

NGSphy documentation

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<http://github.com/merlyescalona/ngsphy>

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1. About NGSphy

NGSphy is a Python open-source tool for the genome-wide simulation of NGS data (read counts or Illumina reads) obtained from thousands of gene families evolving under a common species tree, with multiple haploid and/or diploid individuals per species, where sequencing coverage (depth) heterogeneity can vary among species, individuals and loci, including off-target or uncaptured loci.

2. Citation

If you use NGSphy, please cite:

- Escalona, M, Rocha S and Posada D. NGSphy: phylogenomic simulation of next-generation sequencing data . *Submitted*.
- Sukumaran, J and Holder MT. (2010). DendroPy: A Python library for phylogenetic computing. *Bioinformatics* 26: 1569-1571.

if running ART cite also:

- Huang W, Li L, Myers JR and Marth, GT. (2012) ART: a next-generation sequencing read simulator. *Bioinformatics* 28 (4): 593-594

if using SimPhy cite also:

- Mallo D, De Oliveira Martins L and Posada D. (2016). SimPhy : Phylogenomic Simulation of Gene, Locus, and Species Trees. *Systematic Biology* 65(2): 334-344.

If using single gene tree input, cite also:

- Fletcher, W and Yang Z. (2009) INDELible: A flexible simulator of biological sequence evolution. *Molecular Biology and Evolution*. 26 (8): 1879–88.

3. Getting started

NGSphy is designed to simulate reads (or read counts) from alignments originated from single gene trees or gene-tree distributions (originated from species-tree distributions). It is designed to read directly from [SimPhy](#) (a simulator of gene family evolution) in the case of gene-tree distributions, but it can also be fed with gene trees directly. These trees can contain orthologs, paralogs and xenologs. Alignments are simulated using [INDELible](#) and can represent multiple haploid and/or diploid individuals per species. Then, either Illumina reads (using [ART](#)) or read counts are simulated for each individual, with the depth of coverage allowed to vary between species, individuals and loci. This flexibility allows for the simulation of both off-target (captured but not targeted) and uncaptured (targeted but not captured) loci.

You will need a NGSphy settings file and the required files according to the input mode selected (see below).

- Examples of setting files can be found [here](#) (<https://github.com/merlyescalona/ngsphy/tree/master/data/settings>)
- For installation please go to [Section 4. Installation](#).
- For detailed explanations please search through this manual.
- In [Section 11](#), as well as in the NGSphy's wiki, you can find tutorials for each of the possible input modes (<https://github.com/merlyescalona/ngsphy/wiki/>).

3.1. Input/output files

3.1.1. Input

[Single gene-tree scenario]

- [NGSPhy settings file](#)
- [INDELible control file](#)
- [Newick](#) file with single gene tree
- ancestral sequence file ([FASTA](#)) (optional)
- [reference allele file](#) (optional)

[Species-tree scenario]

- [NGSPhy settings file](#)
- [SimPhy](#) output
- [reference allele file](#) (optional)

3.1.2. Output

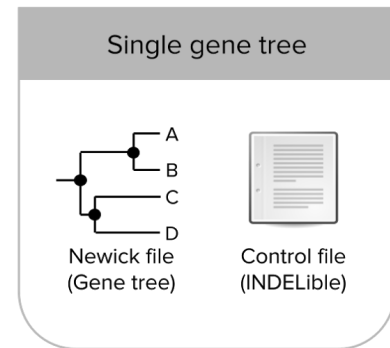
- NGS reads:
 - [FASTQ](#)
 - [ALN](#)
 - [BAM](#)

- read counts:
 - [VCF](#)
- sequence alignments:
 - [FASTA](#)
- coverage variation
 - [CSV](#)
- log files
- bash scripts

3.2. Input modes

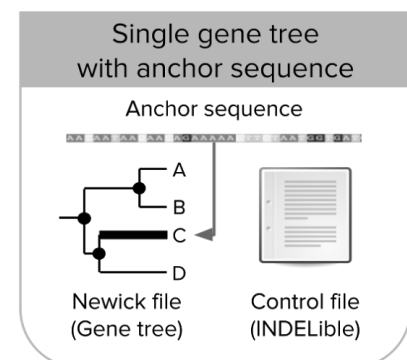
3.2.1. Single gene-tree scenarios:

- **inputmode 1:** allows you to generate DNA sequence alignments from a single gene tree, generate haploid or diploid individuals (by random mating within the same species) and produce NGS reads or read counts [[Tutorial 1](#)].



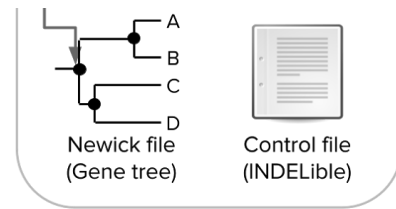
- **inputmode 2:** allows you to simulate DNA sequence alignments from a single gene tree and a known ancestral sequence. DNA sequences are evolved from the ancestral sequence under the specified gene-tree, haploid or diploid individuals and NGS reads or read counts generated [[Tutorial 2](#)].

- **inputmode 3:** allows you to simulate reads/read counts from a single gene tree and a known anchor (tip) sequence. The tree is re-rooted at the anchor sequence before the simulation of the sequence alignments [[Tutorial 3](#)].



3.2.2. Gene-tree/Species-tree distributions

- **inputmode 4:** this mode uses the output from [SimPhy](#) to generate NGS reads or reads counts. SimPhy generates distributions of gene trees and species trees under different conditions. Each species tree is here considered a replicate. For diploids, NGSphy requires that the number of SimPhy gene tree tips per species be even. If this is not the case, NGSphy will issue a warning and stop. Alternatively the user can specify that NGSphy uses the SimPhy replicates that satisfy this requirement [[Tutorial 4](#)].



D

Gene-tree distribution

4. Installation

4.1 Computer requirements

NGSphy has been developed for Linux/MAC environments with Python 2.7.

4.2 NGSphy

To install NGSphy you can:

- a) clone its git repository and download the required third-party software ([Section 4.3](#)):

```
# 1. Clone NGSphy repository
git clone https://github.com/merlyescalona/ngsphy.git
# 2. Move to ngsphy/dist folder
cd ngsphy
# 3. Extract files and install:
python setup.py install --user # if user does not have sudoer permissions
# sudo python setup.py install # if user with sudoer permissions
```

- b) install NGSphy through pip and download the required third-party software ([Section 4.3](#)):

```
pip install --user ngsphy # if user does not have sudoer permissions
# sudo pip install ngsphy # if user with sudoer permissions
```

4.3 Third-party software

4.3.1 ART (for Illumina reads generation)

ART is a set of simulation tools to generate synthetic next-generation sequencing reads. You can download it from:

<http://www.niehs.nih.gov/research/resources/software/biostatistics/art/>
Version ChocolateCherryCake or later.

Following installation instructions from ART, you can download the binaries or compile the source code. If you decide to compile the source code:

```
# 1. Extract files from the compressed tgz
cd /path/to/art-download
tar -xvf artsrcmountainier20160605linuxtgz.tgz
# 2. Change current directory to the extracted one
cd art_src_MountRainier_Linux/
# 3. Make sure you have all the dependencies installed and generate the Makefile
./configure
# 4. Run the Makefile
make
```

4.3.2 INDELible (for sequence generation)

INDELible is an application for sequence simulation. You can download it from:

<http://abacus.gene.ucl.ac.uk/software/indelible/>
Version 1.03.

In order to get INDELible, you will need to register. It is free software, and is distributed under: GNU General Public License as published by the Free Software Foundation, either version 3 of the License, or any later version. For more information go to <http://www.gnu.org/licenses/>.

Once the software is downloaded:

1. Unpack the archive on Unix-like systems using:

```
# 1. Change directory to the download folder.
cd /path/to/indelible-download
# 2. Extract file from the compressed file.
tar -xvzf INDELibleV1.03.tar.gz
```

- a. If you want to compile from source:

```
# 3. Move to the source folder
cd INDELibleV1.03/src/
```

- i. include the following line at to the top of MersenneTwister.h file.

```
#include <unistd.h>
```

- ii. Compile INDELible using:

```
# 4. Compile the program.
g++ -o indelible indelible.cpp -lm
```

- b. If you want to use the binaries, directly:

```
# This is an example for MacOS
# 3. Move to the binaries folder
cd INDELibleV1.03/bin/
# 4. Rename the binary (for proper NGSphy execution)
mv indelible_1.03_OSX_intel indelible
# 5. Modified execution permissions
chmod +x indelible
```

4.3.3. INDELible - NGSphy version (for sequence generation with known ancestral sequence)

This is a version of INDELible that we have modified to be able to use a given ancestral sequence at the root for a single partition. It can be obtained from cloning its repository:

```
# 1. Clone the repository
git clone https://github.com/merlyescalona/indelible.git indelible-ngsphy
# 2. Change directory to indelible-ngsphy source code folder.
cd indelible-ngsphy/
# 3. Compile
make
```

4.3.4. SimPhy (multiple gene trees evolved under a species tree)

SimPhy can be obtained from cloning its repository and installing its dependencies. Detailed, information on how to install SimPhy [here](#).

```
# 1. Clone the repository
git clone https://github.com/adamallo/SimPhy.git
```

4.4. Adding NGSphy and third-party software to the path

Once all software has been installed, it must be added to the path.

- First you have to add the lines below to the `~/.bashrc` file to keep the changes permanently.

```
ART="/path/to/art/executable"  
INDELIBLE="/path/to/indelible/executable"  
INDELIBLENP="/path/to/indelible-ngsphy/executable"  
NGSPHY="/path/to/ngsphy/executable"  
SIMPHY="/path/to/simphy/executable"  
export PATH="$ART:$INDELIBLE:$INDELIBLENP:$NGSPHY:$SIMPHY:$PATH"
```

- Apply changes

```
source ~/.bashrc
```


5. Usage

NGSphy does not have a Graphical User Interface (GUI) and works on the Linux/Mac command line in a non-interactive fashion.

```
usage: ngsphy [-s <settings_file_path>]
              [-l <log_level>] [-v] [-h]
```

5.1. Arguments

- Optional arguments:
 - **-s <settings_file_path>, --settings <settings_file_path>**
Path to the settings file. This is optional, by default NGSphy looks for a settings.txt file in the current working directory. You can also specify a particular settings file with:

```
ngsphy -s my_settings.txt
```
 - **-l <log_level>, --log <log_level>**
Specified hierarchical log levels that will be shown through the standard output. A detailed log will be stored in a separate file when **level == DEBUG**. Possible values:
 - **DEBUG**: shows very detailed information of the program's process.
 - **INFO** (default): shows only information about the state of the program.
 - **WARNING**: shows only system warnings.
 - **ERROR**: shows only execution errors.
- Information arguments:
 - **-v, --version**
Show program's version number and exit.
 - **-h, --help**
Show help message and exit.

NOTE: Example of the settings files can be found under the `data/settings` folder in the NGSphy source.

6. The settings file

NGSphy requires a settings file (by default “**settings.txt**”) that specifies the different options and parameter values for the simulations. A file with a different name can be specified with the **-s/--settings** option. The information in the settings file is organized in 6 optional/required blocks (default values are underlined):

1. **[general]**: general parameters.
2. **[data]**: specifies the type of input data as well as input parameters and files.
3. **[coverage]**: parameters that describe the variation of coverage in the dataset (optional).
4. **[ngs-reads-art]**: specifies ART execution parameters (optional)
5. **[ngs-read-counts]**: specifies parameters for read counts (optional).
6. **[execution]**: describes how the execution of the whole process will be made (optional).

6.1. [general] block

Stores general parameters for each NGSphy run.

```
[general]
path=/home/user/
output_folder_name=NGSphy_output
ploidy=1
```

- **path**
 - purpose: path where output folder will be created.
 - type: string (path).
- **output_folder_name**
 - purpose: name of the output folder where NGSphy results will be stored. If the output folder already exists, the new output folder will get the same base name with a numerical suffix (outputFolder_n), representing the nth time the program with that output folder name was ran.
 - type: string.
 - value: NGSphy_output
- **ploidy**
 - purpose: refers to the ploidy that the resulting individuals will have. So far it is only possible to generate haploid and diploid individuals.
 - type: number (integer).
 - values: 1, 2 (in the closed-interval [1,2]).
- **seed**
 - purpose: random number generator seed.
 - type: number (integer)
 - value in the closed-interval $[0, 2^{32} - 1]$

6.2. [data] block

Defines the input data for NGSphy, which consists of different modes:

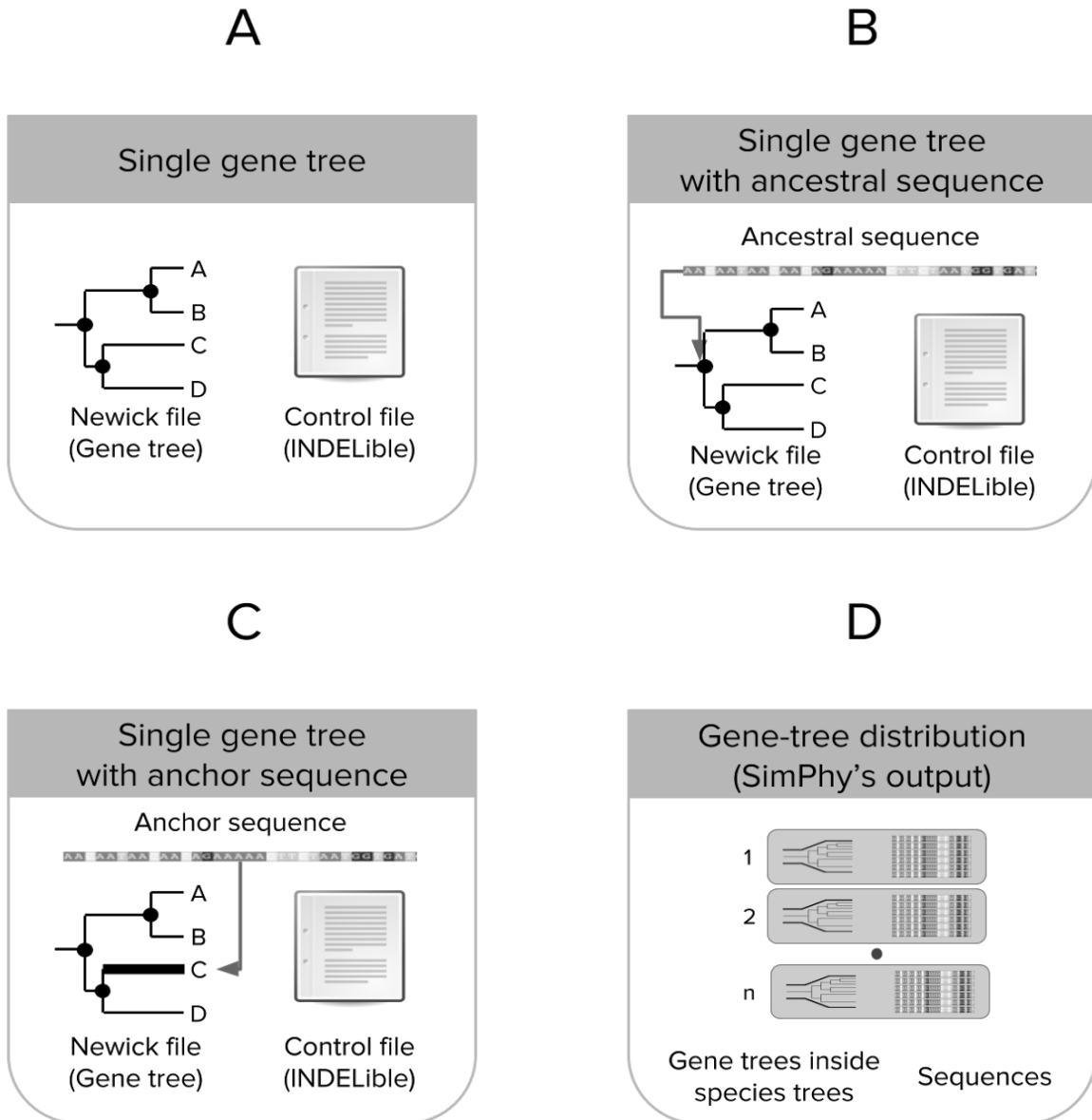


FIGURE 1: Input modes: a) a single gene tree; b) single gene tree with a user-defined ancestral sequence; c) a single gene tree with an anchor sequence and d) gene-tree distributions (SimPhy output [species-tree simulations])

6.2.1. Input data options

Single gene tree	<code>[data]</code> <code>inputmode=1</code> <code>gene_tree_file=/home/myuser/my_gene_tree.tree</code> <code>indelible_control_file=/home/myuser/my_control_indelible.txt</code>
Single gene tree with user-defined ancestral sequence	<code>[data]</code> <code>inputmode=2</code> <code>gene_tree_file=/home/myuser/my_gene_tree.tree</code> <code>ancestral_sequence_file=/home/myuser/my_ancestral.fasta</code> <code>indelible_control_file=/home/myuser/my_control_indelible.txt</code>
Single gene tree with user-defined anchor sequence	<code>[data]</code> <code>inputmode=3</code> <code>gene_tree_file=/home/myuser/my_gene_tree.tree</code> <code>anchor_sequence_file=/home/myuser/my_anchor.fasta</code> <code>anchor_tip_label=1_0_0</code> <code>indelible_control_file=/home/myuser/my_control_indelible.txt</code>
Gene-tree distribution SimPhy output (species-tree simulations)	<code>[data]</code> <code>inputmode=4</code> <code>simphy_folder_path=testSimphy</code> <code>simphy_data_prefix=data</code> <code>simphy_filter=true</code>

- **inputmode**
 - purpose: identifies the type of input.
 - type: number (integer)
 - value: values within the closed interval [1,4]
 1. single gene tree
 2. single gene tree with an ancestral sequence
 3. single gene tree with an anchor sequence
 4. gene-tree distribution (SimPhy output [species-tree simulations])

6.2.2. Single gene tree

- **gene_tree_file**
 - purpose: path of the gene tree in [Newick format](#) . There must be a single path and a single tree in the file. The name of the file, without extension, must be the same as the name of the tree within the INDELible control file, in the **[NGSPHYPARTITION]** option.
 - type: string (path)
 - format: see specification in [Section 6.2.5.](#) (INDELible control file).
- **indelible_control_file**
 - purpose: path for the INDELible control file.
 - type: string (path)
 - format: see specification in [Section 6.2.5.](#) (INDELible control file).

6.2.3. Single gene tree with an user-defined ancestral sequence

- **gene_tree_file**
 - purpose: same as in [Section 6.2.1.](#) (Single gene tree)
 - type: string (path)
- **ancestral_sequence_file**
 - purpose: path to the FASTA file that contains the ancestral sequence.
 - type: string (path)
- **indelible_control_file**
 - purpose: Same as [Section 6.2.1.](#) (Single gene tree)
 - type: string (path)

6.2.4. Single gene tree with an user-defined anchor sequence

- **gene_tree_file**
 - purpose: same as in [Section 6.2.1.](#) (Single gene tree)
 - type: string (path)
- **anchor_sequence_file**
 - purpose: path to the FASTA file that contains the anchor sequence.
 - type: string (path)
- **anchor_tip_label**
 - purpose: tip label of the gene tree that corresponds to the tip that will be used as root.
 - type: string
 - format: see specification in the [Section 6.2.6.](#) (Single gene-tree file labeling)
- **indelible_control_file**
 - purpose: Same as [Section 6.2.1.](#) (Single gene tree)
 - type: string (path)

6.2.4.1. Re-rooting process

If the user wants to use a tip sequence as the root for the alignment simulation (**anchor_sequence**), the gene tree has to be re-rooted (**anchor_tip_label**), so that simulation can proceed using the **anchor_sequence** as the root node. In the example shown in [Figure 2](#), NGSphy would transform the tree on the left into the one on the right, using as anchor tip **2_0_0**. The key observation here is that the branch length from node A to tip **2_0_0** has to become zero. Then, the re-rooted tree plus the anchor (known) sequence are given to [indelible-ngsphy](#), with the INDELible control file (format in [Section 6.2.5. INDELible control file](#)) to simulate the corresponding sequence alignments under the model from the control file.

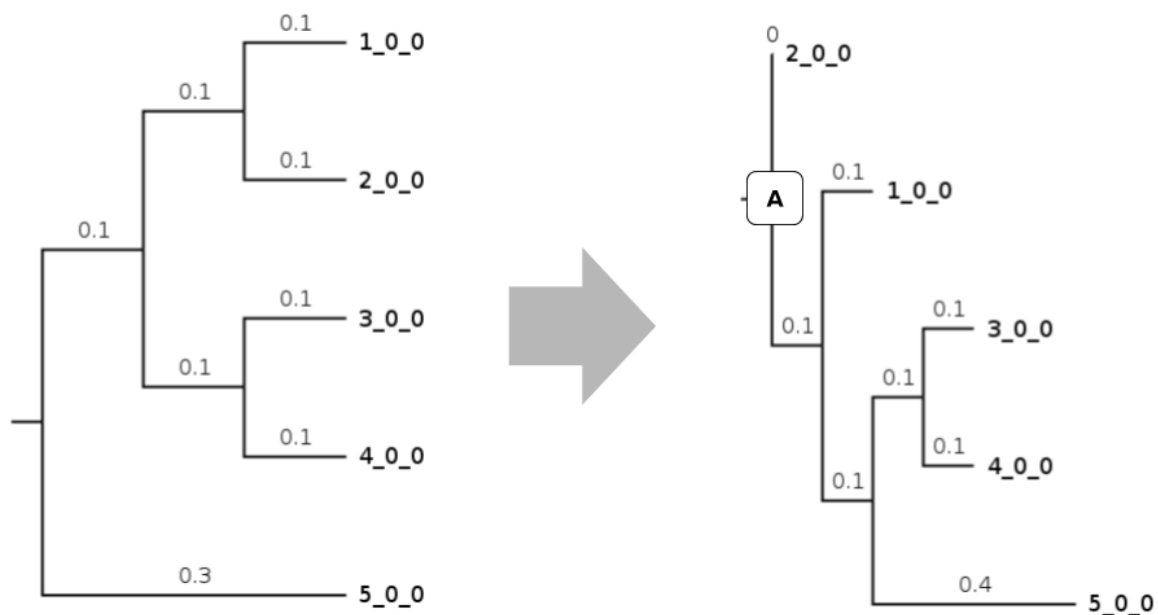


FIGURE 2: Re-rooting process. Number above branches indicate branch length.

6.2.5. Gene-tree distribution (SimPhy output [species-tree simulations])

- **simphy_folder_path**
 - purpose: path to the folder with SimPhy's output
 - type: string (path)
- **simphy_data_prefix**
 - purpose: prefix used in SimPhy's run.
 - type: string
- **simphy_filter [optional]**
 - purpose: filter out the replicates that do not satisfy the required ploidy. For the diploid case the number of gene tree tips per species has to be an even number. See more in [Section 6.2.7](#). (Individual assignment).
 - type: boolean
 - value:
 - **0, false, off:** don't filter
 - **1, true, on:** filter

6.2.5.1. A valid SimPhy output

A detailed description of SimPhy's output can be found in <https://github.com/adamallo/simphy>. The SimPhy output required by NGSphy has to include:

- **<simphy_project_name>.command:** a plain text file with the original command line arguments.

- **<simphy_project_name>.db**: a SQLite database composed by three (3) linked tables with different information about species, locus and gene trees.
- **<simphy_project_name>.params**: a plain text file summarizing the sampled options.
- a set of folders with the multiple sequence alignments and the corresponding trees in FASTA format.

6.2.6. INDELible Control file - NGSphy version

When the input mode is a single gene tree, it is necessary to have a control file to call INDELible. Here, we use a slightly modified version of the INDELible's control file. To properly set up the configuration file for INDELible, users should refer first to INDELible's [manual](http://abacus.gene.ucl.ac.uk/software/indelible/manual/) (<http://abacus.gene.ucl.ac.uk/software/indelible/manual/>). In our version, the file must include the following blocks:

- **[TYPE]**: 1 block
- **[SETTINGS]**: 1 block (optional)
- **[MODEL]**: 1 block
- **[NGSPHYPARTITION]**: 1 block

Including a wrong number of blocks or other type of blocks will result in an error message and will terminate NGSphy execution.

6.2.6.1. Block definitions

- **[TYPE]** standard INDELible specification.
- **[SETTINGS]** standard INDELible specification.
- **[MODEL]** standard INDELible specification.
- **[NGSPHYPARTITION]** this block defines:
 - the gene tree for INDELible (this name has to be the same as the Newick file used as input (see [Section 6.2](#)))
 - the substitution model for INDELible. This name must match the name of the model used in the previous **[MODEL]** block.
 - the sequence length.

For example, we have a gene tree in the Newick file: **tree1.tree**, where sequences will evolve under model **m1**, with a length of 500bp.

```
[NGSPHYPARTITION] tree1 m1 500
```

NOTE: Example of the modified INDELible control files can be found under the `data/indelible` folder in the NGSphy source.

6.2.7. Single gene-tree file format and labeling

Single gene trees in Newick format should have specific tip labels. Tips must follow a specific format in order to be managed by NGSphy. This format indicates species, locus and individual with the scheme (**X_Y_Z**) where:

- **X** stands for the the species identifier, where **X > 0**
- **Y** for the locus identifier, where **Y > 0**
- **Z** for the individual identifier, where **Z > 0**

The gene tree file must be in [Newick format](#), rooted and with branch lengths. If the gene tree is not rooted, it will be forced following [Dendropy specifications](#).

For example, if we have 3 species and 2 gene copies per species the labels would be:

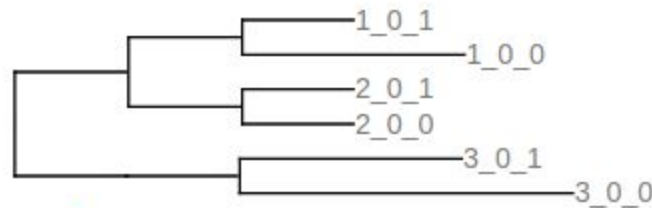


FIGURE 3: Gene tree labeling example.

```
((((1_0_1:1.0,1_0_0:2.0):1.0, (2_0_1:1.0,2_0_0:1.0)),( (3_0_1:2.0,3_0_0:3.0) ));
```

6.2.8. Assignment of individuals

For haploid individuals, each tip in the gene tree provided will correspond to a single individual. For diploid individuals the number of gene-tree tips per species must be even. In this case, the individuals are generated by randomly sampling without replacement two gene copies from a specific gene-family until all gene tree tips have been assigned to an individual.

For the gene-tree distribution input mode only, the outgroup in the gene trees is called “**0_0_0**” and has one gene copy. Therefore, for the generation of diploid individuals, the outgroup will be homozygous, obtained by the duplication of the sequence of its gene copy.

The description of the sequence(s) in the final FASTA file of the individual is formatted as:

```
>project:repID:locusID:sequence_file_prefix:indID:full_sequence_description
```

Where:

- **project**: if using any of the single gene tree input modes, it will be **NGSphy**. For the gene-tree distribution input mode, it will be the name of the SimPhy output folder.
- **repID**: replicate identifier.
- **locID**: locus identifier.
- **indID**: individual identifier.
- **sequence_file_prefix**: if using any of the single gene tree input modes, it will be **ngsphydata**. For the gene-tree distribution input mode, it will be the **simphy_data_prefix**.
- **full_sequence_description**: the description of the original sequence.

6.3. [coverage] block

Sequencing coverage can be specified at three different levels: experiment, individual and locus-wide. It is also possible to mimic the variation in coverage expected for targeted sequencing, including off-target loci and taxon-specific effects.

```
[coverage]
experiment=F:100
individual=LN:1.2,1
locus=LN:1.3,1
offtarget=0.4, 0.01 # 40% loci are off-target, will have 1% of the coverage
notcaptured=0.5
taxon= 1,0.5;2:0.25
```

6.3.1. Sampling notation

The parameters that will define the coverage in NGSphy have to be provided using a specific notation in order to define statistical distributions and dependency between arguments. The sampling notation is structured as a particular statistical distribution (see [code for the statistical distribution](#)), followed by a colon and a list of comma-separated parameter values:

```
distribution_code:param1,param2, ...
```

For example:

- a) Fixed value=100.

```
F:100
```

b) Poisson distribution with mean=100.

P:100

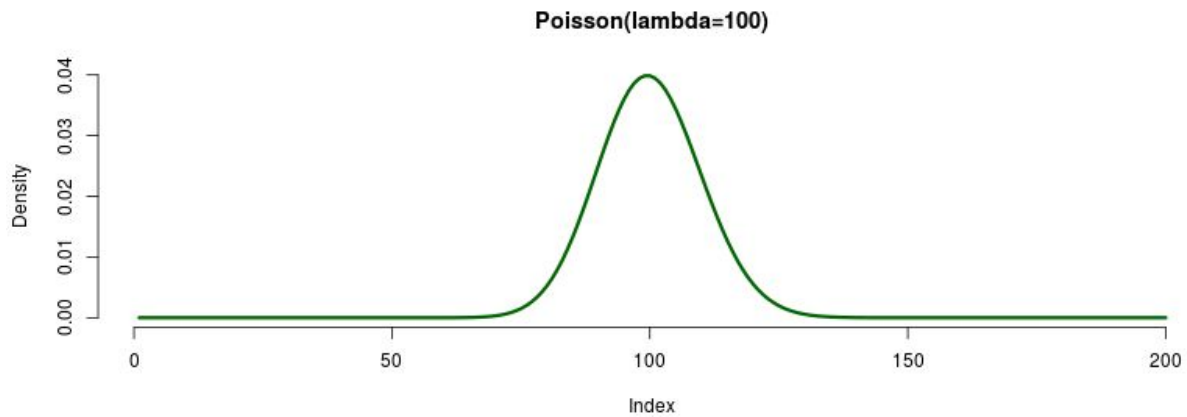


FIGURE 4: Sampling notation example. Poisson distribution.

c) Negative Binomial, mean=100 and overdispersion=10.

NB:100,10

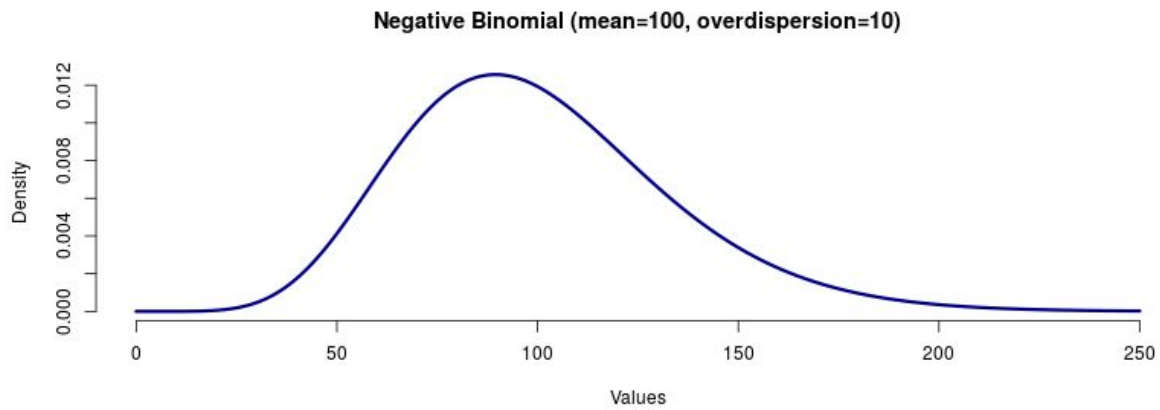


FIGURE 5: Sampling notation example. Negative Binomial distribution.

6.3.2. Statistical distributions

Distribution	Code	Num. parameters	Parameters	Description
Binomial	b/B	2	r,p	trials, probabilities
Exponential	e/E	1	s	scale
Fixed point	f/F	1	v	value
Gamma	g/G	2	sh,sc	shape,scale
Log. Normal	ln/LN	2	mu, sd	mean, standard deviation
Negative Binomial	nb/N B	2	mu, r	mean of the underlying Poisson distribution, overdispersion
Normal	n/N	2	mu, var	mean, variance
Poisson	p/P	1	l	mean
Uniform	u/U	2	min,max	minimum (included), maximum (excluded)

6.3.3. Coverage options

- **experiment**
 - purpose: expected depth of coverage for a specific replicate.
 - type: fixed value or statistical [distribution](#).
- **locus [optional]**
 - purpose: variation of expected coverage between loci.
 - type: fixed value or statistical [distribution](#).
- **individual [optional]**
 - purpose: variation of expected coverage between individuals.
 - type: fixed value or statistical [distribution](#).
- **offtarget [optional]**
 - purpose: related to targeted-sequencing experiments; percentage of loci that will be considered off-target (captured and sequenced but not originally targeted); expected coverage will be 1% of the experiment-wide.
 - type: 1 pair (proportionLoci, proportionCoverage)
 - value:
 - **proportionLoci**: number (float) in the closed interval [0,1].
 - **proportionCoverage**: number (float) in the closed interval [0,1].

- **notcaptured [optional]**
 - purpose: related to targeted-sequencing experiments; fraction of originally targeted loci that will not be captured/sequenced.
 - type: number (float).
 - value: number in the closed interval [0,1].
- **taxon [optional]**
 - purpose: related to targeted-sequencing experiments; decrease in coverage for particular species. It can be due to the phylogenetic distance between a reference species (used to design the probes for the targeted loci) and the individuals from the target-sequencing experiment or to species-specific sample conditions.
 - type: pairs (speciesID,coverageProportion)
 - values:
 - **speciesID**: one or more of the existent species in the tree.
 - **coverageProportion**: value in the closed interval [0,1].
 - format:

```
taxon=speciesID1,coverageProportion1; speciesID2,coverageProportion2 ...
```

6.3.4. Coverage sampling strategy

The **experiment-wide coverage** is sampled for each replicate from the specified statistical distribution, and this value becomes the expected coverage for every loci and individual in that replicate. For example, if experiment=P:100, we might sample a value of 104 for replicate 1, so the expected coverage would be 104x for that particular experiment.

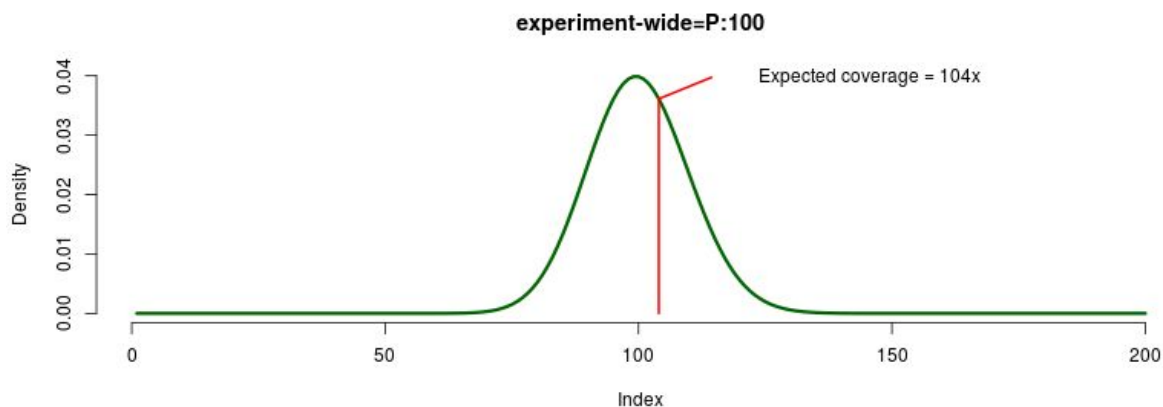


FIGURE 6: Experiment-wide coverage sampling example.

An **individual-wide** coverage multiplier is sampled for each individual within a given replicate. The value indicated in the settings file is in fact a hyper-parameter that controls a specific hyper-distribution from which a single value is sampled per replicate. For that replicate, this value will become the shape of a Gamma distribution with mean = 1, from which a multiplier is sampled for each individual.

In exactly the same manner, a **locus-wide** coverage multiplier is sampled for each locus within a given replicate. The value indicated in the settings file is again a hyper-parameter that controls a specific hyper-distribution from which a single value is sampled per replicate. For that replicate, this value will become the shape of a Gamma distribution with mean = 1, from which a multiplier is sampled for each loci.

For example, imagine we have 2 replicates, 2 loci, 2 individuals and input the following coverage settings:

```
[coverage]
experiment-wide: P:100
locus-wide: LN:1.2,1
individual-wide: E:1
```

First, we sample from a Poisson, with mean=100, to obtain the expected coverage per experiment (rep1c, rep2c).

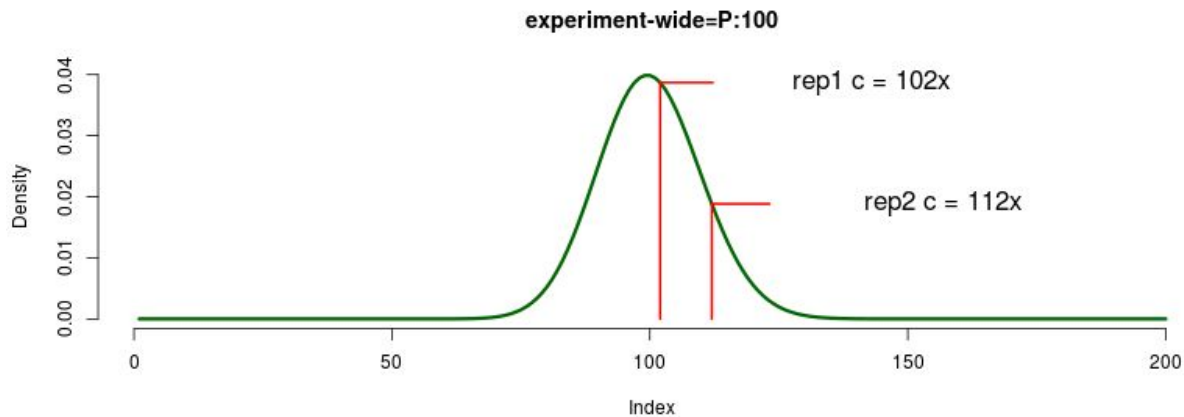


FIGURE 7: Experiment-wide coverage sampling a complex example.

- Coverage variation before locus/individual multipliers:

Replicate	Locus	Expected coverage	
		Individual I	Individual II
1	A	102	102
	B	102	102
2	A	112	112
	B	112	112

- Afterwards, we sample the **locus-wide** rate multipliers from the hyper-distribution, in this case a Log Normal with mean=1.2 and standard deviation =1 ($\text{locrep1}\alpha, \text{locrep2}\alpha$). This, give us the shape of the Gamma distribution with mean 1 from which we sample the rate multipliers, as many as loci ($\text{locAm}, \text{locBm}$).

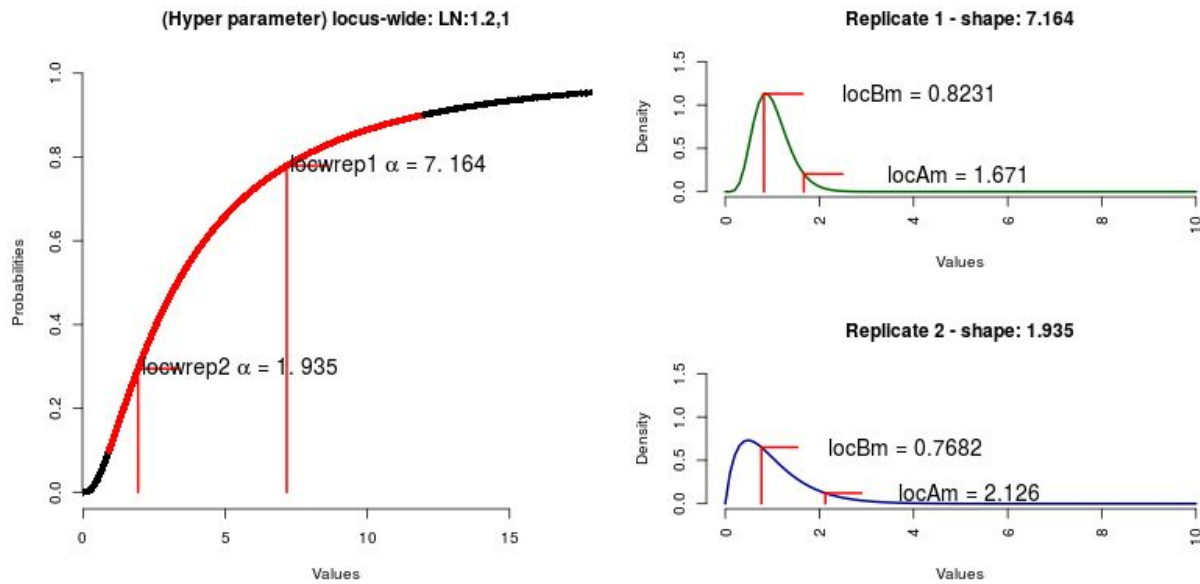


FIGURE 8: Locus-wide coverage sampling.

- Coverage variation after locus-wide multipliers:

Replicate	Locus	Rate multiplier (per loci)	Resulting coverage	
			Individual I	Individual II
1	A	1.6710	170.4420	170.4420
	B	0.8231	83.9562	83.9562
2	A	2.1260	238.1120	238.1120
	B	0.7682	86.0384	86.0384

- Next, we get the **individual-wide** rate multipliers, sampling from the hyper-distribution, an Exponential with rate 1 ($\text{indwrep1}\alpha, \text{indwrep2}\alpha$). This, give us the shape of the Gamma distribution with mean 1 from which we sample the rate multipliers, as many as individuals ($\text{indAm}, \text{indBm}$).

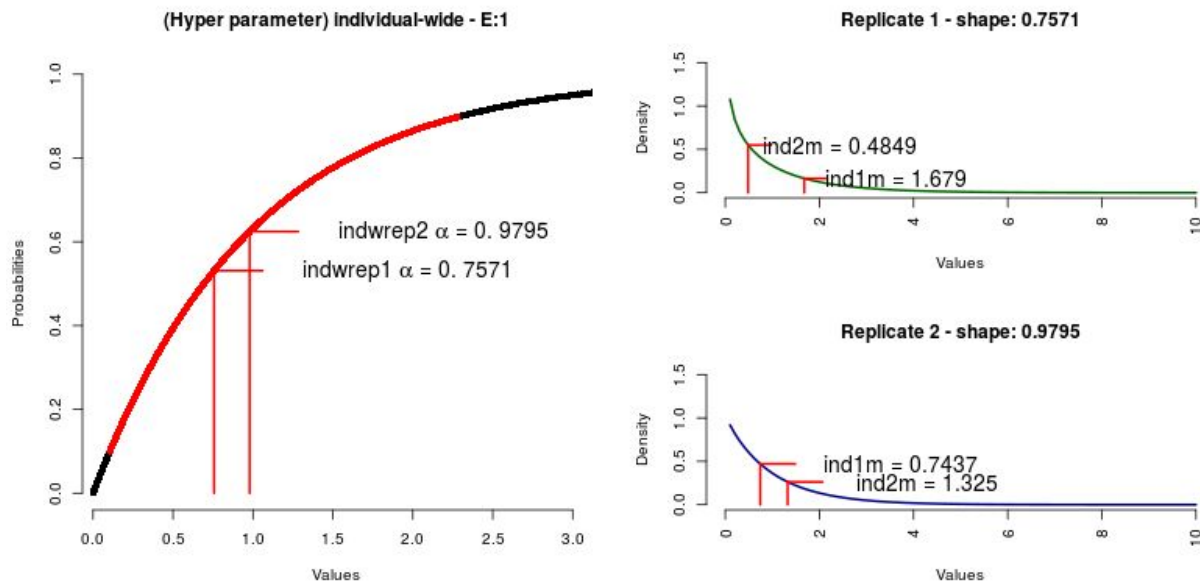


FIGURE 9: Individual-wide coverage sampling.

Finally, we apply all the multipliers. Coverage variation after locus-wide and individual-wide multipliers:

Replicate	Individuals	Rate multiplier (per individual)	Resulting coverage	
			locus A	locus B
1	I	0.4849	82.64733	40.71036
	II	1.6790	286.1721	140.9625
2	I	0.7437	177.0838	63.9867
	II	1.3250	315.4984	114.0009

Targeted sequencing parameters allow the user to emulate the variation in depth of coverage that can occur in a targeted-sequencing experiment. This is possible when using gene tree distributions (SimPhy project) as input data. These parameters identify the on-target/off-target loci as well as the number of loci that may not be captured. While on-target loci will keep their expected coverage, the off-target fraction will have a (user-defined) fraction of this. The not-captured indicates the fraction of targeted loci that will not be captured, and its expected coverage will be 0x. For example, if we have 2 replicates, 3 loci, and input the following coverage:

```
[coverage]
experiment-wide: P:100
off-target=0.33 0.1
notcaptured=0.5 # half of the on-target
```

If we consider the same coverage sampling as before, P:100:

Replicate	Locus	Category	Expected coverage	Rate multiplier	Sampled coverage
1	A	on-target	105	1	105
	B	on-target, not captured	105	0	0
	C	off-target	105	0.1	10.5
2	A	on-target	92	1	92
	B	on-target, not captured	92	0	0
	C	off-target	92	0.1	9.2

Taxon-specific effects allows the user to define of coverage variation for specific taxa. It can be used for example to emulate a decay in coverage, related to the phylogenetic distance of the a species to the reference species used to build the target-loci probes ([Bragg et al. 2016](#)) (this is sometimes called phylogenetic decay) or in a more general context for particular sample conditions (low amount of DNA, museum specimens, etc.).

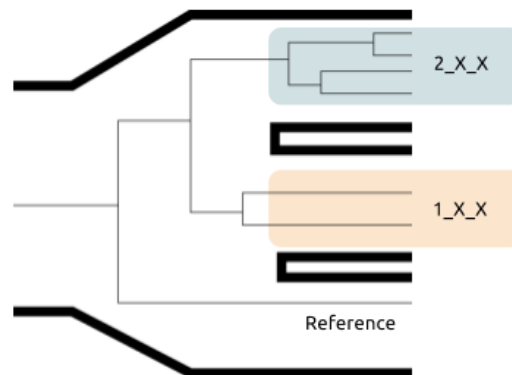


FIGURE 10: Taxon-specific coverage can be incorporated. Different clades (blue and orange) can be assigned a proportion of the experiment-wide defined coverage.

For example:

```
[coverage]
experiment: F:60
taxon=1,0.5; 2,0.25
```

Meaning that, if the expected coverage for the experiment is 60x, individuals from the species speciesID=1, will have a coverage of 30x (50% of the expected coverage) and the individuals from the species speciesID=2, will have coverage of 15x (25% of the expected coverage).

6.4. [ngs-reads-art] block

Defines the options for [ART](#). If the user specifies here any input (in,i) or output (out,o) arguments, these will be ignored since these values will be auto-generated upstream by NGSphy.

For coverage, ART offers considers two different parameterizations:

- **-c, --rcount:** number of reads/read pairs to be generated per sequence/amplicon
- **-f, --fcov:** mean fold of read coverage to be simulated for each amplicon.

Here, these parameters will be treated as Boolean (true,false) while its expected value will be set with the distributions given in the Section [6.3. \[coverage\] block](#). Units will be reads or depth of coverage according to the ART option selected.

Parameter	Possible Values	Units
-c, --rcount	True/on/1	reads
-f, --fcov	False/off/0	depth (x)

IMPORTANT: In NGSphy the coverage is defined per individual not per sequence/amplicon.

6.5. [ngs-read-counts] block

Generates a VCF file per locus per replicate, that contains the variable positions, haplotype/genotype and likelihoods.

```
[ngs-read-counts]
read_counts_error=0.1
reference_alleles_file=/home/myuser/my_reference_alleles.txt
```

- **read_counts_error**
 - purpose: to emulate sequencing error.
 - type: number (float)
 - value: value in the left-closed interval [0,1).
- **reference_alleles_file**
 - purpose: identifiers of the sequences used as reference for the variable sites.
 - type: string (path)

6.5.1. Reference allele file [optional]

Defines which alleles will be used as references to generate the VCF files. The description of the allele sequences follow the labeling explained above in [Section 6.2.5](#) (Single gene-tree file labeling). The content of the file should be formatted as:

```
repID, spID, locID, indID
```

Where:

- **repID**, replicate ID.
- **spID**, species ID (X value of the sequence description)
- **locID**, locus ID (Y value of the sequence description)
- **indID**, gene tree tip ID (Z value of the sequence description).

IMPORTANT: By default, if the reference allele file is not specified (or badly formatted), the reference allele will correspond to the sequence named 1_0_0.

6.5.1.1. Example

The simplest case will be when the input is a single tree and all individuals have the same number of loci. So, let's suppose we want to run NGSphy, with single gene tree inputmode (inputmode=1). The gene tree is the following (as in [Section 6.2.6](#)) :

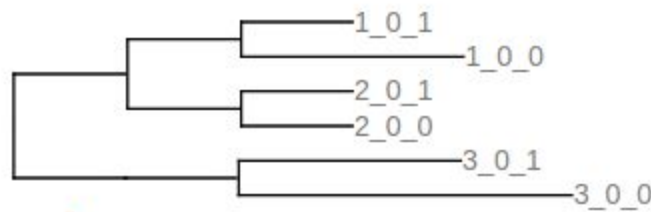


FIGURE 11: Example of gene tree with proper label notation.

Also, we want to generate read counts, with no errors, and we want to use the gene-tree tip with label “**2_0_1**” as the reference allele. The reference allele file should contain:

```
1,2,0,1
```

6.6. [execution] block

This section define how NGSphy is executed. If the user has access to a computational cluster, the ART commands can be converted into jobs for SGE or SLURM schedulers (see [Section 6.6.2](#)). If desired, ART calls can be made by NGSphy transparently to the user (sequentially or in parallel - multi-threading).

```
[execution]
environment=bash
runART=on
running_times=off
threads=4
```

6.6.1. Options

- **environment**
 - purpose: specify in which environment the ART runs are going to be executed ([more details below](#)).
 - type: enumerate (possible environments)
 - values:
 - **bash:** generates a bash file with all the commands used to call ART.
 - **sge:** generates the necessary files to run a job array in a cluster environment running Sun Grid Engine. Includes: seed file, job script and a possible script to launch ART jobs.
 - **slurm:** generates the necessary files to run a job array in a cluster environment running Simple Linux Utility for Resource Management. Includes: seed file, job script and a possible script to launch ART jobs.
- **threads**
 - purpose: number of threads to execute NGSphy.
 - type: number (integer)
 - value: 1
- **runART**
 - purpose: indicate whether the user actually wants to generate NGS reads This will only run on local, under bash environment
 - type: boolean
 - values:
 - **1, true, on:** run ART.
 - **0, false, off:** don't run ART, bash scripts will be generated.
- **running_times:**
 - purpose: obtain the running times file for the NGS mode processes (read counts or ART).
 - type: boolean
 - values:
 - **0, false, off:** don't generate file
 - **1, true, on:** generate file

IMPORTANT: the generation of this file increases the execution time of the program.

NOTES

- If the execution block is missing, a bash script will be generated and ART instances will not be run.
- If the option environment is missing, a bash script will be generated (default behavior) and ART instances will not be run, unless runART option is set.
- If the option runART is missing, ART instances will not be run.
- If the value chosen for the option run is wrong and bash is the value of environment, then ART instances will not be run.
- If the value chosen for the option environment is wrong, behavior will be as if there was no execution section, bash script will be generated and ART instances will not be run.

6.6.2. Cluster execution options (SGE,SLURM)

NGSphy can generate job templates for execution in computational clusters running Sun Grid Engine ([Gentzsch 2001](#), Oracle Corp.) or Simple Linux Utility for Resource Management ([Yoo et al. 2003](#), <https://slurm.schedmd.com/>).

In this case, NGSphy generates two files, project.XXX.sh (job script) and project.seedfile.txt (the seed-file for job arrays)

Where:

- **XXX** will be **sge** or **slurm** according to the selected execution environment.
- **project**: if using any of the single gene tree input modes, will be NGSphy. For the gene-tree distribution input mode, it will be the name of the SimPhy output folder.

To execute this one would type a different command depending on job scheduler (SGE or SLURM)

- SGE:

```
qsub -t 1-100 project.sge.sh
```

- SLURM:

```
sbatch --array 1-100 project.slurm.sh
```

Here there are some arbitrary examples of the files generated:

- SEED FILE

```
art_illumina art_illumina -ss GA2 -amp -p -sam -na -i
/home/user/NGSphy_output/individuals/1/01/testwsimphy_1_1_data_0.fasta -l 50 -f 10
-o /home/user/NGSphy_output/reads/1/01/testwsimphy_1_1_data_0_R
art_illumina art_illumina -ss GA2 -amp -p -sam -na -i
/home/user/NGSphy_output/individuals/1/01/testwsimphy_1_1_data_1.fasta -l 50 -f 10
-o /home/user/NGSphy_output/reads/1/01/testwsimphy_1_1_data_1_R
art_illumina art_illumina -ss GA2 -amp -p -sam -na -i
/home/user/NGSphy_output/individuals/1/01/testwsimphy_1_1_data_2.fasta -l 50 -f 10
-o /home/user/NGSphy_output/reads/1/01/testwsimphy_1_1_data_2_R
...
```

- SGE job script:

```
#!/bin/bash
# SGE submission options
#$ -l num_proc=1      # number of processors to use
#$ -l h_rt=00:10:00  # Set 10 mins - Average amount of time for up to 1000bp
#$ -t 1-{0}          # Number of jobs/files that will be treated
#$ -N art.sims        # A name for the job

command=$(awk 'NR==$SGE_TASK_ID{{print $1}}' $SEEDFILE)
$command
```

- SLURM job script

```
#!/bin/sh
#SBATCH -n 1
#SBATCH --cpus-per-task 1
#SBATCH -t 00:10:00
#SBATCH --mem 4G
#SBATCH --array=1-1000

command=$(awk 'NR==$SLURM_ARRAY_TASK_ID{{print $1}}' $SEEDFILE)
$command
```

IMPORTANT: Take into account that the job script files generated by NGSphy are general templates, and that in most cases they will have to be modified according to the particular cluster environments. It is strongly encouraged to consult the cluster administrator for proper execution.

6.6.3. Running times file

Generated to keep track of the timings for each ART call or each NGS read counts process. File name follows the format:

```
project.info
```

where, **project** will be **NGSphy**, if using any of the single gene tree input modes. Whereas, for the gene-tree distribution input mode, it will be the name of the SimPhy output folder.

Content of the file is formatted as follows:

```
repID,locID,indID,inputFile,cpuTime,seed,outputFilePrefix
```

- **repID**: replicate identifier.
- **locID**: locus identifier.
- **indID**: individual identifier.
- **inputFile**: path of the input file, corresponding to the individual FASTA file.
- **cpuTime**: processing time
- **seed**: if the NGS mode needs a seed for the generation of random numbers, it will be here.
- **outputFilePrefix**: prefix of the file generated.

7. Output

The output of NGSphy will depend on the NGS mode selected (ngs-read-counts or ngs-reads-art). In both cases, the user will get a detailed log file and a folder structure as:

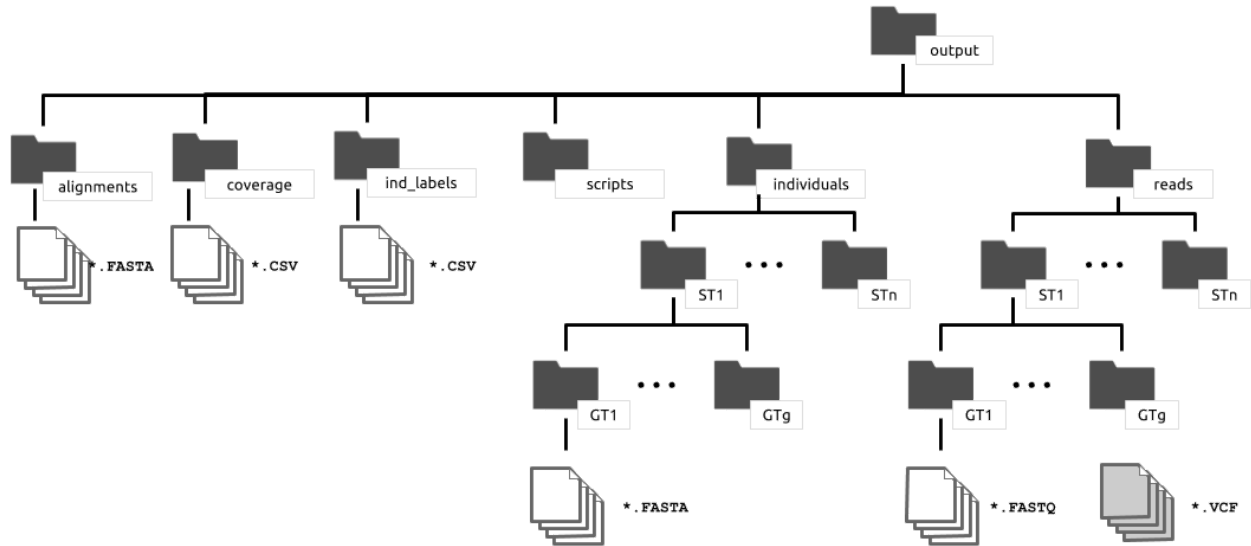


FIGURE 12: Folder structure of the NGSphy output.

Folder structure include:

1. **alignments**: for single gene tree modes, stores the alignments and files generated for the INDELible run.
2. **coverage**: stores tables describing the coverage for each locus and individual, one per replicate.
3. **individuals**: stores the FASTA files with the individual sequences. Structured along the hierarchy replicate > locus > individuals.
4. **ind_labels**: stores the correspondence between sequences and individuals.
5. **reads**: for Illumina reads, stores the ALN/BAM and/or FASTQ files generated by ART. For read counts, stores all the VCF files. Structured as hierarchy:
 - a. replicate > locus > ALN/BAM/FASTQ/VCF files
6. **ref_alleles**: stores the sequences of the references alleles used for the simulation of read counts.
7. **scripts**: stores all the bash scripts generated.

7.1. Alignments

Stores the simulated sequences in FASTA format, both unaligned (*.FASTA) and aligned (*_TRUE.fasta) together with [INDELible/INDELible-NGSphy](#) control file, ancestral sequence, and gene trees.

```
alignments/
|__ngsphy.tree # if inputmode = 3
|__NGSphy.indelible.times # if running_times=1
|__1
|   |__control.txt
|   |__ancestral.fasta # if inputmode in [2,3]
|   |__ngsphydata_1.fasta
|   |__ngsphydata_1_TRUE.fasta
|   |__LOG.txt # default indelible file
|   |__tree.txt # default indelible file
```

7.2. Coverage

This folder will contain comma-separated file (CSV) files with the coverage distribution of each individual per replicate. Each file stores a matrix of shape (number of individuals X number of loci) where each cell corresponds to the depth of coverage of the loci for the specific individual. Format of the filename is:

```
project.repID.csv
```

Where:

- **project**: if using any of the single gene tree input modes, it will be **NGSphy**. For the gene-tree distribution input mode, it will be the name of the SimPhy output folder.
- **repID**: number of the replicate.

Folder structure will look like this:

```
coverage/
|__SimOhyOutput.1.csv
|__SimOhyOutput.2.csv
|__SimOhyOutput.3.csv
|__...
```

7.3. Ind_labels

These will store the correspondence between the original sequences and the generated individuals. Each table is a CSV file named as follows:

```
project.repID.individuals.csv
```


Where:

- **project**: if using any of the single gene tree input modes, it will be **NGSphy**. For the gene-tree distribution input mode, it will be the name of the SimPhy output folder.
- **replID**: number of the replicate.

Folder structure will look like this:

```
ind_labels/  
|__NGSphy.1.individuals.csv
```

7.3.1. Haploid individuals

This folder will contain tables with the correspondence between the individual identifier and the corresponding sequence identifier. CSV file format:

```
repID, indID, spID, locID, geneID  
1, 0, 0, 0, 0  
1, 1, 1, 0, 1  
1, 2, 1, 0, 2
```

Where:

- **replID**: identifier of the replicate to which the gene trees and sequences belong.
- **indID**: identifier of the haploid individual.
- **spID**: identifier of the species.
- **locID**: identifier of the locus.
- **geneID**: identifier of the gene copy.

7.3.2. Diploid individuals

These tables will contain the correspondence between each individual and its two sequences. CSV file format:

```
repID, indID, spID, locID, mateID1, mateID2  
1, 1, 1, 0, 3, 0  
1, 2, 1, 0, 4, 1  
1, 3, 1, 0, 2, 5  
1, 4, 3, 0, 4, 0
```

Where:

- **replID**: identifier of the replicate.
- **indID**: identifier of the generated diploid individual.
- **spID**: identifier of the species.
- **locID**: identifier of the locus.
- **mateID(1&2)**: identifiers of the gene copies paired within each individual.

7.4. Individuals

This folder will store the diploid individual sequence files (i.e., 2 sequences for each locus), hierarchically organized within replicates and loci. For example:

```
individuals/
|__1/
|   |__1/
|       |__prefix_1_1_ind1.fasta
|       |__prefix_1_1_ind2.fasta
|       |__prefix_1_1_ind3.fasta
|       |__2/
|           |__prefix_1_2_ind1.fasta
|           |__prefix_1_2_ind2.fasta
|           |__prefix_1_2_ind3.fasta
|   |__2/
|       |__1/
|           |__prefix_2_1_ind1.fasta
|           |__prefix_2_1_ind2.fasta
|           |__prefix_2_1_ind3.fasta
|       |__2/
|           |__prefix_2_2_ind1.fasta
|           |__prefix_2_2_ind2.fasta
|           |__prefix_2_2_ind3.fasta
```

7.5. Ref_alleles

This folder contains the FASTA files with the reference allele sequences used in the VCF file with the read counts. Folder is structured per replicate. There is a reference allele file per locus. Each file contains a single sequence. The format of each file name:

```
project_REF_repID_locID.fasta
```

Where:

- **project**: if using any of the single gene tree input modes, it will be **NGSphy**. For the gene-tree distribution input mode, it will be the name of the SimPhy output folder.
- **repID**: replicate identifier.
- **locID**: locus identifier.

Folder structure will look like this:

```
ref_alleles/  
  |__1/  
    |__NGSphy_REF_1_1.fasta  
    |__NGSphy_REF_1_2.fasta  
    ...  
  |__2/  
    |__NGSphy_REF_2_1.fasta  
    |__NGSphy_REF_2_2.fasta  
    ...
```

7.6. Scripts

This folder will store all the scripts for ART execution, according to the options in the execution block. If we decide to run NGSphy for any cluster environment, we will have the job script and the seed file. If we choose bash as environment and we do not want to execute the ART commands within NGSphy, we would have a single bash script.

SGE	SLURM
reads/ __project.sge.sh __project.sh	reads/ __project.slurm.sh __project.sh
bash	
reads/ __project.sh	

Where, **project** will be **NGSphy**, if using any of the single gene tree input modes. Whereas, for the gene-tree distribution input mode, it will be the name of the SimPhy output folder.

7.7. NGS mode

Data will be structured per replicate.

7.7.1. NGS reads ART

This folder will store the output of ART. It follows the same folder structure of the [individuals folder](#), but instead of having FASTA files, it will contain the FASTQ files [and alignment and mapping files (ALN and SAM) if requested] generated by ART.

```
reads/
|__1/
|   |__1/
|       |__prefix_1_1_ind1_R1.fq
|       |__prefix_1_1_ind1_R2.fq
|       |__prefix_1_1_ind2_R1.fq
|       |__prefix_1_1_ind2_R2.fq
|       |__2/
|           |__prefix_1_2_ind1_R1.fq
|           |__prefix_1_2_ind1_R2.fq
|           |__prefix_1_2_ind2_R1.fq
|           |__prefix_1_2_ind2_R2.fq
|   |__2/
|       |__1/
|           |__prefix_2_1_ind1_R1.fq
|           |__prefix_2_1_ind1_R2.fq
|           |__prefix_2_1_ind2_R1.fq
|           |__prefix_2_1_ind2_R2.fq
|       |__2/
|           |__prefix_2_2_ind1_R1.fq
|           |__prefix_2_2_ind1_R2.fq
|           |__prefix_2_2_ind2_R1.fq
|           |__prefix_2_2_ind2_R2.fq
```

NOTE: Independently of the environment chosen and the value of the “runART” option, NGSphy will generate the hierarchical folder structure.

7.7.2. NGS read counts

This folder will store the output obtained from the read count simulation. This folder is structured in 2 sub-folders (with and without sequencing errors), each structured per replicate, and containing as many VCF files as loci.

Sub-folders will be:

- **no_error:** VCF files with the simulated read counts without sequencing error.
- **with_error:** VCF files with the simulated read counts with the introduced sequencing error.

```
reads
|__no_error/
|  |__1/
|  |  |__prefix_1_1_TRUE.VCF
|  |  |__prefix_1_2_TRUE.VCF
|  |  |__prefix_1_3_TRUE.VCF
|  |__2/
|  |  |__prefix_2_1_TRUE.VCF
|  |  |__prefix_2_2_TRUE.VCF
|  |  |__prefix_2_3_TRUE.VCF
|__with_error/
|  |__1/
|  |  |__prefix_1_1.VCF
|  |  |__prefix_1_2.VCF
|  |  |__prefix_1_3.VCF
|  |__2/
|  |  |__prefix_2_1.VCF
|  |  |__prefix_2_2.VCF
|  |  |__prefix_2_3.VCF
```

7.8. Other files

7.8.1. Summary log file

In the output folder there will be a file which contains a summary of the parameters used for the simulation. The name of the file will have the format:

```
NGSPHY.YYYYMMDD-HH:mm:ss.summary.log
```

- YYYY: year
- MM: month
- DD: day
- HH: hours
- mm: minutes
- ss: seconds

Here, an example of the output:

```
Settings:
  [GENERAL]
    path                : ./
    output_folder_name   : NGSphy_case1_100x_RC
    ploidy               : 2 (Diploid individuals)
    seed                : 50426717
    numreplicates        : 1
    numlociperreplicate  : 1
    filtered_replicates  : 1
    numindividualsperreplicate : 8
  [DATA]
    inputmode            : 4 (Gene-tree distribution-SimPhy output)
    simphy_folder_path   : SimPhy_usecase
    simphy_data_prefix   : data
  [COVERAGE]
    experiment           : f:100
  [NGS-READ-COUNTS]
    read_counts_error    : 0
    reference_alleles_file : files/my_reference_allele_file.case1.txt
  [EXECUTION]
    environment          : bash
    threads              : 2
    runart               : off
    running_times        : 0
```

7.8.2. Running times file

Stores information related to the time used in each ART run or read-count thread per locus. This file will contain input/output files for each process and its corresponding individual, locus (gene-tree) and replicate (REPID). See more on [Section 6.6.3](#)

Example of the file

```
1,1,0,output/individuals/1/01/test_wrapper_1_01_data_0.fasta, 0.013984,
1479977980,output/reads/1/01/test_wrapper_1_01_data_0_R
1,1,1,output/individuals/1/01/test_wrapper_1_01_data_1.fasta, 0.014757,
1479977980,output/reads/1/01/test_wrapper_1_01_data_1_R
1,1,2,output/individuals/1/01/test_wrapper_1_01_data_2.fasta, 0.013589,
1479977980,output/reads/1/01/test_wrapper_1_01_data_2_R
1,1,3,output/individuals/1/01/test_wrapper_1_01_data_3.fasta, 0.013404,
1479977980,output/reads/1/01/test_wrapper_1_01_data_3_R
1,1,4,output/individuals/1/01/test_wrapper_1_01_data_4.fasta, 0.013775,
1479977980,output/reads/1/01/test_wrapper_1_01_data_4_R
```

7.8.3. Debug file

For each NGSphy run is optional to get a debug log file. If the “-l/--log” option in the command line is set to DEBUG, the file will be generated in the current working directory and under the name:

```
NGSPHY.YYYYMMDD-HH:mm:ss.log
```

- YYYY: year
- MM: month
- DD: day
- HH: hours
- mm: minutes
- ss: seconds

This file stores information of the program execution, at a very detailed level. A debug log file will look like this:

```
13/08/2017 11:21:19 AM - ERROR (__main__|handlingCmdArguments:82):      Something
happened while parsing the arguments.
Please verify. Exiting.
```

8. Additional information

8.1. Motivation

Advances in sequencing technologies have now made very common that datasets for phylogenomic inference consist of large numbers of loci from multiple species and individuals. The use of next-generation sequencing (NGS) for phylogenomics implies a complex computational pipeline where multiple technical and methodological decisions are necessary that might influence the final tree obtained, from coverage to assembly, mapping, variant calling and/or phasing. In order to assess the influence of these variables, here we introduce NGSphy, an open-source tool for the genome-wide simulation of Illumina reads obtained from thousands of gene families evolving under a common species tree, with multiple haploid and/or diploid individuals per species, where sequencing coverage (depth) heterogeneity can be modeled across individuals and loci, including off-target loci and phylogenetic decay. Moreover, parameter values for the different replicates can be sampled from user-defined statistical distributions.

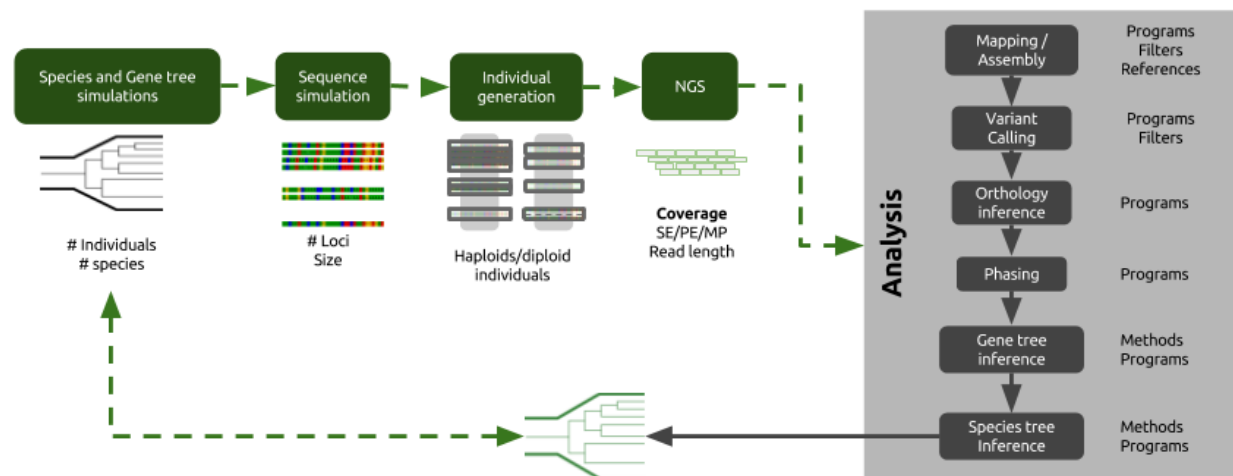


FIGURE 13: A possible analysis pipeline for multilocus, multispecies datasets with multiple individuals with the final goal of exploring the sensitivity of species tree inferences to NGS parameterization variation.

8.2. What can be done with NGSphy? The detailed scenarios

With NGSphy you can generate:

- haploid/diploid individuals from gene-tree distributions
- genome sequences of haploid/diploid individuals from a single gene tree
- genome sequences of haploid/diploid individuals from a single gene tree and an user-defined ancestral sequence
- genome sequences of haploid/diploid individuals from a single gene tree, an user-defined ancestral sequence and an anchor tip.

- NGS Illumina reads of haploid/diploid individuals
- NGS read counts of haploid/diploid individuals
- For the NGS data generation, variation of coverage due to the following:
 - variation across individuals and/or loci
 - targeted-sequencing effects
 - on/off target loci
 - on-target loci not captured
 - taxon-specific variation

8.3. Third-party software involved

8.3.1. ART

- Huang W, Li L, Myers JR, and Marth, GT (2012) ART: a next-generation sequencing read simulator. *Bioinformatics* 28 (4): 593-594

ART (<http://www.niehs.nih.gov/research/resources/software/biostatistics/art/>) is a set of simulation tools to generate synthetic next-generation sequencing reads. ART simulates sequencing reads by mimicking real sequencing process with empirical error models or quality profiles summarized from large recalibrated sequencing data. ART can also simulate reads using user own read error model or quality profiles. ART supports simulation of single-end, paired-end/mate-pair reads of three major commercial next-generation sequencing platforms: Illumina's Solexa, Roche's 454 and Applied Biosystems' SOLiD. ART can be used to test or benchmark a variety of method or tools for next-generation sequencing data analysis, including read alignment, *de novo* assembly, SNP and structural variation discovery. ART outputs reads in the FASTQ format, and alignments in the ALN format. ART can also generate alignments in the SAM alignment or UCSC BED file format.

8.3.2. INDELible

- William Fletcher and Ziheng Yang (2009) INDELible: A flexible simulator of biological sequence evolution. *Molecular Biology and Evolution*. 26 (8): 1879–88. doi:10.1093/molbev/msp098

INDELible (<http://abacus.gene.ucl.ac.uk/software/indelible/>) is an application for biological sequence simulation that combines many features. Using a length-dependent model of indel formation it can simulate evolution of multi-partitioned nucleotide, amino-acid, or codon data sets through the processes of insertion, deletion, and substitution in continuous time.

Nucleotide simulations may use the general unrestricted model or the general time reversible model and its derivatives, and amino-acid simulations can be conducted using fifteen different empirical rate matrices. Substitution rate heterogeneity can be modelled via the continuous and discrete gamma distributions, with or without a proportion of invariant sites. INDELible can also simulate under non-homogenous and non-stationary conditions where evolutionary models are permitted to change across a phylogeny. Unique among indel simulation programs, INDELible

offers the ability to simulate using codon models that exhibit nonsynonymous/synonymous rate ratio heterogeneity among sites and/or lineages.

8.3.3. SimPhy

- Diego Mallo, Leonardo De Oliveira Martins and David Posada (2015). SimPhy : Phylogenomic Simulation of Gene, Locus, and Species Trees. *Systematic Biology.*, November, syv082. doi:10.1093/sysbio/syv082

SimPhy (<https://github.com/adamallo/simphy>) is a program for the simulation of gene family evolution under incomplete lineage sorting (ILS), gene duplication and loss (GDL), replacing horizontal gene transfer (HGT) and gene conversion (GC). SimPhy simulates species, locus and gene trees with different levels of rate heterogeneity, and uses INDELible to evolve nucleotide/codon/aminoacid sequences along the gene trees. The input for SimPhy are the simulation parameter values, which can be fixed or sampled from user-defined statistical distributions. The output consists of sequence alignments and a relational database that facilitate posterior analyses.

8.4. NGSphy workflow

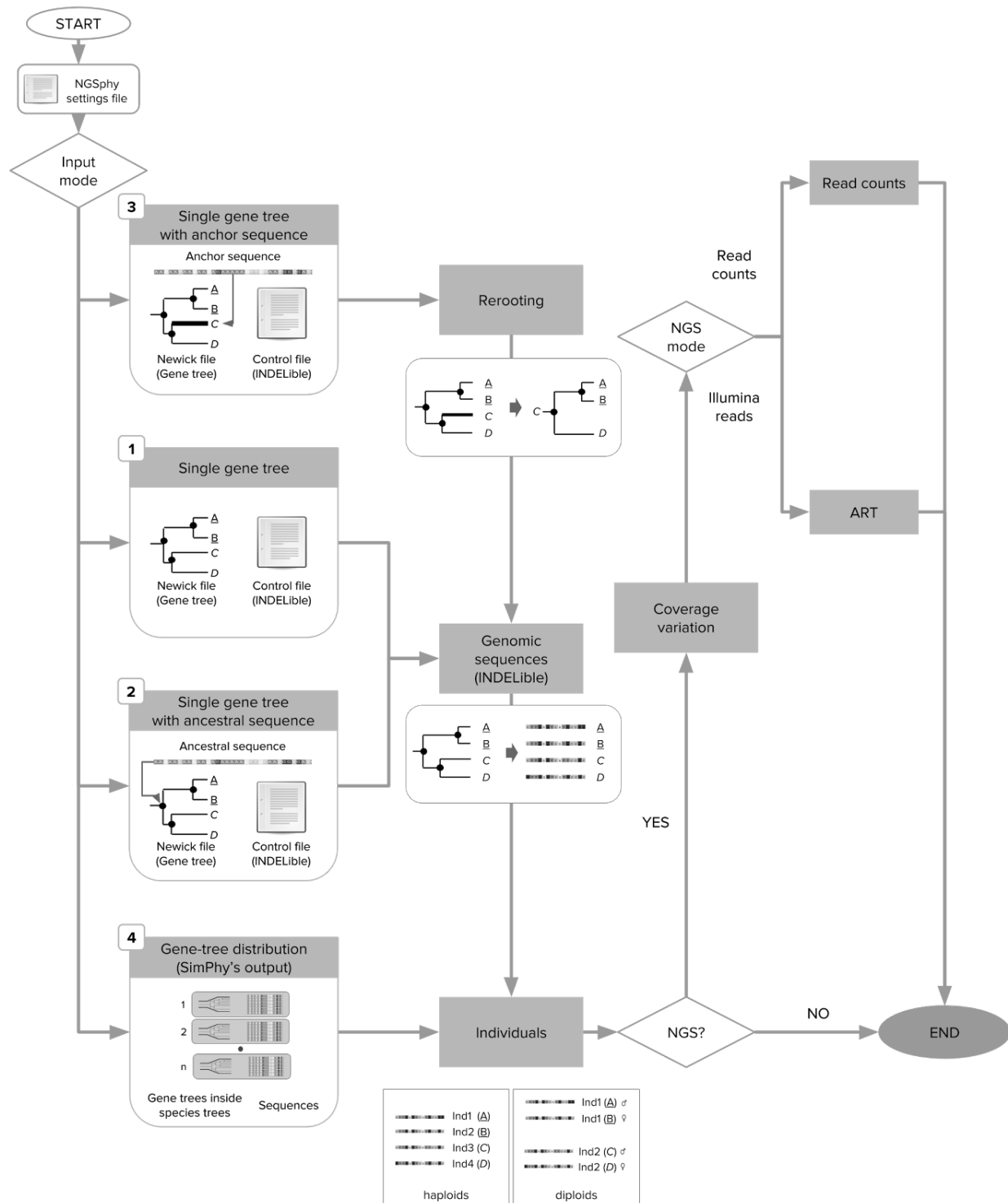


FIGURE 14: NGSphy workflow

NGSphy, verifies all the content of the project, the settings files involved and/or the existence of the corresponding third-party applications in order to run. If the input data corresponds to the single gene tree an user-defined ancestral sequence, first the tree is rooted to the selected gene-tree tip. The next step (for any single gene tree input mode) is to evolve the tree under the specific evolution mode to obtain the expected genome sequences. Then (any input mode), the *generation of individuals*, whether haploid or diploid:

- For haploid individuals, resulting genome sequences are separated into single FASTA files and identified. In addition, a file is generated with the correspondence between the individual generated and the description of the sequence it belongs to.
- For diploid individuals, there is a process of verification that the project content includes species-trees with an even number of individuals per taxa. Sequences are then "paired", individuals being generated by randomly sampling without replacement two sequences within the same gene family and species. Output will include a table for each replicate with the identifiers for the sequences paired and the individuals generated.

Afterwards, the coverage variation matrices will be computed according to the parameters introduced and finally the sequencing data generated, consist on either Illumina reads or read counts (VCF files).

- For the Illumina reads, program calls out ART, the NGS simulator, with the parameters established in the settings file and generates reads from the previously generated individuals. Resulting files depend on the settings introduced, and they are files related to the execution of the ART processes (scripts and text files), and the output of such processes (ALN, BAM and/or FASTQ files).
- For read counts, two scenarios are simultaneously computed, with and without errors.

8.5. Read count simulation

The read count approach is based on the assumption ([Ritz et al., 2011](#)) that the sequencing process is uniform in generating short reads from the target genome, and that the number of reads mapped to a region is expected to be proportional to the number of times the region appears in a DNA sample ([Ji and Chen, 2015](#)). Read counts are produced under a user-defined error rate. First, the variable sites (regarding the reference sequences) are identified. Then, coverage for each position is sampled from a Negative Binomial distribution whose mean and overdispersion parameter are the sampled coverage for the specific locus and individual. For diploid individuals, coverage is further splitted among chromosomes with equal probability. Genotype likelihoods for every site are computed as in GATK ([McKenna et al 2010](#)) (see also [Korneliussen et al. 2014](#)). The output is a set of VCF files, one per locus.

9. Getting help

Most common issues, doubts and questions should be solved by reading this manual. If that is not the case or you find any bug, you can post an issue to this repository for reproducibility purposes, with the following files attached:

- the settings file
- `<simphy_project_name>.command` file or the `indelible_control.txt` file.

10. Development and testing

This software has been developed for Linux/Mac environments and specifically tested under:

- Linux Kernel:

```
4.8.0-58-generic #63~16.04.1-Ubuntu SMP Mon Jun 26 18:08:51 UTC 2017 x86_64 x86_64
x86_64 GNU/Linux
```

- Distribution

```
Ubuntu 16.04.2 LTS
```

- Hardware

```
Dual core Intel Core i5-3427U (-HT-MCP-) cache: 3072 KB
8GB RAM
```

11. Tutorials

Here we find settings files for 4 simple test-case scenarios. They are available in `ngsphy/data/settings` (see *Escalona et al.* submitted for further details). They correspond to the 4 possible input modes. The trees, sequences, reference alleles and INDELible control files needed for their execution can be found, respectively, in:

- `ngsphy/data/trees`
- `ngsphy/data/sequences/`
- `ngsphy/data/reference_alleles/`
- `ngsphy/data/indelible/`

You can use them to check the proper installation of the pipeline and adapt them to your particular case. In addition, there is a script file for each case scenario in: `ngsphy/test`

1. [Generating read counts from a single gene tree](#)
 - a. Corresponds to `inputmode=1`
 - b. Script: `ngsphy.test.1.sh`
2. [Generating Illumina reads from a single gene tree, using an ancestral sequence](#)
 - a. Corresponds to `inputmode=2`
 - b. Script: `ngsphy.test.2.sh`
3. [Generating read counts from a single gene tree, using an anchor sequence](#)
 - a. Corresponds to `inputmode=3`
 - b. Script: `ngsphy.test.3.sh`
4. [Generating Illumina reads from gene tree distributions](#)
 - a. Corresponds to `inputmode=4`
 - b. Script: `ngsphy.test.4.sh`

NOTES:

- All setting files here described assume that:
 - a. executables are accessible from any folder, and are properly (re)named.
 - b. all the requested files are in the same directory, otherwise you will have to change the path related options accordingly.

11.1. Generating read counts from a single gene tree

Here we will generate read counts for the tips of a single gene tree. The read counts will have 0.1% of sequencing error and the expected coverage is 100x. Reference allele file is not given, thus the one with the label 1_0_0 is used (default). Output will be stored in the current working directory. Settings file looks as below and is named `ngsphy.settings.1.txt`

```
[general]
path=.
output_folder_name=NGSphy_output
ploidy=1
[data]
inputmode=1
gene_tree_file=t1.tree
indelible_control_file=control.1.txt
[coverage]
experiment=F:100
[ngs-read-counts]
read_counts_error=0.1
[execution]
environment=bash
running_times=off
threads=2
```

FILE: ngsphy.settings.1.txt

- For the generation of sequences with INDELible:

```
[TYPE] NUCLEOTIDE 1 // nucleotide simulation using algorithm from method 1
[SETTINGS]
  [ancestralprint] NEW // generates a file with the ancestral sequence
  [output] FASTA
[MODEL] m1 // no insertions, no gamma
  [submodel] HKY 2.5 // HKY with kappa=2.5
  [statefreq] 0.1 0.2 0.3 0.4 // frequencies for T C A G
[NGSPHYPARTITION] t1 m1 1000 // t1 with model m1 to generate 1000 bp long sequences
```

FILE: control.1.txt

- The tree has 4 tips, one per individual.

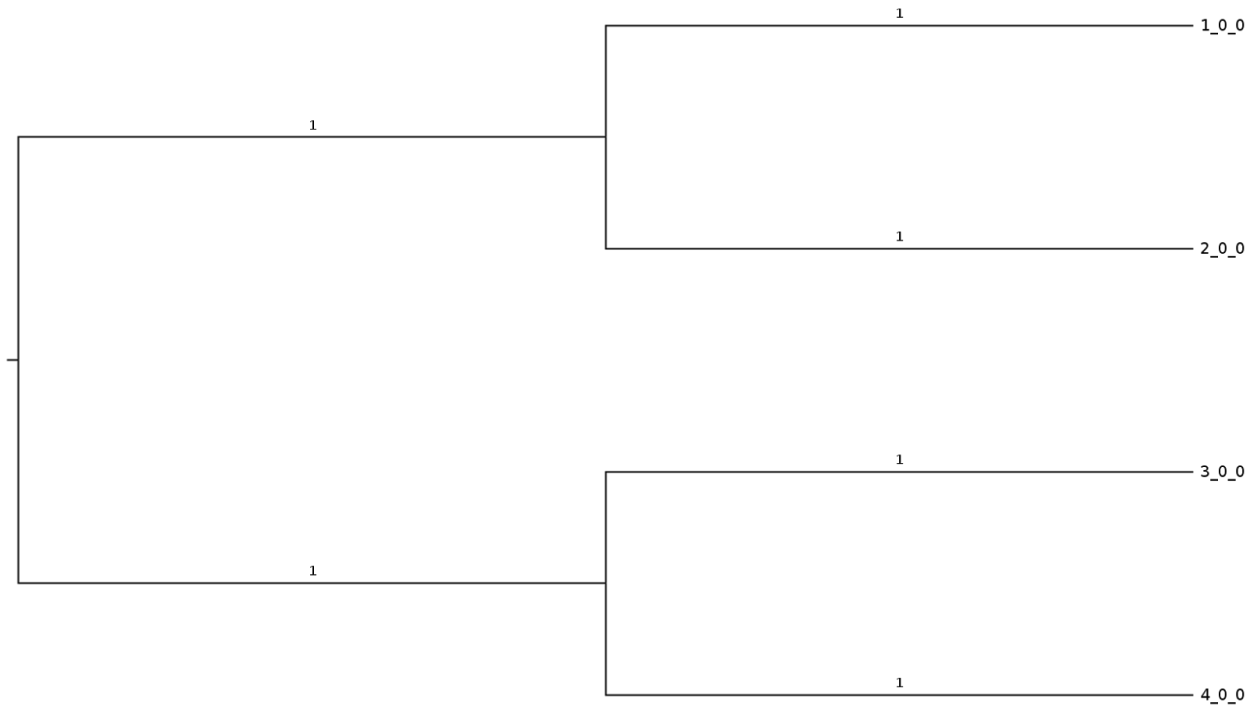


FIGURE 15: Tree file *t1.tree*

```
((1_0_0:1.0, 2_0_0:1.0):1.0,(3_0_0:1.0, 4_0_0:1.0):1.0);
```

FILE: *t1.tree*

11.1.1. Execution

To run this example, use:

```
ngsphy -s ngsphy.settings.1.txt
```

11.1.2. Output

Several output folders/files are produced under the main directory:

- **alignments:** this contains the data used and generated by INDELible.
- **coverage:** the exact coverage for each loci (L) of each individual (I). This is written into a table with dimensions (I x L). In this case, this value was fixed at 100x for all individuals.
- **individuals:** where the sequence files (FASTA) for all loci and individuals are written. There is a subfolder structure reflecting the number of replicates and gene trees. In this case, the single gene-tree *t1* has 4 tips, corresponding to 4 haploid individuals, thus we will have 4 FASTA files, each file containing a single sequence corresponding to an

individual. Here, an example of a single individual file (see more in [Section 6.2.7. Individual assignment](#)):

```
$ cat individuals/1/1/NGSphy_1_1_ngsphydata_0.fasta  
  
>NGSphy:1:1:ngsphydata:0:1_0_0  
GGCCGTGGCCCGGGGTGGGAAACGGCCGACGAAAATGGGGGAATCCAACCAGTGG....
```

- **ind_labels:** it has as many files as replicates, and stores the relation replicate/individual/species/locus/sequence. In this case:

```
$ cat ind_labels/NGSphy.1.individuals.csv  
indexREP,indID,speciesID,locusID,geneID  
1,0,1,0,0  
1,1,2,0,0  
1,2,3,0,0  
1,3,4,0,0
```

- **reads:** stores VCF with the simulated read counts. If sequence error is introduced, the VCF files without errors will also be written.

```
|__reads/  
  |__no_error/ # VCF files without sequencing error  
    |__1/ # replicate identifier  
      |__ngsphydata_1_1_NOERROR.vcf  
  |__with_error/ # VCF files with sequencing error  
    |__1/ # replicate identifier  
      |__ngsphydata_1_1.vcf
```

- **ref_alleles:** FASTA files with the sequences of the reference alleles used for the read count process.

```
|__ref_alleles/  
  |__1/ # replicate identifier  
    |__NGSphy_REF_1_1.fasta # FASTA file with reference allele sequence for  
    replicate 1, locus 1.
```

11.2. Generating Illumina reads from a single gene tree, using an ancestral sequence

Here we will simulate Illumina reads from diploid individuals evolving under a single gene tree with a known ancestral sequence. The Illumina reads will have the following characteristics:

- Machine: HiSeq2000
- 100bp PE reads
- Fragments will have mean length of 250bp (standard deviation 50bp)
- Expected coverage of 50x.

Settings file looks as below and is named `ngsphy.settings.2.txt`

```
[general]
path=.
output_folder_name=NGSphy_output
ploidy=2
[data]
inputmode=2
gene_tree_file=t2.tree
ancestral_sequence_file=my_ancestral.fasta
indelible_control_file=control.2.txt
[coverage]
experiment=F:50
[ngs-reads-art]
fcov=true
amp=true
l=100
m=250
p=true
q=true
s=50
sam=true
ss=HS20
[execution]
environment = bash
runART=off
threads=2
```

FILE: ngsphy.settings.2.txt

INDELible control file:

```
[TYPE] NUCLEOTIDE 1
[SETTINGS]
  [output] FASTA
  [ancestralprint] NEW
[MODEL] m1 // no insertions, no gamma
  [submodel] HKY 0.5 // HKY with kappa=0.5
[NGSPHYPARTITION] t2 m1 500
```

FILE: control.2.txt

The tree, in this case, has 8 tips belonging to 4 individuals of 4 species.

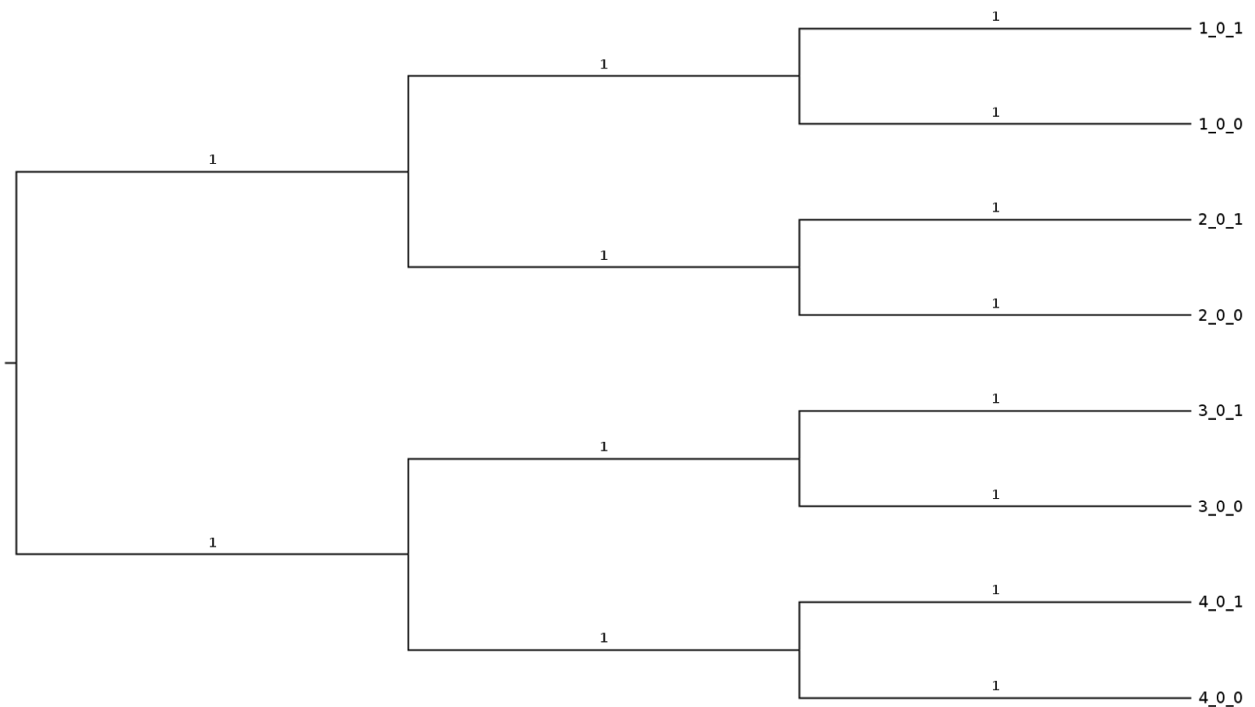


FIGURE 16: Tree file t2.tree

```
((((1_0_1:1.0,1_0_0:1.0):1.0,2_0_1:1.0,2_0_0:1.0):1.0):1.0,((3_0_1:1.0,3_0_0:1.0):1.0, (4_0_1:1.0,4_0_0:1.0):1.0):1.0);
```

FILE: t2.tree

11.2.1. Execution

To run this example, use:

```
ngsphy -s ngsphy.settings.2.txt
```

11.2.2. Output

Several output files are produced under the main directory:

- **alignments:** this contains the data used and generated by INDELib.
- **coverage:** the exact coverage for each loci (L) of each individual (I). This is written into a table with dimensions (I x L). In this case, this value was fixed at 50x for all individuals.
- **individuals:** where the sequence files (FASTA) for all loci and individuals are written. There is a subfolder structure reflecting the number of replicates and gene trees. In this case, the single gene-tree t2 has 8 tips, corresponding to 4 diploid individuals, thus we will have 4 FASTA files, each file containing a 2 sequences corresponding to an individual.
- **ind_labels:** correspondence replicate/individual/species/locus/sequence. In this case:

```
$cat NGSphy.1.individuals.csv
indexREP,indID,speciesID,locusID,mateID1,mateID2
1,0,2,0,0,1
1,1,3,0,0,1
1,2,4,0,1,0
1,3,1,0,1,0
```

- **reads:** stores the FASTQ files generated by ART. In this case the execution has been turned off (runART=off) and we obtain an empty hierarchical folder structure.
- **scripts:** file with all needed command lines to generate the Illumina reads from all the diploid individuals in ART. For medium-big datasets it is convenient to use this feature and run ART separately. The file looks like this:

```
$ cat NGSphy_output/scripts/NGSphy.sh
art_illumina -amp -l 100 -m 250 -p -q -s 50 -sam -ss HS20 --fcov 50.0 --in
/home/user/git/test-ngsphy/test2/NGSphy_output/individuals/1/1/NGSphy_1_1_ngsphydat
a_0.fasta --out
/home/user/git/test-ngsphy/test2/NGSphy_output/reads/1/1/NGSphy_1_1_ngsphydata_0_R
art_illumina -amp -l 100 -m 250 -p -q -s 50 -sam -ss HS20 --fcov 50.0 --in
/home/user/git/test-ngsphy/test2/NGSphy_output/individuals/1/1/NGSphy_1_1_ngsphydat
a_1.fasta --out
/home/user/git/test-ngsphy/test2/NGSphy_output/reads/1/1/NGSphy_1_1_ngsphydata_1_R
art_illumina -amp -l 100 -m 250 -p -q -s 50 -sam -ss HS20 --fcov 50.0 --in
/home/user/git/test-ngsphy/test2/NGSphy_output/individuals/1/1/NGSphy_1_1_ngsphydat
a_2.fasta --out
/home/user/git/test-ngsphy/test2/NGSphy_output/reads/1/1/NGSphy_1_1_ngsphydata_2_R
art_illumina -amp -l 100 -m 250 -p -q -s 50 -sam -ss HS20 --fcov 50.0 --in
/home/user/git/test-ngsphy/test2/NGSphy_output/individuals/1/1/NGSphy_1_1_ngsphydat
a_3.fasta --out
/home/user/git/test-ngsphy/test2/NGSphy_output/reads/1/1/NGSphy_1_1_ngsphydata_3_R
```

11.3. Generating read counts from a single gene tree, using an anchor sequence

In this example we use a sequence from a specific tip of the tree called anchor sequence - to root the tree and start the simulation. We will obtain sequences from the rest of the gene tree tips keeping their relationships, and then read counts for all tips with 0.1% sequencing error and 100x expected coverage. In this case the allele used as reference for the read counts is the anchor sequence (here 2_0_0; in my_anchor_sequence.fasta), but a different one can be specified.

```
[general]
path=.
output_folder_name=NGSphy_output
ploidy=1
[data]
inputmode=3
gene_tree_file=t3.tree
anchor_sequence_file=my_anchor_sequence.fasta
anchor_tip_label=2_0_0
indelible_control_file=control.3.txt
[coverage]
experiment=F:100
[ngs-read-counts]
read_counts_error=0.1
reference_alleles_file=my_reference_allele_file.txt
[execution]
environment=bash
running_times=off
threads=2
```

FILE: ngsphy.settings.3.txt

The tree:

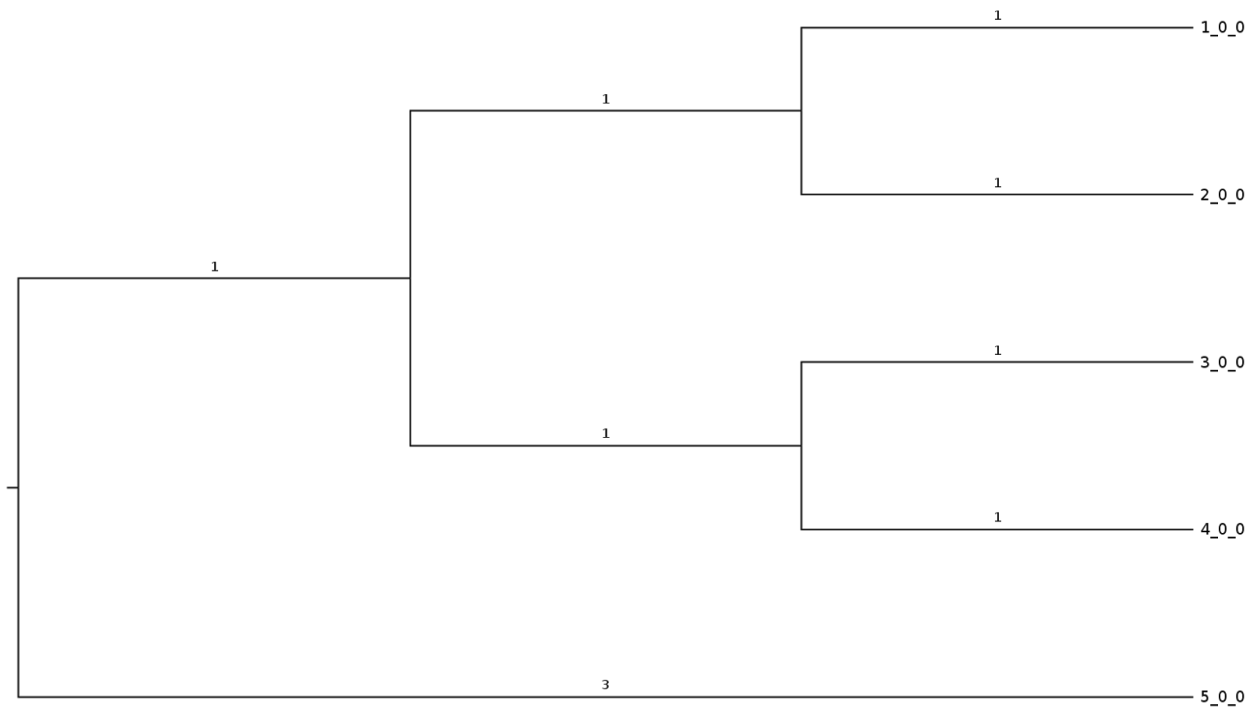


FIGURE 17: Tree file *t3.tree*

```
((((1_0_0:1.0, 2_0_0:1.0):1.0),(3_0_0:1.0, 4_0_0:1.0):1.0),5_0_0:3.0);
```

FILE: *t3.tree*

INDELible control file:

```
[TYPE] NUCLEOTIDE 1
[SETTINGS]
  [ancestralprint] NEW
  [output] FASTA
[MODEL] m1
  [submodel] HKY 0.1
[NGSPHYPARTITION] t3 m1 100
```

FILE: *control.3.txt*

11.3.1. Execution

To run this example, use:

```
ngsphy -s ngsphy.settings.3.txt
```

11.3.2. Output

- **alignments:** this contains the data used and generated by INDELible.
- **coverage:** the exact coverage for each loci (L) of each individual (I). This is written into a table with dimensions (I x L). In this case, this value was fixed at 100x for all individuals.
- **individuals:** where the sequence files (FASTA) for all loci and individuals are written. There is a subfolder structure reflecting the number of replicates and gene trees. In this case, the single gene-tree (t3) has 5 tips, corresponding to 5 haploid individuals, thus we will have 5 FASTA files, each file containing a single sequence corresponding to an individual.
- **ind_labels:** relation replicate/individual/species/locus/sequence.
- **reads:** stores VCF with the simulated read counts.
- **ref_alleles:** FASTA files with the sequences of the reference alleles used for the read count. These files will be generated whether the reference allele matches the anchor sequence or not.

11.4. Generating Illumina reads from gene tree distribution

In this more complex example, we will simulate Illumina reads from a gene tree distribution, which needs to be first obtained with SimPhy.

11.4.1. SimPhy run

For this example we will simulate 2 species tree replicates with a variable height between 200.000 years and 20.000.000 years (u:200000,20000000). These trees will have 5 ingroup + 1 outgroup species. The ingroup species will have 6 individuals per species, while the outgroup is a single individual. Each replicate will have 10 gene trees. The effective population size (10.000) of each species and the substitution rate (0.00001) of each gene tree are fixed. Finally will add heterogeneity at different levels (-h parameters). To run the simulation we use:

```
simphy -rs 2 -rl f:10 -sb ln:-15,1 -st u:200000,20000000 -sl f:5 -so f:1 -sp  
f:100000 -su f:0.00001 -si f:6 -hh ln:1.2,1 -hl ln:1.4,1 -hg f:200 -v 1 -o  
testwsimphy -cs 6656 -od 1 -op 1 -oc 1 -on 1
```

In detail:

Parameter	Value	Description
-rs	2	Number of species tree replicates
-rl	f:10	Number of locus tree per replicate
-rg	f:1	Number of gene trees per replicate
-sb	ln:-15,1	Speciation rate (events/time unit)
-st	u:200000,20000000	Species tree height (time units)
-sl	f:5	Number of taxa
-so	f:1	Ratio between ingroup height and the branch from the root to the ingroup
-sp	f:100000	Tree-wide effective population size
-su	f:0.00001	Tree-wide substitution rate
-si	f:6	Number of individuals per species

-hh	ln:1.2,1	Gene-by-lineage-specific locus tree parameter
-hl	ln:1.4,1	Gene-family-specific rate heterogeneity modifiers
-hg	f:200	Gene-by-lineage-specific rate heterogeneity modifiers
-v	1	verbosity: Global settings summary, simulation progress per replicate (number of simulated gene trees), warnings and errors
-o	testwsimphy	Common output prefix-name (for folders and files)
-cs	6656	Random number generator seed
-od	1	Activates the SQLite database output
-op	1	Activates logging of sampled options
-on	1	Activates the output of the bounded locus subtrees file

To simulate the DNA alignment for every gene tree simulated we now use the script provided with Simphy (INDELible_wrapper.pl) and the following INDELible control file.

```
[TYPE] NUCLEOTIDE 1
[SETTINGS]
    [output] FASTA
    [fastaextension] fasta
[MODEL] complex_common
    [submodel] GTR $(rd:6,16,2,8,20,4)
    [statefreq] $(d:1,1,1,1)
    [rates] 0 $(e:2) 0

[SIMPY-UNLINKED-MODEL] simple_unlinked
    [submodel] HKY $(e:1)
    [statefreq] $(d:1,1,1,1)

[SIMPY-PARTITIONS] simple [1 simple_unlinked 500] //// The first half of the gene
families will evolve under the model "simple_unlinked". Their sequence lengths are
sampled from a Normal with mean=1000 and sd=100.
[SIMPY-EVOLVE] 1 data // One sequence alignment for each gene tree, saved in files
with "dataset" as common prefix (it will generate dataset_1, dataset_2, etc.)
```

FILE: control.4.txt. This file is based on a SimPhy example case. For more information on this example please go to [SimPhy wiki](#)

To run we use:

```
# perl INDELIble_wrapper.pl <simphy_folder> <simphy's_indelible_control_file>
<seed_for_random_number_generation> <num_threads>
perl INDELIble_wrapper.pl testwsimphy/ control.4.txt $RANDOM 2
```

Here, we use the Linux environment variable \$RANDOM, which returns a different random integer in the range [0,32767]. For more information, go [here](#).

After this, we will obtain folders in a hierarchical structure which look like this:

```
|__testwsimphy/
|  |__testwsimphy.command    # SimPhy log files
|  |__testwsimphy.db        # SimPhy log files
|  |__testwsimphy.params    # SimPhy log files
|  |__1/ # Species tree replicate
|    |__data_[1-10].fasta   # INDELIble output
|    |__data_[1-10]_TRUE.phy # INDELIble output
|    |__g_trees[1-10].trees # SimPhy output
|    |__control.txt         # INDELIble_wrapper.pl output
|    |__bounded_locus_subtrees.out # SimPhy output
|    |__LOG.txt             # INDELIble output
|    |__l_trees.trees       # SimPhy output
|    |__s_tree.trees        # SimPhy output
|    |__trees.txt           # INDELIble output
|  |__2/ # Species tree replicate
|    |__data_[1-10].fasta   # INDELIble output
|    |__data_[1-10]_TRUE.phy # INDELIble output
|    |__g_trees[1-10].trees # SimPhy output
|    |__control.txt         # INDELIble_wrapper.pl output
|    |__bounded_locus_subtrees.out # SimPhy output
|    |__LOG.txt             # INDELIble output
|    |__l_trees.trees       # SimPhy output
|    |__s_tree.trees        # SimPhy output
|    |__trees.txt           # INDELIble output
```

11.4.2. Running NGSphy

Now we use NGSphy to generate the Illumina reads from the tips of these gene-trees with the settings file `ngsphy.settings.4.txt` (see below). As we want to generate diploid individuals in this case, NGSphy will randomly “mate” tips (gene-copies) within each taxa/species. Outgroup taxa has always one sequence (when it is generated in SimPhy) and is assumed to be homozygous (so its sequence is duplicated before generating reads/read counts). As the species trees generated have 6 taxa (5 ingroup + 1 outgroup), each ingroup having 6 tips, we will have 16 individuals in total:

- 6 gene-copies * 5 ingroup taxa = 30 sequences = 15 diploid individuals.
- 1 outgroup * 2 (homozygous) = 2 sequences = 1 diploid individual.

Also, we want the base coverage to be 100x for both replicates (`experiment=F:100`), but we want to add some variation among loci and individuals. This variation is in this example modeled with a Log Normal distribution with mean 1.2 and standard deviation 1 for the individuals and a Log Normal distribution with mean 1.3 and standard deviation 1 for the loci. These distributions will model the underlying Gamma distribution that will sample the rate multipliers for the specific individual and locus. For example, the multipliers for the individual variation, for each replicate, might be sampled from distributions like these, first (to the left) the shapes of the Gamma distributions, and finally from the Gamma distribution the multipliers:

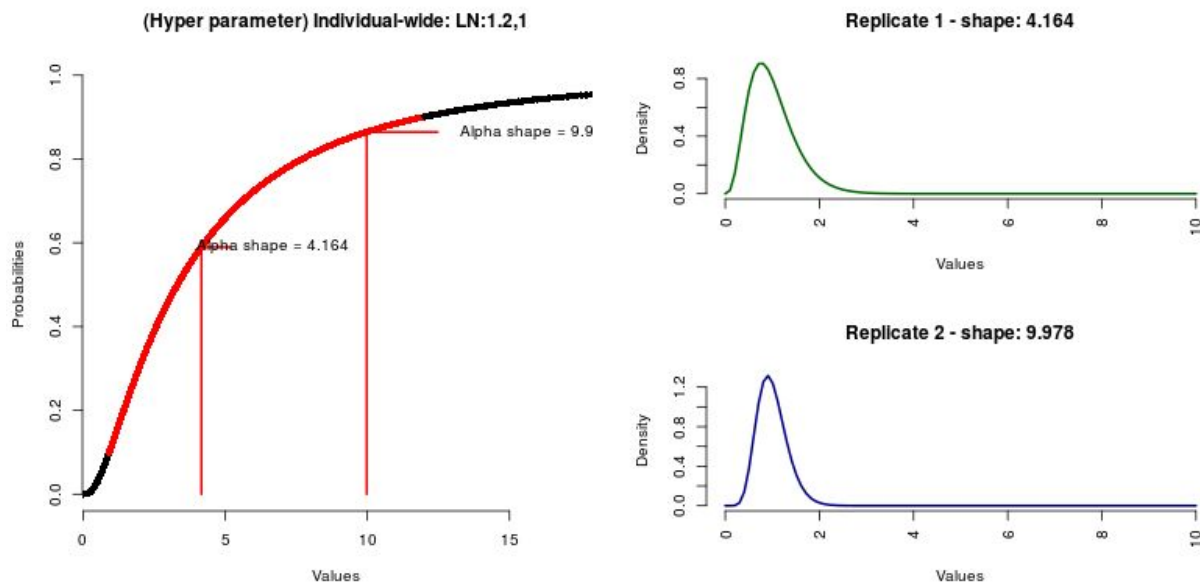


FIGURE 18: Example of possible distributions of the rate multipliers according the settings file `ngsphy.settings.4.txt` to model the coverage variation among individuals.

The file indicates the general parameters, mode of the input (gene tree distribution) and SimPhy related parameters, the sampling process of the expected coverage and the sequencing features.

```
[general]
path=.
output_folder_name=NGSphy_output
ploidy=2
[data]
inputmode=4
simphy_folder_path=./testwsimphy
simphy_data_prefix=data
simphy_filter=true
[coverage]
experiment=F:100
individual=LN:1.2,1
locus=LN:1.3,1
offtarget=0.25, 0.01
notcaptured=0.5
taxon= 1,0.5;2,0.25
[ngs-reads-art]
fcov=true
l=100
m=250
p=true
q=true
s=50
sam=true
ss=HS20
[execution]
environment = bash
runART = on
threads=2
```

FILE: ngsphy.settings.4.txt

11.4.3. Execution

To run this example, use:

```
ngsphy -s ngsphy.settings.4.txt
```

11.4.4. Output

- **coverage:** the exact coverage for each loci (L) of each individual (I). This is written into a table with dimensions (I x L). In this case, we include variation in coverage among loci and individuals as in targeted-sequencing experiments. It is also introduced the simulation of off-target loci (thus with less coverage) and some of the loci are assumed to be not captured/sequenced at all. We have 2 files, one per replicate. From the simulated species trees we get 16 individuals per replicate and 10 gene-trees (loci) each. Hence, the coverage tables have 16 x 10 dimensions (I x L). Content of the coverage file (testwsimphy.1.coverage.csv):

```
$ cat testwsimphy.1.coverage.csv
indID ,L.01 ,L.02 ,L.03 ,L.04 ,L.05 ,L.06 ,L.07 ,L.08 ,L.09 ,L.10
0 ,4.317, 401.540, 467.337, 0.000, 0.0, 222.453, 0.0, 0.0, 190.841, 286.270
1 ,7.646, 711.199, 827.737, 0.0, 0.0, 394.004, 0.0, 0.0, 338.013, 507.035
2 ,6.401, 595.411, 692.976, 0.0, 0.0, 329.857, 0.0, 0.0, 282.982, 424.486
3 ,10.602, 986.243, 1147.850, 0.0, 0.0, 546.378, 0.0, 0.0, 468.733, 703.121
4 ,15.316, 1424.710, 1658.165, 0.0, 0.0, 789.289, 0.0, 0.0, 677.124, 1015.717
5 ,8.363, 777.926, 905.398, 0.0, 0.0, 430.971, 0.0, 0.0, 369.726, 554.606
6 ,3.749, 348.727, 405.870, 0.0, 0.0, 193.195, 0.0, 0.0, 165.740, 248.618
7 ,6.680, 621.410, 723.236, 0.0, 0.0, 344.261, 0.0, 0.0, 295.339, 443.022
8 ,7.538, 701.225, 816.129, 0.0, 0.0, 388.478, 0.0, 0.0, 333.272, 499.924
9 ,3.724, 346.376, 403.134, 0.0, 0.0, 191.892, 0.0, 0.0, 164.623, 246.942
10 ,6.686, 621.916, 723.824, 0.0, 0.0, 344.541, 0.0, 0.0, 295.579, 443.382
11 ,3.334, 310.150, 360.971, 0.0, 0.0, 171.823, 0.0, 0.0, 147.405, 221.115
12 ,18.427, 1714.101, 1994.976, 0.0, 0.0, 949.611, 0.0, 0.0, 814.664, 1222.033
13 ,7.502, 697.831, 812.178, 0.0, 0.0, 386.598, 0.0, 0.0, 331.659, 497.504
14 ,6.712, 624.364, 726.674, 0.0, 0.0, 345.898, 0.0, 0.0, 296.743, 445.128
15 ,0.015, 1.401, 1.631, 0.0, 0.0, 0.776, 0.0, 0.0, 0.666, 0.999
```

- **individuals:** we will have the sequence files for the individuals generated. This case, we asked to generate diploid individuals. We will have 16 individuals per locus, and each file contains 2 sequences.
- **ind_labels:** relation correspondence replicate/individual/species/locus/sequences.
- **reads:** this folder follows a hierarchical structure like the one obtained in SimPhy, and it stores the FASTQ files generated by ART.
- **scripts:** this folder will be empty since ART is, in this example, ran within NGSphy.

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