allow-extra-chr --file yourpop.filter2.number --r2 --ld-window-kb 250 --ld-window-r2 0 –out yourpop
Be sure to replace 'yourpop' with the name of one of your populations, used by Sambar. For example: if your file is called Busen, it becomes 'Busen.filter2.number'.

This command will create three files, ending on 'ld', 'log', and 'nosex'. The ld file will contain LD values for each pairwise combination of snps occuring on the same contig or chromosome within a distance of 1.0 Mb. To plot these estimates, execute on the R command line:
LD_plot(export="pdf", xrange=c(0,1000000),stepsize=100000,addstripchart=FALSE, plotmeans=TRUE)

This function outputs several files to the Diversity directory, including a file called 'LD.boxplot.100K.pdf'. This file shows LD values for each of your populations. You can export the plot in different formats by setting the export flag to 'eps', 'png', or 'wmf'. If wanted you can edit the range and the stepsize.

If your reference genome is assembled up to chromosomes (rather than contigs or scaffolds), you can also execute:
LDperchrom()
This function will export a table with mean LD estimates per chromosome.

7.4 Kinship analyses

SambaR allows to evaluate inbreeding and relatedness statistics using the calckinship function.

Usage

calckinship()

Function arguments

flag	default	description
calcF	TRUE	Logical indicating whether to execute inbreeding calculations.
F_correct_maf	TRUE	Logical indicating whether to calculate inbreeding coefficient using a corrected minor allele frequency (i.e., a minor allele frequency which is independent of the sample under consideration).
overwrite_kindf	TRUE	Logical indicating whether kinship relatedness should be repeated if previous results are still stored.
silent	TRUE	Suppress verbose

Function output

Output figures and tables will be exported to the newly created directory 'Kinship'. For better understanding of the relatedness calculations, consult the chapter 'SambaR calculations' in this manual.

Additional analyses

SambaR also provides users the opportunity to plot the output of PLINK and GCTA relatedness calculations (see boxes with additional analyses below).

ADDITIONAL ANALYSIS: Plot PLINK relatedness (kinship) estimates

SambaR has a built-in function to estimate relatedness, but a more sophisticated method to calculated relatedness is implemented in PLINK. SambaR contains a function to plot sample relatedness measures (pi_hat scores) generated by PLINK. For that purpose, you will find, if you have run the exportsambarfiles() function, within the inputfiles directory files called 'metapop.filter.number.ped', 'metapop.filter.number.map', 'metapop.allinds.filter.number.ped' and 'metapop.allinds.filter.number.map'.

Navigate on the command line of your computer to this directory and execute these plink commands:

plink --file metapop.allinds.filter.number –cow –-allow-extra-chr –genome -out plink.kin.allinds

plink --file metapop.filter.number –cow –-allow-extra-chr –genome -out plink.kin.retainedinds

This will output to the SambaR input files directory a file called 'plink.kin.allinds.genome' and 'plink.kin.retainedinds.genome'. In R, execute:

plinkrelatedness(export="pdf")

This function will export several plots to the Kinship subdirectory:

- Relatedness.plink.between.pdf
- Relatedness.plink.within.pdf
- Relatedness.plink.persample.pdf
- Relatedness.plink.matrix.pdf (with and without axes with sample numbers. The plots without axes have the legend included.)

ADDITIONAL ANALYSIS: Plot GCTA relatedness (kinship) estimates

SambaR contains a function to plot sample relatedness measures generated by GCTA.

Store the output of GTCA in SambaR's inputfiles directory. Needed are 3 files, all with the same name, except for their extensions 'grm.bin', 'grm.id', and 'grm.N.id'.

In R, execute:

do_gctamatrix(export="pdf",gctaprefix="yourprefix")

This function will export heatmaps to the Kinship subdirectory. It expects to find in the input files all individuals have been included in the analysis, also the samples which didn't meet SambaR's filter settings.

ADDITIONAL ANALYSIS: Plot PLINK inbreeding estimates

SambaR contains a function to plot inbreeding statistics generated by PLINK. For that purpose, you will find, if you have run the exportsambarfiles() function, within the inputfiles directory files called 'metapop.filter.number.ped', 'metapop.filter.number.map', 'metapop.allinds.filter.number.ped' and 'metapop.allinds.filter.number.map'.

Navigate on the command line of your computer to this directory and execute this plink command:

plink --file metapop.filter2.miss0.letter –-allow-extra-chr --het

In R, execute:

plotplinkF(export="pdf",addlabels=TRUE)

7.5 Selection analyses

You might be interested in the question if and which loci are under selection. To find out, the selectionanalyses function runs up to 4 selection scans: Fsthet, GWDS, OutFlank, and PCadapt.

Usage

selectionanalyses(do_meta=TRUE,do_pairwise=FALSE,do_pheno=FALSE,export='pdf')

Function arguments

flag	default	Description
do_meta	TRUE	If TRUE, the function will run selection analyses for the metapopulation, considering all populations simultaneously. This option will exclude the selection scan GWDS, as GWDS works for pairwise comparisons only.
do_pairwise	FALSE	If TRUE, the function will run selection analyses for all pairwise population comparisons. Depending on the number of populations, this can result in a high number of analyses.
do_pheno	FALSE	If TRUE, the function will run selection analyses for population pairs which are assigned to different groups (as defined by the inds$type column). The division can be based on either a phenotypic trait or on geographical occurrence. Populations can be split in two groups by adding to the inds dataframe a column called 'inds$type' containing TRUE/FALSE values. For example: if your data contains two marine populations (pop1 and pop2) and two freshwater populations (pop3 and pop4), a division between freshwater and marine populations can be specified with the command: *inds$type<-ifelse(inds$pop=="pop1"|inds$pop=="pop2",TRUE,FALSE)*.
onlypooled	TRUE	If TRUE, the function will run selection analyses for a pooled population comparison (as defined by inds$type column) and not for all pairwise populations comparisons between both groups. To be used in conjunction with do_pheno flag.
export	NULL	File type of output plots. Accepted values are 'eps','pdf','png', and 'wmf').
export_data	FALSE	If TRUE, PED and MAP files will be exported which contain either neutral SNPs (marked by none of the selected outlier scans) or outlier SNPs (marked by at least one of the selected outlier scans).
overwriteped	TRUE	If FALSE, the function will not generate new PED and MAP files. This can save time if rerunning the function, but can lead to errors if run for the first time or if run with different filter settings.
do_pcadapt	TRUE	If TRUE, the function will run the software PCadapt.
do_outflank	TRUE	If TRUE, the function will run the software OutFLANK. OutFLANK throws errors at times, which can only be circumvented by omitting OutFLANK from the analyses by setting this flag to FALSE.
do_fsthet	FALSE	If TRUE, the function will run the software FstHet. The selection scan 'Fsthet' takes a relatively long time to run (like 30 minutes for a dataset of 60 individuals and 50,000 loci), and is therefore excluded from the analyses by default. If TRUE, the function expects to find a genepop file of the original input data in the input data directory (i.e. same directory as where RAW/BIM files are stored.)
add_bayescan	FALSE	If TRUE, the function expects to find Bayescan output files (originally ending on 'baye_fst.txt' or 'g__fst.txt' but renamed to 'pop1_pop2.bayescanout.fst' or 'pheno.bayescanout.fst') in the SambaR inputfiles directory, and will include the information in these files to the output plots and tables.
bayescan_FDR	0.01	Bayescan false discovery rate
overwrite_ bayescan	FALSE	If TRUE, and if the function has been run before, data in snps dataframe will be overwritten.
pheno_labels	c('pheno1', 'pheno2')	Optional vector (consisting of two elements) specifying the group division (e.g. c('marine','freshwater'). To be used in conjuction with the do_pheno flag. Labels will be included in the output plots.
my_correction	'bonferroni'	String which specifies the multiple test correction method. Values can be NULL (default), 'bonferroni', 'holm', and 'BH', the latter denoting Benjamini-Hochberg. If NULL, GWDS will be run using the Bonferroni correction, PCadapt will be run using the Holm correction holm, and OutFLANK will be run using q-values.
do_thin	FALSE	The do_thin flag applies to the GWDS test, and indicates whether the neutral distribution should be inferred from a thinned dataset. If set to TRUE, the GWDS scan will use one SNP per genomic region to infer the neutral distribution. The size of these genomic regions can be adjusted with the gwdsbinsize flag (default is 1000000 bp). The 'do_thin' option should be set to TRUE only if working with dense SNP datasets. When setting the flag 'do_thin' to TRUE for datasets with relatively low number of SNPs (e.g. RADseq SNP datasets), GWDS will return many false positives.
gwdsbinsize	1000000	Thinning parameter applied by GWDS selection scan (see do_thin flag).
silent	TRUE	Suppress verbose

Example

To run selection analyses for the metapopulation:

selectionanalyses(do_meta=TRUE ,export="pdf",do_fsthet=FALSE,my_correction=NULL)

To run selection analyses for all pairwise population comparisons:

selectionanalyses(do_meta=FALSE,do_pairwise=TRUE,export="pdf" do_fsthet=FALSE)

To run selection analyses for a pooled comparison between a predefined population division:

selectionanalyses(do_meta=FALSE,do_pheno=TRUE,onlypooled=TRUE,export="pdf",phenolabels=c("high","low"), do_thin=FALSE)

Function output

The function will create Manhattan plots, Venn diagrams, Fdist plots (He-Fst plot) and piecharts with population specific minor allele frequencies of outlier SNPs.

Identified outliers are potentially under either positive/diversifying selection or balancing selection. (GWDS is the exception, as it can detect diversifying selection only). You can infer the type of selection from the piecharts and the WC_Fst.outliers plots. Note that outliers are just that: outliers. They are either loci under selection or, not unlikely, false positives. Have a look at the allele frequencies of the outlier SNPs and consider whether the allele frequencies are similar or dissimilar enough between/among populations (compared to the differences in other SNPs) to justify the inference of selection. See the section 'Simulate your data' within this manual for a potential way to obtain power and specificity estimates of the selection scans GWDS, PCadapt and OutFLANK in the context of your study system.

GWDS

SambaR's GWDS (Genome Wide Differentiation Scan) implements the Fisher exact fisher test to search for correlations between (groups of) populations and their minor allele frequencies (using R's build in fisher.test function). Afterwards it fits an exponential curve to the histogram of obtained p-values, assuming the distribution is exponential (using R's build in rexp function). Using this exponential curve, a Bonferroni corrected threshold value is deduced (using R's build in qexp function).

SambaR outputs Manhattan plots of the negative log of the p values based on Fisher exact tests. It will also add three columns to the snps dataframe: snps$rfisherp, and snps$rfisherlogp, and snps$rfisherout. SambaR will also output histograms of the p values, and the fitted curve. Users should check if the fit is good. If not, the outcome of the GWDS test might be unreliable.

Trouble shooting

You might run into the one of the following errors:

Calculating FSTs, may take a few minutes...
Error in if (s2 == 0) { : missing value where TRUE/FALSE needed

All loci with Fst above the upper (righthand) trim point were marked as outliers. Re-run with smaller RightTrimFraction or smaller qthreshold.
Error: $ operator is invalid for atomic vectors

Error in quantile.default(pi0, prob = 0.1) :
missing values and NaN's not allowed if 'na.rm' is FALSE

Error in optim(NumberOfSamples, localNLLAllData, lower = 2, method = "L-BFGS-B") :
L-BFGS-B needs finite values of 'fn'

Error in Sample_Mat[, 1] : subscript out of bounds

These errors are all related to OutFlank and unfortunately I don't know how to fix them. The only solution I can offer at the moment is to exclude OutFlank from the analyses. For example:
selectionanalyses(do_meta=TRUE, do_outflank=FALSE)
selectionanalyses(do_meta=FALSE,do_pairwise=TRUE, do_outflank=FALSE)

ADDITIONAL ANALYSIS: Bayescan

The selection scan Bayescan runs on the Unix command line, can therefore not be invoked by SambaR. However, SambaR does accept Bayescan output. To create input files for Bayescan, first create the inds$type column as explained above, and then run:
createbayescaninput(allpairwise=FALSE)

To execute PGDspider and Bayescan, you first have to convert the PED and MAP files to Bayescan input format (called 'geste'). To do so, execute on the command line (assuming java have and PGDSpider have been installed):
java -jar /path/to/PGDSpider2-cli.jar -inputfile pheno.filter2.letter.ped –outputfile pheno.filter2.geste -spid /path/to/PED2Bayescan.spid &

The PED2Bayescan.spid file is included in your SambaR download.
If you receive the message 'Please specify an input file', then PGDSpider is not able to find your input file, maybe because you misspelled it or did not provide the right path.
No need to worry if you receive the error message 'simdata.map not found!'.

Now you are ready to run Bayescan. First create an output directory:
mkdir bayescan_pheno
Then run Bayescan:
/path/to/bayescan_2.1 pheno.filter2.geste -od bayescan_pheno -threads 10 &
If Bayescan can not find the output directory, it will give the somewhat confusing error 'Could not open file'.

To subsequently incorporate Bayescan output in Sambar plots, transfer bayescan output files ending on either 'baye_fst.txt' or 'g__fst.txt' to the SambaR inputfiles directory, rename them with 'pop1_pop2.bayescanout.fst' (or 'pheno.bayescanout.fst'), and execute in R:
selectionanalyses(do_meta=FALSE,do_pheno=TRUE,add_bayescan=TRUE)

ADDITIONAL ANALYSIS: Sliding window Tajima's D

wintajd(my_chrom=25,winsize=1000000,winstep=200000)
This function will export (in pdf format) graphs with sliding window Tajima's D estimates. It will also produce a dataframe called wintaj.

ADDITIONAL ANALYSIS: Find and plot genes close to outlier SNPs

If you used reference mapping rather than denovo mapping, Sambar will have exported to the selection directory txt.files in BED format. These files contain lists with outlier SNPs, and can be used to find nearby genes using the software BEDTOOLS2 . To do so, you need the have the annotation file (in gff format) of the genome which you used as reference.

On Unix command line execute the following command to convert this annotation file from gff format to BED format:

cut -f1,4,5,9 reindeer_coding_gene_annotations.gff > genes.bed

Sort both BED files:

bedtools2/bin/sortBed -i reindeergenes.bed > genes.sorted.bed

bedtools2/bin/sortBed -i outliers.bed > outliers.sorted.bed

To find the 10 closest features (including overlapping features), and to report the distance between SNP and feature (-D a flag), execute:

bedtools2/bin/closestBed -a outliers.sorted.bed -b genes.sorted.bed -D a -k 10 > putative.txt

To subsequently select features which are within 200kb distance from an outlier SNP:

awk '$9 <= 200000' putative.txt | cut -f1-8 | uniq -f7 | cut -f1,6,7,8 > putative. 200kb.bed

It might be that the gene names (4th column of BED file) are uninformative, and that you have to perform a blast with the gene sequence to find the actual gene name.

To plot the positions of the outlier SNPs and the adjacent genes, copypaste the BED-file to the selection subdirectory and execute:

multiplotscaffold(my_bed="putativegenes.within200kb.genenames.bed",background_pop=NULL,doexport=TRUE,x_range=NULL,y_loc=c(0.4,0.65))

This will export pdf files of all contigs/chromosomes containing outlier SNPs to the selection subdirectory. With the background_pop flag you can determine which population is shown on the background. With the x_range flag you can determine the region of the contig/chromosome you want to display. With the y_loc flag you can edit the locations of the gene names.

7.6 Association analyses

The selectionanalyses() function allows you to detect SNPs which have allele frequencies which differ strongly between your populations. Alternatively, you might want to find out if your dataset contains SNPs which have allele frequencies which differ strongly between individuals within populations, for example between males and females, or between healthy and affected individuals. This type of analysis is known as genome wide association scan (GWAS).

To do GWAS with SambaR, first use the addsampleinfo() function to add additional sample specific information to the inds dataframe.
addsampleinfo(samplefile="sample_info.txt",filteronly=TRUE)

Assuming the command above added a column called 'sex' to the inds dataframe, next execute:
assocfisher(pheno="sex",export="pdf",mylabels=c("male","female"))

This will run GWDS (as explained in the previous section) and output to the selection directory a 'WC_Fst.outliers.pdf' file which highlights outlier SNPs (if present) in blue.

ADDITIONAL ANALYSIS: RDA
Create an input file with geographical coordinates, sample names (called 'individual'), and environmental variables, and store this txt file (for example under the name "evofile.txt") in the directory which also contains the PED and MAP input files. Next execute:
runRDA(export="pdf",legendpos="topleft",envfile="evofile.txt",doall=FALSE)

7.7 Population demography (ADDITIONAL ANALYSIS)

NOTE: comparisons have revealed inconsistencies between SFS-vectors generated by SambaR, and SFS-vectors generated by ANGSD, especially regarding the counts of common alleles. This will affect the outcome of Stairwayplot analyses. If these analyses are a key part of your study, you are advised to create SFS vectors using ANGSD (see below for further instructions).

Genetic diversity estimates can be used to estimate current and historic effective population sizes. One program you can use to do so is the Stairway_plot executable (https://sites.google.com/site/jpopgen/stairway-plot). To get this program up and running simply download the latest version and unzip the file. Hamilton users can copy the program from: */ddn/data/fjsq43/Programs/Populationdemography/stairwayplot/stairway_plot_v2*

You don't need your genotype data to run stairway_plot analyses. The program takes all the information it needs from the site frequency spectrum (SFS) vector, which SambaR generated when executing the calcdiversity() function. You need to input the information in this vector into an input file, as will be explained below.

It is for the user to decide which SFS vector (among the several generated by the calcdiversity() function), is most suitable for these analyses.

Instructions

If you navigate within the folder 'stairway_plot_v2' you will find two files called 'two-epoch.blueprint' and 'two-epoch_fold.blueprint'. Since you created a folded SFS vector (i.e. a 'minor allele frequency SFS' rather than a 'derived SFS', because you do not know whether an allele is ancestral or derived), you have to use the second one.

For each of your populations, copy, edit and save the 'two-epoch_fold.blueprint' file. Say for now you save it under the name 'mypop.blueprint'. The lines you have to edit are indicated in bold below.

For L (total number of sequenced sites) you could use the same number as the input to the nrsites argument for the calcdiversity() function. (For more information, see section 7.3 of this manual.) However, this only makes sense if you input the SFS vector which is based on all SNPs, also the SNPs which did not pass the filter settings. If, instead, you prefer to work with the SFS

vector which is based on retained SNPs (rather than on all SNPs), you would have to think yourself of a way to make a justifiable correction of the total number of sites.

On the SFS line simply copy paste the line in the file SFSvector.yourpop.txt ('Diversity' subdirectory).

```
#example blueprint file
#input setting
popid: yourpop                  # id of the population (no white space)
nseq: 42                        # number of sequences (length of SFSvector x 2)
L: 9350000                      # total number of sites
whether_folded: true            # whether the SFS is folded (true or false)
SFS: 1946 1032 951 901 etc      # SFS vector, generated with calcdiversity() function
smallest_size_of_SFS_bin_used_for_estimation: 1          # default is 1; to ignore singletons, change to 2
largest_size_of_SFS_bin_used_for_estimation: 21          # default is nseq/2 for folded SFS
pct_training: 0.67              # percentage of sites for training
nrand: 10 20 30 40              # (nseq-2)/4, (nseq-2)/2, (nseq-2)*3/4, nseq-2, space separated
project_dir: yourpop            # project directory name
stairway_plot_dir: stairway_plot_es # directory to the stairway plot files
ninput: 200                     # number of input files to be created for each estimation
#output setting
mu: 2.5e-8                      # assumed mutation rate per site per generation
year_per_generation: 20         # assumed generation time (in years)
#plot setting
plot_title: yourpop             # title of the plot
xrange: 0.1,10000               # Time (1k year) range; format: xmin,xmax; "0,0" for default
yrange: 0,0                     # Ne (1k individual) range; format: xmin,xmax; "0,0" for default
xspacing: 2                     # X axis spacing
yspacing: 2                     # Y axis spacing
fontsize: 12                    # Font size
```

Now you are ready to run the analyses. The following instructions are for Linux command line.
First make sure Java is added to the environment (Hamilton users, type: *module add java*).
To run the program all you have to do is to execute for all your populations two commands.
First execute:

java -cp stairway_plot_es Stairbuilder mypop.blueprint &

This command will create within seconds new directories and a bash script.
Next execute the bash script:

bash mypop.blueprint.sh & *# note: don't forget the extension (.sh)*

This command will initiate the actual calculation, which takes typically several hours or days to complete, depending on the total number of sites. It prints zero's on the screen whilst running.

Plot results

The Stairway_plot executable automatically generates within the output directory plots (both in png and pdf format) which can be readibly viewed. To generated a multitile plot with shows the output of all populations combined, with colour settings which agree with the other plots

generated by the SambaR-package, SambaR comes with a function which recreates the plots outputted by the Stairway_plot executable.

To make use of this function, transfer the files ending on '.final.summary' to the Demography directory. You should have as many of those files as populations (listed in the populations vector), and the prefices of the files should correspond with the names listed in the populations vector. For example, if according to your populations vector you have three populations, called 'Busen', 'Barff' and 'Norway', SambaR expects to find within the Demography directory three files, called 'Busen.final.summary', 'Barff.final.summary' and 'Norway.final.summary'.

Now execute the function:
*run_plotstairway(mu_rate="2.5*10^-8",Gtime="20",x_range=c(100,17500),exporttype="pdf")*
Set u_rate and gen_time to the correct values (the values used whilst generating the stairwayplot output files). This information is used for labelling the plot. Set x_range for values greater than 0. The values for x_range should be in years (i.e. not in ky or My).

This function will export multiple files called 'stairwayplot.pdf' to the 'Demography' subdirectory. The plot can also be exported in eps format by setting the export flag to eps.

ADDITIONAL ANALYSIS: Generating SFS vectors with ANGSD instead of with SambaR

Another way to acquire SFS vectors is by using the software ANGSD. You can generate this SFS vector, starting from your bam files, in just two commands. For each of your populations run the following command:

/path/to/angsd/angsd –bam listofbamfiles.txt –doSaf 1 –anc /path/to/referencegenome.fa –GL 1 –out yourpopname –fold 1 –minMapQ 20 –minInd 20 –nThreads 4 &

Afterwards run:

/path/to/angsd/misc/realSFS yourpop.saf.idx -p 4 > yourpop.sfs

Note that, in contrast to the SFS vectors generated by SambaR, the SFS vector of ANGSD includes the number of non-segregating sites. Therefore you will notice that the first value of the SFS vector is much higher than the other values. You will also notice that the length of the vector is the number of individuals (as defined in the listofbamfiles.txt) plus 1.

When preparing the blueprint file of the Stairway plot analyses, the L value should be the sum of the SFS vector. On the line of the SFS vector, you should insert the SFS vector except without the first value. L is the sum of the total vector, including the first number.

Because ANGSD uses a different method to estimate the SFS vector (a likelihood method), the value might not be rounded. To make it more realistic, you might want to round the values.

ADDITIONAL ANALYSIS: Circosplot with Bayesass migration rates

SambaR contains functions to create input files for Bayesass3-SNPs, and to subsequently create a circos plot of the migration rates calculated by the software Bayesass3-SNPs. Execute:
exportsambarfiles()
Afterwards, you wil find in the inputfiles directory a file called 'Bayesassinput.immanc.txt'.

Download Bayesass (from: https://github.com/stevemussmann/BayesAss3-SNPs), unzip on Unix command line (type: unzip BayesAss3-SNPs-master.zip), copy the Bayesassinput.immanc.txt file to the newly created Bayesass directory, and execute:
BA3-SNPS-Ubuntu64 --file Bayesassinput.immanc.txt --loci 15000 -u -t -s 10 -i 1000000 -b 100000
This will run Bayesass with the defaults settings of 1 million iterations, a burn-in of 100000 generations, a seed of 10, and with delta (A, F and M) values of 0.10000000000000001, and for 15000 loci. Set the number of loci equal to:
nrow(snps[snps$filter,])

After completion of the run, which can take hours, you will find a file called BA3out.txt. In it you will find a matrix (after the line Migration Rates) which looks similar to:
m[0][0]: 0.8973(0.0348) m[0][1]: 0.0066(0.0028) m[0][2]: 0.0554(0.0209)
m[1][0]: 0.0138(0.0086) m[1][1]: 0.8262(0.0610) m[1][2]: 0.1110(0.0575)
m[2][0]: 0.0016(0.0012) m[2][1]: 0.0043(0.0031) m[2][2]: 0.9895(0.0036)
(If you can't find it, type on command line: grep -A10 -B4 'Migration Rates' BA3out.txt)
This matrix shows proportion of migrants per generation (proportion of population size) – the direction being from column to row (e.g: 0.0514 migrants from pop2 to pop1). Copy paste the matrix into a new file in SambaR's inputfiles directory (e.g. name it 'bayesassmatrix.txt'). Add to the beginning of each row the name of the specific population, followed by a space or tab.

Now, to visualize migration rates in a circos plot execute the following SambaR function:
plotmigration("bayesassmatrix.txt",export="pdf",addlabels=TRUE)
The circosplot will be exported to the Divergence subdirectory.

Potential errors

If you input file doesn't end with a white line, you might get the error:
In read.table(myinputmatrix, stringsAsFactors = FALSE) :
incomplete final line found by readTableHeader on ''
If so, add a white line to the end of input file.

ADDITIONAL ANALYSIS: TreeMix plots

To generate the required input format for the software TreeMix (developed by the Pritchard Lab), run the command:

exporttreemix(snpsfilter=snps$filter,exportname="Treemixinput.snpsfilter.txt")

In a Unix environment, run the software Treemix using the following commands (assuming the outgroup population is called 'OutPop':

gzip treemixinput.snpsfilter.txt

treemix -i Treemixinput.snpsfilter.txt.gz -m 0 -root OutPop -o Treemixout.0

treemix -i Treemixinput.snpsfilter.txt.gz -m 1 -root OutPop -o Treemixout.1

treemix -i Treemixinput.snpsfilter.txt.gz -m 2 -root OutPop -o Treemixout.2

Afterwards transfer all output files to your SambaR working directory, and run in R the commands:

plottreemix(prefix="Treemixout.0",export="pdf",myxmin=0,plotname="Treemixoutput.0")

plottreemix(prefix="Treemixout.1",export="pdf",myxmin=0,plotname="Treemixoutput.1")

plottreemix(prefix="Treemixout.2",export="pdf",myxmin=0,plotname="Treemixoutput.2")

Output plots will be exported to the working directory.

8. How to get the most out of SambaR

Apart from the main wrapper functions described in the previous sections, SambaR contains several functions/utilities which can prove useful for speeding up your analyses. Most of these utilities are described in full detail in sections 8.1 and 8.2. Here I will show an example of a workflow which can help you to get the most out of SambaR and out of your data.

Specify the order of populations

When importing the data, specify the order in which your populations should be listed in output tables and plots:

```
popvector     <- c("East","North","NorthEast","South", "West")
colvector     <- c("darkred","orange","darkorchid4","indianred1","darkgreen")
poporder      <- c("West","North","NorthEast","East","South")
importdata(inputprefix="mydata",colourvector=colvector,pop_order=poporder)
filterdata()
```

Although rather basic, you will find that this option is very useful, and that certain patterns and trends are much better conveyed when your plots and legends show your populations in a certain order (for example geographical order) rather than simply in alphabetical order. This is especially the case for the LEA admixture plots, but also for matrices and heatmaps showing pairwise populations differentiation estimates, and in general for figure legends of any plot.

To redefine the order after importing the data, use the reorderpop function. For example:

```
reorderpop(poporder= c("South","West","North","NorthEast","East"))
```

Run analyses on subsets of individuals/populations

You will find that it can be useful to exclude certain individuals or populations when running a particular analysis. This is especially the case when creating ordination analyses plots, such as PCA and PCoA plots, because it is often impossible to depict all the variation in your data in a single biplot. In many cases, you would have to 'peel the union' to reveal substructures. There are two options to do so. If you would like to remove single individuals, you could simply edit the inds$filter column. For example, to remove the individuals named 'Ind1' and 'Ind8', type:

```
inds$filter     <- inds$filter&inds$name!="Ind1"&inds$name!="Ind8"
```

The second option, which is especially useful (and in fact mandatory) when you want to exclude all individuals belonging to a certain population, is to use the excludepop() function. For

example, if you want to exclude all individuals of populations PopA and PopB from subsequent analyses, type:
excludepop(c("PopA","PopB"))

Now if you run analyses, for example by executing the findstructure(quickrun=TRUE) function, the analysis will only consider the particular subset of individuals that you selected. Make sure to either rename the output plots or move output files into subfolder with a telling name, so you know which individuals/populations are included, and so these plots will not be overwritten when you rerun the analysis for another subset.

To reset the filter setting to the original filter setting – which includes all individuals which passed quality control – simply rerun the excludepop function without specifying any population:
excludepop()

Work with multiple datasets at the same time
To enable users to work with multiple datasets at the same time, SambaR provides the functions 'backupdata' and 'getdata'. Once you finished working with a dataset, make a backup by typing on the command line:
backupdata("datasetA")
Instead of 'datasetA', you can type any name you want, as long as the name does not equal the names of SambaR or other functions. The backupdata function basically renames the mysambar list object to the name that you specified.

Next, you can import a new dataset, as normal. Once you run the importdata and filterdata function, you can also back-up this dataset using the backupdata function, for example:
backupdata("datasetB")

To start working again with the first dataset, all you have to do (instead of importing the data again and rerunning analyses) is to type:
getdata("datasetA")

This command will automatically load the dataset (make this data the 'inds', 'snps', and 'genlight' dataframe), and change the working directory. If you forgot the name under which you stored your dataset, simply type any name you can think of. The function will tell you if such a SambaR list object indeed exists, and if not, which objects are available to choose from.

Understand the SambaR functions

If you have a basic understanding of R commands, and if you have a question about the way SambaR runs a certain function, you could have a look at the SambaR source script.

SambaR consists of main wrapper functions (such as findstructure and selectionanalyses), which call other functions. To better understand the way SambaR executes certain analyses, you could examine which functions are executed by the wrapper functions, and subsequently examine these functions.

Another way to examine a function is to simply type on the command line the name of the function without the rounded brackets. For example:

findstructure

This will not execute the function, but instead print the function on the screen.

If you are interested only in the options/arguments which are available for a particular function, type on the command line:

args(function_name) # e.g.: args(findstructure)

8.1 Manage your data

Export your data

Although SambaR comes with built-in functions for population genetic analyses, it obviously cannot do everything for you. For that reason, the SambaR package contains a function which allows you to export your filtered data in PED and MAP format, which can be used as input for programs which don't run in R.

The function exports the combined dataset as well as all populations separately, meaning you can easily perform calculations on the entire dataset as well as on populations separately.

To export your data to the SambaR directory, execute the following command:
exportdata()

> ***Trouble shooting***
> If the population names listed in the mysambar$populations vector do not correspond with the names in the inds$pop2 column, you will get the error:
> *Error in x@gen[[1]] : subscript out of bounds*

Back up your data

You might find yourself working at multiple projects at the same time. An easy way to switch between datasets is by saving your genlight, inds, snps and population objects in a sambar list object. It might be wise to back up your data anyway. If you make a mistake and somehow delete your data, it allows you to easily retrieve it (see section 'reload your data').

To back up your data, for example under the name 'myproject_1', type:
backupdata("myproject_1")

The data, including the paths to directories and font type vector, is stored in an list object. These types of objects are a bit more difficult to work with than dataframes, but still not that hard. Some commands to observe the backup of your data are:
myproject_1$genlight
summary(myproject_1)

Reload your data

To reload your data to the populations, snps, inds and mygenlight objects (as well as paths to directories and font type vector), type:

getdata("myproject_1")

Be careful! This will overwrite the existing populations, snps, inds and mygenlight objects, so be sure to back up those files first if you haven't do so yet.

Reloading a dataset is obviously only possible if you previously backed up this dataset using the the backupdata()-function. Be sure to use the same prefix as when running the backupdata()-function.

> ***Trouble shooting***
>
> If you forget to add the double quotes whilst trying to run the getdata function, you will get the error:
>
> *Error in get(myprefix, envir = myglobal) : invalid first argument*

Subset your data based on snp names

To obtain a targetted subselection of snps, execute the function:

subselectdata(snp_names=snpnames,name2=TRUE)

In which snpnames should be a vector with names of the snp files as listed in snps$name2 column (i.e.: chr_pos). If you want to search with snps$name column, set name2 to FALSE.

Subset your data based on population names

To subselect individuals from particular populations, execute the function:

subset_pop(include_pops=c("popname1","popname2","etc"))

The include_pops should be a vector with the names of the populations you want to select.

Run this function after the filterdata() function. Don't run the filterdata() function afterwards, because this will partially undo the changes.

To undo the changes, rerun the importdata() function.

Subsample your data randomly

Occasionally you might find that you want to randomly subsample your dataset, for example for to use as a control dataset. To sample your datasets, execute:

subsampledata(nrinds=NULL,nrsnps=NULL,exportprefix=NULL)

in which:

nrinds: how many individuals should subset contain? Should be less or equal to number of rows of inds dataframe.

nrsnps: how many loci should subset contain? Should be less or equal to number of rows of snps dataframe.

exportprefix: if NULL (default), data will be outputted as R objects.
If not NULL, data will be exported as PED and MAP files to SambaR_output directory, and string will be used as prefix.

Output is a sambar list object called 'mysambarsubset'.

Find overlap between datasets

Say you are working with two SNP datasets between two snp datasets generated by different protocols (e.g. different restriction enzymes) but using the same reference genome. You might wonder: do those datasets have SNPs in common? Because if so, you can combined both datasets and execute analyses on the combined dataset.

To find out, and to create the new combined dataset, SambaR comes with a function called findoverlap(). This functions expects two sambar list objects (created with the backupdata function), and if it finds overlapping snps, it outputs a new sambar list object called 'mysambarcombined'. Say that your input sambar list objects are called mydata1 and mydata2, execute the following command:

findoverlap(mydata1,mydata2,mycolours=c("darkgreen","orange","blue","darkred"))

You have to provide the colours of the populations, in alphabetical order of the populations names in the to be combined dataset. Just as a reminder: the default colours used in SambaR plots are, in order of occurence: blue, darkgreen, darkred, orange, purple ('darkorchid4'), brown ('#654321'), yellow, and darkgrey ('gray20').

To start working with the data, type:

getdata("mysambarcombined")

As mentioned above: don't forget the double quotes!

Now you can run the SambaR functions again to do analyses on the combined dataset, starting with the filterdata() function.

8.2 Manage your plots

Change your plot colours

The default colours used in SambaR plots are, in order of occurence: blue, darkgreen, darkred, orange, purple ('darkorchid4'), brown ('#654321'), yellow, and darkgrey ('gray20').
To use different (or more) colours in your plots, you have to assign new colours to your populations in the inds$popcol column. You can do so with the addcol()-function. This function expects as input a vector with colour names or codes. The length of this vector should equal the length of the populations vector.

If for example you have two populations and you want the first population to be shown in red and the second in blue, you should type:
editcol(c("red","blue"))
Execution of this function will update the inds$popcol column, and as such affect all plots created subsequently.

There are many online tools available which are helpful to select colour combinations. One example is colorbrewer2.org.

Change your plot font type

The default font type used by SambaR is R's default font type, which is Helvetica (called 'sans'), a sans serif font accepted by most journals. If you want or need to use another font, you might have to install this font first on your computer. This is for example true for Arial. To install all available fonts, run the following function:
getfonts(importfonts=TRUE)

This function uses the extrafont package to import and load font types. Once you have imported font types onto your computer (which can take up to 10 minutes on my computer), they will be stored safely until you shut down your computer. Every time you start a new R session, you do have to load the fonts though. This takes less than a second.
If you only want to load the fonts – without importing them – run the getfonts() function by setting the importfonts flag to FALSE (default value):
getfonts(importfonts=FALSE)

To subsequently set your desired font type, for example 'serif', type on the command line:
mysambar$myfont <- "serif"

All the functions you subsequently run, will generate plots with 'serif' as font type.

Note that many font types (especially the ones you imported) will only show up in the .pdf and .eps format. When trying to use these font types for the formats .png and .wmf you will get this non fatal warning:
font family not found in Windows font database
If so, the font type will default to Helvetica and the plots will still be generated.

Also note that SambaR currently does not embed the font types, meaning that if you send the plot to someone who has not installed the font type on his or her computer, it will not display properly. Journals will have the font types installed though, so this should not cause major problems.

Importing pdf into Word file

You can insert any pdf object into a word file by choosing 'Adobe Acrobat Document' under the option 'Object' in the 'Insert' tab (within Word). When you do so, make sure that the selected document is not opened in a PDF viewer, otherwise Word will choke. (If you happen to do so by mistake, (force) shutdown Word and try again after reopening the document.)
When inserting the file into word, the file might automatically be opened in a PDF viewer. If so, diagonal black lines will appear on the plot inserted in the Word document. The lines will disappear if you close the file in the PDF-viewer.

How to combine SambaR output plots into high quality multi-tile plots

One option to import, edit and combine pdf files is the free software 'Inkscape'. PDF-files can be imported into Inkscape by dragging and dropping. To export the combined figure, select file -> Document Properties, and in the tab 'Page' select 'Resize page to drawing or selection' (it is recommended first set the left, top, right and bottom margins to another value than 0). The figure can now be exported by saving as a svg file. If desired, an online pdf converted can be used to convert the svg file to pdf format.

9. Understand your output

This section contains descriptions of the columns of the snps (10.1) and inds (10.2) dataframe, and describes some of the calculations underlying SambaR output plots (10.3).

9.1 The snps dataframe

Your snps dataframe could contain the following columns, depending on the functions you run and which options you used:

After running importdata() function:

chr: name of contig/chromosome/scaffold on which SNP was found (if no information in PED-file, 0)

name: SNP name

morgan: position in cM (if no information in PED-file, 0)

pos: position in bp (if no information in PED-file, 0)

minor: identity of minor allele (1, 2, 3, or 4)

major: identity of major allele (1, 2, 3, or 4)

minor2: identity of minor allele (A, C, T, or G)

major2: identity of major allele (A, C, T, or G)

stackID: locus number (if generated with STACKS)

stackbp: position of SNP on locus (if generated with STACKS)

sameread: is SNP located on same read as previous SNP? (if generated with STACKS)

name2: chr + name

placed: if reference genome is assembled to chromosomes, TRUE means that SNP is found on a chromosome, and not an unplaced contig/scaffold. Defaults to TRUE if reference genome is not assembled to chromosome.

autosomal: if reference genome is assembled to chromosomes, TRUE means that SNP is found on an autosomal chromosome, and not an unplaced contig/scaffold and neither on a sex chromosome. Defaults to TRUE if reference genome is not assembled to chromosome.

dist: gap distance between SNP and previous SNP (in bp)

dist2: is SNP located on the same contig/scaffold/chromosome as previous SNP?

samepos: does SNP occur on same position as another SNP (if SNP dataset is generated with STACKS, this does occur occasionally)

uniqpos: inverse of samepos

meandepth: sequencing depth averaged over all samples (present if depthfile is present)

depthfilter: mean read depth of SNP below threshold? Threshold is defined as a bonferroni corrected right tail value, assuming mean loci specific read depths fit a normal distribution after removing outliers. Outliers are defined as the top 0.5% values.

This filter has been introduced to filter out SNPs/reads with unusual high read depths.

After running the filterdata() function:

GC: major allele G or C?

transit: transition (A/G or C/T)?

readpos: position of SNP on read (same as *stackbp*)

readpos2: is SNP positioned outside dubious start/end of read? (see SNPsalongreads plot)

misscount: number of missing datapoints

nonmissallelecount: 2*n_individuals - misscount

miss: misscount/(misscount + nonmisscount)

fmiss0.xx miss < threshold?

minorcount: minor allele count

majorcount: major allele count

maf: minor allele frequency (minorcount/nonmisscount)

maf_pop: minor allele frequency per population (with minor allele respective to all pops)

maf2_pop: minor allele frequency per population (with minor allele respective to pop)

hetero: heterozygosity

AA/Aa/aa: observed genotype counts

expAA/expAa/expaa: expected genotype counts (2*maf*(1-maf))

heteropop: heterozygosity per population

hefilter: maximum heterozygosity of He = 2*maf*(1-maf) + 0.5*maf for maf > 0.05? (An arbitrary threshold to filter out paralogs; see He_vs_maf.png)

distfilter: is gap to previous SNP bigger than certain threshold (default: 500 bp)?

filter: uniqpos + hefilter + (minorcount > 1) + fmiss0.xx + distfilter

filter2: uniqpos + hefilter + (minorcount > 1) + fmiss0.xx. If you didn't provide positional information about your snps (for example because you generated your data using STACKS denovo rather than STACKS refmap), filter2 will equal filter1.

mafdiff_pop: difference in minor allele frequency between adjacent snps per population

After running the findstructure() function:

Hexp_meta: 2*maf*(1-maf)

F_meta: *(*Hexp_meta – hetero)/Hexp_meta

HWEchi2: Hardy Weinberg Equilibrium test chi-squared score

HWE: in Hardy Weinberg equilibrium?

Hdeficit: less heterozygotes than expected?

These columns are repeated with population specific estimates.

After running the calcdistance() function:

WrightFst_pop1_pop2: locus specific Wright Fst estimate

WeirHe_pop1_pop2: locus specific Weir & Cockerham heterozygosity estimate

WeirFst_pop1_pop2: locus specific Weir & Cockerham 1987 Fst estimate

After running the calcdiversity() function:

mac_pop: minor allele count per population

9.2 The inds dataframe

Your inds dataframe could contain the following columns, depending on the functions you run and which options you used:

After running the importdata() function:

name: sample name

pop/pop2: population name (as defined in PED-file)

popcol: population colour (used in subsequent functions for plotting)

meandepth: sequencing depth averaged over all loci (present if depthfile is present)

name2: section until first period in name. If period is absent, first 10 characters.

After running the filterdata() function:

miss: proportion of missing data

fmiss0.xx miss < indmiss

maf: minor allele frequency averaged over segregating loci (respective to population to which sample belongs)

- if maf = 0: all loci homozygous major allele
- if maf = 0.5: either all loci are heterozygous, or 50% homozogous minor and 50% homozygous major, or anything in between
- if maf = 1: all loci homozygous minor allele

maf_all: minor allele frequency average over all loci (both segregating and non-segregating)

nsegsites1: number of segregating loci in population (same for all samples belonging to same population)

nsegsites2: number of segregating loci in population which are nonmissing in sample

hetero: number of heterozygous loci divided by number of segregating loci (respective to population to which sample belongs)

hetero_all: number of heterozygous loci divided by total number of loci (both segregating and non-segregating)

nsites1: number of retained loci (same for all samples)

nsites2: number of retained loci which are nonmissing in sample

expHe: expected heterozygosity: 2*maf*(1 – maf)

expHe_all: expected heterozygosity: 2*maf_all*(1 – maf_all)

filter: fmiss0.xx

F: inbreeding coefficient: (expHe – hetero)/expHe

F_all: inbreeding coefficient: (expHe_all – hetero_all)/expHe_all

After running the findstructure() function:

pr_pop: Bayesian probability of a sample belonging to one of the predefined populations, based on its genotype scores and population specific minor allele frequencies. For example: say you have two loci and two populations (A and B). Population A has minor allele frequencies 0.5 and 0.25 for respectively locus 1 and locus 2, and population B has minor allele frequencies 0.1 and 0.05.
Individual1 has genotype scores 1 and 0, meaning this individual is heterozygous for the first locus and homozygous major allele for the second locus. The probability that this individual belongs to either population A or population B is calculated as follows:
Pr(A|ind1) = Pr(ind1|A)/(Pr(ind1|A)+Pr(ind1|B))
Pr(B|ind1) = Pr(ind1|B)/(Pr(ind1|A)+Pr(ind1|B))
We find:
Pr(ind1|A) = (2*0.5*0.5) * (0.75*0.75) = 0.5625
Pr(ind1|A) = (2*0.9*0.1) * (0.95*0.95) = 0.16245
Therefore:
Pr(A|ind1) = 0.5625/(0.5625 + 0.16245) = 0.776
Pr(B|ind1) = 0.5625/(0.5625 + 0.16245) = 0.224

No columns added by calcdistance() function.

After running the calcdiversity() function:

hetero_all2: as hetero_all, but calculated using different method

theta: expected proportion of differences between a haplotype from individual and haplotype from randomly chosen individual within same population

autozygosity: hetero_all/theta

theta2: mean number of differences between two randomly drawn haplotypes within the population to which individual belongs

harmonic_number: 1/1 + 1/2 + 1/3 + ... + 1/n-1 , with n defined as number of haplotypes (i.e. n_individuals*2 – 1)

Watterson: nsegsites1/harmonic_number (Watterson's estimate of theta2)

TajimaD: theta2 – Watterson

theta2_scaled: theta2 per nucleotide

Watterson_scaled: Watterson's estimate per nucleotide

rare_alleles: does the population to which the individual belongs, contain more or less rare alleles than expected?

TajimaD_scaled: Tajima's D score per nucleotide

genometheta: genome wide estimate of theta: theta*nsnps/nsites (nsites is explained elsewhere in this manual)

genomehe: genome wide estimate of heterozygosity: hetero_all*nsnps/nsites (nsites is explained elsewhere in this manual)

9.3 SambaR calculations

SambaR estimates of number of (segregating) sites in inds dataframe

The inds dataframe contains several columns which lists number of sites for each individual.
In the following description it is assumed that the data stored in mygenlight is saved in a matrix called mymatrix, and that only one row (one individual) is considered:

mymatrix *<- as.matrix(mygenlight)*

myind *<- mymatrix[i,]*

ALL SITES

inds$nsites1

Number of sites retained after filtering and thinning (same for each individual):

length(myind[snps$filter])

or

nrow(snps[snps$filter,])

inds$nsites2

Number of sites per individual retained after filtering and thinning, and after excluding missing data points:

length(myind[snps$filter&!is.na(myind)])

(Same as inds$nsites5 and inds$ndata)

inds$nsites_filter2

Number of sites per individual retained after filtering (no thinning), and after excluding missing data points:

length(myind[snps$filter2&!is.na(myind)])

inds$nsites_nofilter

Number of sites per individual retained after filtering on excessive heterozygosity only, and excluding missing data points:

length(myind[snps$hefilter&!is.na(myind)])

SEGREGATING SITES

For the next estimates, we also calculate for each SNP the number of minor allele copies in the population to which individual i belongs:
mypopmaf <- glMean(mygenlight[inds$filter&inds$pop==popname,])

inds$nsegsites1
Number of segregating sites per population after filtering and thinning (same for individuals belonging to the same population):
length(myind[snps$filter&mypopmaf>0])

inds$nsegsites2
Number of segregating sites per population after filtering and thinning, and after excluding missing datapoints:
length(myind[snps$filter&mypopmaf>0&!is.na(myind)])

inds$nsegsites3
Number of segregating sites per population retained after filtering on excessive heterozygosity only (same for individuals belonging to the same population):
length(myind[snps$hefilter&mypopmaf>0])
(These estimates are displayed in the left tile of the plot pi.pop.stripchart and pi.pop.vioplot.)

inds$nsegsites_filter2
Number of segregating sites per population after filtering (no thinning), and after excluding missing datapoints:
length(myind[snps$hefilter2&mypopmaf>0&!is.na(myind)])

inds$nsegsites_nofilter
Number of segregating sites per population retained after filtering on excessive heterozygosity only , and after excluding missing datapoints:
length(myind[snps$hefilter&mypopmaf>0&!is.na(myind)])
(Same as inds$nsegsites4)

SambaR's calculation of pairwise sequence dissimilarity, nucleotide diversity (π), Dxy, Watterson's theta (θ_W) and Tajima's D illustrated using an example dataset

Implemented in SambaR is the function 'calcpi', which is invoked by several of SambaR's main functions. This function calculates observed pairwise sequence dissimilarities, followed by several population-genetic measures which depend on these estimates. Here we will describe the algorithm of the calcpi function using a small example dataset.

Consider the following genotype dataset for 3 individuals and 2 SNPs (in which 0 codes for homozygous major, 1 for heterozygous, 2 for homozygous minor, and NA for missing data):

		SNP1	***SNP2***
PopA	***Ind1***	0	1
PopA	***Ind2***	2	NA
PopB	***Ind3***	1	0

Pairwise sequence dissimilarity

SambaR calculates the number/proportion of differences and missing data points between and within individuals – taking into account that each sample pair provides four potential sequence comparisons (AB,Ab,aB,ab) – and subsequently estimates sequence dissimilarity, both for 'between individual'-comparisons and 'within individual'-comparisons:

	n_ comparisons	***n_differences***				***n_missing_data_points***				***pairwise sequence***
		SNP1	***SNP2***	***total***	***mean (O)***	***SNP1***	***SNP2***	***total***	***mean (M)***	***O/(nsnps-M)***
Ind1-	4	4	NA	4	1	0	4	4	1	1/(2-1)=1
Ind1-	4	2	2	4	1	0	0	0	0	1/(2-0)=0.5
Ind2-	4	2	NA	2	0.5	0	4	4	1	0.5/(2-
Ind1	1	0	1	1	1	0	0	0	0	1/(2-0)=0.5
Ind2	1	0	NA	0	0	0	1	1	1	0/(2-1)=0
Ind3	1	1	0	1	1	0	0	0	0	1/(2-0)=0.5
total	15			12				9		

Nucleotide diversity (π)

Nucleotide diversity (π), which is the expected proportion of differences between any two randomly drawn sequences, is calculated as:

π = sum(n_differences)/(n_comparisons * n_snps – sum(n_missing_data_points)).

For the overall example dataset, π equals: *12/(15*2 – 9) = 12/21 = 0.5714.*

SambaR also estimates $\boldsymbol{\pi}$ per population:

$\pi_{popA} = (4 + 1 + 0)/(6*2 - 5) = 5/7 = 0.714$

$\pi_{popB} = 0.5$

Dxy

SambaR obtains an estimate of *Dxy*, the mean proportion of differences between two sequences randomly drawn from two different populations, by calculating the mean number of pairwise sequence dissimilarity over all possible sample pairs.

For the example dataset, Dxy for pop1 and pop2 is obtained from averaging the pairwise sequence dissimilarity estimates obtained for Ind1-Ind3 and Ind2-Ind3:

n_differences = 4 + 2 = 6

*n_datapoints = 8*2 = 16*

n_missing_data_points = 0 + 4 = 4

Dxy = n_differences/(ndatapoints - n_missing_data_points) = 6/(16 – 4) = 0.5

Fst_π

$Fst_\pi = (\pi_{between\_pop} - \pi_{within\_pop}) / \pi_{between\_pop} = (Dxy - (\sum\pi_{pop})/n_{pops}) / Dxy$

Fst_π for population pair popA and popB in the example dataset equals (see also the sections on nucleotide diversity and Dxy):

$Fst_\pi = (Dxy - (\sum\pi_{pop})/n_{pops}) / Dxy = (0.5- (0.714+0.5)/2)/0.5 = -0.214$

Negative Fst_π values indicate that individuals within populations are, in terms of their genotypes, more different from each other than individuals from different populations.

Watterson's theta (θ_W)

SambaR calculates Watterson's estimate of theta (θ_W) by dividing the number of segregating sites within a population by the harmonic number (a_n). The number of segregating sites (S) is defined as the number of SNPs with a minor allele frequency above 0. The average number of sequences (n), needed to calculate the harmonic number (a_n), is estimated as twice the number average number of individuals with non-missing data per SNP.

For the example dataset, Watterson's theta for all individuals combined equals:

S = 2

n = 2(3 + 2)/2 = 5*

$a_n = \sum_{i=1}^{n-1} 1/(1/i) = 1/1+1/2+1/3+1/4 = 2.083333$

$\theta_W = 2/2.083333 = 0.96$

Hence for each pair of sequences (consisting of 2 SNPs) 0.96 sites are expected to differ (assuming neutrality). SambaR reports estimates per site ($\theta_{W\_persite}$), which equals 0.48 for the numerical example.

Tajima's D

SambaR calculates Tajima's D scaled per single site as:

$D = \pi - \theta_{W\_persite}$

For the example dataset, SambaR's Tajima's D estimate (for all individuals combined) equals: *0.5714 – 0.48 = 0.0914*

SambaR also calculates significance using a chi squared test on observed and expected number of differences. For the example dataset (all individuals combined) the calculation becomes:

$n\_expected = \theta_W = (ndatapoints - n\_missing\_data\_points)*\theta_{W\_persite} = (15*2 - 9)*0.48 = 10.08$

$X^2 = (O - E)^2/E = (n\_differences - n\_expected)^2/n\_expected = (12 - 10.08)^2/10.08 = 0.37$

Next SambaR uses the R base function pchisq(df=1,lower.tail=FALSE) to derive the corresponding p-value:

$P(X^2 = 0.37, df=1) = 0.55$

What if a population is represented by a single sample?

For practical reasons, SambaR omits from the calculations populations which are represented by 1 sample only. Even without running the analyses, we can predict the outcome:

Nucleotide diversity (pi) is the mean difference between two randomly drawn sequences. If you have 1 sample per population only, this is essentially the heterozygosity (He) of this individual. For larger sample sizes, there will be most likely slight or more pronounced difference between He and pi due to inbreeding (for example due to isolation by distance or population substructure). Only in case of perfect panmixia, which is a theoretical construct, pi equals heterozygosity.

Watterson's theta is defined as the proportion of segregating sites divided by the harmonic number. The harmonic number is defined as 1/1 + 1/2 + ... + 1/(n-1), with n being the number of sequences in the population. If the population is represented by 1 diploid sample only, n=2 and n-1 = 1. In that case the harmonic mean simplifies to 1/1 = 1. Given that in populations which are represented by a single sample the number of segregating sites equals the number of heterozygous sites, Watterson's theta simply equals He/1 = He.

Tajima's D is defined as: D = pi - theta

Therefore, for populations represented by one sample only, the outcome is:

D = He - He = 0

SambaR's estimation of minor allele frequencies

The inds$maf_all estimates give the proportion of minor alleles per individual, considering all non-missing datapoints. The inds$maf estimates give the proportion of minor allele per individual, only considering sites which are segregating within the population to which the population belongs. Consider, as an example, the following example dataset:

		SNP1	***SNP2***	***SNP3***	***SNP4***	***SNP5***
PopA	***Ind1***	0	NA	0	2	1
PopA	***Ind2***	1	1	0	0	0
PopA	***Ind3***	1	0	0	0	1
PopB	***Ind4***	0	0	1	0	0
PopB	***Ind5***	1	1	2	1	0

The individual estimates for Ind1 are:

Maf_all: (0+0+2+1)/(4*2) = 0.375

Maf: (0+2+1)/(3*2) = 0.5

SNP3 has been excluded in the calculation of 'maf_all', but excluded from the 'maf' calculation, because the minor allele is absent from all sampled individuals in PopA. Similarly, SNP5 would be excluded from the calculation of inds$maf for individuals of PopB.

The snps$maf estimates give the proportion of minor alleles per SNP. Estimates are available for the metapopulation (snps$maf) as well as for population specifically (e.g, snps$maf_PopA and snps$maf_PopA).

In the example dataset, SNP2 would have the following scores:

maf: (1+0+0+1)/(4*2) = 0.25

maf_PopA: (1+0)/(2*2) = 0.25

maf_PopB: (0+1)/(2*2) = 0.25

Given that a SNP dataset typically contains many more SNPs than individuals, the variation observed in the snps$maf column will exceed the variation observed in the inds$maf column.

The estimates in the 'SambaR.popdiversity.stats' output table ('maf_all') and ('maf_seg') show the inds$maf_all and inds$maf estimates, average over all individuals within the same population.

The table 'TajimaD.statistics' contains a column named 'mean_maf', which show the maf_PopA and maf_PopB values, averaged over all (retained) SNPs.

SambaR's estimation of genome wide heterozygosity

If users provide to the nrsites flag of SambaR's calcdiversity function (default is NULL), an estimate of the total number of sequenced sites from which the SNP data is derived, SambaR will estimate genome wide estimates (e.g. genome wide heterozygosity and genome wide nucleotide diversity).

For RADseq data, the number of sequenced sites, here denoted as 'N_total', equals the combined length of all polymorphic as well as monomorphic stacks which passed filter settings, and can be obtained from the sumstats summary file.

For resequencing data, N_total equals the total number of monomorphic and polymorphic sites of the filtered data set. This number can for example be obtained by counting the number of lines in the VCF file, excluding the header section. The precondition is that the VCF file contains both monomorphic and polymorphic sites. If the VCF file is generated with bcftools, users should not include the -v option when running the bcftools calls command, otherwise monomorphic sites will not be outputted.

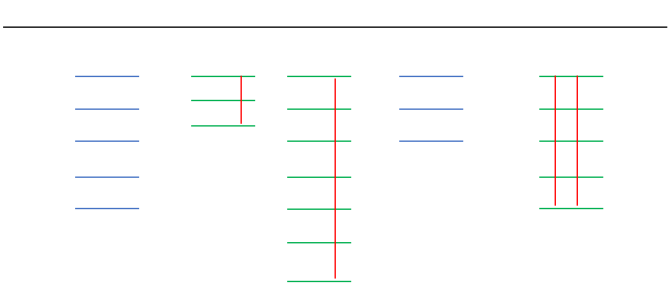
SambaR calculates genome wide heterozygosity (He_{genome}) and proportion of segregating sites (S_{genome}) as:

$$He_{genome} = (n_H/n_{ind} * n_{snps})/n_{total}$$

$$S_{genome} = n_{seg}/n_{total}$$

In which n_H denotes number of heterozygous sites observed for individual i within the SNP dataset, n_{ind} denotes the number of non-missing data points for individual i within the SNP dataset, n_{snps} denotes the total number of snps in the dataset, n_{seg} denotes number of segregating sites for population j, and n_{total} denotes the total number of sequenced sites (sum of total length of both monomorphic and polymorphic STACKS loci. All calculations are based on the full SNP dataset, excluding SNPs with excessive heterozygosity.

The method is illustrated in the scheme below:



Genome

110 bp STACKs loci
green: polymorphic
blue: monomorphic
red: SNP
combined length (n_{total}):
5x110bp = 550 bp

Pop1	Ind1	A	A	C C
		A	G	C T
Pop1	Ind2	A	A	C N
		A	G	G N
Pop2	Ind3	A	A	C T
		T	G	C T
Pop2	Ind4	A	A	C C
		T	G	G T
Pop2	Ind5	A	A	N C
		A	G	N T

SNP data

black: fixed
red: segregating
orange: out of HWE,
excluded from calculations

Because one SNP is excluded from the analysis because of HWE considerations, n_{snps} = 3. Heterozygosity and segregating site calculations are displayed in black and grey respectively:

		all sites non-missing n_{ind}	segregating sites within population n_{seg}	segregating sites non-missing n_{ind2}	Heter-ozygous sites n_H	Heterozygosity within SNP dataset* $H_{SNPS} = n_H / n_{ind}$	Heterozy-gosity for segregating sites** $H_{seg} = n_H / n_{ind2}$	Genome wide Proportion Segregating sites $S = n_{seg}/n_{total}$	Genome wide heterozygosity $(H_{snps}*n_{snps})/ n_{total}$
Pop 1	Ind 1	3	2	2	1	1/3 = 0.333	1/2 = 0.5	0.0036	0.0018
Pop 1	Ind 2	2	2	1	1	1/2 = 0.5	1/1 = 1	0.0036	0.0027
Pop 2	Ind 3	3	3	3	1	1/3 = 0.333	1/3 = 0.333	0.0055	0.0018
Pop 2	Ind 4	3	3	3	3	3/3 = 1	3/3 = 1	0.0055	0.0055
Pop 2	Ind 5	2	3	2	1	1/2 = 0.5	1/2 = 0.5	0.0055	0.0027

**This estimate is stored in the column inds$hetero_all, and is identical to the multi-locus heterozygosity (MLH) estimates returned by the function MLH of the R package 'inbreedR', stored in the column inds$MLH.*

***This estimate is stored in the column inds$hetero. These estimates are higher than (or equal to) the values in the inds$hetero_all column, because non-segregating sites are excluded from the calculation.*

SambaR's estimation of locus specific Fst-values, illustrated using an example dataset

Differences in minor allele frequencies (MAF, or p) between populations can be summarized using a Fst-metric. SambaR relies upon the stamppFst function (of the package 'Stampp') to generate Weir & Cockerham 1984 Fst estimates, along with associated significance values. This function generates for each population pairwise comparison one estimate for all SNPs combined.

In addition, SambaR uses built-in functions to calculate Fst estimates for each single SNP: locus specific Fst estimates. SambaR generates three different Fst measures:

- Fst = Var(p)/(p(1-p)) Wright, 1943
- Fst = $(H_T-H_S)/H_T$ Nei, 1977
- Fst = (f0 – f1)/(1 – f1) Cockerham & Weir, 1987

For illustrative purposes we will consider again the following genotype dataset for 3 individuals and 2 SNPs (in which 0 codes for homozygous major, 1 for heterozygous, 2 for homozygous minor, and NA for missing data):

		SNP1	***SNP2***
PopA	***Ind1***	0	1
PopA	***Ind2***	2	NA
PopB	***Ind3***	1	0

Wright 1943 Fst

This measure was first published by Sewall Wright in 1943 (Wright, 1943, see equation 48 and the following lines).

Var(p), also denoted as s_p^2, or σ_p, denotes the variance in minor allele frequencies among populations. If p denotes the minor allele frequency, and if subscripts 1 and 2 denote either of the two populations considered in a pairwise population comparison, this estimate can be described as:

$Var(p) = \frac{1}{2}*((p_1 - p)^2 + (p_2 - p)^2)$

To standardize this estimate (i.e. make it comparable among SNPs regardless of their mean minor allele frequencies), the value is divided by the maximum value. It can be mathematically shown that for population pairs which are fixated for different alleles (i.e. $p_1=1$ and $p_2=0$ or vice versa), *Var(p)* equals: p(1-p). Therefore, a standardized estimate is given by:

Fst = Var(p)/(p(1-p))

For SNP2 in the example dataset above, the Var(p) and Fst estimates equal:

Var(p) = ½*((0.5 – 0.25)² + (0 – 0.25)²) = 0.0625

(p(1-p)) = 0.25*0.75 = 0.1875

Fst = Var(p)/(p(1-p)) = 0.0625/0.1875 = 1/3

Nei 1977 Fst

This measure was developed by Nei (1977) for multi-allelic loci.

H_T denotes the expected heterozygosity in the overall dataset (total population).

H_S denotes the expected heterozygosity in the subpopulations.

If p denotes the minor allele frequency, and if subscripts 1 and 2 denote either of the two populations considered in a pairwise population comparison, these estimates are equal to:

$H_T = 2*p*(1-p)$

$H_S = ½*(2*p_1*(1-p_1) + 2* p_2*(1-p_2))$

For SNP2 in the example dataset above, the H_T and H_S estimates are equal to:

$H_T = 2*0.25*(1-0.75)$ = 0.125

$H_S = ½*(2*0.5*(1-0.5) + 2*0*1)$ = 0.25

$Fst = (H_T-H_S)/H_T$ = (0.25 – 0.125)/(0.25) = 1/2

Cockerham & Weir 1987 Fst

This measure was published by Cockerham and Weir in 1987, and is not be confused with their Fst metric published in 1984. The original notation used by Cockerham and Weir (1987) was: Fst = $(\theta_1 – \theta_2)/(1 – \theta_2)$. Here we denote θ_1 as f_0 and θ_2 as f_1.

f0 denotes the probability that two randomly drawn alleles from within a (sub)population are identical.

f1 denotes the probability that two randomly drawn alleles from the overall population (the total or metapopulation) are identical.

If p denotes the minor allele frequency, and if subscripts 1 and 2 denote either of the two populations considered in a pairwise population comparison, these estimates are equal to:

$f0 = ½*(p_1^2*(1-p_1)^2 + p_2^2*(1-p_2)^2)$

$f1 = p_1*p_2 + (1-p_1)*(1-p_2)$

For SNP2 in the example dataset above, the f0 and f1 estimates are equal to:

$f0 = ½*(0.5^2 + (1-0.5)^2 + 0^2 + (1-0)^2)$ = 0.75

$f1 = 0.5*1 + 0.5*0$ = 0.5

Hence, there is a 75% probability of drawing two identical alleles when pooling from within one of the populations, compared to a 62.5% probability when drawing two alleles from the metapopulation.
The Fst estimates for SNP2 becomes:
Fst = (f0 – f1)/(1 – f1) = (0.75 – 0.5)/(1-0.5) = 0.5

Note that from its definition it follows that *f1* is an estimate of expected homozygosity in the populations in the absence of population structure. Expected heterozygosity is therefore simply:
He = 1 – f1

The 'selectionanalyses'-function of SambaR will generate Fdist plots, showing locus specific Fst estimates on the y-axis against He (= 1 – f1) estimates on the x-axis. If SNPs are biallelic, and if datasets consist of two populations/groups, this spectrum of possible He-F_{ST} values has the shape of a shark fin. The left boundary is described by F_{ST} = He and represents loci which are segregating in one population only. The right boundary of the 'shark fin'-spectrum represents loci with opposing allele frequencies in either population (e.g 0.3-0.7 in one population and 0.7-0.3 in the other population), and is described by F_{ST} = (2He-1)/He for He>=0.5.

References:

S. Wright, 1943, Isolation by distance, Genetics 28(2): 114-138
M. Nei (1972), Genetic distance between populations, The American Naturalist 196(949): 283-292 (*paper on Nei's genetic distance*)
M. Nei, 1977, F-statistics and analysis of gene diversity on subdivided populations, Annals of Human Genetics 41 (2): 225-233
B. S. Weir and C. C. Cockerham, 1984, Estimating F-Statistics for the Analysis of Population Structure, Evolution 38(6): 1358-1370
C. C. Cockerham and B. S. Weir, 1987, Correlations, descent measures: drift with migration and mutation, Proc. Natl. Acad. Sci. USA 84(23): 8512-8514

SambaR's estimation of inbreeding coefficient F

SambaR uses two methods to calculate individual inbreeding coefficient F, the probability that the two alleles at any locus of a diploid individual are identical by descent (IBD): F_H (Kardos et al., 2015) and the here defined F_π (see below).

F_H

F can be defined as the proportion of expected heterozygous sites (in case of no inbreeding) which are homozygous due to inbreeding. In formula:

$F_H = (obshomo - exphomo)/(n - exphomo)$ (Kardos et al., 2015)

In which:

n: total number of sites (for an individual), excluding missing data points

obshomo: observed number of homozygous sites (for an individual)

exphomo: expected number of homozygous sites (for an individual), estimates as:

$\sum_i^n q_i^2+(1-q_i)^2$

in which q_i denotes the minor allele frequency in the population to which the individual belongs, which is derived from all individuals in the population except for the individual for which F is calculated.

SambaR evaluates significance by applying a chi-squared test on observed and expected number of homozygous sites.

Consider, as an example, a diploid individual A which is genotyped at 10 sites, of which 6 are heterozygous. Through selfing individual A produces individual B. The most likely genotype of individual B consists of (4 + 0.5*6 =) 7 homozygous sites.

If all other individuals in the population are not inbred, and on average have the same number of homozygous and heterozygous sites as individual A, then the expected number of homozygous sites equals 4. The inbreeding coefficient of individual B therefore equals:

$F_H = (obshomo - exphomo)/(n - exphomo) = (7 - 4)/(10 - 4) = 3/6 = 0.5$

The F_H value of 0.5 correctly indicates that individual B results from selfing. It also correctly indicates the proportion of autozygous sites of individual B (i.e. proportion IBD). Assuming the homozygous sites of individual A are IBS (identical by state) but not identical by descent (IBS), the most likely number of autozygous sites of individual B equals (0.5*10 =) 5.

Significance is evaluated using a chi-squared test:

$X^2 = (O - E)^2/E = (obshomo - exphomo)^2/exphomo = (7 - 4)^2/4 = 2.25$

SambaR uses the R base function pchisq(df=1,lower.tail=FALSE) to derive the corresponding p-value:

$P(X^2 = 2.25, df=1) = 0.1336144$

***F*π**

The second method SambaR uses to estimate heterozygosity is based on a comparison between the heterozygosity (He) of an individual (j) and the mean pairwise sequence dissimilarity (here defined as π_j) of this individual with all other individuals in the population:

$F\pi = 1 - He_j/\pi_j$

SambaR evaluates significance by applying a chi-squared test on observed and expected number of heterozygous sites, in which the number of expected heterozygous sites is defined estimated by π_j times the number of sites.

Consider, as an example, an individual with a heterozygosity of 0.23 and a mean pairwise dissimilarity with other individuals of the population of 0.25. SambaR estimates the inbreeding coefficient for this individual as:

$F\pi = 1 - 0.23/0.25 = 0.08$

If the heterozygosity and nucleotide diversity estimates are based on a dataset of 1000 sites, a chi-squared test returns:

$X^2 = (O - E)^2/E = (He_j - \pi_j)^2/\pi_j = (230 - 250)^2/250 = 1.6$

SambaR uses the R base function pchisq(df=1,lower.tail=FALSE) to derive the corresponding p-value:

$P(X^2 = 1.6, df=1) = 0.2059032$

SambaR's estimation of relatedness between samples

Relatedness calculations are based on the proportions of genotype scores (0, 1, 2) of pairs of individuals. For biallelic SNPs in a diploid population, there are three possible genotypes:

0: homozygous major allele

1: heterozygous

2: homozygous minor allele

If two individuals are unrelated, the probability of observing a certain genotype for one individual and at a same time a certain genotype for the second individual, is a function of the proportion of genotypes for these two individuals. For example, if 80 percent of the genotypes of the first individual are 0, whereas for the second individual this percentage equals 85 percent, then the expected proportion of sites for which both individuals are homozygous major (i.e., 0), is 0.8*0.85 = 0.68. Similar calculations can be made for the other 9 possible genotype combinations:

Ind2: **Ind1:**	**0: p2[1]**	**1: p2[2]**	**2: p2[3]**
0: p1[1]	x11 = p1[1]* p2[1]	x12 = p1[1]* p2[2]	x13 = p1[1]* p2[3]
1: p1[2]	x21 = p1[2]* p2[1]	x22 = p1[2]* p2[2]	x23 = p1[2]* p2[3]
2: p1[3]	x31 = p1[3]* p2[1]	x32 = p1[3]* p2[2]	x33 = p1[3]* p2[3]

Strong deviations from the expected two-dimensional genotype proportions can indicate that two samples are related. Relatedness scores measure the observed deviation, and thereby provide an estimate of relatedness. They do so by evaluating the proportion of sites with respectively 0 (darkgrey), 1 (light grey) and 2 (white) alleles being identical by state (IBS) between the two individuals. For example, consider the following hypothetical matrix showing observed and expected (italic) genotype combinations:

Ind2: **Ind1:**	**0: 0.84**	**1: 0.08**	**2: 0.06**
0: 0.86	0.737 *0.7224*	0.07 *0.0672*	0.057 *0.0504*
1: 0.11	0.09 *0.0924*	0.01 *0.0088*	0.014 *0.0066*
2: 0.03	0.016 *0.0252*	0.003 *0.0033*	0.004 *0.0018*

The proportions of sites with IBS=0, IBS=1, and IBS=2 are respectively:

k0 = Proportion of sites with IBS=0
= (x[3,1]+x[1,3])/sum(x)
= (0.016 + 0.057)/1
= 0.073

k1 = Proportion of sites with IBS=1
= (x[1,2]+x[2,1]+x[2,3]+x[3,2])/sum(x)
= (0.07 + 0.09 + 0.014 + 0.003)/1
= 0.177

k2 = Proportion of sites with IBS=2
= (x[1,1]+x[2,2]+x[3,3])/sum(x)
= (0.737+0.01+0.004)/1
= 0.751

Kinship coefficient

The kinship coefficient *r* is defined as:

r = $¼*P_{IBS=1} + ½*P_{IBS=2}$
= $k1/4+k2/2$
= 0.177/4 + 0.751/2
= 0.41975

The kin-coefficient can range between 0 and 0.5 (i.e., 0/4 + 1/2). (See also: *Waples et al, 2018, Allele frequency free inference of close familial relationships from genotypes or low depth sequencing data*)

King-robust score

An alternative measure of relatedness is the king-robust score, which is defined as:

h2 = proportion of sites both individuals are heterozygous

king-robust = $(h2 – 2*P_{IBS=0})/(P_{IBS=1} +2*h2)$
= $(h2 – 2*k0)/(k1+2*h2)$

= (x[2,2]-2*(x[1,3]+x[3,1]))/(x[2,1]+x[1,2]+x[2,3]+x[3,2]+2*x[2,2])
= (0.01*(0.057 + 0.016))/(0.09 + 0.07 + 0.014 + 0.003 + 2*0.01)
= 0.003705584

The king-robust score can range between strongly negative and 0.5. The maximum value of 0.5 occurs when all off-diagonal values are zero. For such matrices, the formula becomes:

king-robust = (x[2,2]-2*0)/(0+0+0+0+2*x[2,2])
= x[2,2]/(2*x[2,2])
= *h2*/(2**h2*)
= 1/2

The king-robust score is sometimes dissected into r0 and r1:

r0 = *k0*/*h2*
= (x[3,1]+x[1,3])/x[2,2]
= (0.016 + 0.057)/0.01
= 7.3

r1 = *h2*/(*k1* + *k2*)
= (x[2,2])/(x[1,2]+x[1,3]+x[2,1]+x[2,3]+x[3,1]+x[3,2])
= 0.01/(0.07 + 0.057 + 0.09 + 0.014 + 0.016 + 0.004)
= 0.03984064

Geno2Dmat

SambaR creates heatmaps showing genotype combination frequencies for each sample pair with a king-robust score above a certain threshold as specified by the king_threshold flag of the calckinship function (by default 0.4). The colour scale (white to dark orange) does not reflect the genotype frequencies, but instead the deviation of observed frequencies from expected frequencies.

As explained above, expected genotype combination frequencies of a sample pair is given by:
e_{ij} = p1[i]*p2[j]
in which p1 and p2 are the genotype frequencies of individual 1 and individual 2 respectively for the three genotypes 0, 1 and 2.

The difference between observed and expected genotype frequencies is calculated as:

$d_{ij} = abs(e_{ij} - o_{ij})$

For example, for the example dataset above, the difference matrix is:

Ind2: **Ind1:**	**0: 0.84**	**1: 0.08**	**2: 0.06**
0: 0.86	abs(0.7224 - 0.737) = **0.0146**	abs(0.0672 - 0.07) = **0.0028**	abs(0.0504 - 0.057) = **0.0066**
1: 0.11	abs(0.0924 - 0.09) = **0.0024**	abs(0.0088 - 0.01) = **0.0012**	abs(0.0066 - 0.014) = **0.0074**
2: 0.03	abs(0.0252 - 0.016) = **0.0092**	abs(0.0033 - 0.003) = **0.0003**	abs(0.0018 - 0.004) = **0.0022**

By default, SambaR distinguish five categories:

<0.02 white

0.02 – 0.04 light orange

0.04 – 0.06 medium orange

0.06 – 0.08 orange

>0.08 dark orange

Thus, in the example dataset above, all fields will be white.

PLINK pi-hat score

The kinship coefficient and king-robust score are based on the concept of 'identity by state' (IBS). However, what we are really want to do is whether alleles are 'identical by descend' (IBD). IBD is more difficult to determine. Alleles with are identical by state are not necessarily identical by descend. Multiple mutation events can introduce the same allele into a population, especially when considering single nucleotides only.

The pi-hat score of the software PLINK (which can be calculated with plink --genome) is a composite of $P_{IBD=1}$ and $P_{IBD=2}$ estimates:

$Pi\_hat = 1/2*P_{IBD=1} + P_{IBD=2}$

PLINK denotes $P_{IBD=0}$, $P_{IBD=1}$ and $P_{IBD=2}$ as Z0, Z1 and Z2 respectively. PLINK estimates $P_{IBS=0}$, $P_{IBS=1}$ and $P_{IBS=2}$ by considering identity by state in the context of the minor allele frequencies within the

population (see Purcell et al., 2017, PLINK: a tool set for whole-genome association and population-based linkage analyses). The rationale behind this is that the sharing of alleles between individuals is especially indicative of kinship if these alleles are rare in the overall population.

SambaR's Bayesian population assignment (BPA) test, illustrated using an example dataset

The BPA test addresses the question: among a set of predefined populations, which population is most likely to be the origin of a particular individual? The BPA test calculates the probability that an individual originates from each of the predefined populations, given the observed genotype of the individual and given the observed population allele frequencies.

Consider, as an analogy, two jars. Jar A contains 10 red and 90 white balls. Jar B contains 1 red and 99 white balls. A ball is drawn from one of these jars, and the ball is red. From which bottle has the ball been drawn? According to Bayes Rule, the posterior probabilities are:
Pr(A|red) = Pr(red|A)/(Pr(red|A)+Pr(red|B)) = 0.1/(0.1+0.01) = 0.91
Pr(B|red) = Pr(red|B)/(Pr(red|A)+Pr(red|B)) = 0.01/(0.1+0.01) = 0.09
Hence, there is a 91% probability the ball has been drawn from jar A, and a 9% probability the ball has been drawn from jar B.

The BPA test uses a similar algorithm to calculate the probabilities that a sample has been drawn from each of the set of predefined populations:
Let Hkj denote the probability that individual k belongs to population j, let MAFij denote the minor allele frequency of locus i in population j (excluding the genotype of individual k), let Oi denote the genotype of individual k for locus i (i.e. number of minor alleles (0, 1, or 2)), and let k denote the number of predefined populations. For any given locus, the probability that an individual belongs to population j can be described as:

$Pr(H_{kj}|O_i) = (Pr(O_i|H_{kj})*Pr(H_{kj}))/Pr(O_i)$ $\{Oi = 0,1,2 \text{ and } 0 \leq MAF_{ij} \leq 1\}$

in which:

$Pr(O_i=0|H_{kj}) = (1-MAF_{ij})^2$

$Pr(Oi=_1|H_{kj}) = 2*MAF_{ij}*(1-MAF_{ij})$

$Pr(O_i=2|H_{kj}) = MAF_{ij}^2$

If assuming a flat prior distribution (i.e. $Pr(H_{kj}) = 1/j$), the formula for $Pr(H_{kj}|O_i)$ can be simplified to:

$Pr(H_{kj}|O_i) = Pr(O_i|H_{kj}) / \sum_j^k Pr(O_i|Hk_j)$

To calculate the probability for the nth locus, SambaR uses a recursive formula:

$Pr(H_{kj}|O_n) = (n-1)*Pr(H_{kj}|O_{n-1})*Pr(H_{kj}|O_i)$

As an example, consider the following genotype dataset for 10 individuals and 2 SNPs (in which 0 codes for homozygous major, 1 for heterozygous, 2 for homozygous minor, and NA for missing data):

		SNP1	***SNP2***
PopA	***Ind1***	2	2
PopA	***Ind2***	0	1
PopA	***Ind3***	1	0
PopA	***Ind4***	0	0
PopA	***Ind5***	0	2
PopB	***Ind6***	1	2
PopB	***Ind7***	2	2
PopB	***Ind8***	2	2
PopB	***Ind9***	1	1
PopB	***Ind10***	2	0

All individuals have been assigned to a set of two populations, PopA or PopB. Ind1 has been assigned to PopA, but what is the probability it does indeed belong to PopA (assuming the other population assignments are correct)?

The population minor allele frequencies, not considering Ind1, are:

	SNP1	***SNP2***
PopA	1/8 = 0.125	3/8 = 0.375
PopB	8/10 = 0.8	7/10 = 0.7

For both SNPs, Ind1 is homozygous for the minor allele (i.e. genotype 0).
Let Pr_sum denote: (Pr(2|popA)+Pr(2|popB).
The posterior probabilities for SNP1 and SNP2 are equal to:

SNP1:

$Pr(popA|2) = Pr(2|popA)/Pr\_sum = 0.125^2/(0.125^2+0.8^2) = 0.02383222$

$Pr(popB|2) = Pr(2|popB)/Pr\_sum = 0.8^2/(0.125^2+0.8^2) = 0.9761678$

SNP2:

$Pr(popA|2) = Pr(2|popA)/Pr\_sum = 0.375^2/(0.375^2+0.7^2) = 0.2229931$

$Pr(popB|2) = Pr(2|popB)/Pr\_sum = 0.7^2/(0.375^2+0.7^2) = 0.7770069$

The combined posterior probabilities are equal to:

*Pr(popA|2 2) = (0.02383222*0.2229931)/(0.02383222*0.2229931+0.9761678 * 0.7770069) = 0.006957837*

*Pr(popB|2 2) = (0.9761678 * 0.7770069 /(0.02383222*0.2229931+0.9761678 * 0.7770069) = 0.9930422*

In conclusion, given the observed genotype of Ind1 and the observed allele frequencies in PopA and PopB, Ind1 belongs with 99.3% probability to PopB and with 0.7% probability to PopA, suggesting that the original population assignment is incorrect.

The 'distinct clustering'-score (dc-score) algorithm, illustrated using an example dataset

SambaR aims to facilitate objective interpretation of ordination analyses by calculating a 'dc-score' for PCA, PCoA, CA and DAPC analyses. The dc-score, or 'distinct clustering'-score, measures the overlap between population clusters in a 2-dimensional space defined by the first and second axis of an ordination analysis (e.g. PCA and PCoA).

The algorithm behind the dc-score is as follows:
Let p1i_j and p2i_j denote the loading of sample i, belonging to population j, on the first and second ordination axes, and let n_j denote the number of individuals belonging to population j.

The mean loadings (population centres) for population j are defined as:
$p1j = (\sum_i^{nj} p1i\_j)/n_j$
$p2j = (\sum_i^{nj} p2i\_j)/n_j$

The mean distance dj of samples belonging to population j from the population centre of population j is defined as:
$d1i\_j = | p1i\_j - p1j |$
$d2i\_j = | p2i\_j - p2j |$
$di\_j = \sqrt{((d1i\_j)^2 + (d1i\_j)^2)}$
$dj = (\sum_i^{nj} di\_j)/n_j$

The mean distance of all samples from their population centres, given n_z populations, is defined as:
$dz = (\sum_j^{z} dj)/n_z$

The distance between population centres for population pair k, dk, is defined as:
$d1k = | p1j_{j=1} - p1j_{j=2} |$
$d2k = | p2j_{j=1} - p2j_{j=2} |$
$dk = \sqrt{((d1k)^2 + (d2k)^2)}$

The mean distance between population centres for all n_m populations pairs, dm, is defined as:
$dm = (\sum_k^m dk)/n_m$
The dc-score is defined as:
$dc = dz/dm$

As an example, consider an ordination analysis on six samples (from three populations) returning the following output:

		axis1	***axis2***
PopA	***Ind1***	-0.02	-0.03
PopA	***Ind2***	0.02	-0.03
PopB	***Ind3***	0.03	0.03
PopB	***Ind4***	0.02	0.02
PopC	***Ind5***	-0.03	0.03
PopC	***Ind6***	-0.01	0.01

To calculate the dc-score, SambaR derives the mean loadings per population ('population centres'):

	axis1	***axis2***
PopA	0	-0.03
PopB	0.025	0.025
PopC	-0.02	0.02

Thirdly, SambaR calculates the mean distance between sample loadings and population centres, and between population centres, and calculates the dc-score as the ratio between both distances:

		axis1	***axis2***	***distance***			***distance***	***dc (dz/dm)***
PopA	***Ind1***	-0.02	-0.03	0.02		***popA-popB***	0.06041523	
PopA	***Ind2***	0.02	-0.03	0.02		***popA-popC***	0.05385165	
PopB	***Ind3***	0.03	0.03	0.007071068		***popB-popC***	0.04527693	
PopB	***Ind4***	0.02	0.02	0.007071068				
PopC	***Ind5***	-0.03	0.03	0.014142136				
PopC	***Ind6***	-0.01	0.01	0.014142136				
mean				dz = 0.01373773			dm = 0.05318127	0.258319

SambaR's calculation of the folded site frequency spectrum (SFS), illustrated using an example dataset

NOTE: comparisons have revealed inconsistencies between SFS-vectors generated by SambaR, and SFS-vectors generated by ANGSD, especially regarding the counts of common alleles. If these analyses are a key part of your study, you are advised to create SFS vectors using ANGSD (see elsewhere in this manual for further instructions).

A SFS is a histogram showing the frequencies/numbers of SNPs binned based on their number of minor allele copies. Say for example that we have the following SFS-vector:
3678 560 400 322 360 78 20 0 0
This SFS-vector tells us that for a given (meta)population the dataset contains in total 5418 SNPs. Of those, 3678 SNPs have 1 copy of the minor allele, 560 have 2 copies of the minor allele, 400 sites have 3 copies of the minor allele, 322 have 4 copies of the minor allele, etc.

It also tells us that the dataset consists of 9 individuals, because assuming diploidy and assuming the data contains biallelic SNPs only (i.e. no SNPs with more than 2 different alleles) the total number of allele copies per SNP (minor and major allele combined) is twice the number of individuals. As the minor allele is by definition the less abundant allele, the minor allele can be represented by maximum half the number of total allele copies, which for diploid individuals equals the number of individuals. Therefore, the length of the SFS-vector equals the number of individuals, which in the example above is 9. In the example above there are no SNPs with 8 or 9 copies of the minor allele, and therefore the vector ends with 2 zero's.

How does SambaR generate SFS-vectors?
SambaR uses two steps to generates folded site frequency spectrum (SFS) vectors:

- The first step involves counting for each SNP and for each population the number of minor allele copies, when only considering individuals which retained filter settings. This counting is performed using the glSum function of the adegenet package. The minor allele is defined based on the total dataset. As a consequence, some populations can carry SNPs with more minor allele copies than major allele copies. If so, SambaR subtracts for these particular SNP the number of minor allele copies from the total number of observed allele copies (ignoring missing data points). This latter number is computed with the glNA-function of the adegenet package. The output is saved in the snps dataframe, in columns

which are names 'mac' (for minor allele count) followed by an underscore and the name of the population.

- The second step consists of binning and counting SNPs based on their number of minor allele copies. You could roughly do so yourself by typing on the command line:
  table(snps$mac_popname) # replace 'popname' with the name of your populations

Consider, as an example, the following genotype dataset for 7 individuals and 4 SNPs (in which 0 codes for homozygous major, 1 for heterozygous, 2 for homozygous minor, and NA for missing data):

		SNP1	***SNP2***	***SNP3***	***SNP4***
PopA	***Ind1***	1	1	0	NA
PopA	***Ind2***	2	0	0	0
PopA	***Ind3***	2	0	0	2
PopA	***Ind4***	0	0	0	0
PopB	***Ind5***	1	1	2	0
PopB	***Ind6***	0	2	2	1
PopB	***Ind7***	0	0	2	0

Step 1. Count number of minor allele copies

Counting the number of minor allele copies results in the following data:

	SNP1	***SNP2***	***SNP3***	***SNP4***
PopA	8-5=3*	1	0	2
PopB	1	3	6-6=0*	1

*Due to population structure, it can occur that within a population the minor allele (which is defined relative to the metapopulation) has in fact more copies than the minor allele. If that is the case, for this population and for this particular SNP the number of major alleles copies are counted (rather than number of minor allele copies).

Step 2. Binning SNPs based on their number of minor allele copies

The second step is to bin and count the SNPs based on their number of minor allele copies present in either population. The following data frame shows the number of SNPs for each bin class:

	Number of minor allele copies				
	0	***1***	***2***	***3***	***4***
PopA	1	1	1	1	0
PopB	1	2	0	1	

Hence the SFS vectors, if including sites which are monomorphic (within populations), are:

PopA: 1,1,1,1,0

PopB: 1,2,0,1

And if excluding sites which are monomorphic (within populations):

PopA: 1,1,1,0

PopB: 2,0,1

The latter two vectors are outputted by SambaR. In other words: the SFS vector generated by SambaR does not include sites with zero copies of the minor allele. As a result, the length of the SFS-vector for each population equals the number of retained individuals within this population. Also note that the sum of the SFS vectors are equal to the number of segregating sites within the respective population.

The site frequency spectrum for small sample sizes will not be as interesting as for big sample sizes. If a population is represented by a single individual, the SFS will be restricted to three bars: 0, 1 and 2 allele copies of the reference allele. Furthermore: high uncertainty of the accuracy of SFS vectors is expected if datasets consist of a small number of SNPs and/or a high proportion of missing data points.

Bootstrapping

SambaR calculates confidence intervals using a bootstrap method. Bootstrap estimates are generated by resampling, with replacement, the mac-vector generated at step 1, with a sample size which equals the length of the mac vector. By default, SambaR performs 100 bootstraps replicated. The obtained 95% confidence intervals (i.e, mean ± 2*sd) are shown in the SFS barplots using whiskers.

How does SambaR deal with missing data when generating SFS vectors?

Because the SFS vector is based on minor allele counts rather than on minor allele frequencies, and hence do not have a built-in correction for missing data, the occurrence of missing data affects the composition of the SFS vector. As an example, say that in the example dataset given above, Ind1 would have genotype 2 for SNP4 (rather than a missing data point), then the SFS-vector of population A would become '1,0,1,1' rather than '1,1,1,0'.

Generally, the presence of missing data results in an overestimate of the number of SNP with low number of minor alleles (i.e., higher bars at the left side of the SFS vector), and an underestimate of the number of SNPs with a high number of minor allele (i.e, lower bars right side of the SFS vector). In other words, the higher the number of missing data points, the less flat the distribution.

The reason is obvious: a minor allele copy can only be counted if the genotype data for this site is available.

A way to correct for this bias, is to impute the data: fill in each missing data point with a 'guessed' genotype. SambaR imputes the data by calculating genotype probabilities from population specific minor allele frequencies. As an example, say that the minor allele frequency is 0.1, then the genotype (AA, Aa, aa) probabilities are respectively 0.81, 0.18 and 0.01. SambaR then generates a random value (using the runif function) between 0 and 1. If the value is between 0 and 0.81, SambaR will assign AA to the missing genotype. If the value is between 0.81 and 0.99, the genotype will be assigned Aa. And if the value is between 0.99 and 1, the assigned genotype will be aa.

Although SambaR generates SFS vectors with and without imputing missing data points, users are recommended to use the SFS vectors generated using imputed datasets, because, as described above, non-imputed datasets will return biases SFS vectors. A folded SFS-vector typically has the shape of the left side of a halfpipe, although appearing almost flat for recently bottlenecked populations.

Filtered or non-filtered data?

In addition to generating SFS vector over imputed and non-imputed datasets, SFS vectors are generated for the entire dataset as well as for retained SNPs only. The first dataset includes all SNPs, whereas the second contains only SNPs which passed the filter settings defined with the filterdata() function.

It is left to the user to decide which dataset (filtered or not filtered) generates the SFS vector which is most sensible or most suitable for the study purposes. However, if deciding to use a filtered dataset, the user might want to consider to set the flag min_mac to 1 when (re)running the filterdata-function. By default, this flag is set 2, meaning that SNPs in which the minor allele is present only once, are not included in the analysis, which will obviously affect the shape of the SFS vector.

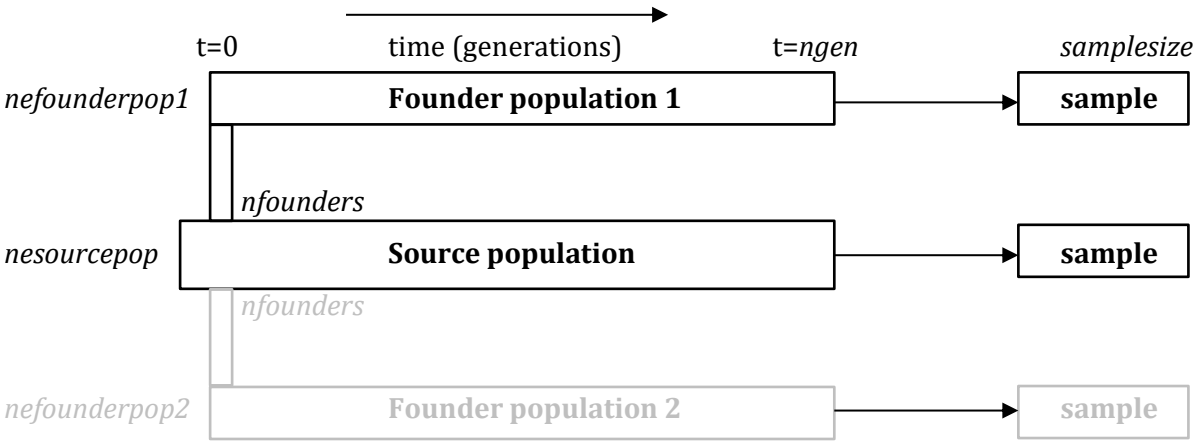
10. Simulate your data

NOTE: the simulation tool included in SambaR has limited utility and has not been tested extensively (although: see the section below titled 'Validation of simulation tool'). You are welcome to run the functions to obtain a rough impression of the expected distribution of population specific minor allele frequencies given the specified demographical scenario and the power and specificity of selection scans. However, you are also advised to also make use of established and more sophisticated methods, such as simcoal and SLIM.

outliersim()

SambaR provides a function, called outliersim, to simulate allele frequencies of neutral and adaptive SNPs in founder populations which are separated in the past from their source population due to a vicariance event, with no possibility for gene flow. The main purpose of these simulations is to estimate the power and specificity of the selection scans GWDS, PCadapt and OutFLANK given a user defined demographic model. It is assumed that all SNPs are unlinked, and that allele frequencies are affected by drift only (neutral alleles) or by a combination of drift and selection (adaptive alleles).

The general demographic model consists of a (diploid) source population which at t = 0 buds of one or two founder populations:



By default the simulations tool will run the simulations for both founder populations, and report three different outcomes: source vs FP1, source vs FP2, and source vs FP1 and FP2 combined. If the user is interested one founder population only, the results for FP2 and the combined output can simply be ignored.

The user can define constant effective population sizes of the source and founders population (*nesourcepop, nefounderpop1, nefounderpop2),* the number of founders (*nfounders*), the number of generations between the founder/vicariance event and the time of sampling (*ngen*), the sample size (number of individuals) per population (*samplesize*), and the number of SNPs (*nloc*).

The demographic model does not allow for gene flow nor time-variant population sizes. Optionally, the user can define a number of SNPs (*nselectedloci*) which experience positive selection in both founder populations with a user defined selection strength (*selcoef*). If the flag do_selectionscan is set to TRUE, selection scans are executed on the simulated data, and the power and specificity of the selection scans are estimated.

An example command:
outliersim(export=TRUE,nloc=10000,ngen=1500,nesourcepop=10000,nefounderpop1=5000,nefounderpop2=5000,nfounders=500,selcoef=0.01,samplesize=30,nselectedloci=100,do_selectionscan=TRUE,mycorrection='none')

The flag mycorrection can be used to specify the desired multiple test correction method, and accepts the values 'none', 'bonferroni', 'holm' and 'BH' (Benjamini-Hochberg).

The manhattan plot generated by outliersim, shows GWDS scores. All SNPs above the threshold (dashed line) are marked as outlier by GWDS. All points in green are marked by PCadapt as outliers. All points in orange are marked by OutFLANK as outliers.

runsim_power()

The outliersim function allows to estimate power and specificity of the selection scans GWDS, PCadapt and OutFlank for one demographic model. SambaR also provides a function, runsim_power, to generate these estimates for a range of scenarios with varying founder Ne and selection coefficient strengths. To execute this function, run:
runsim_power(n_gen=50,n_loci=1000,mycorr="bonferroni",n_selectedloci=100,selcoefvector=c(0.05,0.1),nevector=c(50,100),do_export=TRUE,dohaploid=TRUE,my_comparison=1)

The runsim_power runs the outliersim function for all combinations of founder Ne and selection strength (defined with the nevector and selcoefvector flags) and afterwards displays all power and specificity estimates in heatmaps. By default the power and specificity scores are shown for the pairwise comparison between the source population and founder population 1. Set the flag 'my_comparison' to 2 to obtain the scores for the pairwise comparison between the source

population and founder population 2. Set the flag 'my_comparison' to 3 to obtain the scores for the comparison between the source population and both founder populations combined.

The flag mycorrection can be used to specify the desired multiple test correction (MTC) method, and accepts the values 'none', ' 'bonferroni', 'holm' and 'BH' (Benjamini-Hochberg).

plotfdr()

Another option is to create line plots showing GWDS, OutFLANK and PCadapt power and specificity estimates on the y-axis and founder effective population sizes on the x-axis, for the three different MTC methods combined. These plots make it easier to compare the test performances, although the downside is that the plots are able to show the results for one selection coefficient only (default 0.1). To run the analysis, execute the command:
plotfdr(do_analysis=TRUE,do_export=TRUE,loci_nr=10000,gen_nr=20,selected_nr=1000,vector_ne=c(20,40,60,80,100,120,160,200),samples_nr=30,selection_coefficient=0.1,plot_fdr=FALSE,add_bh=TRUE,myinputmatrix=NULL,my_comp=1)
For precise estimates, set loci_nr to 100.000 and selected_nr to 10.000.

By default the power and specificity scores are shown for the pairwise comparison between the source population and founder population 1. Set the flag 'my_comp' to 2 to obtain the scores for the pairwise comparison between the source population and founder population 2. Set the flag 'my_comp' to 3 to obtain the scores for the comparison between the source population and both founder populations combined.

The plotfdr function will export into the working directory both an output plot and output tables with the power and specificity estimates. If you set the flag plot_fdr to TRUE, the output plot will also show false discovery rate estimates for the simulated data. However, these FDR estimates are not really useful, because the true false discovery rate for your empirical dataset depends on the proportion of SNPs which are under selection. This is an unknown parameter, but likely not 10%, as implied by the example command above. However, if you rerun the function, but this time with the flag do_analysis set to FALSE and with providing to the myinputmatrix flag the name of the output files (without the MTC name suffix), a new plot will be generated which shows the false discovery rate given various proportions of adaptive SNPs:
plotfdr(do_analysis=FALSE,do_export=TRUE,loci_nr=10000,selected_nr=1000,vector_ne=c(20,40,60,80,100,120,160,200),samples_nr=30,plot_fdr=FALSE,myinputmatrix="FDR_vs_correctionmethod.comp3.s0.1.nSNPs100000.samplesize30.ngen20")

The FDR estimates will be based on the Bonferroni correction.
To display power and specificity estimates excluding the Benjamini-Hochberg correction, which stands out from the Holm and Bonferroni correction, set the flag add_bh to FALSE.

Validation of simulation tool

SambaR provides four functions to generate plots which allow users to validate the reliability of the simulation tool.

To generate a plot showing simulated (points) and expected (lines) proportion of retained variation directly after a bottleneck event, depending on minor allele frequency in the source population and the number of founders (ne), execute:
runsim_retained(n_loci=1100,n_selectedloci=1000,sel_coef=0.1,sourcemafmean=c(0.01,0.1,0.4),ne=c(1,2,5),do_heatmap=TRUE,do_export=FALSE)
Expected retained variation is described by the function: $1-((1-maf)^{2ne})$.

To generate a plot showing simulated (points) and expected (lines) fixation probabilities in founder populations, depending on the minor allele frequency (q) in the source population and the selection coefficient (s), execute:
runsim_fixation(n_loci=1100,ne_F=50,n_selectedloci=1000,do_export=FALSE,n_gen=500)
Expected fixation probability is described by the function:

$$\frac{1-exp^{-2*ne\_F*q*(s+0.0001)}}{1-exp^{-2*ne\_F*(s+0.0001)}}$$

To generate a matrix with fixation probabilities as a function of founder population Ne and number of generations, execute:
runsim_fixationtime(n_loci=1100,n_selectedloci=1000,sel_coef=0.1,maf_source=0.15,do_export=FALSE)
The generated matrix can be compared to expectations from theoretical population genetics. The average time to fixation is given by the function 2/s*ln(2*Ne). If the average fixation time given a certain combination of s and Ne is for example around 100 generations, then the fixation simulated probability after 500 generations should be close or equal to 1.

The starting allele frequency distribution of the source population, prior to the founder events, is generated by letting a uniform distribution (with fixed minor allele frequency) for a certain number of generations (default: 200 generations). To check if this burn-in time generates indeed a realistic MAF distribution, execute:

multimafburnin(mymaf_means=c(0.1,0.125,0.15,0.2),maf_vector=NULL)

For comparison, the user needs to provide to the flag maf_vector an input vector with observed minor allele frequencies. Say for example that the source population in your true SNP dataset is called 'Norway', this could be: *snps$maf_Norway[snps$filter].*

11. Solve or work around errors

Please put in some reasonable efforts of trouble-shooting before consulting me for help (which of course, if needed, I am happy to provide). This includes carefully reading the manual (including the red coloured 'trouble shooting' text boxes), and carefully reading the entire screen output, which might provide clues to what is causing the error. Often errors can be circumvented by omitting a particular analysis by setting a particular flag to FALSE, as will be suggested by the screen output.

Whenever one of the main functions runs into an error, rerun the function with the flag silent set to FALSE. For example:
importdata(silent=FALSE)
This will not solve the error, but allows to locate the error. If consulting me for help, please enclose the resulting entire screen output.

Here is another suggestion for a quick and dirty work-around. It is not unlikely that encountered errors are related to non-essential function, for example plotting functions. Therefore, a work-around can be to disable the responsible the lines in the SambaR script, namely by putting a hashtag at the start of that line. How to know which lines to disable?

Say that you encounter an error whilst running:
filterdata(silent=FALSE)

Say furthermore that you get the following the screen output:
plot_indF
Error in plot.new() : figure margins too large

The screen output seems to indicate that the error is related to the function plot_indF(). Search within the SAMBAR script for the string:
if(!silent){cat("plot_indF",sep="\n")}

Next, put hashtags in front of subsequent lines, until the next occurrence of a line which contains the string '*if(!silent)*'. For example:
if(!silent){cat("plot_indF",sep="\n")}
#plot_indF(export="eps",plotname="Inbreeding")
#plot_indF(export="pdf",plotname="Inbreeding")

#plot_indF(export="png",plotname="Inbreeding")
if(!silent){cat("plotscatter_indF",sep="\n")}

Afterwards, reload the SambaR script (user the source function), and try rerunning the filterdata. Chances are that this time the function will complete without errors.

Note that, obviously, this solution only works for certain problems. If disabling vital parts of the process, SambaR can stop working correctly altogether.