inStrain

Release 1.0.0

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Contents

InStrain is a tool for analysis of co-occurring genome populations from metagenomes that allows highly accurate genome comparisons, analysis of coverage, microdiversity, and linkage, and sensitive SNP detection with gene localization and synonymous non-synonymous identification

Source code is available on GitHub.

Publication is available on bioRxiv

See links to the left for *Installation* instructions

Comments and suggestions can be sent to Matt Olm and/or Alex Crits-Christoph

Bugs reports and feature requests can be submitted through GitHub.

CHAPTER 1

Contents

1.1 Installation

1.1.1 Installation

InStrain is written in python. There are a number of ways that is can be installed.

pip

To install inStrain using the PyPi python repository, simply run

```
$ pip install instrain
```

That's it!

Pip is a great package with many options to change the installation parameters in various ways. For details, see pip documentation

bioconda

To inStrain inStrain from bioconda, run

```
$ conda config --add channels bioconda; conda install instrain
```

From source

To install inStrain from the source code, run

```
$ git clone https://github.com/MrOlm/instrain.git

$ cd instrain

$ pip install .
```

Dependencies

inStrain requires a few other programs to run. Not all dependencies are needed for all operations. There are a number of python package dependencies, but those should install automatically when inStrain is installed using pip

Essential

- samtools This is needed for pysam

Optional

- coverM This is needed for the quick_profile operation
- Prodigal This is needed to profile on a gene by gene level

1.1.2 Docker image

A Docker image with inStrain and dependencies already installed is available on Docker Hub at mattolm/instrain. This image also has a wrapper script in it to make it easier to use inStrain with AWS. See the docker folder of the GitHub page for use instructions.

1.2 Glossary & FAQ

1.2.1 Overview

When you sequence any microbial genome(s), you sequence a population of cells. This population may be a nearly clonal population grown up from an isolate in a culture flask, or a highly heterogeneous population in the real world, but there is always real biological genetic heterogeneity within that population. Every cell does not have the same genotype at every single position.

InStrain is a program for measuring, comparing, and interrogating the genetic heterogeneity of microbial populations in and between metagenomic samples.

1.2.2 Glossary of terms used in inStrain

Note: This glossary is meant to give a conceptual overview of the terms used in inStrain. See *Example output* for explanations of specific output data.

community The collection of taxa in a metagenome, i.e. the species diversity of a microbiome.

population The collection of cells for each taxa in a metagenome, i.e. the genetic diversity of each species or subspecies in a microbiome. **inStrain is for characterizing metagenomes at the population level**

ANI Average nucleotide identity. The average nucleotide distance between two genomes or .fasta files. If two genomes have a difference every 100 base-pairs, the ANI would be 99%

conANI Consensus ANI - average nucleotide identity values calculated based on consensus sequences. This commonly reported as "ANI" in other programs. Each position on the genome is represented by the most common allele (also referred to as the consensus allele), and minor alleles are ignored.

popANI Population ANI - a new term to describe a unique type of ANI calculation performed by inStrain that considers both major and minor alleles. If two populations share any alleles at a loci, including minor alleles, it does not count as a difference when calculating popANI. It's easiest to describe with an example: consider a genomic position where the reference sequence is 'A' and 100 reads are mapped to the position. Of the 100 mapped reads, 60 have a 'C' and 40 have an 'A' at this position. In this example the reads share a minor allele with the reference genome at the position, but the consensus allele (most common allele) is different. Thus, this position **would** count as a difference in conANI calculations (because the consensus alleles are different) and **would not** count as a difference in popANI calculations (because the reference sequence is present as an allele in the reads).

nucleotide diversity A measurement of genetic diversity in a population (microdiversity). We measure nucleotide diversity using the method from Nei and Li 1979 (often referred to as 'pi' π in the population genetics world). InStrain calculates nucleotide diversity at every position along the genome, based on all reads, and averages values across genes / genomes. This metric is influenced by sequencing error, but within study error rates should be consistent and this effect is often minor compared to the extent of biological variation observed within samples. The formula for calculating nucleotide diversity is the sum of the frequency of each base squared - [(frequency of A)^2 + (frequency of C)^2 + (frequency of G)^2 + (frequency of T)^2]. This definition is nice because it is not effected by coverage.

microdiversity We use the term microdiversity to refer to intraspecific genetic variation, i.e. the genetic variation between cells within a microbial species.

clonality The opposite of nucleotide diversity (1 - nucleotide diversity). A deprecated term used in older versions of the program.

SNV Single nucleotide variant. A single nucleotide change that is present in a faction of a population. Can also be described as a genomic loci with multiple alleles present. We identify and call SNVs using a simple model to distinguish them from errors, and more importantly in our experience, careful read mapping and filtering of paired reads to be assured that the variants (and the reads that contain them) are truly from the species being profiled, and not from another species in the metagenome (we call it 'mismapping' when this happens). Note that a SNV refers to genetic variation *within a read set*.

SNS Single nucleotide substitution. A single nucleotide change that has a fixed difference between two populations. If the reference genome has a 'A' at some position, but all of the reads have a 'C' at that position, that would be a SNS (if half of the reads have an 'A' and half of the reads have a 'C', that would be an SNV).

divergent site A position in the genome where either an SNV or SNS is present.

SNP Single Nucleotide Polymorphism. In our experience this term means different things to different people, so we have tried to avoid using it entirely (instead referring to SNSs, SNVs, and divert sites).

linkage A measure of how likely two divergent sites are to be inherited together. If two alleles are present on the same read, they are said to be "linked", meaning that they are found together on the same genome. Loci are said to be in "linkage disequilibrium" when the frequency of association of their different alleles is higher or lower than what would be expected if the loci were independent and associated randomly. In the context of microbial population genetics, linkage decay is often used as a way to detect recombination among members of a microbial population. InStrain uses the metrics r2 (r squared) and D' (D prime) to measure linkage.

coverage A measure of sequencing depth. We calculate coverage as the average number of reads mapping to a region. If half the bases in a scaffold have 5 reads on them, and the other half have 10 reads, the coverage of the scaffold will be 7.5

breadth A measure of how much of a region is covered by sequencing reads. Breadth is an important concept that is distinct from sequencing coverage, and give you an approximation of how well the reference sequence you're

using is represented by the reads. Calculated as the percentage of bases in a region that are covered by at least a single read. A breadth of 1 means that all bases in a region have at least one read covering them

contig A contiguous sequence of DNA. Usually used as a reference sequence for mapping reads against. The terms contig and scaffold are used interchangeably by inStrain.

scaffold A sequence of DNA that may have a string of "N"s in it representing a gap of unknown length. The terms contig and scaffold are used interchangeably by inStrain.

iRep A measure of how fast a population was replicating at the time of DNA extraction. Based on comparing the sequencing coverage at the origin vs. terminus of replication, as described in Brown et. al., Nature Biotechnology 2016

mutation type Describes the impact of a nucleotide mutation on the amino acid sequence of the resulting protein. N = non-synonymous mutation (the encoded amino-acid changes due to the mutation). S = synonymous mutation (the encoded amino-acid does not change due to the mutation; should happen ~1/6 of the time by random chance due to codon redundancy). I = intergenic mutation. M = multi-allelic SNV with more than one change (rare).

dN/dS A measure of whether the set of mutations in a gene are biased towards synonymous (S) or non-synonymous (N) mutations. dN/dS is calculated bases on mutations relative to the reference genome. dN/dS > 1 means the bias is towards N mutations, indicating the gene is under active selection to mutate. dN/dS < 1 means the bias is towards S mutations, indicated the gene is under stabilizing selection to not mutate. dN/dS = 1 means that N and S mutations are at the rate expected by mutating positions randomly, potentially indicating the gene is non-functional.

pN/pS Very similar to dN/dS, but calculated at positions with at least two alleles present rather than in relation to the reference genome.

fasta file A file containing a DNA sequence. Details on this file format can be found on wikipedia

bam file A file containing metagenomic reads mapped to a DNA sequence. Very similar to a *.sam* file. Details can be found online

mismapped read A read that is erroneously mapped to a genome. InStrain profiles a population by looking at the reads mapped to a genome. These reads are short, and sometimes reads that originated from one microbial population map to the representative genome of another (for example if they share homology). There are several techniques that can be used to reduce mismapping to the lowest extent possible.

multi-mapped read A read that maps equally well to multiple different locations in the .fasta file. Most mapping software will randomly select one position to place multi-mapped reads. There are several techniques that can be used to reduce multi-mapped reads to the lowest extent possible, including increasing the minimum MAPQ cutoff to >2 (which will eliminate them entirely).

inStrain profile An inStrain profile (aka IS_profile, IS, ISP) is created by running the `inStrain profile` command. It contains all of the program's internal workings, cached data, and is where the output is stored. Additional commands can then be run on an IS_profile, for example to analyze genes, compare profiles, etc., and there is lots of nice cached data stored in it that can be accessed using python.

null model The null model describes the probability that the number of true reads that support a variant base could be due to random mutation error, assuming Q30 score. The default false discovery rate with the null model is 1e-6 (one in a million).

mm The maximum number of mismatches a read-pair can have to be considered in the metric being considered. Behind the scenes, inStrain actually calculates pretty much all metrics for every read pair mismatch level. That is, only including read pairs with 0 mismatchs to the reference sequences, only including read pairs with >= 1 mis-match to the reference sequences, all the way up to the number of mismatches associated with the "PID" parameter. Most of the time when it then generates user-facing output, it uses the highest mm possible and deletes the column label. If you'd like access to information on the mm-level, see the section titled "Dealing with mm"

1.2.3 FAQ (Frequently asked questions)

How does inStrain compare to other bioinformatics tools for strains analysis?

A major difference is inStrain's use of the popANI and conANI, which allow consideration of minor alleles when performing genomic comparisons.

Graphical example of output for sample population structu

Method	Description	Programs	Clonal	Heterogenous	I
Consensus SNP calling	The consenus base at each position of the genome defines the strain in that sample. One possible strain detection per sample.	StrainPhlan MIDAS			
Haplotype phasing	Strains are defined as sets of polymorphic bases that co-vary in frequency accross samples. Each strain is a disntinct genotype. Many possible strain detections per sample.	ConStrain DESMAN			
Characterizing the strain cloud	Distict strains are not defined. Polymophic base frequencies, microdiversity, and variations in coverage are calculated for each position along the genome.	inStrain			

What can inStrain do?

inStrain includes calculation of nucleotide diversity, calling SNPs (including non-synonymous and synonymous variants), reporting accurate coverage / breadth, and calculating linkage disequilibrium in the contexts of genomes, contigs, and individual genes.

inStrain also includes comparing the frequencies of fixed and segregating variants between sequenced populations with extremely high accuracy, out-performing other popular strain-resolved metagenomics programs.

The typical use-case is to generate a *.bam* file by mapping metagenomic reads to a bacterial genome that is present in the metagenomic sample, and using inStrain to characterize the microdiversity present.

Another common use-case is detailed strain comparisons that involves comparing the genetic diversity of two populations and calculating the extent to which they overlap. This allows for the calculation of population ANI values for extremely similar genomic populations (>99.999% average nucleotide identity).

See also:

Installation To get started using the program

module_descriptions For descriptions of what the modules can do

Example output To view example output

preparing_input For information on how to prepare data for inStrain

choosing_parameters For detailed information on how to make sure inStrain is running correctly

When should I use inStrain?

inStrain is intended to be used as a genome-resolved metagenomics approach. Genome-resolved metagenomics involves sequencing and de novo assembly of the actual microbial genomes present in the sample(s) of interest. It is these microbial genomes, and not microbial genomes derived from reference databases, that we will then use as scaffolds on which to map reads from the sample.

inStrain can be run on individual microbial genomes assembled and binned from a metagenome, sets of de-replicated microbial genomes, or entire metagenomic assemblies at once.

When should I probably not use inStrain?

When breadth and coverage of the consensus genome are low. When you wish to compare populations that are <95% ANI with each other. When you are interested in species-level community composition, not intra-population diversity.

How does inStrain work?

The reasoning behind inStrain is that every sequencing read is derived from a single DNA molecule (and thus a single cell) in the original population of a given microbial species. During assembly, the consensus of these reads are assembled into contigs and these contigs are binned into genomes - but by returning to assess the variation in the reads that assembled into the contigs, we can characterize the genetic diversity of the population that contributed to the contigs and genomes.

The basic steps:

1. Map reads to a *.fasta* file to create a *.bam* file
2. Stringently filter mapped reads and calculate coverage and breadth
3. Calculate nucleotide diversity and SNVs
4. Calculate SNV linkage
5. Optional: calculate gene statistics and SNV function
6. Optional: compare SNVs between samples.

What is unique about the way that inStrain compares strains?

Most strain-resolved pipelines compare the dominant allele at each position. If you have two closely related strains A and B in sample 1, with B being at higher abundance, and two closely related strains A and C in sample 2, with C being at higher abundance, most strain comparison pipelines will in actuality compare strain B and C. This is because they work on the principle of finding the dominant strain in each sample and then comparing the dominant strains. InStrain, on the other hand, is able to identify the fact that A is present in both samples. This is because it doesn't just compare the dominant alleles, but compares all alleles in the two populations. See module_descriptions and choosing_parameters for more information.

What is a population?

To characterize intra-population genetic diversity, it stands to reason that you first require an adequate definition of "population". InStrain relies mainly on population definitions that are largely technically limited, but also coincide conveniently with possibly biological real microbial population constraints (see Olm et. al. mSystems 2020 and Jain et. al. Nature Communications 2018). Often, we dereplicate genomes from an environment at average nucleotide identities (ANI) from 95% to 99%, depending on the hetereogeneity expected within each sample - lower ANIs might be preferred with more complex samples. We then assign reads to each genome's population by stringently requiring

that combined read pairs for SNP calling be properly mapped pairs with an similarity to the consensus of at least 95% by default, so that the cell that the read pair came from was at least 95% similar to the average consensus genotype at that position. Within environment, inStrain makes it possible to adjust these parameters as needed and builds plots which can be used to estimate the best cutoffs for each project.

What are inStrain's computational requirements?

The two computational resources to consider when running inStrain are the number of processes given (`-p`) and the amount of RAM on the computer (usually not adjustable unless using cloud-based computing).

Using inStrain v1.3.3, running inStrain on a .bam file of moderate size (1 Gbp of less) will generally take less than an hour with 6 cores, and use about 8Gb of RAM. InStrain is designed to handle large .bam files as well. Running a huge .bam file (30 Gbp) with 32 cores, for example, will take ~2 hours and use about 128Gb of RAM. The more processes you give inStrain the longer it will run, but also the more RAM it will use.

In the log folder InStrain provides a lot of information on where it's spending it's time and where it's using it's RAM.

To reduce RAM usage, you can try the following things:

- Use the `--skip_mm` flag. This won't profile things on the mm level (see the above section), and will treat every read pair as perfectly mapped
- Use the `database_mode` flag. This will do a couple of things to try and reduce RAM usage

1.3 Tutorial

1.3.1 Quick Start

The functionality of inStrain is broken up into several core modules. To get an overview of these modules, run `inStrain -h`

```
$ inStrain -h

                ..:::: inStrain v1.3.2 ::::..

  Matt Olm and Alex Crits-Christoph. MIT License. Banfield Lab, UC Berkeley. 2019

  Choose one of the operations below for more detailed help. See https://instrain.
↪readthedocs.io for documentation.
  Example: inStrain profile -h

  Workflows:
    profile         -> Create an inStrain profile (microdiversity analysis) from a␣
↪mapping.
    compare         -> Compare multiple inStrain profiles (popANI, coverage_overlap,␣
↪etc.)

  Single operations:
    profile_genes   -> Calculate gene-level metrics on an inStrain profile␣
↪[DEPRECATED; USE profile INSTEAD]
    genome_wide     -> Calculate genome-level metrics on an inStrain profile
    quick_profile   -> Quickly calculate coverage and breadth of a mapping using␣
↪coverM
    filter_reads    -> Commands related to filtering reads from .bam files
    plot            -> Make figures from the results of "profile" or "compare"
    other           -> Other miscellaneous operations
```

The most useful and commonly-used modules are `profile` and `compare`, which are described below.

profile

inStrain profile is the main method of the program. It takes a *fasta file* and a *bam file* (consisting of reads mapping to the *.fasta* file) and runs a series of steps to characterize the *nucleotide diversity*, SNS's and :term:'SNV's, :term:'linkage, etc.. By providing a scaffold to bin file and/or a genes file to the profile operation, it will also perform all operations in the `profile_genes` and `genome_wide` modules as well.

The most basic inStrain profile command looks like this:

```
$ inStrain profile .bam_file .fasta_file -o IS_output_name
```

compare

inStrain is able to compare multiple read mappings to the same .fasta file. Each mapping file must first be make into an inStrain profile using something like the above `profile` command. A basic inStrain compare command looks like this:

```
$ inStrain compare -i IS_output_1 IS_output_2 IS_output_3
```

1.3.2 Example inStrain commands

TODO

1.3.3 Tutorial #1) Running inStrain with custom genomes

The following tutorial goes through an example run of inStrain using your own set of genomes. You can follow along with your own data, or use a small set of reads that are included in the inStrain install for testing. They can be found in the folder *test/test_data/* of your install folder, or can be downloaded from the inStrain source code at this link on GitHub. The only files that you'll need for this tutorial are forward and reverse metagenomic reads (*N5_271_010G1.R1.fastq.gz* and *N5_271_010G1.R2.fastq.gz*) and a .fasta file to map to (*N5_271_010G1_scaffold_min1000.fa*). In case you're curious, these metagenomic reads come from a premature infant fecal sample.

Preparing .bam and .fasta files

After downloading the genome file that you would like to profile (*fasta file*) and at least one set of paired reads, the first thing to do is to map the reads to the .fasta file in order to generate a *bam file*.

When this mapping is performed it is important that you map to all genomes simultaneously, so the first thing to do is to combine all of the genomes that you'd like to map into a single .fasta file. In our case our .fasta file already has all of the genomes that we'd like to profile within it, but if you did want to profile a number of different genomes, you could combine them using a command like this

```
$  cat raw_data/S2_002_005G1_phage_Clostridioides_difficile.fasta raw_data/S2_018_
↪020G1_bacteria_Clostridioides_difficile.fasta > allGenomes_v1.fasta
```

Next we must map our reads to this .fasta file to create .bam files. In this tutorial we will use the mapping program Bowtie 2

```
$ mkdir bt2

$ bowtie2-build ~/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa␣
↪bt2/N5_271_010G1_scaffold_min1000.fa

$ bowtie2 -p 6 -x bt2/N5_271_010G1_scaffold_min1000.fa -1 ~/Programs/inStrain/test/
↪test_data/N5_271_010G1.R1.fastq.gz -2 ~/Programs/inStrain/test/test_data/N5_271_
↪010G1.R2.fastq.gz > N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sam
```

At this point we have generated a .sam file, the precursor to .bam files. Lets make sure it's there and not empty

```
$ ls -lht

total 34944
-rw-r--r--  1 mattolm  staff    16M Jan 23 11:56 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.sam
drwxr-xr-x  8 mattolm  staff   256B Jan 23 11:54 bt2/
```

Perfect. At this point we could convert the .sam file to a sorted and indexed .bam file, but since inStrain can do that for us automatically we won't bother.

Preparing genes file

If we want inStrain to do gene-level profiling we need to give it a list of genes to profile. **Note - this is an optional step that is not required for inStrain to work in general, but without this you will not get gene-level profiles**

We will profile our genes using the program prodigal, which can be run using the following example command

```
$ prodigal -i ~/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa -d␣
↪N5_271_010G1_scaffold_min1000.fa.genes.fna
```

Preparing for genome-level characterization

In the step above ("Preparing .bam and .fasta files"), we combined all of our genomes into a single .fasta file for mapping. However we likely want to profile the microdiversity of the individual genomes in that .fasta file. In order to do that we need to tell inStrain which scaffolds belong to which genomes.

There are two ways of providing this information. One is to give inStrain a list of the .fasta files that went into making the concatenated .fasta file. The other is to provide inStrain with a "scaffold to bin" file, which lists the genome assignment of each scaffold in a tab-delimited file. In this case we're going to use the scaffold to bin file provided by inStrain (called "N5_271_010G1.maxbin2.stb"). Here's what it looks like

```
$ head ~/Programs/inStrain/test/test_data/N5_271_010G1.maxbin2.stb
N5_271_010G1_scaffold_0        maxbin2.maxbin.001.fasta
N5_271_010G1_scaffold_1        maxbin2.maxbin.001.fasta
N5_271_010G1_scaffold_2        maxbin2.maxbin.001.fasta
N5_271_010G1_scaffold_3        maxbin2.maxbin.001.fasta
N5_271_010G1_scaffold_4        maxbin2.maxbin.001.fasta
```

Running inStrain profile

Now that we've gotten everything set up, it's time to run inStrain. To see all of the options, run

```
$ inStrain -h
```

A long list of arguments and options will show up. For more details on what these do, see *User Manual*. The **only** arguments that are absolutely required, however, are a .sam or .bam mapping file, and the .fasta file that the mapping file is mapped to.

Note: In this case we're going to have inStrain profile the mapping, call genes, make the results genome wide, and plot the results all in one command. It is possible to do these all as separate steps, however, using the subcommands "inStrain profile", "inStrain profile_genes", "inStrain genome_wide", and "inStrain plot". See :doc:`user_manual

---

` for more information.

Using all of the files we generated above, here is going to be our inStrain command

```
$ inStrain profile N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sam ~/Programs/
↪inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa -o N5_271_010G1_scaffold_
↪min1000.fa-vs-N5_271_010G1.IS -p 6 -g N5_271_010G1_scaffold_min1000.fa.genes.fna -s␣
↪~/Programs/inStrain/test/test_data/N5_271_010G1.maxbin2.stb
```

You should see the following as inStrain runs (should only take a few minutes)

```
You gave me a sam- I'm going to make it a .bam now
Converting N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sam to N5_271_010G1_
↪scaffold_min1000.fa-vs-N5_271_010G1.bam
samtools view -S -b N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sam > N5_271_
↪010G1_scaffold_min1000.fa-vs-N5_271_010G1.bam
sorting N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.bam
samtools sort N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.bam -o N5_271_010G1_
↪scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam
Indexing N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam
samtools index N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam N5_271_
↪010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam.bai
*************************************************
    ..:: inStrain profile Step 1. Filter reads ::..
*************************************************

Getting read pairs: 100%|| 178/178 [00:00<00:00, 715.57it/s]
Making read report
/Users/mattolm/.pyenv2/versions/3.6.9/lib/python3.6/site-packages/numpy/core/
↪fromnumeric.py:3335: RuntimeWarning: Mean of empty slice.
  out=out, **kwargs)
/Users/mattolm/.pyenv2/versions/3.6.9/lib/python3.6/site-packages/numpy/core/_methods.
↪py:161: RuntimeWarning: invalid value encountered in double_scalars
  ret = ret.dtype.type(ret / rcount)
Filtering reads
1,727 read pairs remain after filtering
*************************************************
.:: inStrain profile Step 2. Profile scaffolds ::..
*************************************************

Profiling scaffolds: 100%|| 23/23 [00:06<00:00,  3.44it/s]
Storing output
*************************************************
  .:: inStrain profile Step 3. Profile genes ::..
*************************************************
```

(continues on next page)

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```
20.67703568161025% of the input 1093 genes were marked as incomplete
161 scaffolds with genes, 169 in the IS, 153 to compare
Running gene-level calculations on scaffolds: 100%|| 153/153 [00:18<00:00,  8.16it/s]
*************************************************
.:: inStrain profile Step 4. Make genome-wide ::..
*************************************************

Scaffold to bin was made using .stb file
85.66% of scaffolds have a genome
93.82% of scaffolds have a genome
*************************************************
 .:: inStrain profile Step 5. Generate plots ::..
*************************************************

making plots 1, 2, 3, 4, 5, 6, 7, 8, 9
85.66% of scaffolds have a genome
Plotting plot 1
Plotting plot 2
85.66% of scaffolds have a genome
Plotting plot 3
57.37% of scaffolds have a genome
Plotting plot 4
97.33% of scaffolds have a genome
Plotting plot 5
93.82% of scaffolds have a genome
Plotting plot 6
Plotting plot 7
97.33% of scaffolds have a genome
Plotting plot 8
93.96% of scaffolds have a genome
Plotting plot 9
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$

    ..:: inStrain profile finished ::..

Output tables........ /Users/mattolm/Programs/testing_house/tutorial/N5_271_010G1_
↪scaffold_min1000.fa-vs-N5_271_010G1.IS/output/
Figures.............. /Users/mattolm/Programs/testing_house/tutorial/N5_271_010G1_
↪scaffold_min1000.fa-vs-N5_271_010G1.IS/figures/

See documentation for output descriptions - https://instrain.readthedocs.io/en/latest/

$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
```

The last note shows you where the plots and figures have been made. Here's a list of the files that you should see

```
$ ls -lht N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.IS/output/
total 512
-rw-r--r--  1 mattolm  staff   545B Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_genomeWide_mapping_info.tsv
-rw-r--r--  1 mattolm  staff   602B Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_genomeWide_scaffold_info.tsv
-rw-r--r--  1 mattolm  staff    25K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_SNP_mutation_types.tsv
-rw-r--r--  1 mattolm  staff   125K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_gene_info.tsv
-rw-r--r--  1 mattolm  staff    19K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_mapping_info.tsv
```

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```
-rw-r--r--  1 mattolm  staff    14K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_linkage.tsv
-rw-r--r--  1 mattolm  staff    26K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_SNVs.tsv
mattolm@Matts-MacBook-Pro-3:~/Programs/testing_house/tutorial$ caffold_min1000.fa-vs-
↪N5_271_010G1.IS_scaffold_info.tsv

$ ls -lht N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.IS/figures
total 7792
-rw-r--r--  1 mattolm  staff   432K Jan 23 15:17 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_GeneHistogram_plot.pdf
-rw-r--r--  1 mattolm  staff   422K Jan 23 15:17 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_LinkageDecay_types_plot.pdf
-rw-r--r--  1 mattolm  staff   448K Jan 23 15:17 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_ScaffoldInspection_plot.pdf
-rw-r--r--  1 mattolm  staff   419K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_ReadFiltering_plot.pdf
-rw-r--r--  1 mattolm  staff   421K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_LinkageDecay_plot.pdf
-rw-r--r--  1 mattolm  staff   420K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_MajorAllele_frequency_plot.pdf
-rw-r--r--  1 mattolm  staff   419K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_readANI_distribution.pdf
-rw-r--r--  1 mattolm  staff   443K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_genomeWide_microdiveristy_metrics.pdf
-rw-r--r--  1 mattolm  staff   419K Jan 23 15:16 N5_271_010G1_scaffold_min1000.fa-vs-
↪N5_271_010G1.IS_CoverageAndBreadth_vs_readMismatch.pdf
```

For help interpreting these output files, see *Example output*

1.3.4 Tutorial #2) Comparing strains with inStrain compare

InStrain compare allows you to compare genomes that have been profiled by multiple mappings. To compare a genome in multiple samples, you must first map reads from multiple samples to the **same** .fasta file, then run run `inStrain profile` on each mapping.

In Tutorial #1 we profiled reads mapped to the .fasta file "N5_271_010G1_scaffold_min1000.fa". Provided in the inStrain test_data folder is also a different set of reads mapped to the same .fasta file. We've also already run inStrain on this mapping for you! The resulting inStrain profile is the folder *N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G2.IS/*

To compare these inStrain profiles we will use the following command

```
$ inStrain compare -i N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.IS/ ~/Programs/
↪inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G2.IS/ -o N5_
↪271_010G1_scaffold_min1000.fa.IS.COMPARE -p 6

 Loading N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.IS/
 Loading /Users/mattolm/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_
↪min1000.fa-vs-N5_271_010G2.IS/
 Warning! Your inStrain folder is from version 1.0.0, while the installed version is␣
↪1.2.1.
 If you experience weird behavior, this might be why
 169 of 178 scaffolds are in at least 2 samples
 Profiling scaffolds: 100%|| 169/169 [00:22<00:00,  7.38it/s]
```

You should now have the following output file created

```
$ ls -lht N5_271_010G1_scaffold_min1000.fa.IS.COMPARE/output/
total 64
-rw-r--r--  1 mattolm  staff    30K Jan 23 15:20 N5_271_010G1_scaffold_min1000.fa.IS.
↪COMPARE_comparisonsTable.tsv
```

This file shows the comparison values between scaffolds, however. To make these on the genome level, we can run `inStrain genome_wide`

```
$ inStrain genome_wide -i N5_271_010G1_scaffold_min1000.fa.IS.COMPARE/ -s ~/Programs/
↪inStrain/test/test_data/N5_271_010G1.maxbin2.stb
Scaffold to bin was made using .stb file
89.62% of scaffolds have a genome
```

Now we should also have a table that compares these genomes on the genome level

```
$ ls -lht N5_271_010G1_scaffold_min1000.fa.IS.COMPARE/output/
total 72
-rw-r--r--  1 mattolm  staff   556B Jan 23 15:23 N5_271_010G1_scaffold_min1000.fa.IS.
↪COMPARE_genomeWide_compare.tsv
-rw-r--r--  1 mattolm  staff    30K Jan 23 15:20 N5_271_010G1_scaffold_min1000.fa.IS.
↪COMPARE_comparisonsTable.tsv
```

Finally, we can also plot these results using the `inStrain plot` function

```
$ inStrain plot -i N5_271_010G1_scaffold_min1000.fa.IS.COMPARE/
making plots 10
89.62% of scaffolds have a genome
Plotting plot 10
Done!
```

This should make a figure in the figures folder

```
$ ls -lht N5_271_010G1_scaffold_min1000.fa.IS.COMPARE/figures/
total 936
-rw-r--r--  1 mattolm  staff   419K Jan 23 15:25 N5_271_010G1_scaffold_min1000.fa.IS.
↪COMPARE_inStrainCompare_dendrograms.pdf
```

As before, for help interpreting this output see *Example output* .

1.3.5 Tutorial #3) Running inStrain using public reference genomes

TODO

We are working on a tutorial on the use of inStrain with publicaly available reference genomes but it's not complete yet. If this is something you'd like to do, reach out via email or GitHub and we we'll do our best to speed-up the creation of this tutorial.

1.4 User Manual

1.4.1 Generating inStrain input

There are two main inputs to inStrain: a *fasta file* containing reference genome sequences, and a *bam file* containing reads mapped to these sequences. Additionally and optionally, by providing a genes *.fna* file inStrain can calculate

gene-level metrics, and by providing a scaffold-to-bin file inStrain can calculate metrics on a genome level. Here we go over some considerations involved in generating these inputs.

Preparing the .fasta file

A *fasta file* contains the DNA sequences of the contigs that you map your reads to. Choosing what *fasta file* you will use (consensus / reference genomes) is important and will affect the interpretation of your *inStrain* results. Below we describe the three most common strategies.

Please note that the *fasta file* provided to inStrain must always be the same as, or a subset of, the *fasta file* used to create the *bam file* (i.e. the *fasta file* that reads were mapped to).

Using *de novo* assembled genomes (recommended)

This strategy involves assembling genomes from the metagenomic samples that you'd like to profile. This is the recommended workflow for running *inStrain*:

1. Assemble reads into contigs for each sample collected from the environment. Recommended software: IDBA_UD, MEGAHIT, metaSPADES.
2. Bin genomes out of each assembly using differential coverage binning. Recommended software: Bowtie2 (for mapping), MetaBAT, CONCOCT, DasTOOL (for binning).
3. Dereplicate the entire set of genomes that you would like to profile (all genomes from all environments) at 97-99% identity, and filter out low quality genomes. Recommended software: dRep, checkM.
4. Create a bowtie2 index of the representative genomes from this dereplicated set and map reads to this set from each sample: Recommended software: Bowtie2
5. Profile the resulting mapping *.bam* files using inStrain.
6. Use *inStrain genome_wide* to calculate genome-level microdiveristy metrics for each originally binned genome.

An important aspect of this workflow is to **map to many genomes at once**. Mapping to just one genome at a time is highly discouraged, because this encourages *mismapped reads* from other genomes to be recruited by this genome. By including many (dereplicated) genomes in your bowtie2 index, you will be able to far more accurately filter out *mismapped reads* and reduce false positive SNPs.

Using a single genome .fasta file

If your .fasta file is a single genome, the main consideration is that it should be a good representative genome for some organism in your sample. Ideally, it was assembled directly from that sample, isolated from that sample, or you have some other evidence that this genome is highly representation of a species in that sample. Regardless, you should check your *inStrain plot* output and *scaffold_info.tsv* output file to be sure that your inStrain run had decent coverage and breadth of coverage of the genome that you use before attempting to interpret the results.

Remember, your .fasta file can be a subset of the .fasta file that was used to create the .bam file. You can create a .bam with all dereplicated genomes from your environment, but then just pass a .fasta file for only the genomes of particular interest. This approach is recommended as opposed to creating a *bam file* for just each genome, as it reduces *mismapped reads*

Using a metagenomic assembly

You can also pass inStrain an entire metagenomic assembly from a sample, including both binned and unbinned contigs. In this case, the output inStrain profile will include population information for each contig in the set. To break

it down by microbial genome / species, you can include a scaffold to bin file to generate results by genome.

Preparing the .bam file

InStrain is designed primarily for paired-end Illumina read sequencing, though un-paired reads can also be used by adjusting the run-time parameters. We recommend using the program Bowtie2 to map your reads to your genome.

Bowtie2 default parameters are what we use for mapping, but it may be worth playing around with them to see how different settings perform on your data. It is important to note that the `-X` flag (capital X) is the expected insert length and is by default `500`. In many cases (e.g., 2x250 bp or simply datasets with longer inserts) it may be worthwhile to increase this value up to `-X 1000` for passing to Bowtie2. By default, if a read maps equally well to multiple genomes, Bowtie2 will pick one of the positions randomly and give the read a MAPQ score of 1. Thus, if you'd like to remove *multi-mapped reads*, you can set you minimum mapQ score to 2.

Other mapping software can also be used to generate .bam files for inStrain. However, some software (e.g. BBmap and SNAP) use the fasta file scaffold descriptions when generating the .bam files, which causes problems for inStrain. If using mapping software that does this, include the flag `--use_full_fasta_header` to let inStrain account for this.

Note: If the reads that you'd like to run with inStrain are not working, please post an issue on GitHub. We're happy to upgrade inStrain to work with new mapping software and/or reads from different technologies.

Preparing the genes file

You can run prodigal on your *fasta file* to generate an .fna file with the gene-level information. This .fna file can then be provided to inStrain profile to get gene-level characterizations.

Example:

```
$ prodigal -i assembly.fasta -d genes.fna
```

Preparing a scaffold-to-bin file

After running `inStrain profile`, most results are presented on a scaffold-by-scaffold basis. There are a number of ways of telling *inStrain* which scaffold belongs to which genome, so that results can be analyzed on a genome-by-gene level as well.

1. Individual .fasta files. As recommended above, if you want to run *inStrain* on multiple genomes in the same sample, you should first concatenate all of the individual genomes into a single *.fasta* file and map to that. To view the results of the individual genomes used to create the concatenated .fasta file, you can pass a list of the individual .fasta files the `-s` arguement.
2. Scaffold-to-bin file. This is a text file consists of two columns, with one column listing the scaffold name, and the second column listing the genome bin name. Columns should be separated by tabs. The script parse_stb.py can help you create a scaffold-to-bin file from a list of individual .fasta files, or to split a concatenated .fasta file into individual genomes. The script comes packaged with the program dRep, and can be installed with the command `pip install drep`.
3. Nothing. If all of your scaffolds belong to the same genome, by running `inStrain profile` without any *-s* options it will summarize the results of all scaffolds together as if they all belong to the same genome.

1.4.2 Description of inStrain modules and arguments

The functionality of inStrain is broken up into modules. To see a list of available modules, check the help:

```
$ inStrain -h

                ..:::: inStrain v1.3.2 ::::..

  Matt Olm and Alex Crits-Christoph. MIT License. Banfield Lab, UC Berkeley. 2019

  Choose one of the operations below for more detailed help. See https://instrain.
↪readthedocs.io for documentation.
  Example: inStrain profile -h

  Workflows:
    profile         -> Create an inStrain profile (microdiversity analysis) from a␣
↪mapping.
    compare         -> Compare multiple inStrain profiles (popANI, coverage_overlap,␣
↪etc.)

  Single operations:
    profile_genes   -> Calculate gene-level metrics on an inStrain profile␣
↪[DEPRECATED; USE profile INSTEAD]
    genome_wide     -> Calculate genome-level metrics on an inStrain profile
    quick_profile   -> Quickly calculate coverage and breadth of a mapping using␣
↪coverM
    filter_reads    -> Commands related to filtering reads from .bam files
    plot            -> Make figures from the results of "profile" or "compare"
    other           -> Other miscellaneous operations
```

profile

Module description

The most complex part of inStrain, and must be run before any other modules can be. The input is a *fasta file* and a *bam file*, and the output is an *IS_profile*. The functionality of `inStrain profile` is broken into several steps.

First, all reads in the .bam file are filtered to only keep those that map with sufficient quality. All non-paired reads will be filtered out by default, and an additional set of filters are applied to each read pair (not the individual reads). Command line parameters can be adjusted to change the specifics, but in general:

- Pairs must be mapped in the proper orientation with an expected insert size. The minimum insert distance can be set with a command line parameter. The maximum insert distance is a multiple of the median insert distance. So if pairs have a median insert size of 500bp, by default all pairs with insert sizes over 1500bp will be excluded. For the max insert cutoff, the median_insert for all scaffolds is used.
- Pairs must have a minimum mapQ score. MapQ scores are confusing and how they're calculated varies based on the mapping algorithm being used, but are meant to represent both the number of mismatches in the mapping and how unique that mapping is. With bowtie2, if the read maps equally well to two positions on the genome (*multi-mapped read*), its mapQ score will be set to 2. The read in the pair with the higher mapQ is used for the pair.
- Pairs must be above some minimum nucleotide identity (ANI) value. For example if reads in a pair are 100bp each, and each read has a single mismatch, the ANI of that pair would be 0.99

Next, using only read pairs that pass filters, a number of microdiveristy metrics are calculated on a scaffold-by-scaffold basis. This includes:

- Calculate the coverage at each position along the scaffold
- Calculate the *nucleotide diversity* at each position along the scaffold in which the coverage is greater than the min_cov argument.
- Identify *SNSs* and *SNVs*. The criteria for being reported as a *divergent site* are 1) More than min_cov number of bases at that position, 2) More than min_freq percentage of reads that are a variant base, 3) The number of reads with the variant base is more than the *null model* for that coverage.
- Calculate *linkage* between *divergent sites* on the same read pair. For each pair harboring a *divergent site*, calculate the linkage of that site with other *divergent sites* within that same pair. This is only done for pairs of *divergent sites* that are both on at least MIN_SNP reads
- Calculate scaffold-level properties. These include things like the overall coverage, breadth of coverage, average nucleotide identity (ANI) between the reads and the reference genome, and the expected breadth of coverage based on that true coverage.

Finally, this information is stored as an *IS_profile* object. This includes the locations of *divergent sites*, the number of read pairs that passed filters (and other information) for each scaffold, the linkage between SNV pairs, ect.

Module parameters

To see the command-line arguments for inStrain profile, check the help:

```
$ inStrain profile -h
usage: inStrain profile [-o OUTPUT] [--use_full_fasta_header] [-p PROCESSES]
                        [-d] [-h] [--version] [-l MIN_READ_ANI]
                        [--min_mapq MIN_MAPQ]
                        [--max_insert_relative MAX_INSERT_RELATIVE]
                        [--min_insert MIN_INSERT]
                        [--pairing_filter {paired_only,all_reads,non_discordant}]
                        [--priority_reads PRIORITY_READS]
                        [--detailed_mapping_info] [-c MIN_COV] [-f MIN_FREQ]
                        [-fdr FDR] [-g GENE_FILE] [-s [STB [STB ...]]]
                        [--mm_level] [--skip_mm_profiling] [--database_mode]
                        [--min_scaffold_reads MIN_SCAFFOLD_READS]
                        [--min_genome_coverage MIN_GENOME_COVERAGE]
                        [--min_snp MIN_SNP] [--store_everything]
                        [--scaffolds_to_profile SCAFFOLDS_TO_PROFILE]
                        [--rarefied_coverage RAREFIED_COVERAGE]
                        [--window_length WINDOW_LENGTH] [--skip_genome_wide]
                        [--skip_plot_generation]
                        bam fasta

REQUIRED:
  bam                   Sorted .bam file
  fasta                 Fasta file the bam is mapped to

I/O PARAMETERS:
  -o OUTPUT, --output OUTPUT
                        Output prefix (default: inStrain)
  --use_full_fasta_header
                        Instead of using the fasta ID (space in header before
                        space), use the full header. Needed for some mapping
                        tools (including bbMap) (default: False)

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
```

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```
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
  --version             show program's version number and exit

READ FILTERING OPTIONS:
  -l MIN_READ_ANI, --min_read_ani MIN_READ_ANI
                        Minimum percent identity of read pairs to consensus to
                        use the reads. Must be >, not >= (default: 0.95)
  --min_mapq MIN_MAPQ   Minimum mapq score of EITHER read in a pair to use
                        that pair. Must be >, not >= (default: -1)
  --max_insert_relative MAX_INSERT_RELATIVE
                        Multiplier to determine maximum insert size between
                        two reads - default is to use 3x median insert size.
                        Must be >, not >= (default: 3)
  --min_insert MIN_INSERT
                        Minimum insert size between two reads - default is 50
                        bp. If two reads are 50bp each and overlap completely,
                        their insert will be 50. Must be >, not >= (default:
                        50)
  --pairing_filter {paired_only,all_reads,non_discordant}
                        How should paired reads be handled?
                        paired_only = Only paired reads are retained
                        non_discordant = Keep all paired reads and singleton reads␣
↪that map to a single scaffold
                        all_reads = Keep all reads regardless of pairing status (NOT␣
↪RECOMMENDED; See documentation for deatils)
                         (default: paired_only)
  --priority_reads PRIORITY_READS
                        The location of a list of reads that should be
                        retained regardless of pairing status (for example
                        long reads or merged reads). This can be a .fastq file
                        or text file with list of read names (will assume file
                        is compressed if ends in .gz (default: None)

READ OUTPUT OPTIONS:
  --detailed_mapping_info
                        Make a detailed read report indicating deatils about
                        each individual mapped read (default: False)

VARIANT CALLING OPTIONS:
  -c MIN_COV, --min_cov MIN_COV
                        Minimum coverage to call an variant (default: 5)
  -f MIN_FREQ, --min_freq MIN_FREQ
                        Minimum SNP frequency to confirm a SNV (both this AND
                        the FDR snp count cutoff must be true to call a SNP).
                        (default: 0.05)
  -fdr FDR, --fdr FDR   SNP false discovery rate- based on simulation data
                        with a 0.1 percent error rate (Q30) (default: 1e-06)

GENE PROFILING OPTIONS:
  -g GENE_FILE, --gene_file GENE_FILE
                        Path to prodigal .fna genes file. If file ends in .gb
                        or .gbk, will treat as a genbank file (EXPERIMENTAL;
                        the name of the gene must be in the gene qualifier)
                        (default: None)
```

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```
GENOME WIDE OPTIONS:
  -s [STB [STB ...]], --stb [STB [STB ...]]
                        Scaffold to bin. This can be a file with each line
                        listing a scaffold and a bin name, tab-seperated. This
                        can also be a space-seperated list of .fasta files,
                        with one genome per .fasta file. If nothing is
                        provided, all scaffolds will be treated as belonging
                        to the same genome (default: [])

READ ANI OPTIONS:
  --mm_level            Create output files on the mm level (see documentation
                        for info) (default: False)
  --skip_mm_profiling   Dont perform analysis on an mm level; saves RAM and
                        time; impacts plots and raw_data (default: False)

PROFILE OPTIONS:
  --database_mode       Set a number of parameters to values appropriate for
                        mapping to a large fasta file. Will set:
                        --min_read_ani 0.92 --skip_mm_profiling
                        --min_genome_coverage 1 (default: False)
  --min_scaffold_reads MIN_SCAFFOLD_READS
                        Minimum number of reads mapping to a scaffold to
                        proceed with profiling it (default: 1)
  --min_genome_coverage MIN_GENOME_COVERAGE
                        Minimum number of reads mapping to a genome to proceed
                        with profiling it. MUST profile .stb if this is set
                        (default: 0)
  --min_snp MIN_SNP     Absolute minimum number of reads connecting two SNPs
                        to calculate LD between them. (default: 20)
  --store_everything    Store intermediate dictionaries in the pickle file;
                        will result in significantly more RAM and disk usage
                        (default: False)
  --scaffolds_to_profile SCAFFOLDS_TO_PROFILE
                        Path to a file containing a list of scaffolds to
                        profile- if provided will ONLY profile those scaffolds
                        (default: None)
  --rarefied_coverage RAREFIED_COVERAGE
                        When calculating nucleotide diversity, also calculate
                        a rarefied version with this much coverage (default:
                        50)
  --window_length WINDOW_LENGTH
                        Break scaffolds into windows of this length when
                        profiling (default: 10000)

OTHER  OPTIONS:
  --skip_genome_wide    Do not generate tables that consider groups of
                        scaffolds belonging to genomes (default: False)
  --skip_plot_generation
                        Do not make plots (default: False)
```

compare

Module description

Compare provides the ability to compare multiple *inStrain profiles* (created by running `inStrain profile`).

Note: *inStrain* can only compare :term:‘inStrain profiles<inStrain profile>‘that have been mapped to the same .fasta file

`inStrain compare` does pair-wise comparisons between each input *inStrain profile*. For each pair, a series of steps are undertaken.

1. All positions in which both IS_profile objects have at least *min_cov* coverage (5x by default) are identified. This information can be stored in the output by using the flag *–store_coverage_overlap*, but due to it's size, it's not stored by default
2. Each position identified in step 1 is compared to calculate both *conANI* and *popANI*. The way that it compares positions is by testing whether the consensus base in sample 1 is detected at all in sample 2 and vice-verse. Detection of an allele in a sample is based on that allele being above the set *-min_freq* and *-fdr*. All detected differences between each pair of samples can be reported if the flag *–store_mismatch_locations* is set.
3. The coverage overlap and the average nucleotide identify for each scaffold is reported. For details on how this is done, see *Example output*

Module parameters

To see the command-line options, check the help:

```
$ inStrain compare -h
usage: inStrain compare -i [INPUT [INPUT ...]] [-o OUTPUT] [-p PROCESSES] [-d]
                        [-h] [--version] [-c MIN_COV] [-f MIN_FREQ] [-fdr FDR]
                        [-s SCAFFOLDS] [--store_coverage_overlap]
                        [--store_mismatch_locations]
                        [--include_self_comparisons] [--greedy_clustering]
                        [--g_ani G_ANI] [--g_cov G_COV] [--g_mm G_MM]

REQUIRED:
  -i [INPUT [INPUT ...]], --input [INPUT [INPUT ...]]
                        A list of inStrain objects, all mapped to the same
                        .fasta file (default: None)
  -o OUTPUT, --output OUTPUT
                        Output prefix (default: instrainComparer)

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
  --version             show program's version number and exit

VARIANT CALLING OPTIONS:
  -c MIN_COV, --min_cov MIN_COV
                        Minimum coverage to call an variant (default: 5)
  -f MIN_FREQ, --min_freq MIN_FREQ
                        Minimum SNP frequency to confirm a SNV (both this AND
                        the FDR snp count cutoff must be true to call a SNP).
                        (default: 0.05)
  -fdr FDR, --fdr FDR   SNP false discovery rate- based on simulation data
                        with a 0.1 percent error rate (Q30) (default: 1e-06)

OTHER OPTIONS:
```

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```
  -s SCAFFOLDS, --scaffolds SCAFFOLDS
                        Location to a list of scaffolds to compare. You can
                        also make this a .fasta file and it will load the
                        scaffold names (default: None)
  --store_coverage_overlap
                        Also store coverage overlap on an mm level (default:
                        False)
  --store_mismatch_locations
                        Store the locations of SNPs (default: False)
  --include_self_comparisons
                        Also compare IS profiles against themself (default:
                        False)

GREEDY CLUSTERING OPTIONS [THIS SECTION IS EXPERIMENTAL!]:
  --greedy_clustering   Dont do pair-wise comparisons, do greedy clustering to
                        only find the number of clsuters. If this is set, use
                        the parameters below as well (default: False)
  --g_ani G_ANI         ANI threshold for greedy clustering- put the fraction
                        not the percentage (e.g. 0.99, not 99) (default: 0.99)
  --g_cov G_COV         Alignment coverage for greedy clustering- put the
                        fraction not the percentage (e.g. 0.5, not 10)
                        (default: 0.99)
  --g_mm G_MM           Maximum read mismatch level (default: 100)
```

Other modules

The other modules are not commonly used, and mainly provide auxiliary functions or allow you run certain steps of `profile` after the fact. It is recommended to provide a genes file and/or a scaffold-to-bin file during `inStrain profile` rather than using `profile_genes` or `genome_wide`, as it is more computationally efficient to do things that way.

profile_genes

After running *inStrain profile* on a sample, you can providing a file of gene calls to calculate gene-level metrics after the fact. This is less computationally efficient than providing the genes file to `profile` in the first place, however. See above for information about creating the input file.

To see the command-line options, check the help:

```
$ inStrain profile_genes -h
usage: inStrain profile_genes [-g GENE_FILE] -i IS [--store_everything]
                              [-p PROCESSES] [-d] [-h] [--version]

GENE PROFILING OPTIONS:
  -g GENE_FILE, --gene_file GENE_FILE
                        Path to prodigal .fna genes file. If file ends in .gb
                        or .gbk, will treat as a genbank file (EXPERIMENTAL;
                        the name of the gene must be in the gene qualifier)
                        (default: None)

INPUT / OUTPUT:
  -i IS, --IS IS        an inStrain profile object (default: None)
  --store_everything    Store gene sequences in the IS object (default: False)
```

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```
SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
  --version             show program's version number and exit
```

genome_wide

After running *inStrain profile* on a sample, you can provide information to analyze things on the genome level after the fact. This is less computationally efficient than providing the stb file to `profile` in the first place, however. See above for information about creating the input file.

To see the command-line options, check the help:

```
$ inStrain genome_wide -h
usage: inStrain genome_wide [-s [STB [STB ...]]] -i IS [--store_everything]
                            [--mm_level] [--skip_mm_profiling] [-p PROCESSES]
                            [-d] [-h] [--version]

GENOME WIDE OPTIONS:
  -s [STB [STB ...]], --stb [STB [STB ...]]
                        Scaffold to bin. This can be a file with each line
                        listing a scaffold and a bin name, tab-seperated. This
                        can also be a space-seperated list of .fasta files,
                        with one genome per .fasta file. If nothing is
                        provided, all scaffolds will be treated as belonging
                        to the same genome (default: [])

INPUT / OUTPUT:
  -i IS, --IS IS        an inStrain profile object (default: None)
  --store_everything    Store gene sequences in the IS object (default: False)

READ ANI OPTIONS:
  --mm_level            Create output files on the mm level (see documentation
                        for info) (default: False)
  --skip_mm_profiling   Dont perform analysis on an mm level; saves RAM and
                        time; impacts plots and raw_data (default: False)

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
  --version             show program's version number and exit
```

quick_profile

This is a quirky module that is not really related to any of the others. It is used to quickly profile a *bam file* to pull out scaffolds from genomes that are at a sufficient breadth.

To use it you must provide a *.bam* file, the *.fasta* file that you mapped to to generate the *.bam* file, and a *scaffold to bin* file (see above section for details). The *stringent_breadth_cutoff* removed scaffolds entirely which have less breath than this (used to make the program run faster and produce smaller output). All scaffolds from genomes with at least

the *breadth_cutoff* are then written to a file. In this way, you can then choose to run inStrain profile only on scaffolds from genomes that known to be of sufficient breadth, speeding up the run and reducing RAM usage (though not by much).

On the backend, this module is really just calling the program coverM **'coverM<https://github.com/wwood/CoverM>'_**

To see the command-line options, check the help:

```
$ inStrain quick_profile -h
usage: inStrain quick_profile [-p PROCESSES] [-d] [-h] [--version]
                              [-s [STB [STB ...]]] [-o OUTPUT]
                              [--breadth_cutoff BREADTH_CUTOFF]
                              [--stringent_breadth_cutoff STRINGENT_BREADTH_CUTOFF]
                              bam fasta

REQUIRED:
  bam                   Sorted .bam file
  fasta                 Fasta file the bam is mapped to

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
  --version             show program's version number and exit

OTHER OPTIONS:
  -s [STB [STB ...]], --stb [STB [STB ...]]
                        Scaffold to bin. This can be a file with each line
                        listing a scaffold and a bin name, tab-seperated. This
                        can also be a space-seperated list of .fasta files,
                        with one genome per .fasta file. If nothing is
                        provided, all scaffolds will be treated as belonging
                        to the same genome (default: [])
  -o OUTPUT, --output OUTPUT
                        Output prefix (default: QuickProfile)
  --breadth_cutoff BREADTH_CUTOFF
                        Minimum genome breadth to pull scaffolds (default:
                        0.5)
  --stringent_breadth_cutoff STRINGENT_BREADTH_CUTOFF
                        Minimum breadth to let scaffold into coverm raw
                        results (done with greater than; NOT greater than or
                        equal to) (default: 0.0)
```

plot

This module produces plots based on the results of *inStrain profile* and *inStrain compare*. In both cases, before plots can be made, *inStrain genome_wide* must be run on the output folder first. In order to make plots 8 and 9, *inStrain profile_genes* must be run first as well.

The recommended way of running this module is with the default *-pl a*. It will just try and make all of the plots that it can, and will tell you about any plots that it fails to make.

See *Example output* for an example of the plots it can make.

To see the command-line options, check the help:

```
$ inStrain plot -h
usage: inStrain plot -i IS [-pl [PLOTS [PLOTS ...]]] [-p PROCESSES] [-d] [-h]

REQUIRED:
  -i IS, --IS IS        an inStrain profile object (default: None)
  -pl [PLOTS [PLOTS ...]], --plots [PLOTS [PLOTS ...]]
                        Plots. Input 'all' or 'a' to plot all
                        1) Coverage and breadth vs. read mismatches
                        2) Genome-wide microdiversity metrics
                        3) Read-level ANI distribution
                        4) Major allele frequencies
                        5) Linkage decay
                        6) Read filtering plots
                        7) Scaffold inspection plot (large)
                        8) Linkage with SNP type (GENES REQUIRED)
                        9) Gene histograms (GENES REQUIRED)
                        10) Compare dendrograms (RUN ON COMPARE; NOT PROFILE)
                         (default: a)

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit
```

other

This module holds odds and ends functionalities. As of version 1.3.1, all it can do is convert old *IS_profile* objects (>v0.3.0) to newer versions (v0.8.0). As the code base around *inStrain* matures, we expect more functionalities to be included here.

To see the command-line options, check the help:

```
$ inStrain other -h
usage: inStrain other [-p PROCESSES] [-d] [-h] [--old_IS OLD_IS]

SYSTEM PARAMETERS:
  -p PROCESSES, --processes PROCESSES
                        Number of processes to use (default: 6)
  -d, --debug           Make extra debugging output (default: False)
  -h, --help            show this help message and exit

OTHER OPTIONS:
  --old_IS OLD_IS       Convert an old inStrain version object to the newer
                        version. (default: None)
```

1.5 Example output

InStrain produces a variety of output in the IS folder depending on which operations are run. Generally, output that is meant for human eyes to be easily interpretable is located in the `output` folder.

1.5.1 inStrain profile

A typical run of inStrain will yield the following files in the output folder:

scaffold_info.tsv

This gives basic information about the scaffolds in your sample at the highest allowed level of read identity.

Table 1: scaffold_info.tsv

scaffold	length	coverage	breadth	nucl_diversity	coverage_median	coverage_std	coverage_SEM	breadth_minCov	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	conANI_reference	[illegible]	[illegible]	divergent_sites	consensus_divergent_sites	population_divergent_sites
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.0	1.0	1.0	0	0	0	0	0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.0	1.0	1.0	0	0	0	0	0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.0	0.0	0.0	0	00	0	0	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]										

scaffold The name of the *scaffold* in the input .fasta file

length Full length of the *scaffold* in the input .fasta file

coverage The average depth of coverage on the scaffold. If half the bases in a scaffold have 5 reads on them, and the other half have 10 reads, the coverage of the scaffold will be 7.5

breadth The percentage of bases in the scaffold that are covered by at least a single read. A breadth of 1 means that all bases in the scaffold have at least one read covering them

nucl_diversity The mean *nucleotide diversity* of all bases in the scaffold that have a nucleotide diversity value calculated. So if only 1 base on the scaffold meats the minimum coverage to calculate nucleotide diversity, the nucl_diversity of the scaffold will be the nucleotide diversity of that base. Will be blank if no positions have a base over the minimum coverage.

coverage_median The median depth of coverage value of all bases in the scaffold, included bases with 0 coverage

coverage_std The standard deviation of all coverage values

coverage_SEM The standard error of the mean of all coverage values (calculated using scipy.stats.sem)

breadth_minCov The percentage of bases in the scaffold that have at least min_cov coverage (e.g. the percentage of bases that have a nucl_diversity value and meet the minimum sequencing depth to call SNVs)

breadth_expected This tells you the breadth that you should expect if reads are evenly distributed along the genome, given the reported coverage value. Based on the function breadth = -1.000 * e^(0.883 * coverage) + 1.000. This is useful to establish whether or not the scaffold is actually in the reads, or just a fraction of the scaffold. If your coverage is 10x, the expected breadth will be ~1. If your actual breadth is significantly lower then the expected breadth, this means that reads are mapping only to a specific region of your scaffold (transposon, prophage, etc.)

nucl_diversity_median The median *nucleotide diversity* value of all bases in the scaffold that have a *nucleotide diversity* value calculated

nucl_diversity_rarefied The average *nucleotide diversity* among positions that have at least `--rarefied_coverage` (50x by default). These values are also calculated by randomly subsetting the reads at that position to `--rarefied_coverage` reads

nucl_diversity_rarefied_median The median rarefied *nucleotide diversity* (similar to that described above)

breadth_rarefied The percentage of bases in a scaffold that have at least `--rarefied_coverage`

conANI_reference The *conANI* between the reads and the reference genome

popANI_reference The *popANI* between the reads and the reference genome

SNS_count The total number of *SNSs* called on this scaffold

SNV_count The total number of *SNVs* called on this scaffold

divergent_site_count The total number of *divergent sites* called on this scaffold

consensus_divergent_sites The total number of *divergent sites* in which the reads have a different consensus allele than the reference genome. These count as "differences" in the conANI_reference calculation, and `breadth_minCov * length` counts as the denominator.

population_divergent_sites The total number of *divergent sites* in which the reads do not have the reference genome base as any allele at all (major or minor). These count as "differences" in the popANI_reference calculation, and `breadth_minCov * length` counts as the denominator.

mapping_info.tsv

This provides an overview of the number of reads that map to each scaffold, and some basic metrics about their quality. The header line (starting with #; not shown in the table below) describes the parameters that were used to filter the reads

Table 2: mapping_info.tsv

scaffold	pass_pairing_filter	filtered_pairs	unfiltered_priority_reads	filtered_priority_reads	pass_min_mapq	mean_insert_distance	median_insert	unfiltered_pairs	pass_min_read_ani	unfiltered_reads	mean_pair_length	pass_max_insert	pass_min_insert	unfiltered_singletons	mean_mapq_score	mean_PID	filtered_singletons	mean_mistmaches
all_scaffolds	[illegible]	[illegible]	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
N5_271_010G1_scaffold_0	[illegible]	[illegible]	[illegible]	0	162.0	[illegible]	[illegible]	[illegible]	[illegible]	364	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
N5_271_010G1_scaffold_5	[illegible]	[illegible]	[illegible]	5	140.0	[illegible]	[illegible]	[illegible]	[illegible]	346	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

scaffold The name of the *scaffold* in the input .fasta file. For the top row this will read `all_scaffolds`, and it has the sum of all rows.

pass_pairing_filter The number of individual reads that pass the selecting pairing filter (only paired reads will pass this filter by default)

filtered_pairs The number of pairs of reads that pass all cutoffs

unfiltered_priority_reads The number of reads that pass the pairing filter because they were part of the `priority_reads` input file (will only be non-0 if a priority reads input file is provided).

filtered_priority_reads The number of priority reads that pass the rest of the filters (will only be non-0 if a priority reads input file is provided).

pass_min_mapq The number of pairs of reads mapping to this scaffold that pass the minimum mapQ score cutoff

mean_insert_distance Among all pairs of reads mapping to this scaffold, the mean insert distance. Note that the insert size is measured from the start of the first read to the end of the second read (2 perfectly overlapping 50bp reads will have an insert size of 50bp)

median_insert Among all pairs of reads mapping to this scaffold, the median insert distance.

unfiltered_pairs The raw number of pairs of reads that map to this scaffold. Only paired reads are used by inStrain

pass_min_read_ani The number of pairs of reads mapping to this scaffold that pass the min_read_ani cutoff

unfiltered_reads The raw number of reads that map to this scaffold

mean_pair_length Among all pairs of reads mapping to this scaffold, the average length of both reads in the pair summed together

mean_mapq_score Among all pairs of reads mapping to this scaffold, the average mapQ score

pass_max_insert The number of pairs of reads mapping to this scaffold that pass the maximum insert size cutoff- that is, their insert size is less than 3x the median insert size of all_scaffolds. Note that the insert size is measured from the start of the first read to the end of the second read (2 perfectly overlapping 50bp reads will have an insert size of 50bp)

unfiltered_singletons The number of reads detected in which only one read of the pair is mapped.

pass_min_insert The number of pairs of reads mapping to this scaffold that pass the minimum insert size cutoff

mean_PID Among all pairs of reads mapping to this scaffold, the average percentage ID of both reads in the pair to the reference .fasta file

mean_mistmaches Among all pairs of reads mapping to this scaffold, the mean number of mismatches

filtered_singletons The number of reads detected in which only one read of the pair is mapped AND which make it through to be considered. This will only be non-0 if the filtering settings allows non-paired reads.

SNVs.tsv

This describes the *SNVs* and *SNSs* that are detected in this mapping. While we should refer to these mutations as *divergent sites*, sometimes SNV is used to refer to both SNVs and SNSs

Table 3: SNVs.tsv

scaffold	position	position_coverage	allele_count	ref_base	con_base	var_base	ref_freq	con_freq	var_freq	A	C	T	G	gene	mutation	mutation_type	cryptic	class
N5_271_010G1_scaffold_120	1174	5	2	C	C	A	0.6	0.6	0.4	2	3	0	0			I	False	SNV
N5_271_010G1_scaffold_120	1195	6	1	T	C	A	0.0	1.0	0.0	0	6	0	0			I	False	SNS
N5_271_010G1_scaffold_120	1401	8	2	A	A	C	0.75	0.75	0.25	6	2	0	0	N5_271_010G1_scaffold_120_1	[illegible]	[illegible]	False	SNV
N5_271_010G1_scaffold_120	1426	9	2	G	G	T	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	N5_271_010G1_scaffold_120_1	[illegible]	[illegible]	False	SNV
N5_271_010G1_scaffold_120	1481	6	2	C	T	C	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	N5_271_010G1_scaffold_120_1	[illegible]	[illegible]	False	con_SNV
N5_271_010G1_scaffold_120	1484	6	2	G	A	G	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	N5_271_010G1_scaffold_120_1	[illegible]	[illegible]	False	con_SNV
N5_271_010G1_scaffold_120	1488	5	1	T	C	T	0.2	0.8	0.2	0	4	1	0	N5_271_010G1_scaffold_120_1	[illegible]	S	False	SNS
N5_271_010G1_scaffold_120	1801	5	1	T	A	T	0.2	0.8	0.2	4	0	1	0	N5_271_010G1_scaffold_120_1	[illegible]	[illegible]	False	SNS
N5_271_010G1_scaffold_120	1897	7	2	G	G	T	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]			I	False	SNV

See the module_descriptions for what constitutes a SNP (what makes it into this table)

scaffold The scaffold that the SNV is on

position The genomic position of the SNV

position_coverage The number of reads detected at this position

allele_count The number of bases that are detected above background levels (according to the *null model*. An allele_count of 0 means no bases are supported by the reads, an allele_count of 1 means that only 1 base is supported by the reads, an allele_count of 2 means two bases are supported by the reads, etc.

ref_base The reference base in the .fasta file at that position

con_base The consensus base (the base that is supported by the most reads)

var_base Variant base; the base with the second most reads

ref_freq The fraction of reads supporting the ref_base

con_freq The fraction of reds supporting the con_base

var_freq The fraction of reads supporting the var_base

A, C, T, and G The number of mapped reads encoding each of the bases

gene If a gene file was included, this column will be present listing if the SNV is in the coding sequence of a gene

mutation Short-hand code for the amino acid switch. If synonymous, this will be S: + the position. If nonsynonymous, this will be N: + the old amino acid + the position + the new amino acid.

mutation_type What type of mutation this is. N = nonsynonymous, S = synonymous, I = intergenic, M = there are multiple genes with this base so you cant tell

cryptic If an SNV is cryptic, it means that it is detected when using a lower read mismatch threshold, but becomes undetected when you move to a higher read mismatch level. See "dealing with mm" in the advanced_use section for more details on what this means.

class The classification of this divergent site. The options are *SNS* (meaning allele_count is 1 and con_base does not equal ref_base), *SNV* (meaning allele_count is > 1 and con_base *does* equal ref_base), con_SNV (meaning allele_count is > 1, con_base does not equal ref_base, and ref_base *is* present in the reads; these count as differences in conANI calculations), pop_SNV (meaning allele_count is > 1, con_base does not equal ref_base, and ref_base *is not* present in the reads; these count as differences in popANI and conANI calculations), DivergentSite (meaning allele count is 0), and AmbiguousReference (meaning the ref_base is not A, C, T, or G)

linkage.tsv

This describes the *linkage* between pairs of SNPs in the mapping that are found on the same read pair at least min_snp times.

Table 4: linkage.tsv

scaffold	position_A	position_B	distance	r2	d_prime	r2_normalized	d_prime_normalized	allele_A	allele_a	allele_B	allele_b	countAb	countAB	countaB	countab	total
N5_271_010G1	59	scaffold_93	0.021739130434782608					C	T	G	A	0	3	4	20	27
N5_271_010G1	70	scaffold_93	0.012820512820512851					C	T	T	A	0	2	4	22	28
N5_271_010G1	80	scaffold_93	0.016722408026755853					C	T	G	A	0	2	5	21	28
N5_271_010G1	84	scaffold_93	0.765217391304347826					A	T	G	C	4	0	1	22	27
N5_271_010G1	101	scaffold_93	0.009070294784580067					C	T	C	A	0	2	2	19	23
N5_271_010G1	126	scaffold_93	0.017543859649122807					C	T	A	T	0	2	3	16	21
N5_271_010G1	133	scaffold_93	0.008333333333333352					C	T	G	T	0	1	3	17	21
N5_271_010G1	70	scaffold_93	0.010869565217391317					C	T	T	A	0	2	3	21	26
N5_271_010G1	80	scaffold_93	0.641025641025641					G	A	G	A	2	0	1	25	28

Linkage is used primarily to determine if organisms are undergoing horizontal gene transfer or not. It's calculated for pairs of SNPs that can be connected by at least `min_snp` reads. It's based on the assumption that each SNP as two alleles (for example, a A and b B). This all gets a bit confusing and has a large amount of literature around each of these terms, but I'll do my best to briefly explain what's going on

scaffold The scaffold that both SNPs are on

position_A The position of the first SNP on this scaffold

position_B The position of the second SNP on this scaffold

distance The distance between the two SNPs

r2 This is the r-squared linkage metric. See below for how it's calculated

d_prime This is the d-prime linkage metric. See below for how it's calculated

r2_normalized, d_prime_normalized These are calculated by rarefying to `min_snp` number of read pairs. See below for how it's calculated

allele_A One of the two bases at position_A

allele_a The other of the two bases at position_A

allele_B One of the bases at position_B

allele_b The other of the two bases at position_B

countab The number of read-pairs that have allele_a and allele_b

countAb The number of read-pairs that have allele_A and allele_b

countaB The number of read-pairs that have allele_a and allele_B

countAB The number of read-pairs that have allele_A and allele_B

total The total number of read-pairs that have have information for both position_A and position_B

Python code for the calculation of these metrics:

```
freq_AB = float(countAB) / total
freq_Ab = float(countAb) / total
freq_aB = float(countaB) / total
freq_ab = float(countab) / total

freq_A = freq_AB + freq_Ab
freq_a = freq_ab + freq_aB
freq_B = freq_AB + freq_aB
freq_b = freq_ab + freq_Ab

linkD = freq_AB - freq_A * freq_B

if freq_a == 0 or freq_A == 0 or freq_B == 0 or freq_b == 0:
    r2 = np.nan
else:
    r2 = linkD*linkD / (freq_A * freq_a * freq_B * freq_b)

linkd = freq_ab - freq_a * freq_b

# calc D-prime
d_prime = np.nan
if (linkd < 0):
    denom = max([(-freq_A*freq_B),(-freq_a*freq_b)])
    d_prime = linkd / denom

elif (linkD > 0):
    denom = min([(freq_A*freq_b), (freq_a*freq_B)])
    d_prime = linkd / denom

################
# calc rarefied

rareify = np.random.choice(['AB','Ab','aB','ab'], replace=True, p=[freq_AB,freq_Ab,
↪freq_aB,freq_ab], size=min_snp)
freq_AB = float(collections.Counter(rareify)['AB']) / min_snp
freq_Ab = float(collections.Counter(rareify)['Ab']) / min_snp
freq_aB = float(collections.Counter(rareify)['aB']) / min_snp
```

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(continued from previous page)

```
freq_ab = float(collections.Counter(rareify)['ab']) / min_snp

freq_A = freq_AB + freq_Ab
freq_a = freq_ab + freq_aB
freq_B = freq_AB + freq_aB
freq_b = freq_ab + freq_Ab

linkd_norm = freq_ab - freq_a * freq_b

if freq_a == 0 or freq_A == 0 or freq_B == 0 or freq_b == 0:
    r2_normalized = np.nan
else:
    r2_normalized = linkd_norm*linkd_norm / (freq_A * freq_a * freq_B * freq_b)


# calc D-prime
d_prime_normalized = np.nan
if (linkd_norm < 0):
    denom = max([(-freq_A*freq_B),(-freq_a*freq_b)])
    d_prime_normalized = linkd_norm / denom

elif (linkd_norm > 0):
    denom = min([(freq_A*freq_b), (freq_a*freq_B)])
    d_prime_normalized = linkd_norm / denom

rt_dict = {}
for att in ['r2', 'd_prime', 'r2_normalized', 'd_prime_normalized', 'total', 'countAB
↪', \
            'countAb', 'countaB', 'countab', 'allele_A', 'allele_a', \
            'allele_B', 'allele_b']:
    rt_dict[att] = eval(att)
```

gene_info.tsv

This describes some basic information about the genes being profiled

Table 5: gene_info.tsv

scaffold	gene	gene_length	coverage	breadth	breadth_minCov	nucl_diversity	start	end	direction	partial	dNdS_substitutions	pNpS_variants	SNV_count	SNV_S_count	SNV_N_count	SNS_count	SNS_S_count	SNS_N_count	divergent_site_count
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	283	-1	False			0.0	0.0	0.0	0.0	0.0	0.0	0.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		0.0	0.0	0.0	0.0	0.0	0.0	0.0	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	335	-1	False			0.0	0.0	0.0	0.0	0.0	0.0	0.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		[illegible]	[illegible]	[illegible]	[illegible]	0.0	0.0	0.0	9.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		[illegible]	[illegible]	[illegible]	[illegible]	0.0	0.0	0.0	4.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False			5.0	0.0	5.0	0.0	0.0	0.0	5.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		0.0	2.0	2.0	0.0	0.0	0.0	0.0	2.0
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	False		[illegible]	[illegible]	[illegible]	[illegible]	0.0	0.0	0.0	4.0

scaffold Scaffold that the gene is on

gene Name of the gene being profiled

gene_length Length of the gene in nucleotides

breadth The number of bases in the gene that have at least 1x coverage

breadth_minCov The number of bases in the gene that have at least min_cov coverage

nucl_diversity The mean *nucleotide diversity* of all bases in the gene that have a nucleotide diversity value calculated. So if only 1 base on the scaffold meats the minimum coverage to calculate nucleotide diversity, the nucl_diversity of the scaffold will be the nucleotide diversity of that base. Will be blank if no positions have a base over the minimum coverage.

start Start of the gene (position on scaffold; 0-indexed)

end End of the gene (position on scaffold; 0-indexed)

direction Direction of the gene (based on prodigal call). If -1, means the gene is not coded in the direction expressed by the .fasta file

partial If True this is a partial gene; based on not having *partial=00* in the record description provided by Prodigal

dNdS_substitutions The *dN/dS* of *SNSs* detected in this gene. Will be blank if 0 N and/or 0 S substitutions are detected

pNpS_variants The *pN/pS* of *SNVs* detected in this gene. Will be blank if 0 N and/or 0 S SNVs are detected

SNV_count Total number of *SNVs* detected in this gene

SNV_S_count Number of synonymous *SNVs* detected in this gene

SNV_N_count Number of non-synonymous *SNVs* detected in this gene

SNS_count Total number of *SNSs* detected in this gens

SNS_S_count Number of synonymous *SNSs* detected in this gens

SNS_N_count Number of non-synonymous *SNSs* detected in this gens

divergent_site_count Number of *divergent sites* detected in this gens

genome_info.tsv

Describes many of the above metrics on a genome-by-genome level, rather than a scaffold-by-scaffold level.

Table 6: genome_info.tsv

genome	coverage	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
all_scaffolds	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

genome The name of the genome being profiled. If all scaffolds were a single genome, this will read "all_scaffolds"

coverage Average *coverage depth* of all scaffolds of this genome

breadth The *breadth* of all scaffolds of this genome

nucl_diversity The average *nucleotide diversity* of all scaffolds of this genome

length The full length of this genome across all scaffolds

true_scaffolds The number of scaffolds present in this genome based off of the scaffold-to-bin file

detected_scaffolds The number of scaffolds with at least a single read-pair mapping to them

coverage_median The median *coverage* amoung all bases in the genome

coverage_std The standard deviation of all coverage values

coverage_SEM The standard error of the mean of all coverage values (calculated using scipy.stats.sem)

breadth_minCov The percentage of bases in the scaffold that have at least min_cov coverage (e.g. the percentage of bases that have a nucl_diversity value and meet the minimum sequencing depth to call SNVs)

breadth_expected This tells you the breadth that you should expect if reads are evenly distributed along the genome, given the reported coverage value. Based on the function breadth = -1.000 * e^(0.883 * coverage) + 1.000. This is useful to establish whether or not the scaffold is actually in the reads, or just a fraction of the scaffold. If your coverage is 10x, the expected breadth will be ~1. If your actual breadth is significantly lower then the expected breadth, this means that reads are mapping only to a specific region of your scaffold (transposon, prophage, etc.)

nucl_diversity_rarefied The average *nucleotide diversity* among positions that have at least `--rarefied_coverage` (50x by default). These values are also calculated by randomly subsetting the reads at that position to `--rarefied_coverage` reads

conANI_reference The *conANI* between the reads and the reference genome

popANI_reference The *popANI* between the reads and the reference genome

iRep The *iRep* value for this genome (if it could be successfully calculated)

iRep_GC_corrected A True / False value of whether the iRep value was corrected for GC bias

linked_SNV_count The number of *divergent sites* that could be *linked* in this genome

SNV_distance_mean Average distance between linked *divergent sites*

r2_mean Average r2 between linked SNVs (see explanation of linkage.tsv above for more info)

d_prime_mean Average d prime between linked SNVs (see explanation of linkage.tsv above for more info)

consensus_divergent_sites The total number of *divergent sites* in which the reads have a different consensus allele than the reference genome. These count as "differences" in the conANI_reference calculation, and `breadth_minCov` * `length` counts as the denominator.

population_divergent_sites The total number of *divergent sites* in which the reads do not have the reference genome base as any allele at all (major or minor). These count as "differences" in the popANI_reference calculation, and `breadth_minCov` * `length` counts as the denominator.

SNS_count The total number of *SNSs* called on this genome

SNV_count The total number of *SNVs* called on this genome

filtered_read_pair_count The total number of read pairs that pass filtering and map to this genome

reads_unfiltered_pairs The total number of pairs, filtered or unfiltered, that map to this genome

reads_mean_PID The average ANI of mapped read pairs to the reference genome for this genome

reads_unfiltered_reads The total number of reads, filtered or unfiltered, that map to this genome

divergent_site_count The total number of *divergent sites* called on this genome

1.5.2 inStrain compare

A typical run of inStrain will yield the following files in the output folder:

comparisonsTable.tsv

Summarizes the differences between two inStrain profiles on a scaffold by scaffold level

Table 7: comparisonsTable.tsv

scaffold	name1	name2	coverage_overlap	compared_bases_count	percent_genome_compared	length	consensus_SNPs	population_SNPs	popANI	conANI
N5_271_010G1_scaffold_98	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	61	0.052905464586938842	1153	0	0	1.0	1.0
N5_271_010G1_scaffold_133	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	78	0.07414448669201152	1052	0	0	1.0	1.0
N5_271_010G1_scaffold_144	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	172	0.16715257531584066	1029	0	0	1.0	1.0
N5_271_010G1_scaffold_158	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	36	0.03574975073783164	1007	0	0	1.0	1.0
N5_271_010G1_scaffold_57	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	24	0.018320611687229	1310	0	0	1.0	1.0
N5_271_010G1_scaffold_139	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	24	0.023121387283236997	1038	0	0	1.0	1.0
N5_271_010G1_scaffold_92	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	336	0.28693424423356996	1171	0	0	1.0	1.0
N5_271_010G1_scaffold_97	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	22	0.019014693517199654	1157	0	0	1.0	1.0
N5_271_010G1_scaffold_100	N5_271_010G1.sorted.bam vs N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-scaffold_min1000.fa vs N5_271_010G1.sorted.bam	1.0	21	0.018292682148268292677	1148	0	0	1.0	1.0

scaffold The scaffold being compared

name1 The name of the first *inStrain profile* being compared

name2 The name of the second *inStrain profile* being compared

coverage_overlap The percentage of bases that are either covered or not covered in both of the profiles (covered = the base is present at at least min_snp coverage). The formula is length(coveredInBoth) / length(coveredInEither). If both scaffolds have 0 coverage, this will be 0.

compared_bases_count The number of considered bases; that is, the number of bases with at least min_snp coverage in both profiles. Formula is length([x for x in overlap if x == True]).

percent_genome_compared The percentage of bases in the scaffolds that are covered by both. The formula is length([x for x in overlap if x == True])/length(overlap). When ANI is np.nan, this must be 0. If both scaffolds have 0 coverage, this will be 0.

length The total length of the scaffold

consensus_SNPs The number of locations along the genome where both samples have the base at >= 5x coverage, and the consensus allele in each sample is different. Used to calculate *conANI*

population_SNPs The number of locations along the genome where both samples have the base at >= 5x coverage, and no alleles are shared between either sample. Used to calculate *popANI*

popANI The average nucleotide identity among compared bases between the two scaffolds, based on population_SNPs. Calculated using the formula popANI = (compared_bases_count - population_SNPs) / compared_bases_count

conNI The average nucleotide identity among compared bases between the two scaffolds, based on consensus_SNPs. Calculated using the formula conANI = (compared_bases_count - consensus_SNPs) / compared_bases_count

pairwise_SNP_locations.tsv

Lists the locations of all differences between profiles

Table 8: pairwise_SNP_locations.tsv

scaffold	position	name1	name2	consensus_SNP	population_SNP	con_base_1	ref_base_1	var_base_1	position_coverage_1	A_1	C_1	T_1	G_1	con_base_2	ref_base_2	var_base_2	position_coverage_2	A_2	C_2	T_2	G_2
N5_27	8213	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	C	C	T	7.0	0.0	0.0	7.0	0.0	A	G	G	6.0	3.0	0.0	0.0	3.0
N5_27	9706	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	C	C	T	6.0	0.0	2.0	4.0	0.0	C	T	T	7.0	0.0	4.0	3.0	0.0
N5_27	24736	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	C	C	T	6.0	0.0	3.0	3.0	0.0	T	T	C	7.0	0.0	3.0	4.0	0.0
N5_27	27940	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	T	T	A	6.0	0.0	0.0	2.0	0.0	T	A	A	9.0	4.0	0.0	5.0	0.0
N5_27	27608	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	A	A	G	8.0	2.0	0.0	0.0	6.0	A	G	G	6.0	5.0	0.0	0.0	1.0
N5_27	26000	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	A	A	G	6.0	4.0	0.0	0.0	2.0								
N5_27	24970	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	A	A	G	6.0	3.0	0.0	0.0	2.0								
N5_27	27108	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	A	A	G	6.0	3.0	0.0	0.0	2.0	G	A	A	15.0	6.0	0.0	0.0	9.0
N5_27	27100	N5_27 vs- N5_27	N5_27 vs- N5_27	False	False	C	C	T	6.0	4.0	2.0	0.0	0.0	C	C	A	6.0	2.0	4.0	0.0	0.0

scaffold The scaffold on which the difference is located

position The position where the difference is located (0-based)

name1 The name of the first *inStrain profile* being compared

name2 The name of the second *inStrain profile* being compared

consensus_SNP A True / False column listing whether or not this difference counts towards *conANI* calculations

population_SNP A True / False column listing whether or not this difference counts towards *popANI* calculations

con_base_1 The consensus base of the profile listed in `name1` at this position

ref_base_1 The reference base of the profile listed in `name1` at this position (will be the same as `ref_base_2`)

var_base_1 The variant base of the profile listed in `name1` at this position

position_coverage_1 The number of reads mapping to this position in `name1`

A_1, C_1, T_1, G_1 The number of mapped reads with each nucleotide in `name1`

con_base_2, ref_base_2, . . . The above columns are also listed for the `name2` sample

genomeWide_compare.tsv

A genome-level summary of the differences detected by inStrain compare. Generated by running `inStrain genome_wide` on the results of `inStrain compare`

Table 9: genomeWide_compare.tsv

genome	name1	name2	coverage_overlap	compared_bases_count	consensus_SNPs	population_SNPs	popANI	conANI	percent_compared
all_scaffolds	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam	1.0	100712	0	0	1.0	1.0	0.3605549091560011
all_scaffolds	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G2.sorted.bam	0.6852932[illegible]	[illegible]	156	5	0.9999[illegible]	0.9987[illegible]	[illegible]20307886
all_scaffolds	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G2.sorted.bam	N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G2.sorted.bam	1.0	104563	0	0	1.0	1.0	0.5214821444553835

genome The genome being compared

name1 The name of the first *inStrain profile* being compared

name2 The name of the second *inStrain profile* being compared

coverage_overlap The percentage of bases that are either covered or not covered in both of the profiles (covered = the base is present at at least min_snp coverage). The formula is length(coveredInBoth) / length(coveredInEither). If both scaffolds have 0 coverage, this will be 0.

compared_bases_count The number of considered bases; that is, the number of bases with at least min_snp coverage in both profiles. Formula is length([x for x in overlap if x == True]).

percent_genome_compared The percentage of bases in the scaffolds that are covered by both. The formula is length([x for x in overlap if x == True])/length(overlap). When ANI is np.nan, this must be 0. If both scaffolds have 0 coverage, this will be 0.

length The total length of the genome

consensus_SNPs The number of locations along the genome where both samples have the base at >= 5x coverage, and the consensus allele in each sample is different. Used to calculate *conANI*

population_SNPs The number of locations along the genome where both samples have the base at >= 5x coverage, and no alleles are shared between either sample. Used to calculate *popANI*

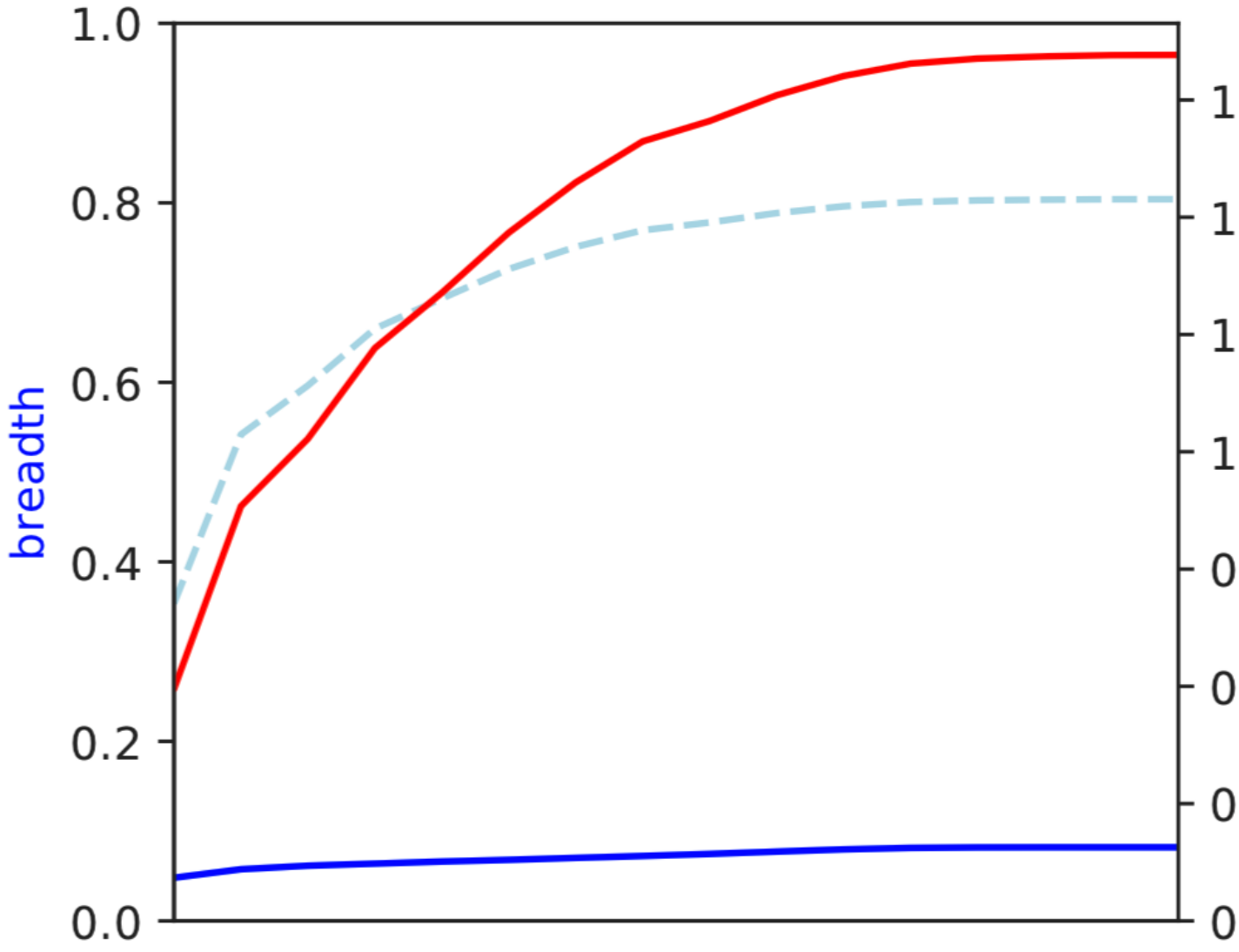
popANI The average nucleotide identity among compared bases between the two scaffolds, based on population_SNPs. Calculated using the formula popANI = (compared_bases_count - population_SNPs) / compared_bases_count

conNI The average nucleotide identity among compared bases between the two scaffolds, based on consensus_SNPs. Calculated using the formula conANI = (compared_bases_count - consensus_SNPs) / compared_bases_count

1.5.3 inStrain plot

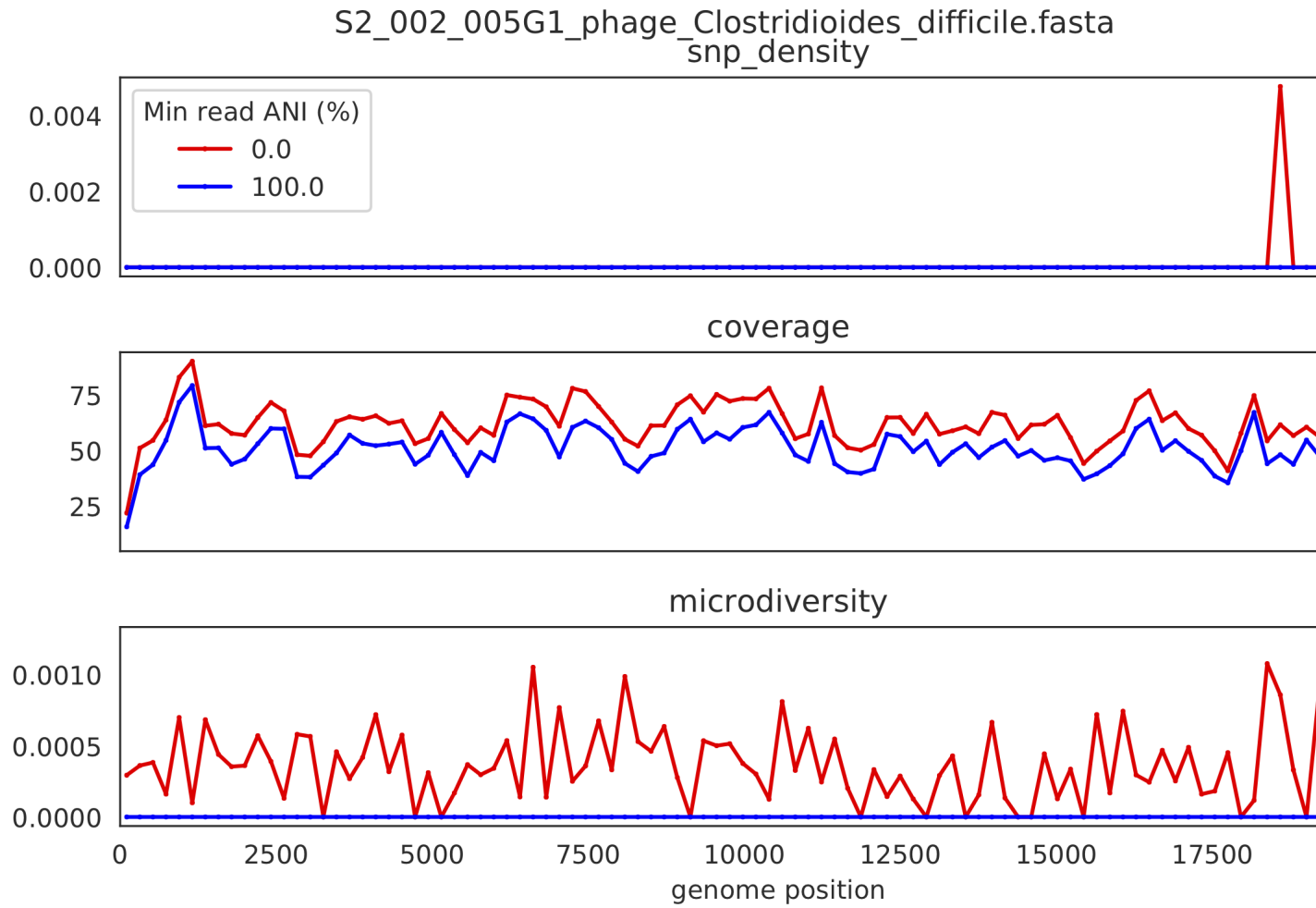
This is what the results of inStrain plot look like.

1) Coverage and breadth vs. read mismatches



Breadth of coverage (blue line), coverage depth (red line), and expected breadth of coverage given the depth of coverage (dotted blue line) versus the minimum ANI of mapped reads. Coverage depth continues to increase while breadth of plateaus, suggesting that all regions of the reference genome are not present in the reads being mapped.

2) Genome-wide microdiversity metrics



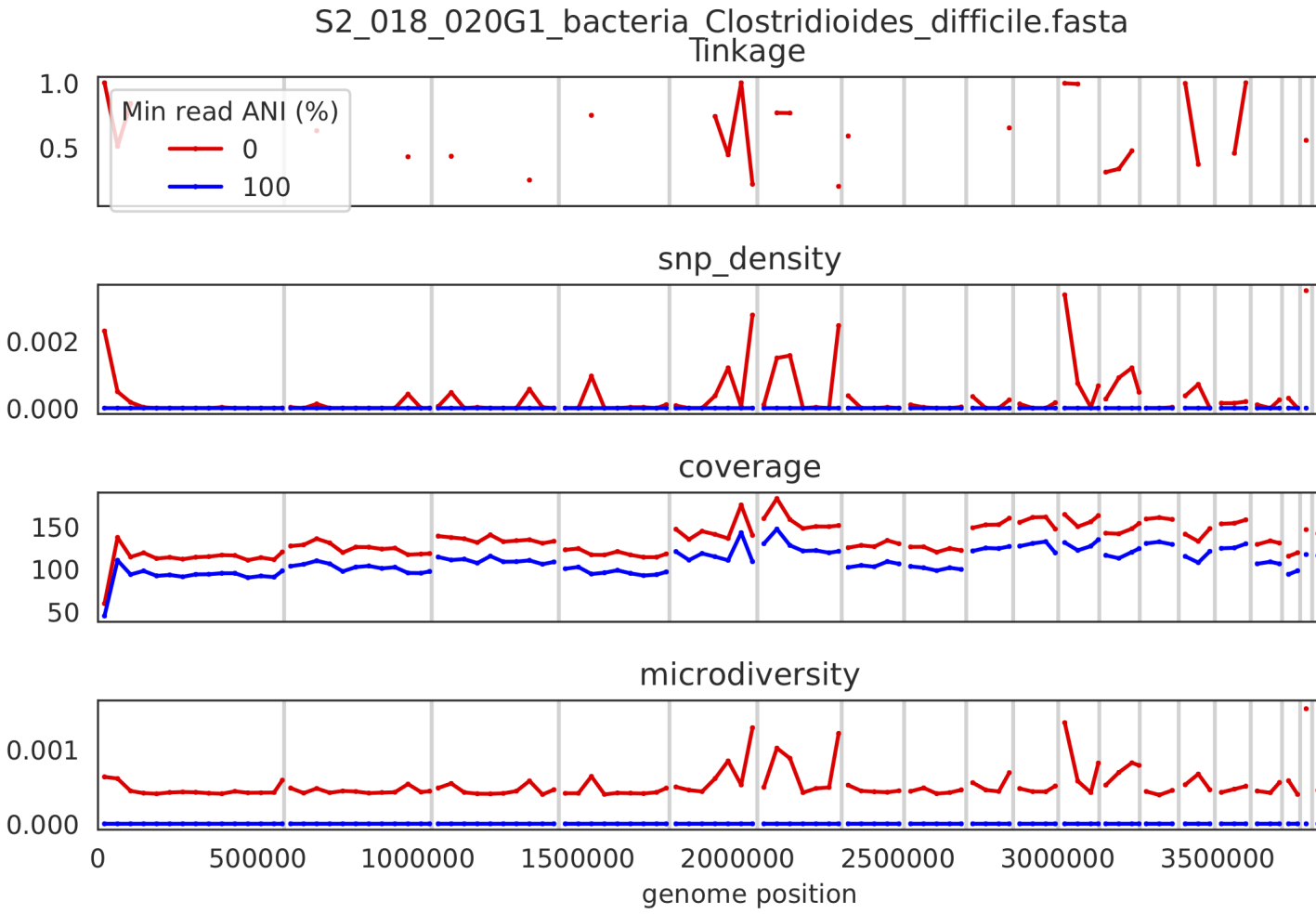
SNV density, coverage, and nucleotide diversity. Spikes in nucleotide diversity and SNV density do not correspond with increased coverage, indicating that the signals are not due to read mis-mapping. Positions with nucleotide diversity and no SNV-density are those where diversity exists but is not high enough to call a SNV

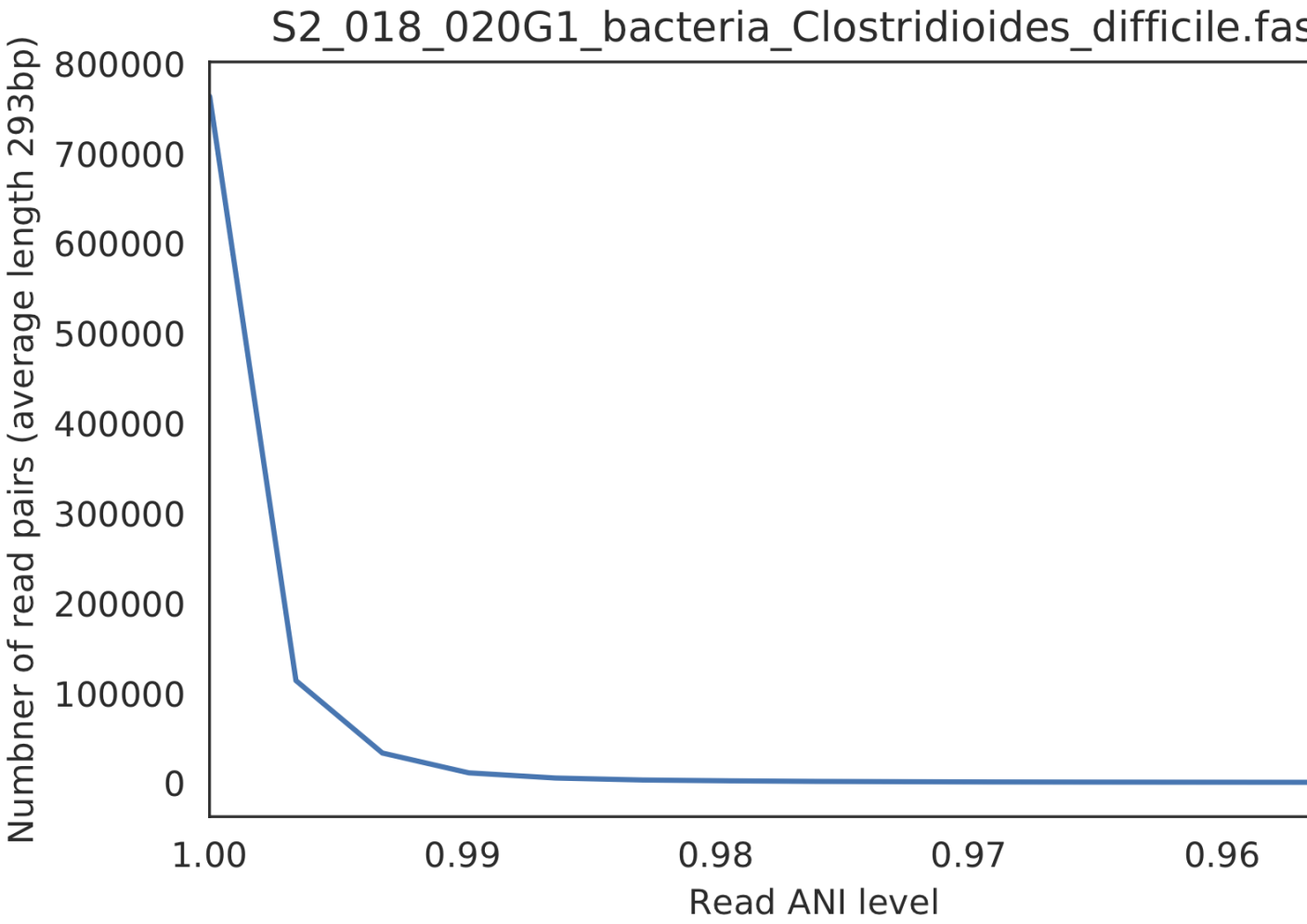
3) Read-level ANI distribution

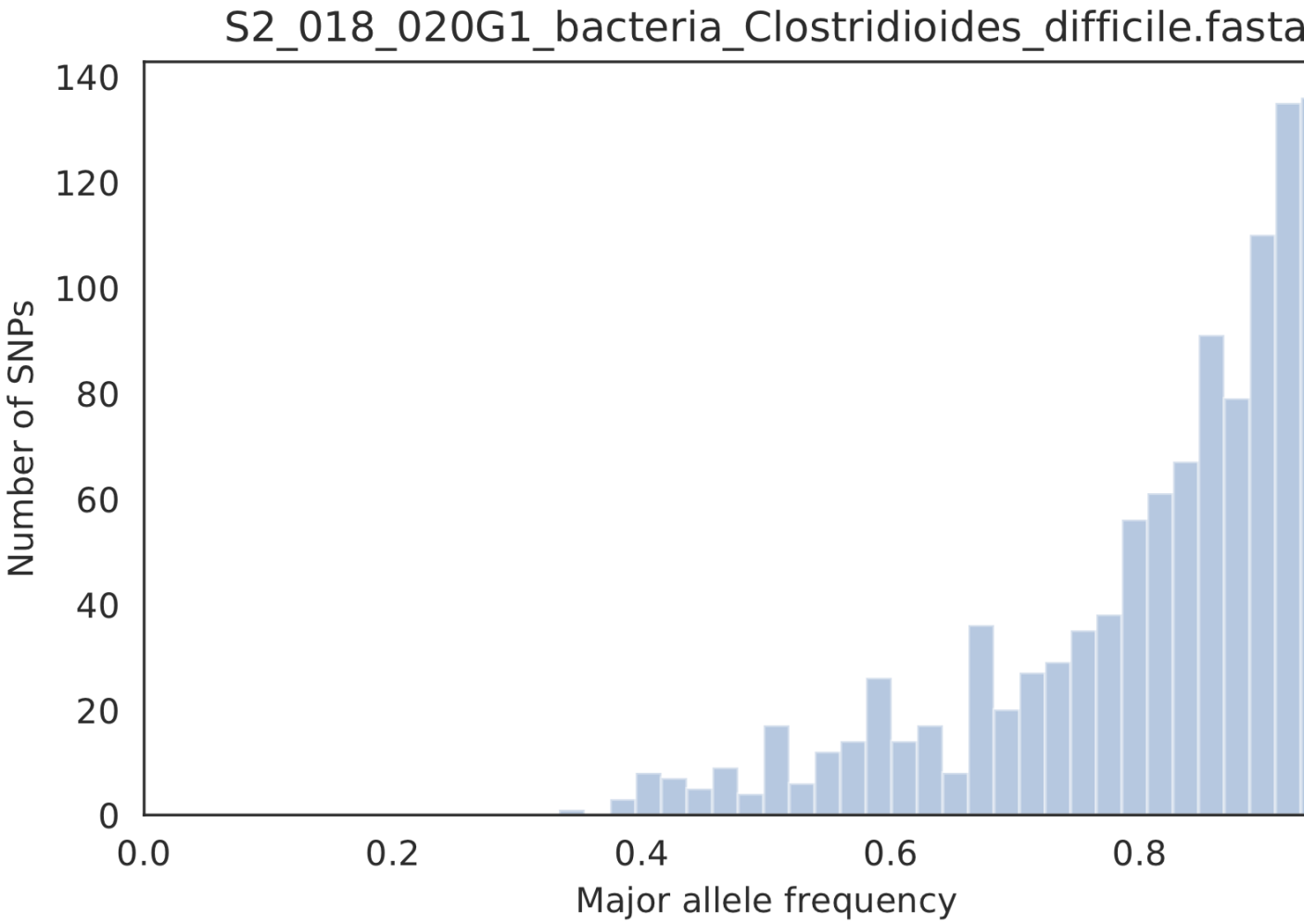
Distribution of read pair ANI levels when mapped to a reference genome; this plot suggests that the reference genome is >1% different than the mapped reads

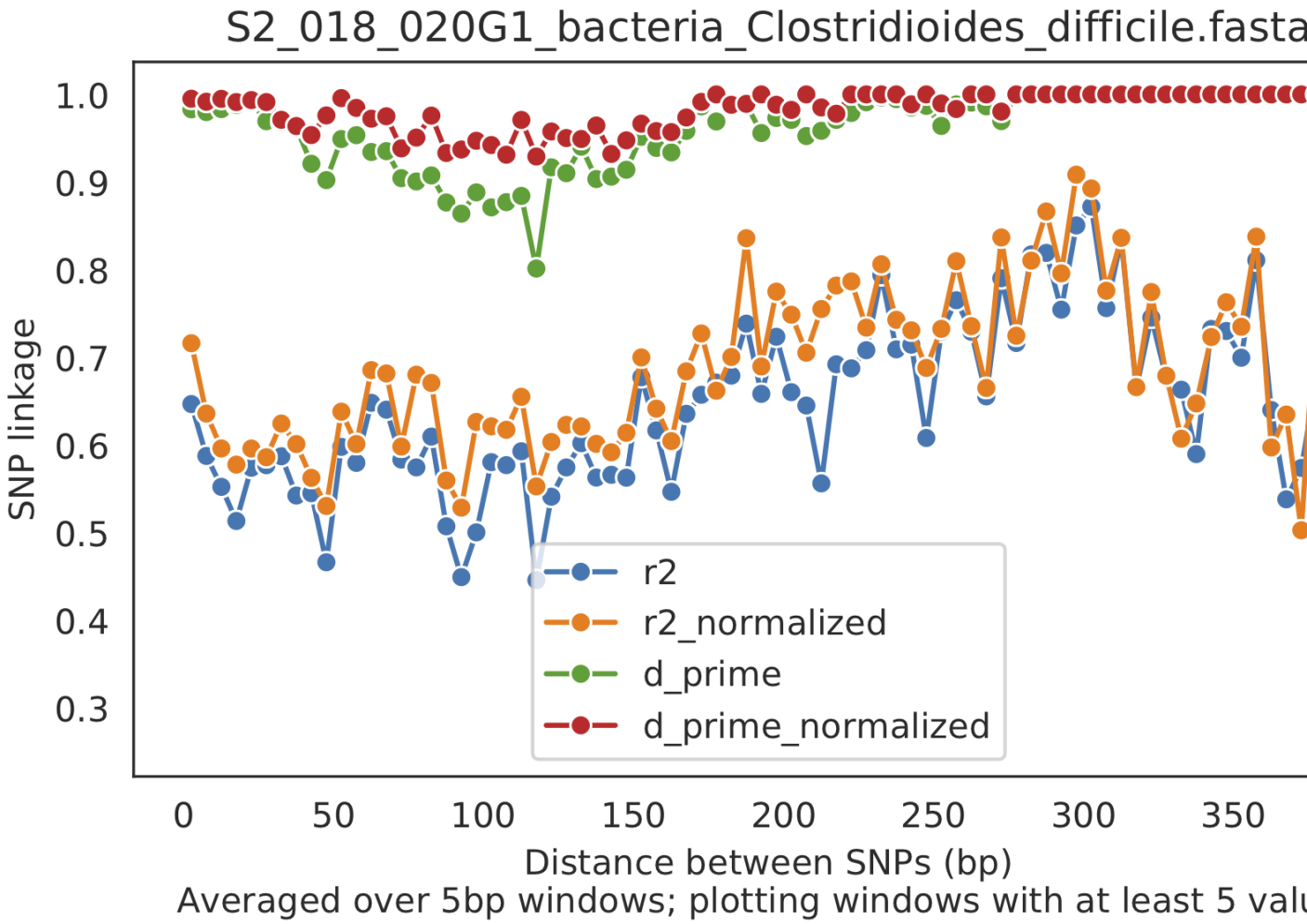
4) Major allele frequencies

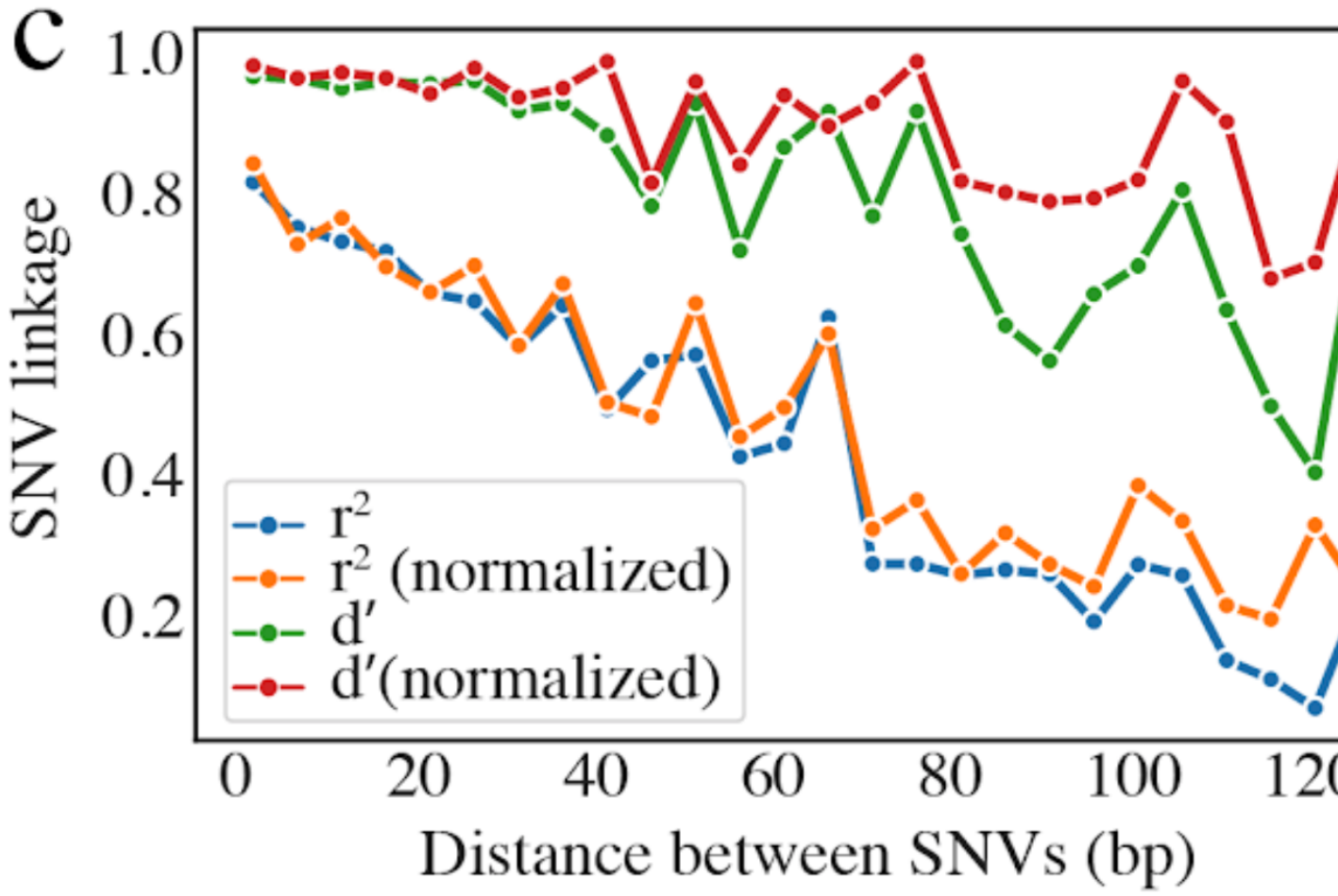
Distribution of the major allele frequencies of bi-allelic SNVs (the Site Frequency Spectrum). Alleles with major frequencies below 50% are the result of multiallelic sites. The lack of distinct puncta suggest that more than a few distinct strains are present.


S2_018_020G1_bacteria_Clostridioides_difficile.fasta
Tinkage
1.0
0.5
Min read ANI (%)
0
100
snp_density
0.002
0.000
coverage
150
100
50
microdiversity
0.001
0.000
0
500000
1000000
1500000
2000000
2500000
3000000
3500000
genome position


S2_018_020G1_bacteria_Clostridioides_difficile.fas
Numbner of read pairs (average length 293bp)
800000
700000
600000
500000
400000
300000
200000
100000
0
1.00
0.99
0.98
0.97
0.96
Read ANI level


S2_018_020G1_bacteria_Clostridioides_difficile.fasta
Number of SNPs
0
20
40
60
80
100
120
140
0.0
0.2
0.4
0.6
0.8
Major allele frequency

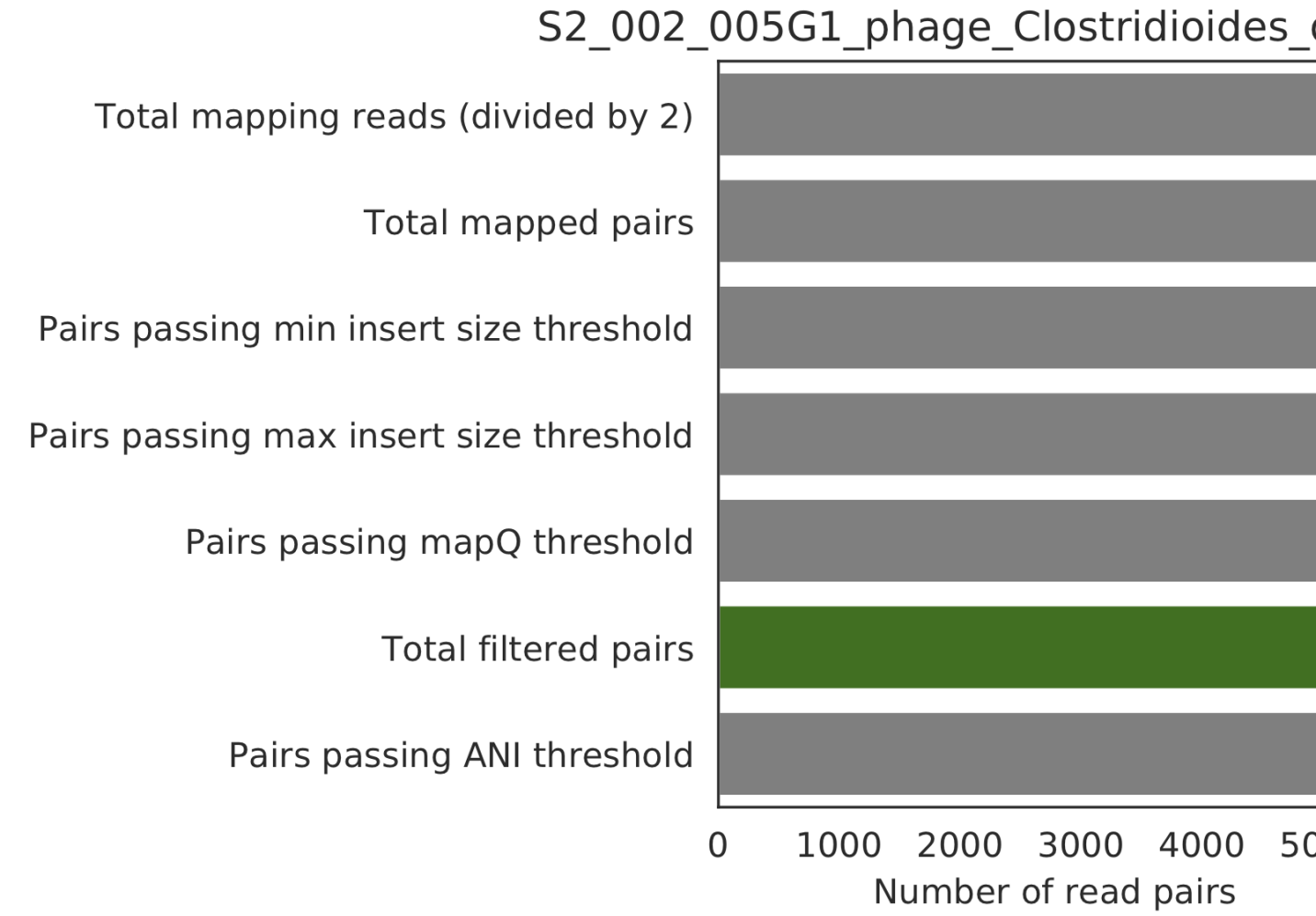

S2_018_020G1_bacteria_Clostridioides_difficile.fasta
SNP linkage
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
r2
r2_normalized
d_prime
d_prime_normalized
0
50
100
150
200
250
300
350
Distance between SNPs (bp)
Averaged over 5bp windows; plotting windows with at least 5 valu


C
1.0
0.8
0.6
0.4
0.2
SNV linkage
r²
r² (normalized)
d′
d′(normalized)
0
20
40
60
80
100
120
Distance between SNVs (bp)

5) Linkage decay

Metrics of SNV linkage vs. distance between SNVs; linkage decay (shown in one plot and not the other) is a common signal of recombination.

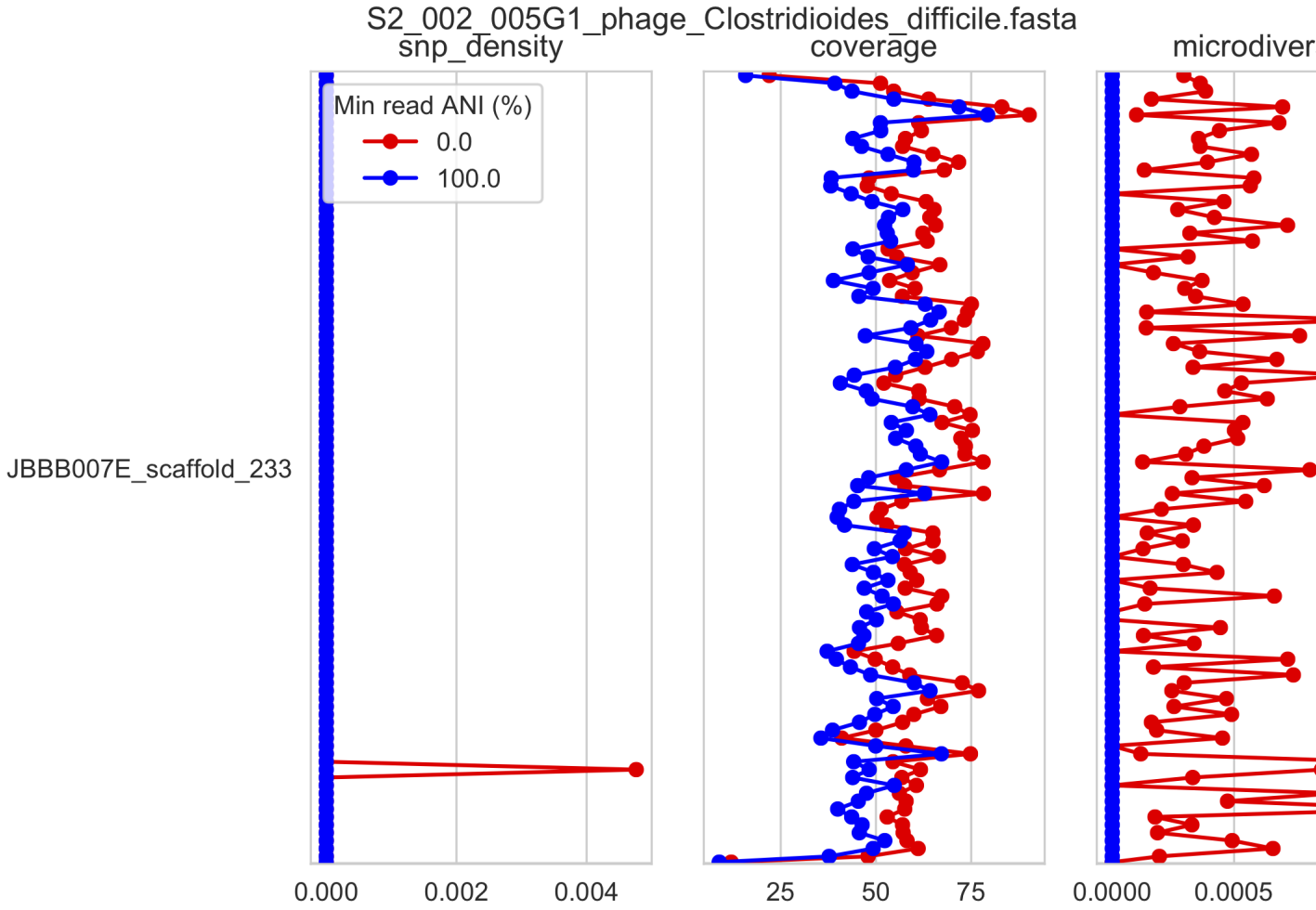
6) Read filtering plots

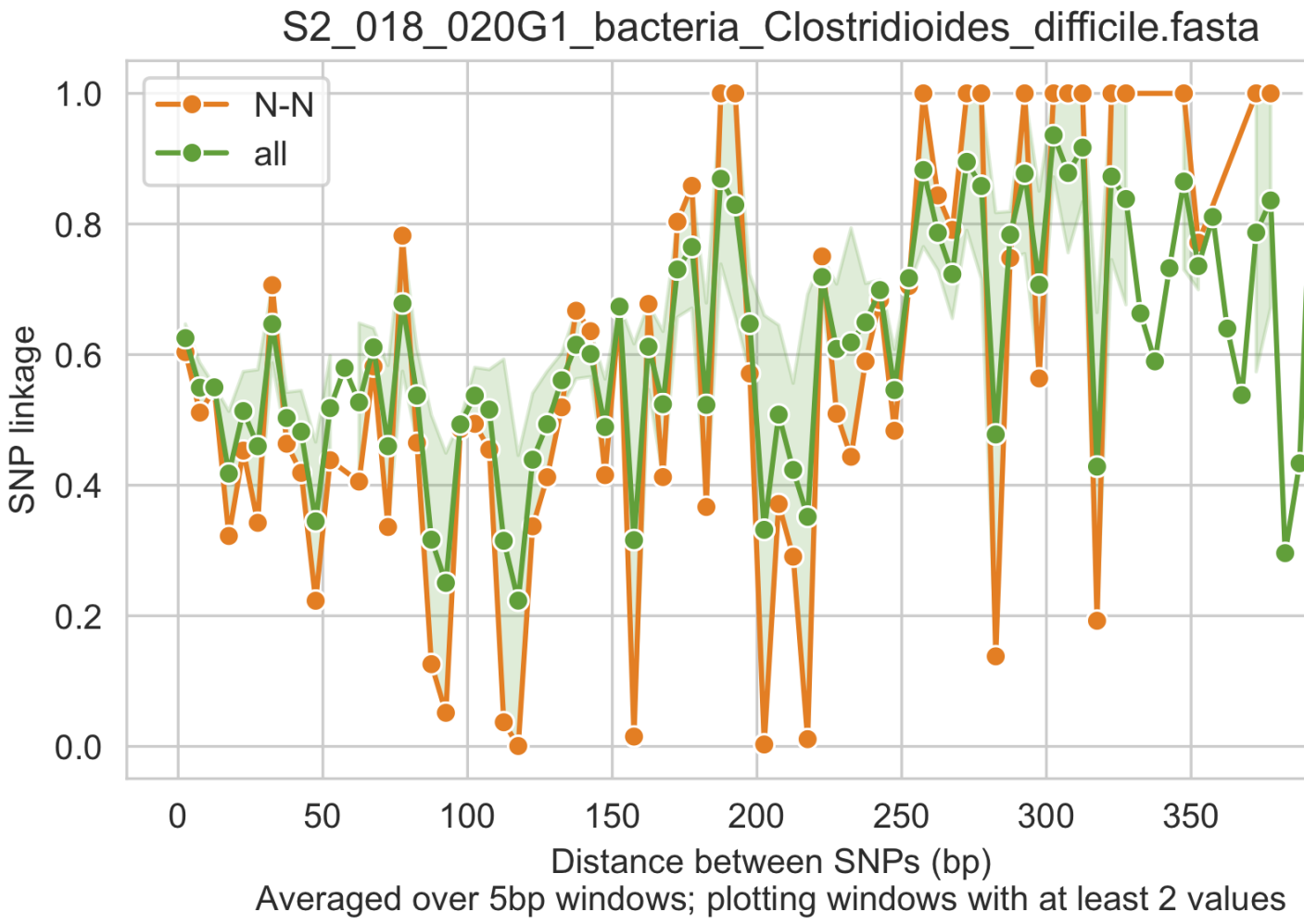


Bar plots showing how many reads got filtered out during filtering. All percentages are based on the number of paired reads; for an idea of how many reads were filtered out for being non-paired, compare the top bar and the second to top bar.

7) Scaffold inspection plot (large)

This is an elongated version of the genome-wide microdiversity metrics that is long enough for you to read scaffold names on the y-axis

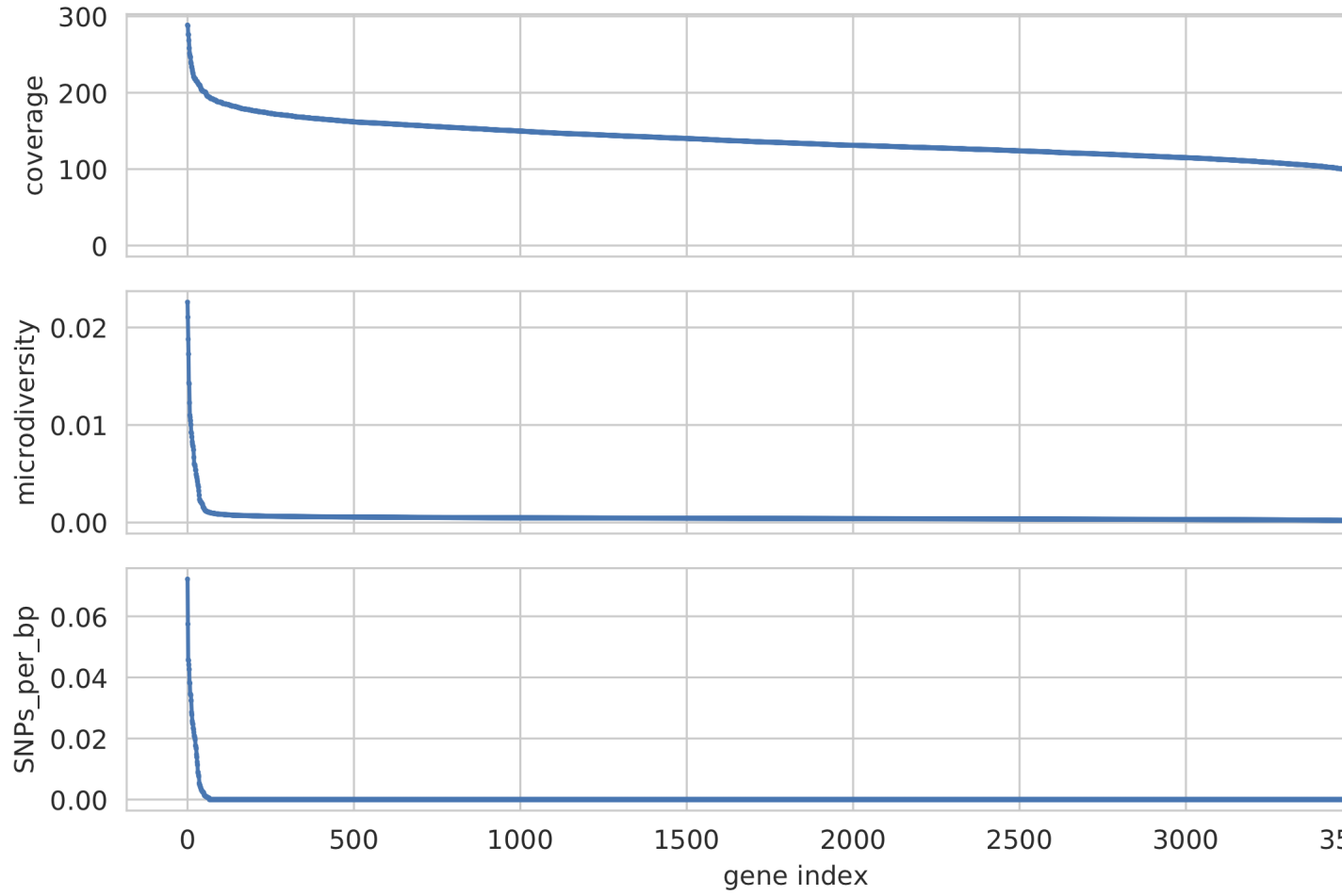

S2_002_005G1_phage_Clostridioides_difficile.fasta
snp_density
coverage
microdiver
Min read ANI (%)
0.0
100.0
JBBB007E_scaffold_233
0.000
0.002
0.004
25
50
75
0.0000
0.0005


S2_018_020G1_bacteria_Clostridioides_difficile.fasta
N-N
all
SNP linkage
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
300
350
Distance between SNPs (bp)
Averaged over 5bp windows; plotting windows with at least 2 values

8) Linkage with SNP type (GENES REQUIRED)

Linkage plot for pairs of non-synonymous SNPs and all pairs of SNPs

9) Gene histograms (GENES REQUIRED)



Histogram of values for all genes profiled

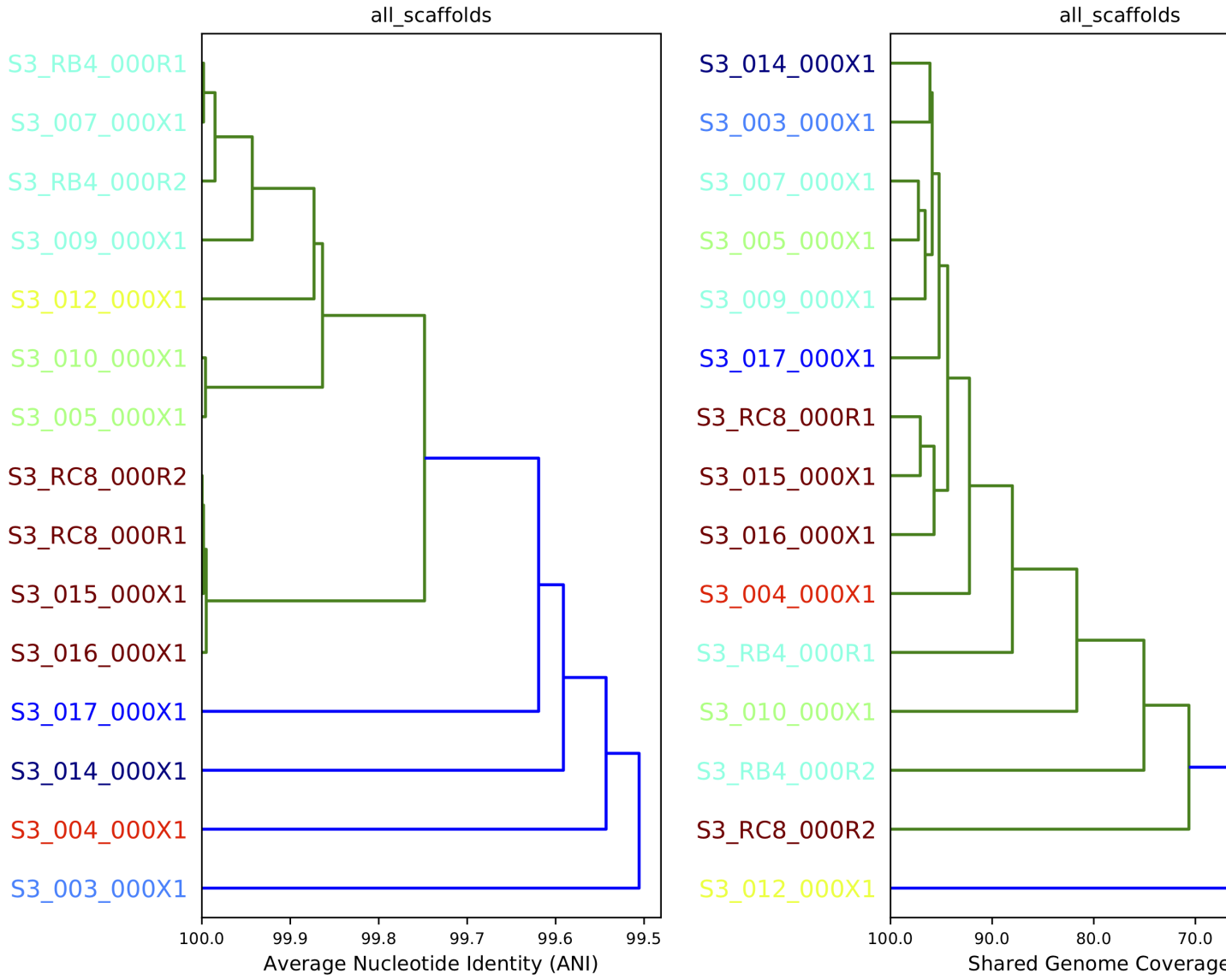
10) Compare dendrograms (RUN ON COMPARE; NOT PROFILE)

A dendrogram comparing all samples based on popANI and based on shared_bases.

1.6 Important concepts and API

1.6.1 API for accessing raw data

inStrain stores much more data than is shown in the output folder. It is kept in the `raw_data` folder, and is mostly stored in compressed formats. This data can be easily accessed using python, as described below.


all_scaffolds
S3_RB4_000R1
S3_007_000X1
S3_RB4_000R2
S3_009_000X1
S3_012_000X1
S3_010_000X1
S3_005_000X1
S3_RC8_000R2
S3_RC8_000R1
S3_015_000X1
S3_016_000X1
S3_017_000X1
S3_014_000X1
S3_004_000X1
S3_003_000X1
100.0
99.9
99.8
99.7
99.6
99.5
Average Nucleotide Identity (ANI)
all_scaffolds
S3_014_000X1
S3_003_000X1
S3_007_000X1
S3_005_000X1
S3_009_000X1
S3_017_000X1
S3_RC8_000R1
S3_015_000X1
S3_016_000X1
S3_004_000X1
S3_RB4_000R1
S3_010_000X1
S3_RB4_000R2
S3_RC8_000R2
S3_012_000X1
100.0
90.0
80.0
70.0
Shared Genome Coverage

To access the data, you first make an SNVprofile object of the inStrain output profile, and then you access data from that object. For example, the following code accessed the raw SNP table

```
import inStrain
import inStain.SNVprofile

IS = inStain.SNVprofile.SNVprofile(``/home/mattolm/inStrainOutputTest/``)
raw_snps = IS.get('raw_snp_table')
```

You can use the example above (`IS.get()`) to access any of the raw data described in the following section. There are also another special things that are accessed in other ways, as described in the section "Accessing other data"

Basics of raw_data

A typical run of inStrain will yield a folder titled "raw_data", with lots of individual files in it. The specifics of what files are in there depend on how inStrain was run, and whether or not additional commands were run as well (like profile_genes).

There will always be a file titled "attributes.tsv". This describes some basic information about each item in the raw data. Here's an example

Table 10: attributes.tsv

name	value	type	description
location	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test	value	Location of SNVprofile object
version	1.3.3	value	Version of inStrain
Rdic	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/Rdic.json	dictionary	Read_pair -> matches
mapping_info	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/mapping_info.csv.gz	pandas	Report on mapped reads
fasta_loc	/Users/mattolm/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa	value	Location of fasta file used during profile
scaffold2length	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/scaffold2length.json	dictionary	scaffold -> scaffold length
object_type	profile	value	Type of SNVprofile (profile or compare)
bam_loc	/Users/mattolm/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa-vs-N5_271_010G1.sorted.bam	value	Location of bam file
scaffold_list	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/scaffold_list.txt	list	List of scaffolds that were profiled
raw_linkage_table	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/raw_linkage_table.csv.gz	pandas	Raw table of linkage information
raw_snp_table	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/raw_SNP_table.csv.gz	pandas	Contains raw SNP information on a mm level
cumulative_scaffold_table	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/cumulative_scaffold_table.csv.gz	pandas	Cumulative coverage on a mm level. Formerly scaffoldTable.csv
cumulative_snv_table	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/cumulative_SNP_table.csv.gz	pandas	Cumulative SNP counts on a mm level. Formerly snpLocations.pickle
scaffold_2_mm_2_read_2_snvs	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/scaffold_2_mm_2_read_2_snvs.pickle	pickle	scaffold -> mm -> read_2_read linkage
genes_coverage	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/genes_coverage.csv.gz	pandas	Coverage of individual genes
genes_clonality	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/genes_clonality.csv.gz	pandas	Clonality of individual genes
genes_SNP_count	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/genes_SNP_count.csv.gz	pandas	SNP density and SNP counts of individual genes
SNP_mutation_types	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/SNP_mutation_types.csv.gz	pandas	The nature of SNPs
covT	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/covT.hdf	special	Scaffold -> hdf5 -> position based coverage
clonT	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/clonT.hdf	special	Scaffold -> hdf5 -> position based clonality
clonTR	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/clonTR.hdf	special	Scaffold -> hdf5 -> rarefied position based clonality
genes_fileloc	/Users/mattolm/Programs/inStrain/test/test_data/N5_271_010G1_scaffold_min1000.fa.genes.fna	value	Location of genes file that was used to call genes
genes_table	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/genes_table.csv.gz	pandas	Location of table associated genes_file
scaffold2bin	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/scaffold2bin.json	dictionary	Dictionary scaffold -> bin
bin2length	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/bin2length.json	dictionary	Dictionary bin -> total length
genome_level_info	/Users/mattolm/Programs/inStrain/test/test_backend/testdir/test/raw_data/genome_level_info.csv.gz	pandas	Table of genome level information

This is what the columns correspond to:

name The name of the data. This is the name that you put into `IS.get()` to have inStrain retrieve the data for you. See the section "Accessing raw data" for an example.

value This lists the path to where the data is located within the raw_data folder. If the type of data is a value, than this just lists the value

type This describes how the data is stored. Value = the data is whatever is listed under value; list = a python list; numpy = a numpy array; dictionary = a python dictionary; pandas = a pandas dataframe; pickle = a piece of data that's stored as a python pickle object; special = a piece of data that is stored in a special way that inStrain knows how to de-compress

description A one-sentence description of what's in the data.

Warning: Many of these pieces of raw data have the column "mm" in them, which means that things are calculated at every possible read mismatch level. This is often not what you want. See the section "Dealing with mm" for more information.

Accessing other data

In addition to the raw_data described above, there are a couple of other things that inStrain can make for you. You access these from methods that run on the IS object itself, instead of using the `get` method. For example:

```
import inStrain
import inStain.SNVprofile

IS = inStain.SNVprofile.SNVprofile(``/home/mattolm/inStrainOutputTest/``)
coverage_table = IS.get_raw_coverage_table()
```

The following methods work like that:

get_nonredundant_scaffold_table() Get a scaffold table with just one line per scaffold, not multiple mms

get_nonredundant_linkage_table() Get a linkage table with just one line per scaffold, not multiple mms

get_nonredundant_snv_table() Get a SNP table with just one line per scaffold, not multiple mms

get_clonality_table() Get a raw clonality table, listing the clonality of each position. Pass *nonredundant=False* to keep multiple mms

Dealing with "mm"

Behind the scenes, inStrain actually calculates pretty much all metrics for every read pair mismatch level. That is, only including read pairs with 0 mis-match to the reference sequences, only including read pairs with >= 1 mis-match to the reference sequences, all the way up to the number of mismatches associated with the "PID" parameter.

For most of the output that inStrain makes in the output folder, it removes the "mm" column and just gives the results for the maximum number of mismatches. However, it's often helpful to explore other mismatches levels, to see how parameters vary with more or less stringent mappings. Much of the data stored in "read_data" is on the mismatch level. Here's an example of what the looks like (this is the cumulative_scaffold_table):

```
,scaffold,length,breadth,coverage,coverage_median,coverage_std,bases_w_0_coverage,
↪mean_clonality,median_clonality,unmaskedBreadth,SNPs,breadth_expected,ANI,mm
0,N5_271_010G1_scaffold_102,1144,0.9353146853146853,5.106643356643357,5,2.
↪932067325774674,74,1.0,1.0,0.6145104895104895,0,0.9889923642060382,1.0,0
```

(continues on next page)

(continued from previous page)

```
1,N5_271_010G1_scaffold_102,1144,0.9353146853146853,6.421328671328672,6,4.
↪005996333777764,74,0.9992001028104149,1.0,0.6748251748251748,0,0.9965522492489882,1.
↪0,1
2,N5_271_010G1_scaffold_102,1144,0.9423076923076923,7.3627622377622375,7,4.
↪2747074564903285,66,0.9993874800638958,1.0,0.7928321678321678,0,0.998498542620078,1.
↪0,2
3,N5_271_010G1_scaffold_102,1144,0.9423076923076923,7.859265734265734,8,4.
↪748789115369562,66,0.9992251555869703,1.0,0.7928321678321678,0,0.9990314705263914,1.
↪0,3
4,N5_271_010G1_scaffold_102,1144,0.9423076923076923,8.017482517482517,8,4.
↪952541407151938,66,0.9992251555869703,1.0,0.7928321678321678,0,0.9991577528529144,1.
↪0,4
5,N5_271_010G1_scaffold_102,1144,0.9458041958041958,8.271853146853147,8,4.
↪9911156795536105,62,0.9992512780077317,1.0,0.8024475524475524,0,0.9993271891539499,
↪1.0,7
```

As you can see, the same scaffold is shown multiple times, and the last column is `mm`. At the row with mm = 0, you can see what the stats are when only considering reads that perfectly map to the reference sequence. As the mm goes higher, so do stats like coverage and breadth, as you now allow reads with more mismatches to count in the generation of these stats. In order to convert this files to what is provided in the output folder, the following code is run:

```
import inStrain
import inStain.SNVprofile

IS = inStain.SNVprofile.SNVprofile(``/home/mattolm/inStrainOutputTest/``)
scdb = IS.get('cumulative_scaffold_table')
ScaffDb = scdb.sort_values('mm')\
            .drop_duplicates(subset=['scaffold'], keep='last')\
            .sort_index().drop(columns=['mm'])
```

The last line looks complicated, but it's very simple what is going on. First, you sort the database by `mm`, with the lowest mms at the top. Next, for each scaffold, you only keep the row with the lowest mm. That's done using the `drop_duplicates(subset=['scaffold'], keep='last')` command. Finally, you re-sort the DataFrame to the original order, and remove the `mm` column. In the above example, this would mean that the only row that would survive would be where mm = 7, because that's the bottom row for that scaffold.

You can of course subset to any level of mismatch by modifying the above code slightly. For example, to generate this table only using reads with <=5 mismatches, you could use the following code:

```
import inStrain
import inStain.SNVprofile

IS = inStain.SNVprofile.SNVprofile(``/home/mattolm/inStrainOutputTest/``)
scdb = IS.get('cumulative_scaffold_table')
scdb = scdb[scdb['mm'] <= 5]
ScaffDb = scdb.sort_values('mm')\
            .drop_duplicates(subset=['scaffold'], keep='last')\
            .sort_index().drop(columns=['mm'])
```

Warning: You usually do not want to subset these DataFrames using something like `scdb = scdb[scdb['mm'] == 5]`. That's because if there are no reads that have 5 mismatches, as in the case above, you'll end up with an empty DataFrame. By using the drop_duplicates technique described above you avoid this problem, because in the cases where you don't have 5 mismatches, you just get the next-highest mm level (which is usually what you want)

A note for programmers

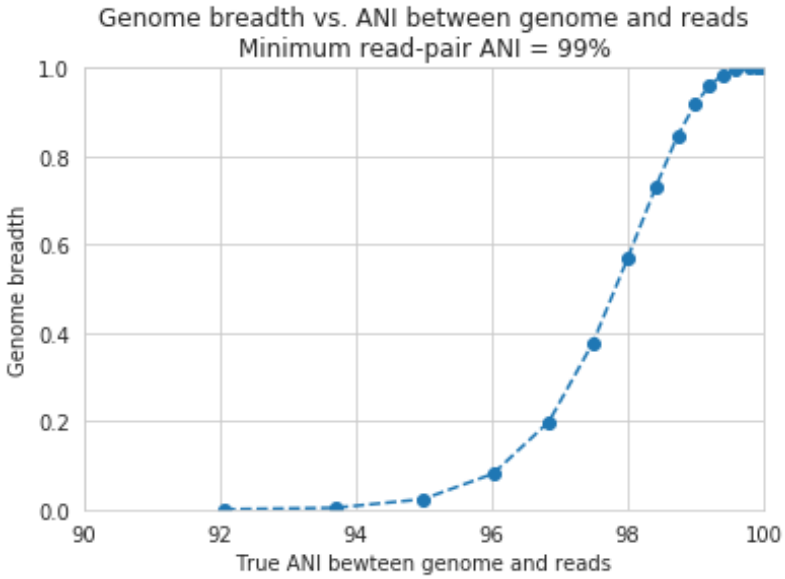
If you'd like to edit inStrain to add functionality for your data, don't hesitate to reach out to the authors of this program for help. Additionally, please consider submitting a pull request on GitHub so that others can use your changes as well.

1.6.2 Important concepts

Reference genome selection

inStrain relies on mapping reads from a sample to a reference genome. How similar the reference genome is to the reads, and the minimum read ANI threshold that you set, are very important and will determine much of what you get out of inStrain.

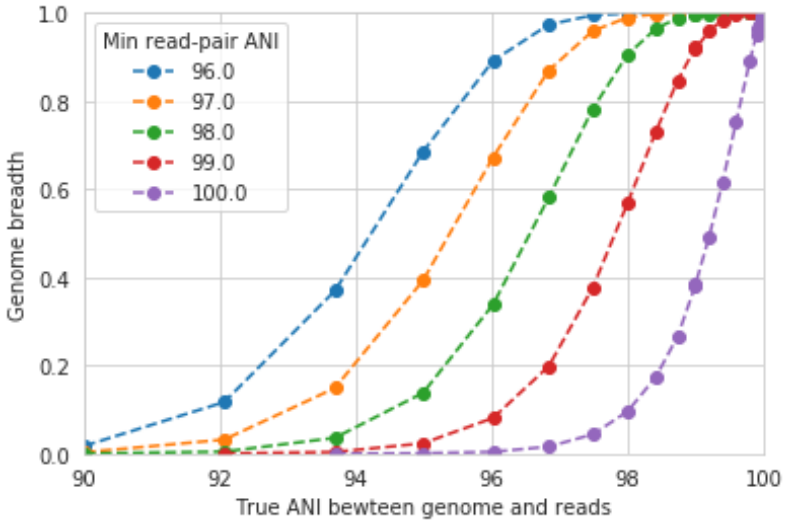
Below are a series of plots made by introducing a known number of mutations into an E. coli genome, simulating reads from these mutated genomes (at 20x coverage) with known ANI differences from the original reference genome, mapping the synthetic reads back to the original reference genome, and running inStrain.



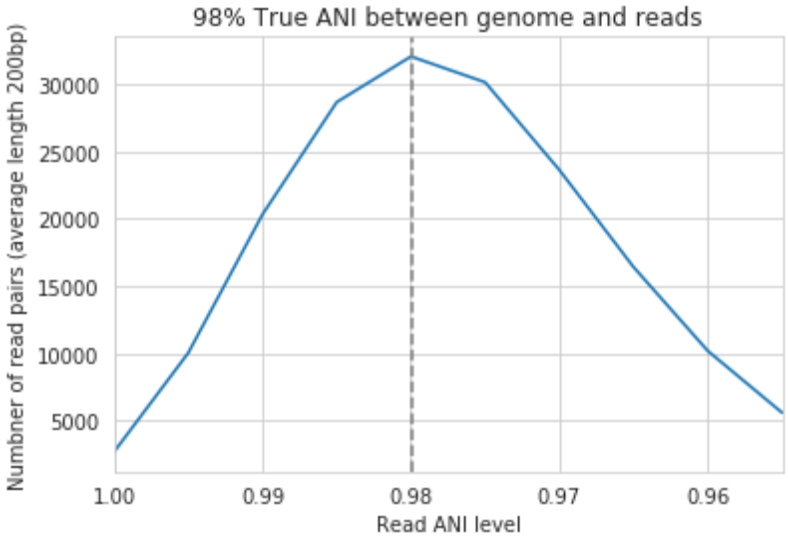
In the above plot, inStrain was run with a minimum read ANI of 0.99 (inStrain profile parameter *-l* or *–min_read_ani*). The reported genome breadth is reported on the y-axis. At 20x coverage, you should see 100% genome breadth (meaning that every base of the reference genome is covered by at least one read). However, when the reference genome is sufficiently different from the reads, the breadth is much lower. This is because when the read pair differs from the reference base by more than 99% ANI, it gets filtered out, and no longer maps to the genome. This can be exemplified a bit better by showing a variety of read filtering thresholds simultaneously:

The line drawn in the first figure is now in red on this second figure. As you can see, the more you relax the minimum read ANI, the more you can align reads to more distantly related reference genomes.

Warning: You don't want your minimum read pair ANI to be too relaxed, because then you risk mapping reads that don't actually belong to the population represented by your reference genome ("non-specific" mapping). You can also avoid non-specific mapping by increasing the size of your reference genome dataset (more on that below)



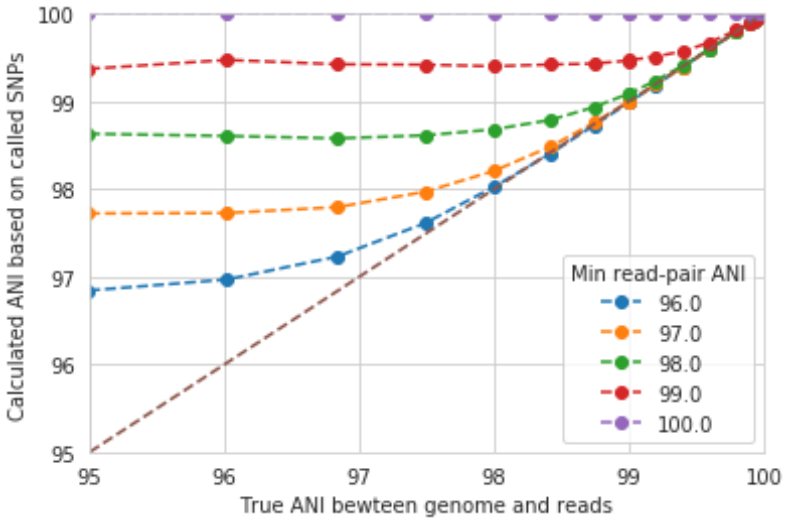
An important takeaway from the above figure is that the minimum read ANI should be at least 3% lower than the expected differences between your reads and the reference genome. If you look at the genome that's 96% ANI from the reads, for example, you see that none of the minimum read ANI levels get the correct breadth of 1. If you look at the genome that's 98% ANI from the reads, you can see that having a minimum read ANI of 96% is the only one that's actually near 100% breadth. This can also be visualized by looking at the distribution of ANI values of read pairs mapping to the 98% genome:



Most read pairs have 98%, as expected, but there is a wide distribution of read ANI values. This is because SNPs are not evenly spread along the genome, a fact that is even more true when you consider that real genomes likely have even more heterogeneity in where SNPs occur than this synthetic example.

The fact that the reads fail to map to heterogenous areas of the genome is also more problematic than it originally seems. It means that the area of the genome that are most similar to the sample reads will recruit reads during read mapping, but the (potentially interesting) areas with more SNPs will not. This is exemplified in the figure below:

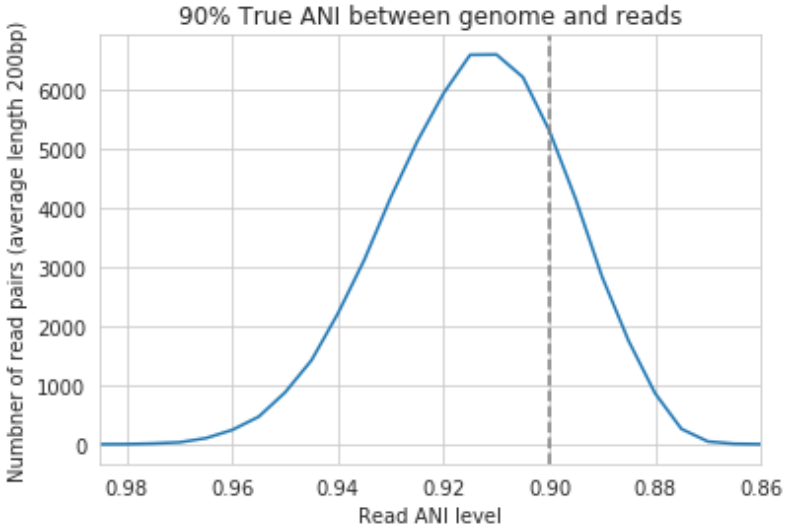
The y-axis in this figure shows the inStrain calculated ANI; that is, the number of identified SNPs divided by the number of bases with at least 5x coverage. If you look at red line, where only reads with at least 99% ANI are mapped, the ANI of reads mapping to the genome is almost always overestimated. This is because reads are only mapping to a



small fraction of the genome (see the breadth in the second figure), and the small fraction of the genome that the reads are mapping to are the regions with a small number of SNPs.

By staring at this figure like I have, you'll notice that the correct ANI is identified when the minimum read pair ANI is 2-3% lower than the actual difference between the reads and the genome. 96% minimum ANI reads correctly identify the ANI of the 98% genome, for example.

Finally, in case you're wondering what the maximum read ANI is that bowtie2 is able to map, the answer is that it's complicated:



When mapping to a genome that is 90% ANI to the reads, you no longer see a peak at 90% as you do in the 98% example. This is because bowtie2 doesn't have a string ANI cutoff, it just maps what it can. This likely depends on where the SNPs are along the read, whether they're in the seed sequence that bowtie2 uses, etc. While bowtie2 can map reads that are up to 86% ANI with the reference genome, I wouldn't push it past 92% based on this graph.

Note: In conclusion, you want your reference genome to be as similar to your reads as possible, and to set your minimum read-pair ANI to at least ~3% lower than the expected different from the reads and the reference genome. The inStrain default is 95% minimum read pair ANI, which is probably ideal in the case that you've assembled your

reference genome from the sample itself. If you plan on using inStrain to map reads to a genome that you downloaded from a reference database, you may want to lower the minimum read-pair ANI to as low as ~92%, and ensure that the genome your mapping to is at least the same species as the organism in your reads (as genomes of the same species share ~95% ANI)

Mapping to multiple reference genomes

Mapping to multiple genomes simultaneously to avoid mis-mapping

There are a number of ways to avoid mis-mapped reads (reads from a different population mapping to your reference genome). One method is to filter out distantly related reads, including by using the minimum read-pair ANI threshold (*-l*, *–min_read_ani*) or by using the mapQ score cutoff (more on that later). Another method is to include multiple reference genomes in the *.fasta* file that you map to, which gives the mapping software a chance to better place your reads.

When bowtie2 maps reads, by default, it only maps reads to a single location. That means that if a read maps at 98% ANI to one scaffold, and 99% ANI to another scaffold, it will place the read at the position with 99% ANI. If the read only maps to one scaffold at 98% ANI, however, bowtie2 will place the read there. Thus, by including more reference genome sequences when performing the mapping, reads will end up mapping more accurately overall.

Based on the above information, if you'd like to run inStrain on multiple reference genomes for the same set of reads, you should concatenate the genomes first and map to the concatenated genome set. You can then use inStrain genome_wide to get information on each genome individually.

Note: You can get an idea of the extent of mis-mapping going on in your sample by looking at the variation in coverage across the genome. If you see a region of the genome with much higher coverage than the rest, it is likely that that region is recruiting reads from another population. Looking at these wavy coverage patterns can be confusing, however. Here is a link for more information on this phenomenon.

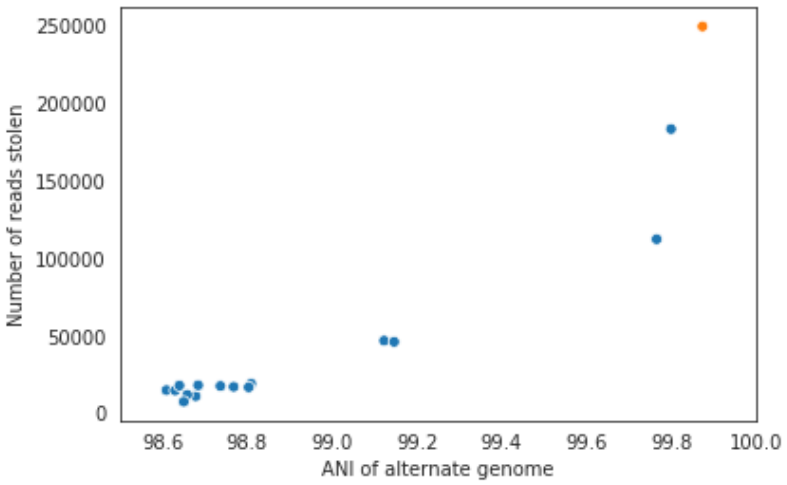
Warning: It is possible to include too many genomes in your reference .fasta file, however. You generally don't want to have genomes that are over 98% ANI to each other in your reference genome set, because then the genomes can steal reads from each other. More on that below.

Read stealing due to including closely related genomes in the reference .fasta file

If bowtie2 finds a read that maps equally well to multiple different positions in your .fasta file, it will randomly choose one of the two positions to place the read at. Because of this, you really don't want to have multiple positions in your .fasta file that are identical. At these positions it is impossible for the alignment algorithm to known which reference sequence the read should actually map to. You can then end up with "read stealing", where closely related genomes will steal reads from the true reference genome.

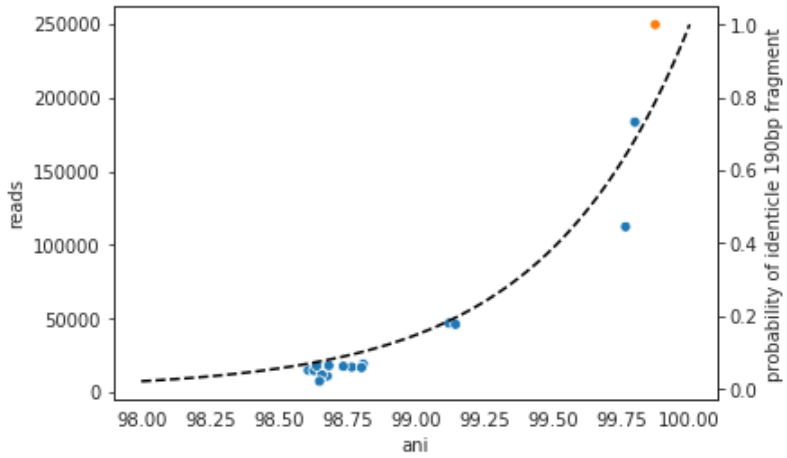
In the below example, thousands of bacterial genomes were dereplicated at 99.8% ANI and combined into a single .fasta file. One genome was randomly chosen to profile, and reads from the sample from which that genome was assembled were mapped to this concatenation of all genomes together and to that one genome individually. We then profiled the difference in read mapping when mapping to the two different .fasta files. Specifically, we looked at reads that mapped to the genome of interest when mapping to that genome individually, and mapped elsewhere when mapping to all genomes concatenated together.

Each dot represents a genome in the concatenated genome set. The position on the x-axis indicates that genomes ANI to the genome of interest (orange dot), and the position on the y-axis indicates the number of reads that were stolen



from the genome of interest. The number of reads that were stolen from the genome of interest is the number of reads that mapped to the genome of interest when it was mapped to as an individual .fasta file, but that now map to a different genome when reads were mapped to a concatenation of many genomes together.

As you can see, the more closely related an alternate genome is to a genome of interest, the more likely it is steal reads. This makes sense, because assuming that the genomes represented by blue dots are not actually present in the sample (likely true in this case), the only way these genomes have reads mapped to them is be having regions that are identical to the genome that is actually present in the sample. In fact, you can even calculate the probability of having an identical region as long as a pair of reads (190bp in this case) based on the genome ANI using the formula: Probability of identical 190bp fragment = (genome ANI) ^ 190. We can then overlay this onto the above plot:

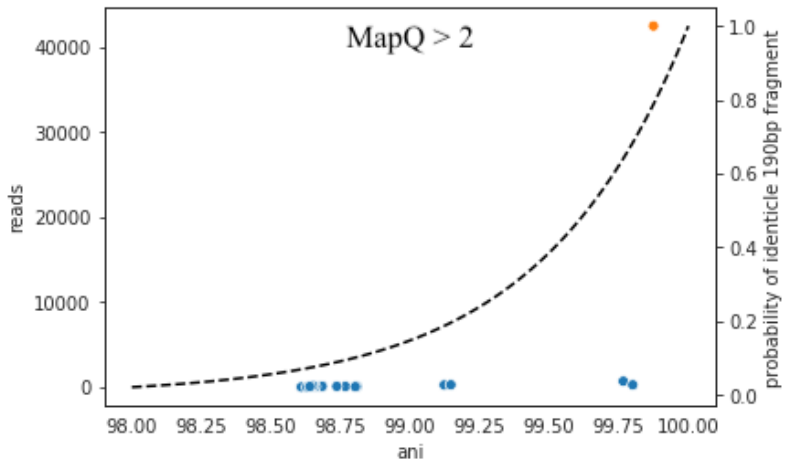


This simple formula fits the observed trend remarkably well, providing pretty good evidence that simple genome-ANI-based read stealing is what is going on.

Note: In the above example, read stealing approaches 0 at around 98% ANI. Thus, when dereplicating your genome set (using dRep for example), using a threshold of 98% or lower is a good idea.

As a final check, we can also filter reads by MapQ score. A MapQ is assigned to each read mapped by bowtie2, and is meant to signify how well the read mapped. MapQ scores are incredibly confusing (see the following link for more information), but MapQ scores of 0 and 1 have a special meaning. If a read maps equally well to multiple different

locations on a .fasta file, it always gets a MapQ score of 0 or 1. Thus, by filtering out reads with MapQ scores < 2, we can see reads that map uniquely to one genome only.



Just as we suspected, read no longer map to these alternate genomes at all. This provides near conclusive evidence that the organisms with these genomes are not truly in the sample, but are merely stealing reads from the genome of the organisms that is there by having regions of identical DNA. For this reason it can be smart to set a minimum MapQ score of 2 to avoid mis-mapping, but at the same time, look at the difference in the number of reads mapping to the correct genome when the MapQ filter is used- 85% of the reads are filtered out. Using MapQ filters is a matter of debate depending on your specific use-case.

Other considerations

A final aspect to consider is de novo genome assembly. When multiple closely related genomes are present in a sample, the assembly algorithm can break and you can fail to recover genomes from either organism. A solution to this problem is to assemble and bin genomes from each metagenomic sample individually, and dereplicate the genome set at the end. For more information on this, see the publication "dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication"

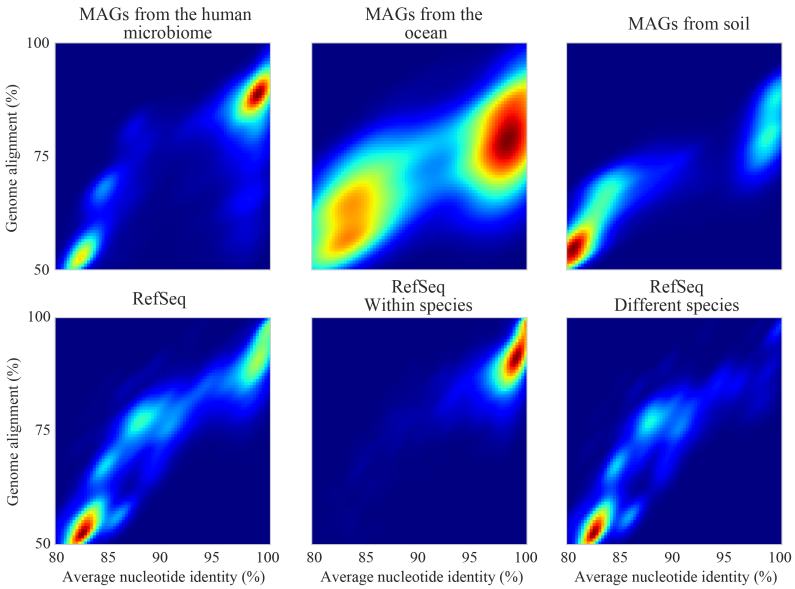
Assuming you de-replicate your genomes at 98% before mapping to run inStrain, another matter to consider is how you define detection of a genome in a sample. The following figure shows the expected genome overlap between genomes of various ANI values from different environments (adapted from "Consistent metagenome-derived metrics verify and define bacterial species boundaries")

As you can see, genomes from that share >95% ANI tend to share ~75% of their genome content. Thus, using a breadth detection cutoff of somewhere around 50-75% seems to be reasonable.

Note: Based on the above information we recommend the following pipeline. 1) Assemble and bin genomes from all samples individually. 2) Dereplicate genomes based on 97-98% ANI. 3) Concatenate all dereplicated genomes into a single .fasta file, and map reads from all original samples to this concatenated .fasta file. 4) Use inStrain to profile the strain-level diversity of each microbial population (represented by a genome in your concatenated .fasta file)

Detecting closely related organisms with inStrain compare

To compare strains with inStrain, one must first generate two inStrain profiles (using the command *inStrain profile*) based on mapping reads to the same .fasta file. *inStrain compare* then compares the reads mapped from both samples



to the same .fasta file to calculate an extremely precise and accurate ANI value for the populations in the two samples. In order for this to work well, however, there are a number of things that you must keep in mind.

Same as *inStrain profile*, *inStrain compare* requires the user to think about the minimum read-pair ANI that should be considered. It will use the read-pair ANI selected during the *inStrain profile* commands by default, but the user can also access many other min read-pair ANI values using the ANI (see section *Dealing with "mm"* below for more information)

Below are a series of plots generated from synthetic data. In these plots, a reference genome was downloaded from NCBI and mutated to a series of known ANI values. Synthetic reads were generated from each of these mutated genomes, mapped back to the original genome, and then *inStrain profile* was run on the resulting .bam file. Synthetic reads were also generated from the original genome and mapped back to it as well. Finally, *inStrain compare* was run to compare the .bams resulting the mutated genomes to the original genome. This allows us to compare the (pop)ANI value reported by inStrain compare to the true ANI value (generated by introducing a known number of mutations).

Note: The ANI values reported from inStrain compare are referred to as popANI values

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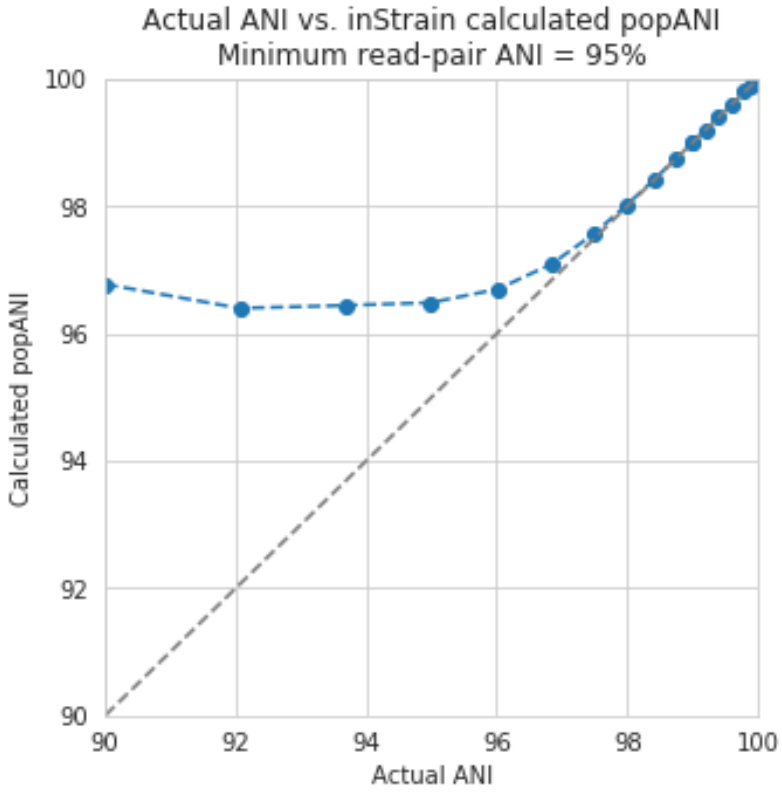
As you can see, the calculated popANI value is incorrect when the actual ANI different is large. This makes sense based on the section above. When mapping reads from an organism that is 90% ANI to the .fasta file that you're mapping to, many read-pairs will have an ANI of over 90%, and thus be thrown out when using a 95% read-pair ANI cutoff. This can also be exemplified by looking at the fraction of the genome that is compared when comparing genomes of increasing ANI.

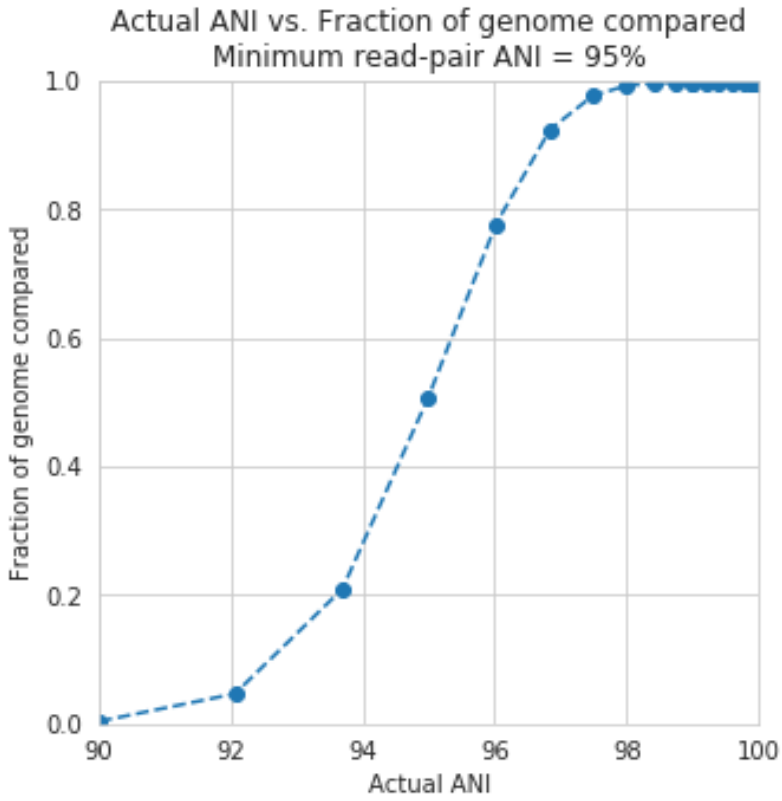
As expected, when comparing genomes of low ANI values with a read-pair ANI threshold of 95%, only a small amount of the genome is actually being compared. This genome fraction represents the spaces of the genome that happen to be the most similar, and thus the inStrain calculated ANI value is overestimated. It's also worth noting that when comparing genomes 95% ANI away from each other, only 50% of the genome bases can be compared when you filter read-pairs at a minimum of 95% ANI. You can also visualize how a lack of genome breadth of coverage leads to errors in the ANI calculation in another way:

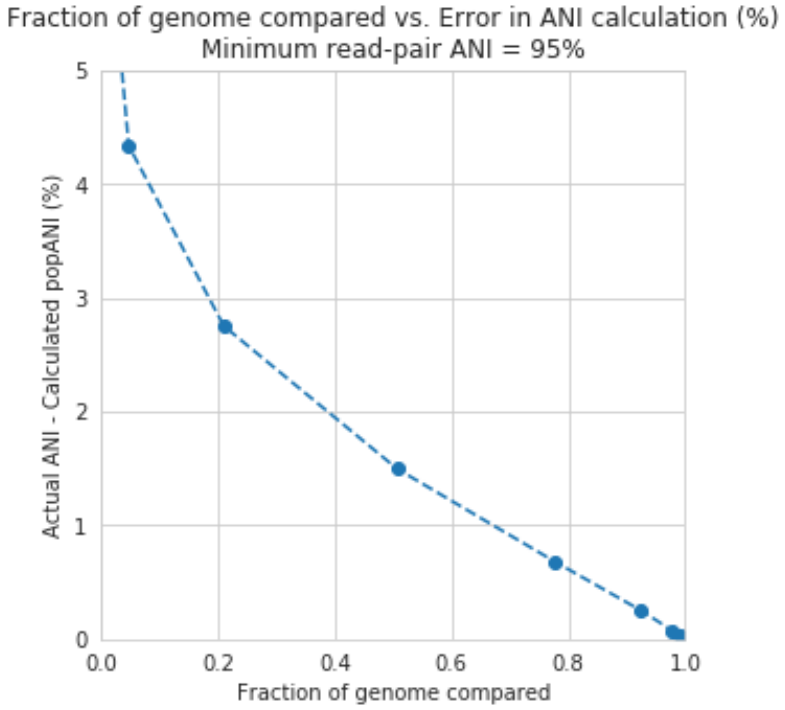
Now that we understand all of this, lets visualize lots of minimum read-pair ANI cutoffs simultaneously

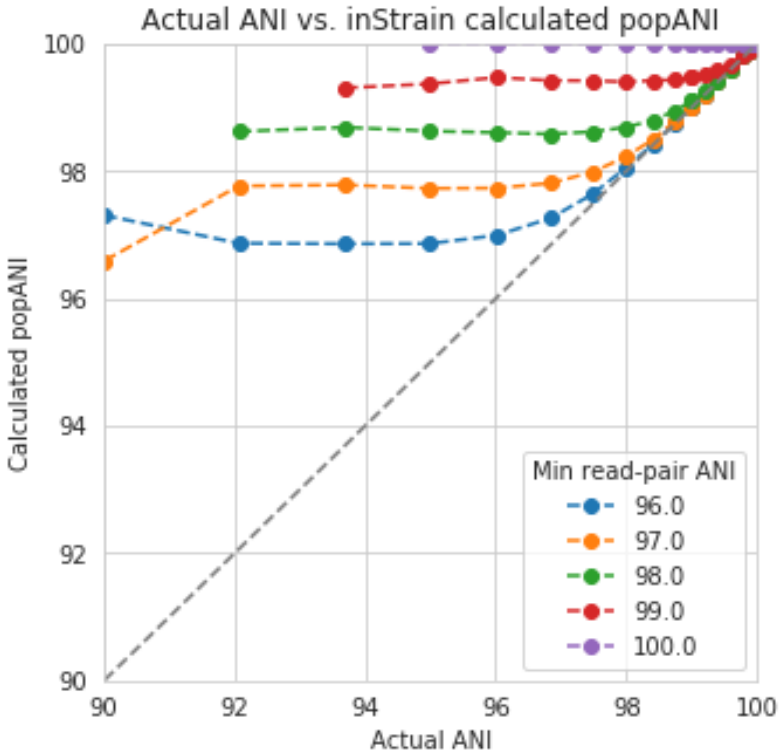
There are a couple of things to point out here.

1) Having a lower minimum read-pair ANI cutoff lets you accurately detect more distant ANI values. This makes sense given the logic above.


Actual ANI vs. inStrain calculated popANI
Minimum read-pair ANI = 95%
Calculated popANI
Actual ANI
100
98
96
94
92
90
90
92
94
96
98
100


Actual ANI vs. Fraction of genome compared
Minimum read-pair ANI = 95%
Fraction of genome compared
Actual ANI
1.0
0.8
0.6
0.4
0.2
0.0
90
92
94
96
98
100


Fraction of genome compared vs. Error in ANI calculation (%)
Minimum read-pair ANI = 95%
Actual ANI - Calculated popANI (%)
Fraction of genome compared
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0

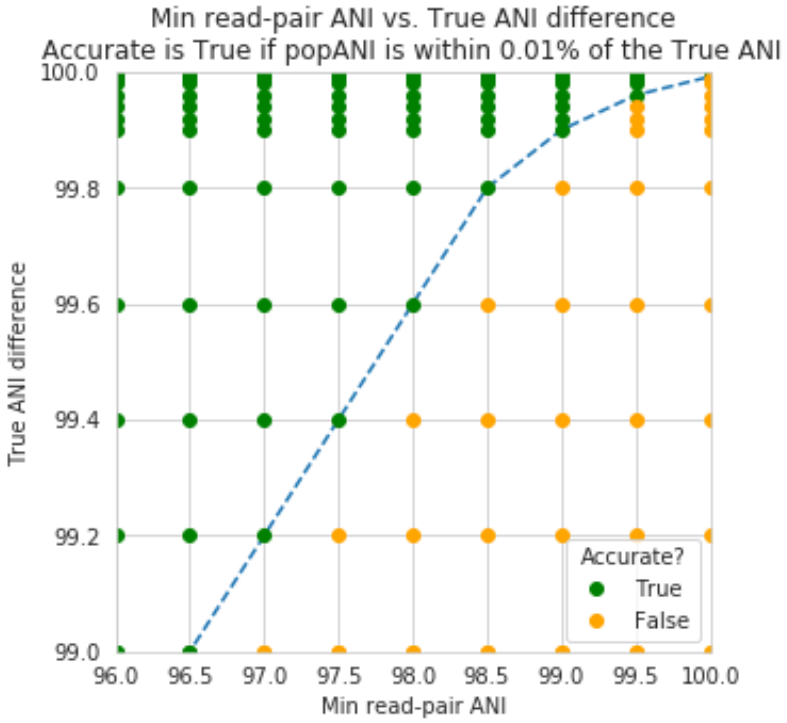

Actual ANI vs. inStrain calculated popANI
Calculated popANI
Actual ANI
90
92
94
96
98
100
Min read-pair ANI
96.0
97.0
98.0
99.0
100.0

2) There is a ceiling to how much the ANI is overestimated. If your minimum read-pair ANI is 96%, you think even very distantly related things have an ANI of ~96.5% ANI. If the minimum ANI threshold is 98%, you think distantly related things are ~98.5% ANI.

3) To get an accurate ANI value, you need to set your minimum read-pair ANI cutoff significantly below the ANI value that you wish to detect.

All of this begs the question, why would you ever set your minimum ANI threshold above 90% or so? If you're comparing clonal genomes, that would be a good idea. However, in most real scenarios, you want to set your minimum ANI threshold as high as possible to avoid mis-mapped reads, which will artificially increase your reported popANI.

Finally, this brings is to perhaps the most confusing yet import figure of this whole section. If I want to identify nearly identical genomes in two samples, what should I set my minimum ANI threshold to?



The above figure shows a range of minimum read-pair ANI thresholds on the x-axis, and a range of True ANI differences between genomes on the y-axis. Dots are colored green if the reported popANI is within 0.01% ANI of the True ANI, and colored yellow if they are not. As you can see, when you want to identify genomes that are extremely closely related (>99.9%), pretty much all minimum read-pair ANI thresholds values work. This is because if the genomes are that similar, there are going to be few reads that are thrown out due to have too many SNPs. This figure looks a bit more odd when you consider an "accurate" comparison to be one with 0.001% of the actual ANI

However, you also need to keep in mind that you want to have high breadth of coverage for each of the reads mapped to the reference genome. If the reference genome is not perfect, you need to relax your ANI threshold even more

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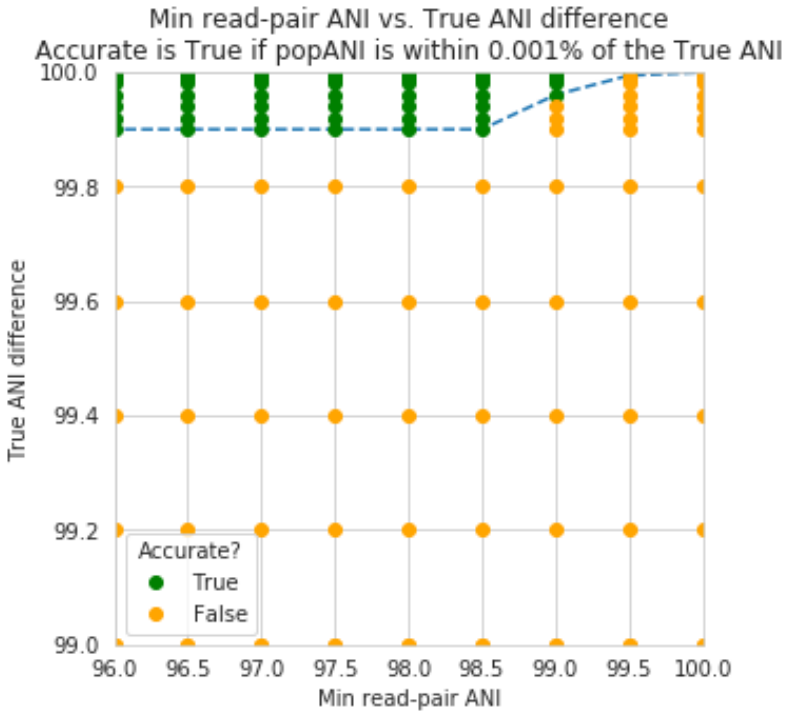
Note: In conclusion: If you have a reference genome that closely represents the true organism, and you want to identify extremely similar genomes (>99.999% ANI), a minimum read-pair ANI threshold of 98% is probably good. If you are working with a de-replicated set of genomes that you're mapping to, however (as recommended above), a minimum read-pair ANI threshold of 95% is probably better.



1.7 Acknowledgements

1.7.1 People

InStrain was developed by Matt Olm and Alex Crits-Christoph in the Banfield Lab at the University of California, Berkeley.

Special thanks to all those who have provided feedback on GitHub and otherwise, especially early adopters Keith Bouma-Gregson, Ben Siranosian, Yue "Clare" Lou, Naïma Madi, and Antônio Pedro Camargo.

1.7.2 Software

InStrain relies on several previously published programs and python modules to run - see here and here for a full list of dependencies. Of special importance are samtools (the basis for parsing .bam files) and coverM (the heart of `quick_profile`).

While not a direct dependency, the open-source program anvi'o was used as significant inspiration for several implementation details, especially related to multiprocessing efficiency and memory management.

1.7.3 Citation

The manuscript describing inStrain is available on bioRxiv and can be cited as follows:

```
Olm, M.R., Crits-Christoph, A., Bouma-Gregson, K., Firek, B., Morowitz, M., Banfield,␣
↪J., 2020. InStrain enables population genomic analysis from metagenomic data and␣
↪rigorous detection of identical microbial strains. BioRxiv. https://doi.org/10.1101/
↪2020.01.22.915579
```

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