phyloscanner

available from github.com/BDI-pathogens/phyloscanner

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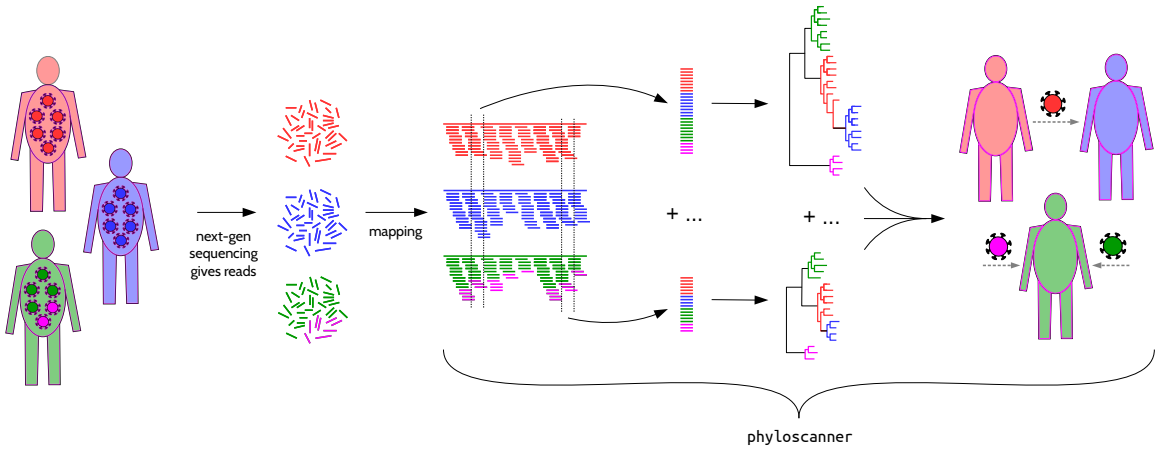


`phyloscanner` analyses pathogen genetic diversity and relationships between and within hosts at once, in windows along the genome using mapped *reads* (fragments of DNA produced by next-generation sequencing), or using phylogenies with multiple sequences per host previously generated by the user via any method. An example showing its action on simulated data (which is included with the code) can be seen at the GitHub repository linked to above.

If you have a query about `phyloscanner`, ask it publicly at the google group. If you have a problem getting the code to run or think you've found a bug, please create a *New issue* on the GitHub repository. The usual bug-reporting etiquette applies. For non-trivial bugs, to allow us to reproduce the problem it's helpful to have input that causes the problem. This needn't be all your data. For example if one read in a bam file is causing a problem, you could make a new bam file containing only that read, allowing us to see the problem very quickly. Isolate the problem as much as you can first – help us to help you! If you get an error when you run `phyloscanner` as part of a larger piece of code you've written yourself (e.g. a job submission script to a cluster), please don't make us debug that – the error might be in your code rather than ours. Isolate the problem to the relevant `phyloscanner` command.

If you use `phyloscanner`, please cite it [**?**] and its dependencies: `SAMtools` [**?**], `MAFFT` [**?**], `RAxML` [**?**] and `ggtree` [**?**]. Details of these are found in the file `CitationDetails.bib` in `phyloscanner`'s `InfoAndInputs` subdirectory.

Throughout the manual we'll use *phylogeny* and *tree* interchangeably. Forgive us.

Contents

A tool in two parts

Conceptually, `phyloscanner` can be neatly split into two steps. Firstly, inferring phylogenies that contain both within- and between-host diversity, in windows along the genome, using mapped reads as input. Secondly, analysing those phylogenies or other similar ones (i.e. containing within- and between-host diversity, but created however the user likes) in order to infer interesting things like transmission and multiply infected individuals, and give summaries of diversity.

This conceptual split is reflected in the code: `phyloscanner_make_trees.py` runs the first step and `phyloscanner_analyse_trees.R` runs the second step. Why didn't we merge the two steps into one?

- For some uses, step one may not be needed. For example if you don't have mapped reads, but have sequence data in another format. We presented an example of this in the `phyloscanner` paper: a dataset consisting of multiple whole genomes of *Streptococcus pneumoniae* for each person carrying the bacterium, obtained by separately sequencing multiple colonies.

- For some uses, step two may not be needed. Having extracted the within- and between-host diversity information in each window (first as a multiple sequence alignment and then as a phylogeny), you might want to do your own totally different analysis of it.

- Each step is controlled by parameters and options to tailor its usage to different kinds of data, and we don't (yet) have a way to automatically optimise these for each data set, so you will need to have a little play to explore what's appropriate in your case. If you are running both steps, it's best to think first about getting step one right first, and then getting step two right. Step one is primarily about bioinformatics, step two about phylogenetic analysis.

Note that to run either `phyloscanner_make_trees.py` or `phyloscanner_analyse_trees.R`, like running any executable file, you must either specify the full path to the executable (e.g. `/path/to/my/phyloscanner/code/phyloscanner_make_trees.py`) or add the directory that contains the executable to the `$PATH` environment variable in your terminal (google this if needed). In the manual we'll refer to scripts named like `tools/SomeCode`; such scripts live in the `tools` subdirectory inside the main `phyloscanner` code directory.

Fun fact: step one was written by Chris, step two mostly by Matthew. We sat next to each other while coding, and we hope that's reflected in how easy it is to make the two steps work together. If it isn't, let us know.

Part I
Inferring within- and between-host phylogenies in windows along the genome, using mapped reads

1 The basic command

As input to `phyloscanner_make_trees.py`, you need to have generated files of mapped reads in bam format – bam files henceforth. The bam format does not include the sequence to which the reads were mapped – the *reference* – which we also need. With the initial `$` traditionally indicating that what follows is a command to be run on the command line / in a terminal (i.e. don't type that initial `$`), the basic `phyloscanner_make_trees.py` command looks like

```
$ phyloscanner_make_trees.py ListOfMyInputFiles.csv --windows 1,300,301,600,...
```

where

- `ListOfMyInputFiles.csv` is a plain-text, comma-separated-variable (csv) format file in which the first column contains the bam files, the second column contains the corresponding reference files. An optional third column, if present, contains *aliases* – things to rename each bam file to in `phyloscanner_make_trees.py` output; if not present the base name of the bam, i.e. the file name not including the directory path, will be used. e.g. This input file list might look like
  `PatientA.bam,PatientA_ref.fasta,A`
  `PatientB.bam,PatientB_ref.fasta,B`
  Bam file base names, and aliases if present, must be unique and free of whitespace. Quotation marks are interpreted as wrapping/protecting fields, allowing them to contain commas that are not field separators. You can generate this input file list however you like, including manually. The following block of code illustrates how you might generate it automatically from the command line. It also checks that your files exist, which `phyloscanner_make_trees.py` does anyway, but it's always best to catch your errors soonest.

  ```
  $ for ID in PatientA PatientB PatientC; do
  $   bam=MyBamsDir/"$ID".bam
  $   ref=MyRefsDir/"$ID".fasta
  $   if [[ ! -f "$bam" ]]; then
  $     echo "$bam does not exist" >&2
  $     break
  $   elif [[ ! -f "$ref" ]]; then
  $     echo "$ref does not exist" >&2
  $     break
  $   fi
  $   echo "$bam","$ref","$ID"
  $ done > MyPhyloscannerInputFile.csv
  ```

  If those IDs were stored in a plain-text file `MyIDs.txt` separated by whitespace, you could replace the first line of that loop with
  `for ID in $(cat MyIDs.txt); do`

- The `--windows` (or `-W`) option is used to specify an even number of comma-separated positive integers: these are the coordinates of the windows to analyse, interpreted pairwise, i.e. the first two are the left and right edges of the first window, the third and fourth are the left and right edges of the second window, ... i.e. in the above example we have windows 1-300, 301-600, ...

You shouldn't run multiple `phyloscanner_make_trees.py` commands simultaneously in the same directory, as it writes to and reads from files with fixed names, so the commands could clash. If you're running

multiple jobs in parallel on a computing cluster that starts jobs in the same place, ensure that each job runs in a different directory (e.g. using the `mkdir` and `cd` commands to make and change into a directory specific to that job).

1.1 The Python version you use

Note that `phyloscanner_make_trees.py` can either be run like this:
`phyloscanner_make_trees.py [input and options]`
or like this:
`python phyloscanner_make_trees.py [input and options]`
In the former case, the version of Python used to execute `phyloscanner_make_trees.py` is whatever version gets called when you simply run the command `python` in the terminal. In the latter case, you can explicitly replace `python` by a specific version of Python on your machine, for example `python2.7` or `/usr/bin/python`. The version of Python that's used must (a) be Python 2, not Python 3, and (b) contain the Python dependencies described in `phyloscanner/InfoAndInputs/InstallationNotesForMakingTrees.sh`. The version of Python used can be specified the same way for all of the Python tools in the `tools` subdirectory.

2 What do the window coordinates mean exactly?

By default the references used for mapping, together with any extra references if included using the `--alignment-of-other-refs` option, are all aligned together and window coordinates are interpreted with respect to the alignment (i.e. position n refers to the nth column of that alignment, which could be a gap for some of the sequences). This alignment can be found in the file `RefsAln.fasta` after running `phyloscanner_make_trees.py`, should you want to inspect it and possibly run again. You can manually specify window coordinates with respect to this alignment, using the `--windows` option, or have windows automatically chosen using `--auto-window-params`, which attempts to minimise the affect of insertions and deletions in the references on your window width and overlap preferences. Alternatively, if you are using the `--alignment-of-other-refs` option to include extra references, you can use `--pairwise-align-to` to name one of these references to be a kind of *reference reference*: instead of aligning of all the bam file references to each other, they will be sequentially and separately pairwise-aligned to your named reference, and window coordinates are interpreted with respect to that named reference (i.e. position n refers to the position of the nth base of the named reference, not counting any gaps inside the reference). Using the `--pairwise-align-to` option is expected to more stable than `--windows` or `--auto-window-params` if your bam file references are many and diverse, since pairwise alignment is easier than multiple sequence alignment. It also has the advantage that when running `phyloscanner_make_trees.py` more than once with different bam files, the coordinates mean the same thing each time.

3 What windows should I choose?

I'm glad you asked. It's important.

3.1 Start & end

You might as well fully cover the genomic region you're interested in. That requires choosing where to start and where to end. If you're interested in the whole genome, the start is 1 and the end is the genome length, or more precisely the length of the alignment of all references together (or the length of your named reference if you used the `--pairwise-align-to` option). You may know that your reads don't start right at the beginning of the genome. If this is the case, a good place to start your first window would be the genome position at which you start having reads.

If your reads were generated by amplifying the sample using primers, the primers should ideally have been trimmed from the reads as part of whatever bioinformatic pipeline produced your input bam files. (This can be done for example using `fastaq`, which is called as part of the `shiver` pipeline.) Then a sensible choice for the start of the first window would be the first position after the first primer, and a sensible choice for the end of the last window would be the last position before the last primer.

You might as well fully cover the genomic region you're interested in. Let's revisit that. In what part of the genome would you like to do phylogenetics? Perhaps all of it. Then again, remember that phylogenetics assumes neutral evolution at every included site. Though `phyloscanner_make_trees.py` allows individual specified sites to be excised after alignment of all the reads from all the bam files, there may be regions of the genome where selected sites are so dense that it's simpler to skip the region altogether. There may also be regions of the genome where there are so many insertions and deletions (indels) that you're sceptical that the reads will align correctly in the first place. You can choose to specify no windows covering such regions.

3.2 Window width

As well as choosing where your first window should start and where your last one should end, you need to choose how wide each window is. On the one hand, increasing the width of a window increases its phylogenetic resolution. To see this effect using just some existing reference sequences (forgetting momentarily about any read data you have), the script `tools/CalculateTreeSizeInGenomeWindows.py` could be useful: it calculates trees from references in sliding windows along the genome. (It then calculates patristic distances, for a purpose discussed later in section **??**.) You may be able to see from visual inspection, or from some automated analysis of your own, that one's ability to construct well-resolved phylogenies increases with window width. On the other hand, a read must fully span a window to be included in it, so the wider you make a window the fewer reads it will contain. To quantify this effect, you can run

```
$ tools/EstimateReadCountPerWindow.py ListOfMyInputFiles.csv
```

(where `ListOfMyInputFiles.csv` is the same file as we discussed at the top of section **??**). This command uses the information in each bam about the number of reads and how long[1] they are; it then estimates the number of reads expected to span a window of width W by assuming reads are distributed randomly over the whole genome (ignoring the actual read location information in the bam files). If reads are found to be paired, the calculation is also done in the context of `phyloscanner_make_trees.py`'s `--merge-paired-reads` option, which merges each pair of reads into a single longer read if they overlap with each other (this will give exactly the same result as considering reads separately if reads in a pair never overlap). The calculation is done as a function of W, showing how the number of reads decreases monotonically[2]. A good choice of window width, roughly, would be as large as you can get before the number of reads per window gets too low to make useful within-host phylogenies. Skip ahead to section **??** if that's good enough for you and you don't want to think about it any more.

The `--explore-window-widths` option of `phyloscanner_make_trees.py` was written with the following balancing act in mind. If a window is too wide, too few reads overlap it, as discussed. However if it is too small, there aren't enough positions at which a variant can be observed and so few *unique* reads will be observed in the window. Somewhere between these two extremes therefore maximises the number of unique reads. The `--explore-window-widths` option reports, for each in a list of window widths to try, a two-dimensional matrix of count values – the number of unique reads found in each bam and in each successive window along the genome. One way of visually summarising this matrix of counts as a function of window widths is to give the file containing this data as the argument to `tools/PlotExplorationOfWindowWidths.py`, which calculates and plots various percentiles of the matrix of counts. e.g. for a given window width, the 50th percentile of the counts means that half of all the windows from all the bams had more unique sequences than this, and half had fewer. Anecdotally, for our HIV data, I did not find that maximising the number of unique sequences per window gave the best results: manually examining phylogenies revealed that a larger window width produced more revealing

[1] In this particular instance we define read length as the length of the mapping reference covered by the read, from its start to its end. This can differ from the actual read length due to insertions or deletions in the read relative to the reference. Defining the read length this way is appropriate since we're interested in reads spanning windows, and windows get defined relative to the reference. This length will also differ from the nominal read length of your sequencing run (which is typically one fixed value) if the reads have been trimmed as part of bioinformatic processing, e.g. removing primers, adapters, low-quality bases, or bits of the read that could not be mapped.

[2] The number of positions at which we could place a window of width W equals the length of the reference $-W+1$. The number of positions at which we could place a window of width W such that it is wholly inside a read is the length of the read $-W+1$. The probability of a randomly placed read overlapping a window of width W is therefore (read length $-W+1$) / (reference length $-W+1$). The number of reads and their different lengths are fixed in the data; increasing W will decrease this probability (except for reads that span the entire reference).

trees. But this option can still be helpful for quantifying the decline in how many unique sequences you get as window width is increased out towards the largest possible values.

The regular `--explore-window-widths` option calculates the number of unique reads after all read processing has been done, including aligning the reads from all bams using mafft, and other options you may have specified that affect how many unique reads survive, for example `--excision-coords`, `--merging-threshold`, `--min-read-count`, `--quality-trim-ends`, `--min-internal-quality`, and `--discard-improper-pairs` options. The first two of these can result in two or more unique reads being merged into one; the rest can simply discard some reads. You could choose values for the associated parameters immediately and then use `--explore-window-widths`, or else come back to `--explore-window-widths` later on once you've got the hang of `phyloscanner_make_trees.py` and investigated the effect of those other options in your data.)

There is also the `--explore-window-widths-speedy` option which is almost identical: the difference is that this counts the number of unique reads in the window straight away after they are extracted from the bam, before any processing of them has been done (including aligning them all with `mafft`). If you don't care about read processing steps that change the number of unique reads, use this because it's faster.

Power users might want to optimise their own measure of phylogenetic information as a function of window width; one of the first metrics to pop into your head might be the mean bootstrap of all nodes in the tree. That's not advised because within a sample there may be many very similar sequences, and the set of nodes connecting these may have poor bootstrap support, but this is not something that ought to be penalised. Also in theory you might be able to increase the window width until only a single read is found spanning the window in each patient; your bootstraps might then be great, because between-host diversity is greater than that within-host, but you've thrown out all the within-host information.

3.3 Window overlap

How much should neighbouring windows overlap?

- A good, simple answer to this is zero, i.e. each window starts right after the previous one ends. e.g. 1-99, 100-199, 200-299, ...
- Negative overlap means unused space in between windows, e.g. 1-99, 200-299, 400-499, ... The only reason for this is if you want to exclude certain parts of the genome. (Though it's hard to imagine that the regions you want to exclude are perfectly regularly spaced.)
- Positive overlap means the next window begins before the current one ends, e.g. 1-99, 50-149, 100-199, ... There's a redundancy in doing this, because you're analysing the same information multiple times. However there's a little bit of stochasticity involved when performing algorithmic multiple sequence alignment then phylogenetic inference, so even if two windows overlap heavily some minor differences in the results could arise by chance. Positive overlap may therefore smooth out some of this stochasticity, at the expense of additional compute time. (You'll also need to take into account the non-independency of overlapping windows if you interpret results from multiple windows in a probabilistic framework.)

4 What output files are produced?

By default, those files that are produced for each window are grouped into subdirectories by file type.

- `RefsAln.fasta`. This contains the mapping reference from each bam file, together with any extra references included with `--alignment-of-other-refs`. This alignment is not created if `--pairwise-align-to` is used.
- `AlignedReads/AlignedReadsInWindow_X_to_Y.fasta` (where X and Y are window coordinates). Contains the reads from all bam files in the window X-Y, after processing and alignment, but before excision of any positions specified with `--excision-coords`. Reads are automatically assigned a name of the form *A_read_M_count_N*, where A identifies the bam from which the read came, N is the number of times that read was found (either identical copies of the same sequence, or merged similar sequences if `--merging-threshold` is used), and M is the rank of the read when they are ordered by count (i.e. $M = 1$ is the most common read, $M = 2$ the second most common etc.)

- `AlignedReads/AlignedReads_PositionsExcised_InWindow_X_to_Y.fasta`. Only produced if you specified some positions to excise with `--excision-coords`, and at least one of those positions was inside[3] the window X-Y. If this file exists, the tree in window X-Y is based on this alignment (not `AlignedReadsInWindow_X_to_Y.fasta`). Note that excising positions may result in reads that were previously distinct becoming identical – if they differed only at the excised positions. This is checked after positions are excised, and reads from the same bam that have become identical are merged with an updated count. Similarly, if the merging of similar reads (in addition to identical reads) has been specified, the merging is rerun after excision of positions. If the excision of positions results in reads becoming merged, clearly there will not be a one-to-one correspondence between reads before and after excision: after merging there may be fewer unique reads, though with the same total count.

- `Consensuses/Consensuses_InWindow_X_to_Y.fasta`. In this alignment, the set of unique reads retained from each bam file is collapsed into a single sequence: their consensus (taking the most common base/gap at each position, weighting each unique read by its count). Note that in general this consensus could differ from the corresponding part of a consensus called using all reads mapped along the full length of the reference, because the only reads retained by `phyloscanner_make_trees.py` in a window are those fully spanning it. This process can result in some columns containing only gaps; these columns are kept, so that the coordinates of this file match those of `AlignedReadsInWindow_X_to_Y.fasta`.

- `Consensuses/Consensuses_PositionsExcised_InWindow_X_to_Y.fasta`. The consensus as previously, but with any specified positions to excise excised. Only produced if there are such positions.

- `DiscardedReads/DiscardedReads_A.bam`, where A identifies one of the input bams. Only produced if `--inspect-disagreeing-overlaps` is used; see that option's description in **??**. These files will be accompanied by `DiscardedReads_A_ref.fasta` (the reference for the bam file).

- `DuplicationData/DuplicateReadCountsRaw_InWindow_X_to_Y.csv`. Each row in this csv-format file corresponds to one instance of exactly the same unique read being found in two different bams, *before* any processing of the reads (merging, excising positions etc.). The first two columns show the two bams involved, and the third and fourth columns show the count of the read in the two bams. If a read is exactly duplicated in N different bams, then there will be $N(N-1)/2$ associated lines in this file – one for each different pair of bams. See also the information in the next point. These files will not be produced if `--dont-check-duplicates` is specified.

- `DuplicationData/DuplicateReadCountsProcessed_InWindow_X_to_Y.csv`. Each row in this csv-format file corresponds to one instance of exactly the same unique read being found in at least two different bams, *after* all processing of the reads (merging, excising positions etc.). If the same read is found in N different bams, there will be one row for this, containing N fields separated by commas (each row in general therefore has a different number of columns, though 2 is the most common). Each field is the name of the read, assigned based on the bam in which it was found. These files will not be produced if `--dont-check-duplicates` is specified.
  Why is information about the duplication of reads checked and recorded twice, in these two different files? Duplication information *after* processing is easiest to work with, because it is the processed reads that are used to infer phylogenies. However instances of exact duplication *before* any processing of the reads is a better indicator of cross-sample contamination. Firstly because the options for similarity-based merging of reads within each bam, and for a minimum read count for inclusion, result in fewer unique reads to compare between bams. Secondly because excising positions makes reads shorter and increases the possibility for genuine cross-sample duplication (i.e. not contamination). Anecdotally, we found that being trigger-happy with deleting all codons associated with HLA escape in a conserved part of the HIV genome resulted in considerable duplication in the reads after processing that was not present before. Note that during both similarity-based merging of reads and excision of positions (which is followed by merging newly identical reads), we lose track of the original identity of reads, i.e. we don't know what the correspondence is between reads before processing and after processing.

[3] Note that if `--pairwise-align-to` is used, the window coordinates X and Y mean the same thing as the excision coordinates. If `--pairwise-align-to` is *not* used, the window coordinates X and Y mean positions X and Y in `RefsAln.fasta` as discussed in section **??**; therefore a position to excise, Z, being inside the window X-Y is not the same as $X \leq Z \leq Y$.

- `DuplicationData/DuplicateReads_contaminants_InWindow_X_to_Y.fasta`. Only produced if `--contaminant-count-ratio` is used; see that option's description in section **??**.
- `ReadNames/ReadNames_InWindow_X_to_Y.csv.gz`. Not produced if `--no-read-names` is used. This csv file shows the correspondence between the name of each sequence present in the `phyloscanner_make_trees.` output for window X to Y, and the names of all reads in the input bam files that went into that output sequence. In general, a sequence in the output is made from more than one read from the input bam files because identical reads are always collapsed into a single sequence with an associated count, and optionally similar reads can be merged too. To save space, these files are automatically gzipped, and have a ragged csv format in which each row generally has a different number of fields. The first field is always the name of the sequence in the output, then subsequent fields are the names of the corresponding reads from the input bam files. If this file is of interest, you might also like `tools/ExtractNamedReadsFromBam.py`, which is run separately from the command line (run it initially with `--help` for more information).
- `RecombinationData/RecombinantReads_InWindow_X_to_Y.fasta`. Only produced if `--check-recombination` is used; see that option's description in section **??**.

Some temporary files are also written to the working directory; by default these are deleted. (They are kept with `--keep-temp-files`.)

5 Optional arguments

Information about all optional arguments and what they do can also be seen by running `phyloscanner_make_trees.py` with the `--help` option. That will give information guaranteed to be synchronised to your current version of the code, as well as showing all option shorthands (e.g. `--help` = `-h`); however we go into slightly more detail here. We particularly encourage you to familiarise yourself with the *Window options* and *Recommended options* below, which are particularly important for running `phyloscanner_make_trees.py` well.

5.1 Window options

You must choose exactly one of these: `--windows`, `--auto-window-params`, `--explore-window-widths`.

- `--windows`: used to specify a comma-separated series of paired coordinates defining the boundaries of the windows. e.g. specifying `1,300,301,600,601,900` would define windows 1-300, 301-600, 601-900.
- `--auto-window-params`: used to specify 2, 3 or 4 comma-separated integers controlling the automatic creation of regular windows.
  - The first integer is the width you want windows to be. If the `--pairwise-align-to` option is *not* being used, we create an alignment containing all references, as mentioned already. In this case, we weight each column in this alignment by its non-gap fraction, so that windows in which many references have gaps become correspondingly wider. If the `--pairwise-align-to` option *is* being used, width is simply interpreted with respect to the named reference (i.e. a width of W means each window contains W bases from that reference).
  - The second is the overlap between the end of one window and the start of the next. This can be negative, implying unused space in between windows; the recommended value of 0 means each window starts right after the previous one ends.
  - The third integer, if specified, is the start position for the first window. If not specified, this is 1.
  - The fourth integer, if specified, is the end position for the last window. If not specified, windows will continue up to the end of the reference or references.
- `--explore-window-widths`: use this option to explore how the number of unique reads found in each bam file in each window, all along the genome, depends on the window width. After this option specify a comma-separated list of integers. The first integer is the starting position for stepping

along the genome, in case you're not interested in the very beginning. Subsequent integers are window widths to try. For example, if you specified 1000,100,150,200 we would count the number of unique reads in windows 1000-1099, 1100-1199, 1200-1299, ... and in 1000-1149, 1150-1299, 1300-1449 ... and in 1000-1199, 1200-1399, 1400-1599, ... where the dots denote continuation to the end of the genome. Output is written to the file specified with the `--explore-window-width-file` option.

- `--explore-window-width-file`: used to specify an output file for window width data, when the `--explore-window-widths` option is used. Output is in csv format.

- `--explore-window-widths-speedy`: exactly the same as `--explore-window-widths`, except that the number of unique reads is calculated before read processing (including alignment) instead of after. See Section **??** for further discussion.

5.2 Recommended options

- `--alignment-of-other-refs`: used to specify an alignment of reference sequences for inclusion with the reads, for comparison. These references need not be those used to produce the bam files. This option is required if `phyloscanner` is to analyse the trees it produces (i.e if you are going to run `phyloscanner_analyse_trees.R` after `phyloscanner_make_trees.py`). The full set of processed, unaligned reads extracted from each window are aligned to the corresponding section of the reference alignment using `mafft --add`.

- `--pairwise-align-to`: by default, `phyloscanner_make_trees.py` figures out where corresponding windows are in different bam files by creating a multiple sequence alignment containing all of the mapping references used to create the bam files (plus any extra references included with `--alignment-of-other-refs`), and window coordinates are interpreted with respect to this alignment. However using this option, the mapping references used to create the bam files are each separately pairwise aligned to one of the extra references included with `--alignment-of-other-refs`, and window coordinates are interpreted with respect to this reference. The reference to use should be specified after this option. Using this option is necessary if you want to run the `tools/CalculateTreeSizeInGenomeWindows.py` script and feed its output into `phyloscanner_analyse_trees.R` via its `--normRefFileName` option (see section **??**).

- `--x-raxml`: use this option to tell `phyloscanner_make_trees.py` how to run `RAxML`, including both the executable (with the path to it if needed), and the options. If you do not specify anything, we will try to find the fastest `RAxML` executable available (first `raxmlHPC-AVX`, then `raxmlHPC-SSE3`, then `raxmlHPC`) and use the options `-m GTRCAT -p 1 --no-seq-check`. You will need to specify something if the path to your `RAxML` executable is not contained in your `PATH` environment variable (i.e. if you need to specify the path to the executable in order to run it), or if you want to use different `RAxML` options. `-m` tells `RAxML` which evolutionary model to use, and `-p` specifies a random number seed for the parsimony inferences; both are compulsory. You may include any other `RAxML` options in this command (including multi-threading, provided you use one of the multi-threaded executables). The set of things you specify with `--x-raxml` need to be surrounded with one pair of quotation marks (so that they're kept together as one option for `phyloscanner_make_trees.py` and only split up for `RAxML`). If you include a path to your `RAxML` executable, it may not include whitespace, since whitespace is interpreted as separating `RAxML` options. Do not include options relating to bootstraps: use `phyloscanner_make_trees.py`'s `--num-bootstraps` and `--bootstrap-seed` options instead. Do not include options relating to the naming of files.

- `--merge-paired-reads`: this is only relevant for paired-read data for which the mates in a pair (sometimes) overlap with each other, but is very useful for such data. With this option, overlapping mates in a pair are merged into a single (longer) read. This allows wider windows to be used, enhancing the phylogenetic resolution in a single window. The two reads will only be merged if they agree exactly on which bases are in the region where the reads overlap, and the positions these bases have been mapped to; if there is any disagreement about the overlap region, that pair is discarded. Discarded pairs can be inspected by using `--inspect-disagreeing-overlaps`. If reads are paired but never overlap, trying to merge them will do nothing except increase `phyloscanner_make_trees.py`'s run time and memory usage; if you're not sure about read pair

overlap (i.e. you don't know the *insert size distribution* for your data), run `tools/EstimateReadCountPerWindow.py` as discussed at the start of section **??**.

5.3 Read quality options

- `--discard-improper-pairs`: discard all reads that are were flagged, at the time of mapping, as improperly paired: in the wrong orientation, or one mate unmapped, or too far apart. For paired-read data.
- `--quality-trim-ends`: used to specify a quality threshold for trimming the ends of reads. We trim each read inwards until a base of this quality is met.
- `--min-internal-quality`: used to specify an internal quality threshold for reads. Reads are allowed at most one base below this quality; any read with two or more bases below this quality are discarded. (If used in conjunction with the `--quality-trim-ends option`, the trimming of the ends is done first.)
- `--min-read-count`: used to specify a minimum count for each unique read. Reads with a count (i.e. the number of times that sequence was observed, after merging if merging is being done) less than this value are discarded. The default value of 1 means all reads are kept. You might want to discard rare reads to protect against sequencing error. Retaining fewer reads will also speed up all subsequent processing and analysis of the reads.

5.4 Other assorted options

- `--excision-coords`: used to specify a comma-separated set of integer coordinates that will be excised from the aligned reads before phylogenies are made. Useful for sites of non-neutral evolution, which distort phylogenies. Requires the `--excision-ref` flag.
- `--excision-ref`: used to specify the name of a reference (which must be present in the file you specify with `--alignment-of-other-refs`) with respect to which the coordinates specified with `--excision-coords` are interpreted. If you are also using the `--pairwise-align-to` option, you must specify the same reference there and here.
- `--merging-threshold-a`: when multiple reads in the same bam file have exactly the same sequence, `phyloscanner_make_trees.py` always collapses these to a single read with an associated *count* (i.e. the number of times that exact sequence was found in distinct reads in the bam file). With this option, similar reads are merged as well as identical reads. Use this option to specify a positive integer as the threshold for merging. If read X differs from read Y by this threshold or less, and X has the smaller count, we keep only read Y and update its count to be the sum of the two counts. Note that with `--merging-threshold-a`, if X is similar enough to Y for merging, but Y has already been merged into Z, X will only be merged into Z too if X and Z are similar enough (contrast with `--merging-threshold-b` below). What this algorithm does is to start with the most common read, see if any less common reads are similar enough to into it, and if so remove those reads and use their count to bolster the more common read. The procedure is repeated starting with the second most common read, skipping any reads that have already been 'used up' in merging; then the third most common read and so on.
- `--merging-threshold-b`: similar to `--merging-threshold-a` above, except that if X is similar enough to Y for merging, but Y has already been merged into Z, X will automatically be merged into Z. This algorithm effectively partions the set of reads into groups such that each member of a group is similar enough to at least one other group member (and each group can be split no further, i.e. no read is similar enough to any read outside its own group), and uses only the read with the highest count to represent each group. For the same value of the threshold, merging `b` will merge as much or more than merging `a` (i.e. `b` will tend to result in fewer unique reads).
- `--check-recombination`: calculate a metric of recombination for each sample's set of reads in each window. How the metric is calculated: for each possible set of three sequences, one is considered the putative recombinant and the other two the parents. For each possible crossover point (the

point at which recombination occurred), we calculate d_L as the difference between the Hamming distance from the recombinant to one parent and the Hamming distance from the recombinant to the other parent, looking to the left of the crossover point only; similarly we calculate d_R looking to the right of the crossover point only. d_L and d_R are signed integers, such that their differing in sign indicates that the left and right sides of the recombinant look like different parents. We maximise the difference between d_L and d_R (over all possible sets of three sequences and all possible crossover points), take the smaller of the two absolute values, and normalise it by half the length of the alignment of sequences. The resulting metric is constrained to be between 0 and 1, inclusive. The maximum possible score of 1 is obtained if and only if the two parents disagree at every site, the crossover point is exactly in the middle, and either side of the crossover point the recombinant agrees perfectly with one of the parents e.g.
AAAAAAA
AAAACCC
CCCCCCC
Calculation time scales cubically with the number of unique sequences each sample has per window, and so the option is turned off by default. You can save time by only turning it on only after you've settled on the values of other parameters that affect the number of unique sequences per window (notably window width, a merging threshold and a minimum read count).

- `--num-bootstraps`: used to specify the number of bootstraps to be calculated for `RAxML` trees (by default, none, i.e. only the maximum-likelihood tree is calculated).

- `--bootstrap-seed`: used to specify the random-number seed for running `RAxML` with bootstraps. The default is 1.

- `--max-gap-frac-for-refs`. If `--alignment-of-other-refs` is used, an external reference sequence will be excluded from the current window if its fractional gap content in the current window (in the alignment including only the references, not the reads) exceeds this threshold. The default value is 0.5; this option allows a different value to be specified. The purpose of this option is to exclude references with too much missing information in a given window, to prevent poor imputation of their missing sequence from messing up phylogenetic inference.

- `--output-dir`: used to specify the name of a directory into which output files will be moved. If it does not exist, it will be created; however we cannot create a new directory inside a directory that does not yet exist. Temporary and output files are always created in the working directory, i.e. the directory in which the `phyloscanner_make_trees.py` command was run, but with this option the output files are copied to the specified directory at the end.

- `--time`: print the times taken by different steps.

- `--x-mafft`: used to specify the command you need in order to run `mafft`; the default is simply `mafft`. Specifying an alternative is useful for two reasons. Firstly if your `mafft` executable is not in the `$PATH` environment variable for your terminal (google this if you don't know what it means), you will need to include the directory where this executable lives, e.g.
`/path/to/where/I/installed/mafft/mafft`. Secondly this allows you to include some of `mafft`'s options when you run it. If you want to include some options, the set of things you specify with `--x-mafft` need to be surrounded with one pair of quotation marks, so that they're kept together as one option for `phyloscanner_make_trees.py` and only split up for `mafft`. Your `mafft` executable's path may not contain whitespace, as whitespace is interpreted as separating the executable from options. Note that we always use the `mafft` options `--quiet --preservecase`, and you should not include `--add`, since we use this automatically depending on the kind of alignment we're doing.

- `--x-mafft2`: this is only applicable if you are also using the `--pairwise-align-to option`. By default, the command we use to do pairwise alignment of references (distinct from all other kinds of alignment, e.g. of the reads) is whatever executable was specified using `--x-mafft` (which is just `mafft` by default if you didn't specify anything using `--x-mafft`) plus the options `` `--localpair --maxiterate 1000' ``. This means slower but more accurate alignment is used, which is more appropriate for alignment two whole genomes, compared to aligning a large number of short reads. Use this option to override the default command for pairwise alignment of references, specifying

something else instead (the desired executable plus any desired options). For example, if you specify neither `--x-mafft` nor `--x-mafft2`, the command used for pairwise reference alignment will be `mafft --localpair --maxiterate 1000 --quiet --preservecase`
If you specify just `--x-mafft /bin/my/dir/mafft`, the pairwise reference command will be `/bin/my/dir/mafft --localpair --maxiterate 1000 --quiet --preservecase`
If you specify `--x-mafft2 /bin/my/dir/mafft --maxiterate 2000`, the pairwise reference command will be
`/bin/my/dir/mafft --maxiterate 2000 --quiet --preservecase`

- `--x-samtools`: used to specify the command you need in order to run `samtools`. The default is simply `samtools`.
- `--keep-output-together`: by default, subdirectories are made for different kinds of output. With this option, all output files will be in the same directory (either the working directory, or whatever you specify with `--output-dir`).
- `--keep-temp-files`: keep temporary files we create on the way (these are deleted by default).

5.5 Options for bioinformatic interrogation

Options for detailed bioinformatic interrogation of the input bam files, not intended for normal usage.

- `--inspect-disagreeing-overlaps`: with –merge-paired-reads, those pairs that overlap but disagree are discarded. With this option, these discarded pairs are written to a bam file (one per patient, with their reference file copied to the working directory) for your inspection.
- `--exact-window-start`: normally `phyloscanner_make_trees.py` retrieves all reads that fully overlap a given window, i.e. starting at or anywhere before the window start, and ending at or anywhere after the window end. If this option is used *without* `--exact-window-end`, the reads that are retrieved are those that start at exactly the start of the window, and end anywhere (ignoring all the window end coordinates specified). If this option is used *with* `--exact-window-end`, for a read to be kept it must start at exactly the window start AND end at exactly the window end. If `--merge-paired-reads` is also used, this explanation applies to inserts (read pairs) instead of individual reads.
- `--exact-window-end`: with this option, the reads that are retrieved are those that end at exactly the end of the window. Read the `--exact-window-start` help.
- `--recover-clipped-ends`: the default behaviour of `phyloscanner_make_trees.py` is to keep only reads that fully span the window in question. A read which is long enough in principle to reach the edge of the window but is not mapped at its end, i.e. the end is clipped, will therefore not be included. With this option, clipped ends are recovered by considering any bases at the ends of the read that are unmapped to be mapped instead to 1 more than the base to their left (at the right end) or 1 less than the base to their right (at the left end), iterating out from the centre. e.g. a 9bp read mapped to positions None,None,10,11,13,14,None,None,None (i.e. clipped on the left by 2bp, and on the right by 3bp, with a 1bp deletion in the middle), is taken to be mapped instead to positions 8,9,10,11,13,14,15,16,17. In this example, if the window left edge is 8 or 9 and the right edge is 15, 16 or 17, the read with its clipped ends recovered spans the window but the read without clipped ends does not. WARNING: mapping software clips the ends of reads for a reason, namely that that stretch of sequence does not look anything like the reference at that point. The clipped sequence could be just junk, or genuine sample from a distant part of the genome (i.e. the read is chimeric); in this case the clipped sequence should be discarded and not recovered. As such, this option should not be used as part of normal `phyloscanner_make_trees.py` usage. Its intended usage is specifically the following: you have identified a window in a bam file in which reads are clipped, but you believe the reads to be correct, i.e. the clipping is an artefact of the mapper being unable to find the correct local alignment. You should combine this option with `--no-trees` because the inclusion of clipped sequence, which by definition is very different, increases the chance of misalignment. You should inspect the aligned reads manually before doing anything else (and hopefully get some insight into how the reference in this window should be changed in order to have

subsequent remapping get the local alignment right, in particular by contrasting the reference with the consensus of the aligned reads).

5.6 Partial processing options

Options to only partially run `phyloscanner_make_trees.py`, stopping early or skipping steps.

- `--align-refs-only`: align the mapping references used to create the bam files (plus any extra reference sequences specified with `--alignment-of-other-refs`), then quit without doing anything else. The point of this is to allow inspection of that alignment, whose coordinates are used to interpret window coordinates (unless `--pairwise-align-to` is used).
- `--no-trees`: process and align the reads from each window, then quit without making trees.
- `--dont-check-duplicates`: don't compare reads between samples to find duplicates – a possible indication of contamination.
- `--read-names-only`: quit after writing the read names to a file (which means the reads are not aligned). (By default this check is done.)
- `--no-read-names`: do not record the correspondence between each unique sequence retained by phyloscanner, and the reads that went into this sequence (as they are named in the bam file), as is done by default.
- `--coordinate-correspondence-only`: stop straight away after producing `WindowCoordinateCorrespondence.csv` which shows the correspondence in window coordinates.

5.7 Deprecated options

Left in `phyloscanner_make_trees.py` for backward compatibility or interest.

- `--contaminant-count-ratio`: used to specify a numerical value which is interpreted in the following way: if a sequence is found exactly duplicated between any two bam files, and is more common in one than the other by a factor at least equal to this value, the rarer sequence is deleted and goes instead into a separate contaminant read fasta file. This is considered deprecated because including the exact duplicates in the tree and dealing with them during tree analysis is more convenient – `phyloscanner_make_trees.py` does not need to be rerun if you change your mind about count thresholds for removing duplicates – it also allows the offending duplicates to be inspected in the tree.
- `--flag-contaminants-only`: for each window, just flag contaminant reads then move on (without aligning reads or making a tree). Only makes sense with the `--contaminant-count-ratio` flag.
- `--forbid-read-repeats`: using this option, if a read with the same name is found to span each of a series of consecutive, overlapping windows, it is only used in the first window. *Consecutive* means next to each other in the order you specified. For example, if you specified windows 10-20, 15-25, 20-30 and 31-40, and there was a read that spanned all four windows (i.e. it started at or before position 10 and ended at or after position 40), it would be used in window 10-20, not used in 15-25 because it spanned the last window, not used in 20-30 because it spanned the last window (even though it was skipped there), and used in 31-40 because this window does not overlap with the last one. NB with paired read data, mates in a pair have the same name; using this option without the `--merge-paired-reads` option will mean at most one of the two mates will be used (in a given window and consecutive overlapping windows), and with the `--merge-paired-reads` option mates will be merged into a single read, which is used only the first time it is encountered in consecutive overlapping windows.
- `--keep-overhangs`: keep the part of the read that overhangs the edge of the window. (By default this is trimmed, i.e. only the part of the read inside the window is kept.) Keeping overhangs means that, within each bam file, reads that are identical inside the window but have different overhangs will not be merged into a single sequence (with a count greater than 1). Differences in overhangs may be SNPs, or simply because the overhangs start or end at different points; this option is

therefore a bit weird, because it's nice to merge all reads that are identical inside the window of interest.

- `--ref-for-coords`: if the `--pairwise-align-to` option is not used, then a multiple sequence alignment is created with all the mapping references (used to create the bam files) plus any extra references included with `--alignment-of-other-refs`. By default, window coordinates are interpreted with respect to this alignment, i.e. they are in the alignment coordinates. With this option (–ref-for-coords), the multiple sequence alignment is still created but window coordinates are interpreted with respect to a named reference, which must be one of those included with `--alignment-of-other-refs`. Use this option to specify the name of the reference. This option is deprecated because the `--pairwise-align-to` option also interprets window coordinates with respect to a named reference, but without needing to construct a multiple sequence alignment – using just pairwise alignment.

- `--recombination-gap-aware`: by default, when calculating Hamming distances for the recombination metric, positions with gaps are ignored. This means that e.g. the following three sequences would have a metric of zero:
  `A-AAAAA`
  `A-AAA-A`
  `AAAAA-A`
  With this option, the gap character counts as a fifth base and so (dis)agreement in gaps contributes to Hamming distance. This increases sensitivity of the metric to cases where indels are genuine signals of recombination, but decreases specificity, since misalignment may falsely suggest recombination.

- `--recombination-norm-diversity`: by default, the normalising constant for the recombination metric is half the number of sites in the window; with this option it's half the number of sites in the window that are polymorphic for that bam file. Richard Neher thoughtfully suggested this, however anecdotal investigation suggested it was too trigger-happy in flagging totally recombinant sequences – a metric value of 1 – in regions with very little diversity.

- `--read-names-simple`: produce a file for each window and each bam, simply listing the names of the reads that phyloscanner used (but not the correspondence between these and the set of unique sequences retained by phyloscanner after any merging etc.). This is not affected by the `--no-read-names` option.

Part II
Analysing within- and between-host phylogenies

The tree analysis procedure takes as input one or more phylogenies, and analyses them to reconstruct transmission, and identify multiply infected individuals and contaminants. In order, the following steps are performed:

1. Identify and exclude tree tips corresponding to reads that should not be used to reconstruct transmission (blacklisting)
2. Perform an ancestral state reconstruction on each tree in turn, and use this to identify host subgraphs (contiguous regions of the phylogeny that have been assigned to the same host)
3. Calculate per-host summary statistics, and graph them across the genome if multiple trees are given as input
4. In each tree, determine the relationship between each pair of hosts determined by the relative positions of their subgraphs
5. Summarise these pairwise relationships over all trees (if more than one tree was given).

The code forms an `R` package entitled `phyloscannerR` which appears as a subfolder in the `phyloscanner` directory. The package manual can also be found in this directory and it is suggested that advanced perform the analysis within `R` itself and consult consult that. This package and its dependencies must be installed prior to use, whether within `R` or at the UNIX command line; all dependencies are available in CRAN except for `ggtree` [**?**], `treeio`, and `RBGL`, which are part of Bioconductor. At present `phyloscannerR` itself is not listed on CRAN but we intend to submit it in the fullness of time.

To install `phyloscannerR` (and the Bioconductor packages), use the command line to navigate to the "phyloscannerR" subdirectory of the "phyloscanner" directory, then start `R` and enter the following commands:

```
> install.packages("devtools")
> install.packages("BiocManager")
> library(devtools)
> BiocManager::install("ggtree")
> BiocManager::install("RBGL")
> install("../phyloscannerR", dependencies = T)
```

(Obviously if you have already installed `ggtree` then the second and third lines can be skipped.)

A single command line script, `phyloscanner_analyse_trees.R`, can be used to perform a full analysis. Each step identified above also has its own standalone script, which can be used to, for example, analyse multiple trees in parallel on a cluster. We do not document all the command line options for these additional scripts here, and the user is directed to their `--help` options.

6 The basic command

The basic command for `phyloscanner_analyse_trees.R` is

```
$ phyloscanner_analyse_trees.R TreeInput OutputString ReconstructionModeArguments
```

`OutputString` is simply a string identifying all file output. The tree input can be either a single tree file, a directory containing tree files, or a single string that begins all files (see below). For full instructions regarding `ReconstructionModeArguments`, see section **??**. For a quick start, `s,0` gives a parsimony reconstruction with no within-host diversity penalty. Be aware that this version of the algorithm will not do any kind of check for contaminant genetic reads.

7 Name format for input files

Tree files should be in Newick or Nexus format. These can be the product of `phyloscanner_make_trees.py`, but need not be; the script will work on any set of trees if the tip labels can be interpreted properly (see below). Each tree should reside in a single file.

There are three ways in which input tree files can be identified:

- As a directory containing all the tree files. Every file in this directory that has a certain file extension (normally "`.tree`" but this can be controlled using the `--treeFileExtension option`) will be considered an input file.
- As a directory containing all the tree files and a string that begins every tree file.
- As a single tree file (containing a single tree) for analysis of one phylogeny only.

Unless this is a single-tree analysis, the file name of each tree must contain a unique identifier. If the genome window approach is used, as is automatically the case when dealing with `phyloscanner_make_trees.py` output, this should be a string of the form "`X_to_Y`" where X and Y are the coordinates of the beginning and end of the genomic window. The `phyloscanner_analyse_trees.R` pipeline will automatically check for these strings and use them if it finds them. (A different string determining window coordinates can also be given; see **??** below.) If it does not find them, then:

- If the input trees were specified as the contents of a directory, then tree IDs are just their file names.
- If the input trees were specified as a path and prefix, the IDs will be the text appearing between the end of that prefix and the beginning of the file extension.

Other, supplementary, input data for `phyloscanner_analyse_trees.R` (such as for blacklisting purposes; see section **??** below) data is often also provided in the form of (at most) one file per tree. These are expected to have matching IDs. File names will be scanned for text of the form "`X_to_Y`" if tree IDs follow that pattern and matched accordingly. **If input is derived from `phyloscanner_make_trees.py` then this will be the case and you can probably ignore the remainder of this paragraph.** If the IDs are not of that form, usually because you are providing `phyloscanner_analyse_trees.R` with your own trees, then each supplementary data file name must *contain* the ID of the tree it corresponds to, which is either the text between prefix and file extension or the entire file name. As including one file name as a substring of another is a little odd, we recommend the "path and prefix" version.

For input, tree files are by default assumed to have `.tree` as a file extension, CSV files `.csv` and alignment files `.fasta`, but this can be overridden with the `--treeFileExtension`, `-csvFileExtension` and `-alignmentFileExtension` options.

8 Output

The default output of `phyloscanner_analyse_trees.R` varies depending on whether it is run on one or multiple trees.

If the input is a single tree file, the default output is an annotated PDF version of that tree, a CSV file of summary statistics for the hosts present in that tree, and a CSV file outlining the relationship between each pair of hosts in that tree.

If the input is multiple trees, then the default output is an annotated PDF version of all trees, a CSV file of summary statistics for every host across all the trees, a PDF containing graphs of those statistics across all trees, and a CSV file summarising which pairs of hosts meet the criteria for being closely related.

9 Preparing the trees

Two options determine processing of the phylogeny before any further operations are performed. A named outgroup can be specified with `--outgroupName`; this is recommended unless the input trees are known to be correctly rooted. It is always assumed that the lineage represented by the root of the entire phylogeny was not present in any sampled host, even if no outgroup is given. Trees output by `phyloscanner_make_trees.py` will need to be re-rooted.

The output of many phylogeny reconstruction packages, including `RAxML`, is a binary phylogeny. While multifurcations may seem to appear when the trees are viewed by eye, these will have a hidden binary branching order. The branches connecting the nodes within these "multifurcations" may all be of an extremely short but nonzero minimum length (in `RAxML` output) or actually zero length (in, e.g., PhyML output). Since this branching order is largely arbitrary, it is recommended that these regions of the tree be collapsed into genuine multifurcating nodes for `phyloscanner_analyse_trees.R`. A length threshold to determine pairs of nodes that should be so collapsed can be specified with `--multifurcationThreshold`. This is normally numerical, but if `g` is given it will be guessed from the trees itself. If the trees have interpretable genome window coordinates, the smallest branch length will be compared to the branch length corresponding to one SNP. If the former is smaller than 0.25 times the latter, the former will be used as the threshold; if not, the tree is assumed to contain no multifurcations and no collapsing is done. If no genome window coordinates are available, the shortest branch length is simply used as the threshold. Before specifying `g` the user should ideally ensure that the trees genuinely do contain apparent multifurcations.

10 Tip label and file suffix regular expressions

The tip labels in the input phylogenies are expected to follow a regular expression that identifies host IDs, and optionally also read identifiers and read counts. The exact format is specified using a regular expression with three capture groups: for host ID, read ID, and count. The count is expected to be an integer. If a third group is not found then it assumed that every tip represents only a single read. The default regular expression is `"^(.*)_read_([0-9]+)_count_([0-9]+)$"`, but the user can specify an alternative with the `--tipRegex` option. In plainer language, this is of the form "NAME_read_X_count_Y" where NAME is the host name, X the read identifier and Y the read count. The tip names of the outgroup (if it exists), and of any other reference sequences that are included, should **not** match this regular expression.

The default regex is appropriate for analysing trees output by `phyloscanner_make_trees.py`, when each bam contains reads from a different host. A different regular expression may be required if the input is not from `phyloscanner_make_trees.py`. It may also be required for trees output by `phyloscanner_make_trees.py` if multiple bam files contain sequences derived from the same host, and part of the bam file name identifies the host. For example if you had three bam files named PatientA-1.bam, PatientA-2.bam and PatientB-1.bam, where tips from the first two are from the same host. Changing the tip regex to `"^(.*)-[0-9+]\\.bam_read_([0-9]+)_count_([0-9]+)$"` means that the first group in the regex will match "PatientA", "PatientA" and "PatientB" for the three bams respectively; all tips from the first two bams are then associated to the same host, PatientA.

Similarly, the ID for each tree (see above) may contain genome window coordinates, and a regular expression is used to identify these. Two capture groups are expected for the start and end of the window. The default is `"^(?:.*\\D)?([0-9]+)_to_([0-9]+).*$"`. In plainer language "X_to_Y" where X is the window start and Y the end (exactly as specified in the previous section), followed by any number of non-digit characters and preceded by either nothing or a string ending in a non-digit character. An alternative can be specified with the `--fileNameRegex` option. If an alternative regexp has only one capture group, that is treated as a single number that specifies the position of a tree relative to all other trees (a genome window midpoint, for example).

11 Branch length normalisation

Two hosts may be classified as closely linked by `phyloscanner_analyse_trees.R` on the grounds that they have host subtrees separated by a patristic distance that lies below a given threshold. However because we are often interested in a genomic window approach, a fixed distance threshold for all trees may not be appropriate because of variations in nucleotide diversity in different areas of the genome. This is because a distance threshold suggestive of a close epidemiological relationship in one window will not be in others. To remedy this, `phyloscanner_analyse_trees.R` can be asked to normalise branch lengths across windows.

One approach to generating appropriate normalisation values over the genome is implemented by the script `tools/CalculateTreeSizeInGenomeWindows.py`. Run from the command line (see it's `--help` option for details on how), and taking a whole-genome alignment of existing reference sequences as input,

it infers phylogenies in sliding windows, and characterises the size of the tree by taking the median of the set of patristic distances between all pairs of references. One such value is obtained per window; one value is then assigned to each integer position in the genome by taking the mean of the values from those windows spanning this position (with windows chosen to overlap, so that multiple values for each position should smooth some stochasticity in phylogenetic inference). Note that you must specify one sequence in the alignment whose coordinates will be used to define genomic position; this sequence must match the one you specified with `--pairwise-align-to` in `phyloscanner_make_trees.py` if we are to use this information for branch length normalisation. This ensures that the coordinates in the output of `tools/CalculateTreeSizeInGenomeWindows.py` and the coordinates in the output of `phyloscanner_make_trees.py` (e.g. in the per-window file names) mean the same thing.

Q: what alignment of existing reference sequences should you use for this? A: ideally, one which is in some sense representative of between-host diversity. We find all pairwise patristic distances, so if your set of sequences is biased to be overly representative of a particular subset, these patristic distances will become biased towards zero. That said, all we're interested in here is how patristic distances change across the genome, not their absolute values.

The output from `tools/CalculateTreeSizeInGenomeWindows.py`, or alternatively any other csv file containing genomic positions with associated normalisation constants, can be used with the `--normRefFileName` option. This will scale all normalisation constants by the same factor such that their mean is 1. A normalisation constant is then determined for each window by taking the mean of the values for all positions in the window. This constant for the window is used to scale all branch lengths in the tree for subsequent processing by `phyloscanner_analyse_trees.R`. The similar option `--normStandardiseGagPol`, specific to HIV genomes, instead scales normalisation constants so that the mean of the constants on the *gag* and *pol* genes is 1; distances anywhere in the genome are then interpretable as standard distances in *gag/pol*.

The `--normalisationConstants` option is used to directly specify the normalisation to be used for each tree file. If the argument is numerical, it is used as a normalising constant for every tree. If it is instead the path to a CSV file, then this file is expected to consist of a first column listing all input tree file names, and a second of normalising constants. The presence of `--normalisationConstants` overrides any other normalisation options, which will be disregarded.

The standalone script `normalisation_lookup_writer.R` can be used to separately write a CSV file for use with the `--normalisationConstants` option using the procedure described in this section. This is **not** normally necessary for use of `phyloscanner_analyse_trees.R`, but can be used as an argument for other standalone scripts.

The normalisation is used for the parsimony reconstruction (see section **??**) and for the identification of closely-related hosts (see sections **??** and **??**). However, output trees have the same branch lengths as input trees, and summary statistics (see section **??**) are calculated using raw branch lengths.

11.1 Parallelising `tools/CalculateTreeSizeInGenomeWindows.py`

The `--threads` option of `tools/CalculateTreeSizeInGenomeWindows.py` can be used to specify multiple threads, allows multiple windows to be analysed in parallel using multiple cores on the same machine. Alternatively, power users may want to massively parallelise, e.g. over multiple different machines on a computing cluster. Read on if interested; skip ahead to section **??** if not. First choose your desired start, end, window width and increment parameters. Then generate a set of `tools/CalculateTreeSizeInGenomeWindows.py` commands, with each command running only a single window, namely the window after the one of the previous command. For example say you wanted to start at position 1000, end at 9000, have a window width of 300 and an increment of 10. Your first window is $1000 - 1299$, your second is $1010 - 1309$, ... and these can be run as separate commands thus:

```
$ tools/CalculateTreeSizeInGenomeWindows.py MyAln.fasta MyChosenSeqName \
  1000 300 output_1000-1299 -E 1299
$ tools/CalculateTreeSizeInGenomeWindows.py MyAln.fasta MyChosenSeqName \
  1010 300 output_1010-1309 -E 1309
...
```

Generating these commands is easily done as part of a loop, e.g.

```
$ for start in $(seq 1000 10 8700); do
```

```
$   end=$((start + 299))
$   command="tools/CalculateTreeSizeInGenomeWindows.py MyAln.fasta MyChosenSeqName"\
" $start 300 output_${start}-${end} -E $end"
$ done
```

and each command could be put inside a separate file to be run as a separate job. In this example, output files will be produced named `output_1000-1299_ByWindow.csv`, `output_1000-1299_ByWindow.csv`, ...; these can be combined with

```
$ cat output_*_ByWindow.csv | sort -n | uniq > output_all_ByWindow.csv
```

(the `sort` and `uniq` commands preventing the csv header repeating). Finally, running

```
$ tools/FromPerWindowStatsToPerPositionStats.py output_all_ByWindow.csv \
  > output_all_ByPosition.csv
```

converts the per-window tree sizes to per-position tree sizes, as required as input for the `--normRefFileName` option of `phyloscanner_analyse_trees.R`.

(We used this approach for parallelisation, as we covered the HIV genome with windows of width 301 bp and an increment of 1 bp, necessitating 9179 windows. Running these as 9179 separate commands in parallel on a cluster sped things up considerably.)

12 Blacklisting

Blacklisting refers to the exclusion of some tips in the phylogeny from the full analysis. This may be done for a variety of reasons:

- Tips from suspected contaminant reads (i.e. for which we suspect the true host is not the recorded host) should not be used to reconstruct transmission
- Possible multiple infections may complicate an analysis and the user may prefer to deal only with the largest collection of reads from such a host
- Uneven tip or read counts between different hosts have the potential to bias the inference of transmission. The user may wish to remove some tips in order to mitigate this.

`phyloscanner` includes a number of utilities to perform blacklisting, all of which can be run as part of `phyloscanner_analyse_trees.R`. The script does all blacklisting internally, but other scripts in the `tools` directory of the `phyloscanner` code can be used to generate lists of suspect tips in an input phylogeny. In addition, a script entitled `phyloscanner_clean_alignment.R` may be used to perform blacklisting and remove blacklisted sequences from the input alignments without performing a full `phyloscanner` run; see the `--help` for this for more details.

The various types of blacklisting are listed below. A CSV file listing every tip that was blacklisted in every tree, and why, will be output if the `--blacklistReport` option is given to `phyloscanner_analyse_trees.R`.

12.1 Duplicate blacklisting

This procedure blacklists reads from one host which are identical to reads from another, but are present in sufficiently small numbers that it would be suspected that they are merely contaminants. It is enabled with the `--duplicateBlacklist` option to `phyloscanner_analyse_trees.R`, which takes a single argument identifying the duplicate output files from `phyloscanner_make_trees.py`. Tips are blacklisted if the corresponding sequence is exactly identical to the sequence for a tip from another host, and either the raw read count from the former tip, or the ratio of the read count from the former tip to the read count from the latter tip, is less than a specified threshold. The raw read count is specified with the `-rwt` option and the ratio threshold with the `-rtt` option. It is acceptable to specify both. As the default values for both are 0, nothing will be blacklisted unless at least one is given a positive value.

`phyloscanner_analyse_trees.R` will append "_X_DUPLICATE" to the tip names of tips blacklisted by this procedure, which will appear in annotated tree output. The standalone R script is `duplicate_blacklister.R`.

12.2 Parsimony-based blacklisting for contaminants

While in many cases contaminant reads will be identical to those from another host in the dataset, the user may not always be so fortunate; the contaminant may be from another dataset entirely, and hence have no exact matches amongst the reads that are used to reconstruct the phylogeny. Similarly, a variant may be sequenced from the dataset that is never assigned the correct host. The `--parsimonyBlacklistK` option to `phyloscanner_analyse_trees.R` can be used to identify such tips, using the same parsimony procedure that is employed for ancestral state reconstruction (see section **??**, and the `phyloscanner` paper). The single additional numerical argument to `--parsimonyBlacklistK` is the value of k used to calculate a within-host diversity penalty.

We use this procedure because excessive amounts of within-host diversity may be just as suggestive of contamination as of a genuine multiple infection. For each host in turn, the tree is pruned so that only the tips from that host, and the outgroup, remain. (If no such outgroup is given, `phyloscanner_analyse_trees.R` will attempt to find one.) The reconstruction is them performed on this pruned tree, using the k parameter specified with `--parsimonyBlacklistK`, and the result used to see whether the tips of the tree were grouped into one subgraph or more than one subgraph. If the former, then the phylogeny does not suggest sufficient diversity within this host to suggest either a multiple infection or contamination. If the latter, then one or the other is likely to be true. We suggest that a subgraph is more likely to be contamination when it contains very few reads. The arguments used in duplicate blacklisting are reused here:

- If the total number of reads associated with the tips in a subgraph is smaller than the raw threshold (`--rawBlacklistThreshold`), then all tips in the subgraph are blacklisted.
- If the proportion of reads from the host that belong to a subgraph is smaller than the ratio threshold (`--ratioBlacklistThreshold`), then all tips in that subgraph are blacklisted.

The former threshold is applied even if there is only one subgraph, in which case it is assumed that so few reads exist from the host in question that all of them could well be contaminants, with genuine sequencing failing in this part of the genome.

`phyloscanner_analyse_trees.R` will append "_X_CONTAMINANT" to the tip names of tips blacklisted by this procedure, which will appear in annotated tree output. The standalone R script is `parsimony_based_blacklister.R`.

12.3 Dual infection blacklisting

If the parsimony-based blacklisting procedure identifies a host with suspicious amounts of within-host diversity where the smaller subgraphs have too many reads to be flagged as likely contaminants, a dual infection (or larger multiple infection) may be suspected. The current version of `phyloscanner_analyse_trees.R` is only able to summarise the relationships of such a host with the neighbours of all its subgraphs in the transmission tree; it cannot separate the neighbours of each subgraph. This may result in spurious inferences being drawn, where a close neighbour to one subgraph and a close neighbour to another are identified as closely related to each other. If this behaviour is regarded as particularly undesirable, a suggested stopgap remedy is to assume that the subgraph with the largest read count represents the same infection across the entire genome, and blacklist the rest.

The `--dualBlacklist` flag, which can be used only if `--parsimonyBlacklistK` is also specified, will do this blacklisting. `phyloscanner_analyse_trees.R` will append "_X_DUAL" to the tip names of tips blacklisted by this procedure, which will appear in annotated tree output. The standalone R script is `dual_host_blacklister.R`.

12.4 Minimum count blacklisting

This type of blacklisting is used to entirely eliminate hosts from windows where the set of reads, or distinct reads, associated with them is too small. A minimum count for either can be specified using the `--minReadsPerHost` and `--minTipsPerHost` options.

12.5 User blacklisting

The user may wish to specify his or her own blacklist before `phyloscanner_analyse_trees.R` is run. A separate plain text file for each window should be prepared, with a blacklisted tip on each line. Unless only one window is being analysed, file names must contain tree IDs as described in section **??**. The path to these files and the string beginning each should be specified with the `--userBlacklist` option. These tips will be blacklisted from the start, and `phyloscanner_analyse_trees.R` will append "_X_USER" to tip labels.

12.6 Downsampling

The intention of the downsampling procedure is to ensure that the same numbers of reads are included in the analysis from each host in the dataset. This is done by taking a random sample of reads, without replacement, and blacklisting tips for which the corresponding reads was never selected at all. The `--maxReadsPerHost` option is used to specify a number of reads to downsample to. If `--blacklistUnderrepresented` is specified then hosts without enough reads in totality are blacklisted, but if it is absent then all tips from those hosts are retained.

`phyloscanner_analyse_trees.R` rewrites tip labels to reflect the number of times a tip was randomly selected. "_X_DOWNSAMPLED" is appended to tips that are blacklisted because the corresponding reads were never selected, in which case the read count in the tip label will have been rewritten to 0. "_X_UNDERREPRESENTED" is appended to those that are blacklisted because they are from a host without enough reads in total (if `-dsb` is given). A standalone R script to perform downsampling and produce lists of discarded tips (which can subsequently be used as user blacklists) is `downsample_reads.R`.

12.7 Pruning the blacklist

Default behaviour is to leave blacklisted tips in the phylogeny; they are not annotated with any host and are instead given the unsampled state in the parsimony reconstruction, but they remain so as to be examined in the annotated tree output. The `--pruneBlacklist` option to `phyloscanner_analyse_trees.R` can be used to prune these tips instead, in which case they will not appear in output at all.

13 Parsimony reconstruction

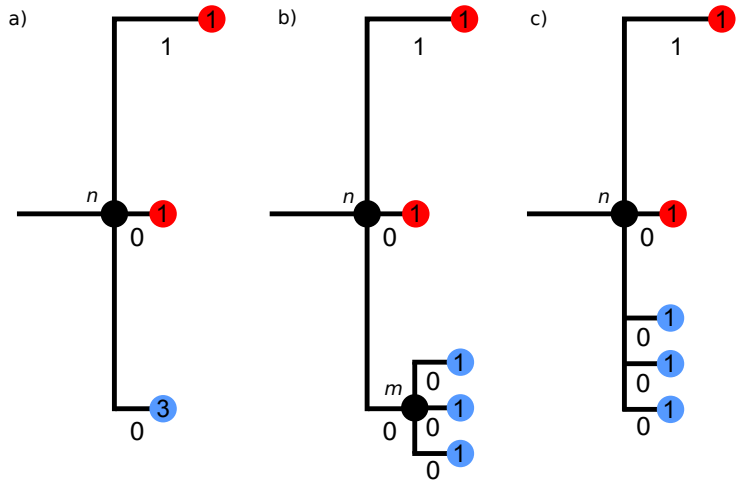
The core of the `phyloscanner_analyse_trees.R` analysis is an ancestral state reconstruction of hosts to internal nodes of each phylogeny. We offer several algorithms for this; the exact one is specified with the final compulsory argument to `phyloscanner_analyse_trees.R`. For full details, see the supplementary information of the `phyloscanner` paper.

- A fast, non-rigorous (for more than two hosts) adaptation of the procedure outlined in Romero-Severson et al. (PNAS 2016). This is specified by using the option "`r`". It will not reliably reconstruct multiple infections.
- A parsimony reconstruction using the Sankoff algorithm that allows for an additional "unassigned" state and can be configured to penalise the reconstruction of single infections to hosts which imply unreasonable amounts of diversity. This is specified by two arguments, separated by commas. The first is "`s`". The second is the value of the k parameter that penalises within-host diversity; if this is set to 0 then no penalty is applied and the procedure is equivalent to standard parsimony with the unassigned state. It is recommended that users explore their data to pick suitable values of k, as it is likely to be pathogen- and sequencing method-dependent. As a rule of thumb, it should be the reciprocal of a patristic distance so large that it would be surprising to encounter a host with an infection so diverse that a phylogeny built from pathogen sequences from just that host had a branch that long or longer, if the host was infected only once.

The latter parsimony reconstruction algorithm uses a very large matrix for large trees and occasionally `R` will run out of memory; the argument `--useff` will cause the script to use the `ff` library instead, which solves many memory issues at the cost of speed.

Of concern in either of the two proper parsimony reconstruction algorithms is used is how to handle terminal branches of zero or negligible length. In particular, if a tip with a read count of n is connected

to its parent node by such a branch, should that tip be considered as one tip or n tips for the parsimony reconstruction? As an illustrated example, see below. Phylogenetic software output, with minimum-length branches collapsed to zero (see above) might appear as in diagram a) in Fig. **??** below, where colours represent hosts and the numbers in the coloured tips read counts, while numbers on branches are branch lengths (with zero-length branches displayed with nonzero lengths for clarity). The question is whether a parsimony reconstruction should consider this clade as b), where all the reads represented by the blue tip in a) form separate tips but their MRCA m is not the MRCA n of the entire clade, or b), where the MRCAs are the same. Under simple parsimony, the cost of reconstructing n as red is 1 in b) and 3 in c), but the cost of reconstructing it as blue is 2 in both. By default, `phyloscanner_analyse_trees.R` treats this situation as b), which is the same as ignoring read counts in the parsimony reconstruction - as it will always be most parsimonious to reconstruct m as blue to avoid turning one transition into three, the three blue tips can be ignored and the colour of m fixed, so we are back to diagram a) - but the `--readCountsMatterOnZeroLengthBranches` option, if specified, will treat it as c) instead.



Annotated versions of all input trees will be output by `phyloscanner_analyse_trees.R`, in PDF format by default. The `--outputNexusTree` option can be used to make it provide these trees in Nexus format instead.

The standalone R script to perform parsimony reconstruction is `split_hosts_to_subtrees.R`.

14 Summary statistics

By default, `phyloscanner_analyse_trees.R` produces a CSV file of summary statistics with the following columns:

- id: the host ID for the statistics in this row.
- file_suffix: the file suffix of the associated tree file.
- xcoord: used to draw the graphs (see below); this is the midpoint of the genome window if the windowed approach is used, and the position of the tree in alphanumeric ordering if not.
- Tips: the number of tips from this host in this tree.
- Reads: the number of reads from this host in this tree (if read counts were not detected then this column will be identical to the tip counts, as each tip is assumed to represent one read).
- Subgraphs: the number of subgraphs that the parsimony reconstruction identified for this host in this tree.
- clades: the number of separate monophyletic clades from this host in this tree.
- overall.rtt: the mean patristic distance from tips from this host to the MRCA of the tips from this host, weighted by read counts if these are given

- largest.rtt: the mean patristic distance from tips from the largest (by read count) subgraph from the host to the MRCA of that subgraph, weighted by read counts if these are given.
- max.branch.length: the length of the longest branch in the tree obtained by pruning all tips from the tree except the ones from this host.
- max.patristic.distance: the largest patristic distance between any pair of tips from this host.
- global.mean.pat.distance: the mean patristic distance between tips from this host.
- subgraph.mean.pat.distance: the mean patristic distance between tips in the largest subgraph.
- recombination.metric: this column only appears if the recombination metric files output by `phyloscanner_make_trees.py` have been given to `phyloscanner_analyse_trees.R` using the `--recombinationFiles` argument. It gives the value of the recombination metric for this host in this tree.
- prop.gp.1 to prop.gp.X: A variable number of columns recording what proportion of the reads from this host occur in each subgraph, ordered by size. The total number of columns is the largest number of subgraphs present for any host in any tree (and so many columns will be 0 for most hosts).

Also generated, if more than one phylogeny is given as input, is a pdf file graphing these statistics across all trees for every host. Each host occupies a separate page in this file, with five or six graphs per host:

1. Number of reads and number of tips
2. Number of subgraphs and number of clades
3. Mean root-to-tip distance in the largest subgraph and in the whole tree
4. Mean pairwise patristic distance in the largest subgraph and in the whole tree
5. The proportion of reads appearing in each subgraph
6. (If recombination metric files are specified with `-R`) The value of the recombination metric across the genome

If the script could detect genome window coordinates from the file IDs, and the starts and ends of those windows occur regularly across the genome, these graphs will be annotated with light grey rectangles for windows where a host is missing from a single window, and dark grey rectangles where a window appears to be entirely missing (i.e. it had no tree file).

The standalone R script to produce summary statistics is `summary_statistics.R`.

15 Classification of pairwise relationships

The parsimony reconstruction has annotated all internal nodes in each phylogeny with either a host or the unassigned state, and from this information, host subgraphs have been identified. The next step in the script is to document the relationships between each pair of hosts in every tree. By default (when the script is run on multiple tree files) this is done silently, but two types of output file can be generated.

The `--collapsedTrees` option produces the collapsed tree (see the `phyloscanner` paper), in csv format. This file has four columns:

- The collapsed tree node ID, which is the host ID or "unassigned_region" followed by `-S` and an identifying number
- The collapsed tree node ID of that node's unique host, or "root" if it has none
- The ID for the host, or "unassigned_region", associated with this node
- The ID for the host, or "unassigned_region", associated with the parent node

If the script is run on a single tree file only, the classification csv file is produced by default, but the collapsed tree is not.

The `--allClassifications` option produces per-tree csv files describing all pairwise relationships between hosts. The columns of this file are as follows:

- host.1, host.2: the pair of hosts. The order does not signify anything on its own but is relevant to interpretation of the path.classification column.
- adjacent: This is TRUE if there exist two nodes, one each from subgraphs from the two hosts, such that the path between the two traverses only nodes that are either also from these host subgraphs, or unassigned. (In other words, it is possible to move through the phylogeny from one host to the other without moving through the subgraph of another sampled patient.)
- contiguous: This is TRUE if for any two nodes, one each from subgraphs from the two hosts, the path between the two intersects no nodes that are not either also from these host subgraphs, or unassigned. This is similar to adjacent but a little stronger.
- paths12, paths21: paths12 is the number of nodes from host.2 in the collapsed tree which have an ancestor that is from host.1, and paths21 is the opposite.
- nodes1, nodes2: these are the number of collapsed tree nodes for host.1 and host.2 respectively.
- path.classification: this describes the topological relationship between the two hosts in the cablollapsed tree:
  - "noAncestry" indicates that no nodes from host.1 are ancestral to nodes from host.2 or vice versa
  - "anc" indicates that there is only one node from host.2 and it is a descendant of a node from host.1
  - "desc" indicates that there is only one node from host.1 and it is a descendant of a node from host.2
  - "multiAnc" indicates that there is more than one node from host.2 and all are descended from a node from host.1
  - "multiDesc" indicates that there is more than one node from host.1 and all are descended from a node from host.2
  - "complex" indicates that none of the above are true (which is suggestive of an ancestral relationship and often direct transmission, but with unknown direction)
- min.distance.between.subgraphs: this is the smallest patristic distance between a node in a host.1 subgraph and a host.2 subgraph
- normalised.min.distance.between.subgraphs: If the tree branch lengths were normalised, this is the above distance but divided by the normalisation constant for this tree

The standalone script to classify pairwise host relationships is `classify_relationships.R`.

16 Pairwise relationship summary

The next stage in the script is to gather the pairwise relationships between hosts in each window and summarise this across the whole run. Pairs are inferred to be related in a tree if they are both adjacent and (optionally) within the patristic distance threshold specified by the `--distanceThreshold` option. If `--distanceThreshold` is not given then it is assumed to be infinite and adjacency is the only criterion that will be used to identify related pairs. If tree branch lengths were normalised then the distance threshold is applied to normalised distances.

By default, two outputs are produced. The first is a `.csv` file whose first three columns are a pair of hosts and a classification of the topological relationship between the pair. Rows for pairs which are never related at all or never show a particular topological relationship are omitted.

The `--windowThreshold` option is used to specify the minimum proportion of trees in which a pair of hosts were found to be related to make rows for that pair appear in the output file. The default is 0.5, meaning that only pairs that are related in at least half of the trees appear. If this is instead set to 0, all relationships are reported if they ever occur at all.

The `.csv` file has the following columns:

- host.1, host.2: the two hosts.
- ancestry: the type of ancestry (see below).
- ancestry.tree.count: the number of trees in which this pair of hosts are related (by the definition given above) and the relationship is of the type given under "ancestry".
- both.exist: the number of trees in which both hosts are present.
- fraction: the number of windows showing this relationship over the number of windows where both occur ("count" over "both.exist"), as text.
- any.relationship.tree.count: the number of trees in which this pair of hosts are related (by the definition given above).

The ancestry relationships are as follows:

- "noAncestry": no node in the collapsed tree from host.1 is descended from a node from host.2 or vice versa.
- "trans": there is just one collapsed tree node from host.2 and it is descended from a node from host.1. Since they must be adjacent, this makes host.1 its immediate ancestor.
- "multi_trans": there is more than one collapsed tree node from host.2 and all are descended from a node from host.1. This classification only appears if the `--allowMultiTrans` option is given; if not these relationships count as "complex". This is because this relationship is less indicative of the direction of transmission than "trans"; it may simply indicate that the tips from the two hosts are very intermingled, and it is prone to bias introduced by differences in tip counts. By default, the script is conservative and does not take this relationship as a signal of directionality. However, as with "complex", it is quite strongly indicative of direct transmission (Romero-Severson et al. PNAS 2016).
- "complex": where none of the above are true; if `--allowMultiTrans` is not specified then "multi_trans" relationships are also allocated "complex".

The standalone script to summarise these relationships is `transmission_summary.R`.

Unless the `--skipSummaryGraph` option is specified, a PDF file displaying a simplified summary of between-patient relationships will also be produced. The dimensions of this plot (in inches) can be controlled with the `--summaryPlotDimensions` options; the default here is 25×25 inches. If the output plot is too small and cluttered, try using a larger number. The output here will simplify the between-host relationships as in figure 4B of the `phyloscanner` paper, such that there is only one link between any pair. Links will appear according to one of two rules:

- The simplest method is simply to count the proportion of windows in which a given relationship appears, and put thresholds on those proportions. Note that this does not take into account windows in which reads from a host are entirely missing, and are less likely to identify relationships involving a host as its number of missing windows increases.
- As an alternative, a multinomial model for calculating a score for a link, and a score for direction, adjusted for windows in which one or other host is missing. The is described in Ratmann et al. [**?**]. As with the proportion, these scores are between 0 and 1.

The default is the former; the multinomial version is enabled using the `--multinomial` option. In either case, two thresholds are used to identify linked pairs and the direction of transmission. A link will appear as an arrow if the proportion of windows showing transmission in that direction is above the latter, but simply as a line otherwise. Links with arrows are labelled with two numbers separated by a

forward slash: the first is the score (proportion of trees or adjusted value) for the directional relationship implied by the arrow, while the second is the score for any relationship at all. Links without arrows are just labelled with the score for any relationship. The default thresholds are 0.5 for linkage and 0.33 for direcion, but this can be overridden with the `--windowThreshold` and `--directionThreshold` options. It is required that `--directionThreshold` be less than or equal to `--windowThreshold`.

Graph visualisation is a complicated matter, and we cannot guarantee that this PDF file will always display a satisfactory arrangement of nodes and labels. Users wishing to make their own graphs may use an analysis package such as Cytoscape with the summary CSV file as input. A standalone script, `simplify_graph.R`, can be used to simplify the information in this file in the manner described above. See the command line help for that script for more information.

17 Miscellaneous options

Remaining command line options are as follows:

- `--verbose`: Specifies the level of verbose output. The default is "1", which gives minimal output to the command line. "0" gives no output and "2" full output.
- `--noProgressBars`: if present and `--verbose` is given, progress bars will not appear (useful when the output stream is written to file).
- `--outputDir`: the directory to write output files into; if not specified this is the current working directory.
- `--pdfWidth`: the width of the pdf tree file output
- `--pdfRelHeight`: the height, per tip, of the pdf tree file output
- `--pdfScaleBarWidth`: The width of the scale bar in the pdf tree file output (in the same units as the branch lengths in the tree)
- `--outputRDA`: If present, an R list containing the objects used in the calculations is written as a workspace image at the end of the script. This can be explored in `R` using the `phyloscannerR` package.
- `--RDAonly`: If this option is given then only the RDA output will appear. It assumes `--outputRDA` even if that is not given, and overrides `--blacklistReport`, `--allClassifications` and `--collapsedTrees`
- `--seed`: Two parts of the process may use randomisation: the downsampling, and occasional tie-breaking in parsimony reconstruction. This option sets the random number seed to the value provided at the start of `phyloscanner_analyse_trees.R`.
- `--overwrite`: This option tells `phyloscanner_analyse_trees.R` to overwrite any existing output file with the same names.