Task 2
Georeferencing genomic data

Introduction

Phylogenetic trees based on whole genome data tell us the about the relationships of bacterial isolates to each other on a very fine scale. When we combine that high resolution information about the evolutionary relationships of isolates with geographical data it can inform our understanding of the current distribution of the pathogen and allow us to **infer the epidemiological processes** that have acted on the pathogen over time. The simplest example of this would be if a phylogeny showed that a pathogen was **geographically constrained** (e.g. isolates from the same region always cluster together). This might indicate that the pathogen is not highly mobile, whereas a pathogen with a phylogeny that shows isolates from distant regions are equally likely to be related to each other as isolates from nearby is likely to be highly mobile across regional borders. Geographical referencing of genomic data can also be **combined with temporal information** to study the movement of pathogens in space and time in real time for use in outbreak detection and monitoring.

For this task, you will be split into teams. Using the skills you learned in the structured modules, your team will use the all the **mapped sequences (.fa)** produced in Module 7 and to identify **single nucleotide polymorphism (SNP)** sites based on the reference sequence strain SL1344 and subsequently construct a **phylogenetic tree.** You will use the software **FigTree** to view and interpret your phylogeny and geo-reference your data using **Microreact** to develop your own hypotheses about the pathogen distribution**.** In addition, you will use the software **ARIBA (A**ntibiotic **R**esistance **I**dentification **B**y **A**ssembly**)** to investigate resistance genes in these strains and a free visualisation tool, **Phandango** to compare with the pathogen distribution observed in your tree.

The aims of this exercise are:

1) Introduce the biology & workflow
2) Gain experience in building and interpreting phylogenetic trees
3) Introduce concepts and tools for geo-referencing metadata
4) Show how **Next Generation Sequencing data** can be used to describe the evolution and distribution of a pathogen across a geographical area
5) Combine phylogenetic analysis and comparative genomic techniques
6) Demonstrate the value of shared data resources
7) Gain experience in presenting the results of **Next Generation Sequencing Data** analysis

Background

Biology
To learn about phylogenetic reconstruction and geo-referencing for epidemiological inference, we will work with some software that has already been introduced as well as some new software introduced in this module. We will work with real data from *Salmonella enetrica* serovar Typhimurium sampled from regional labs in England and Wales, United Kingdom in 2015.

***Salmonella enetrica* serovar Typhimurium**
Salmonella enterica is a diverse bacterial species that can cause disease in both human and animals. Human infections caused by *Salmonella* can be divided into two, typhoidal *Salmonella* or non-typhoidal *Salmonella* (NTS). The former include Typhi and Paratyphi serovars that cause typhoid. NTS comprises of multiple servoars that cause self-limiting gastroenteritis in humans and is normally associated with zoonotic *Salmonella* reservoirs, typically domesticated animals, with little or no sustained human-to-human transmission.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium), unlike the classical views of NTS, can cause an invasive form of NTS (iNTS), with distinct clinical representations to typhoid and gastroenteritis and normally characterized by a nonspecific fever that can be indistinguishable from malaria and in rare cases is accompanied by diarrhoea (Okoro *et al. Nature Genetics,* 2012).

Whole genome sequence analysis of this organism of provides some insight into the short-term microevolution of *S.* Typhimurium. Understanding the level of diversity in this time-period is crucial in attempting to identify if this is an outbreak or sporadic infection.

Your task

The Global Health Authority (GHA) has asked you to provide an overview of *Salmonella enterica* serovar Typhimurium*in* England and Wales, using retrospective samples. In teams you will develop a whole-genome sequencing based tree from all 24 sequences and correlate this to the geography of the city. You will also look into the distribution of antimicrobial resistance and investigate the genetic basis for the resistance phenotype you identified in the laboratory. At the end of the task each group will present their findings.

The five teams are the will have been assigned in the previous day.

The following division of responsibilities in your teams is recommended. If there are extra people, then they should help with the tree builder and both should work on the georefencing task once you have a tree.

- **Tree Builder** – SNP-calling and phylogenetic inference
- **Antimicrobial Resistance Investigator** – ARIBA and Phandango
- **Geo-referencer and Reporter** – geo-referencing with Microreact
- **Presentation** – All group members

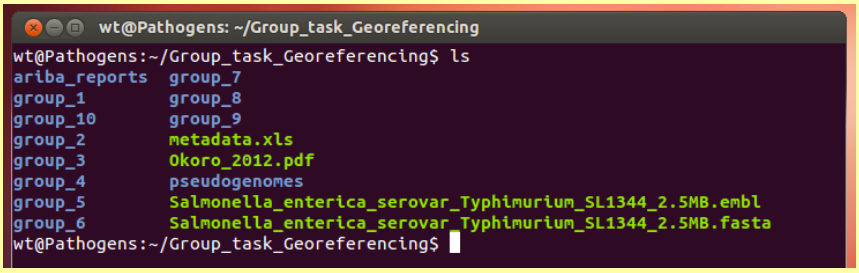
GENERAL INFORMATION

Data provided

As a team you will create a phylogenetic tree of the *S.* Typhimurium isolates from England and Wales. You will geo-reference this information, as well as antimicrobial resistance data, against the address of the isolates to form ideas about the distribution and epidemiology of the pathogen. Additionally, the genetic basis for the antimicrobial resistance will be explored.

To achieve this, each team is provided with the following files in the Task folder:

- A metadata table (**metadata.xls**) which contains information on the isolates including the date and address of collection.
- Your sequence data folder in each of your **groups folders**, which contain symlinked or symbolic linked sequenced data, fastq.gz (unix command: **ln –s**). These act like 'hyperlink' data. Symlinked data is often used to save space when you do not want to copy large files.
- An **ariba_reports** folder that contains a summary the resistance reports from ARIBA to save time. You are encouraged to run the ARIBA analysis on the samples allocated to your group.
- *S.* Typhimurium **fasta** and **embl** files, which you will use as a reverence.
- A **pseudogenomes** folder that contains an .fa file
- And a **PDF** of the literature reference cited on page 2.



assigned group	sample	MLST	year	month	Address	longitude	latitude
1	Stm_1	19	2015	May	Bệnh viện quận 8, 82 Cao Lỗ, phường 4, Quận 8		
	Stm_2	19	2015	May	Hanh Phuc International Hospital,97, Nguyen Thi Minh Khai Street, Ben Nghe Ward, Quận 1		
	Stm_3	19	2015	April	Bệnh viện Nhân dân 115, 527 Sư Vạn Hạnh, 12th Ward, Quận 10, Ho Chi Minh City		
2	Stm_4	19	2015	March	Bệnh viện Nhân dân 115, 527 Sư Vạn Hạnh, 12th Ward, Quận 10, Ho Chi Minh City		
	Stm_5	19	2015	June	Bệnh viện quận 8, 82 Cao Lỗ, phường 4, Quận 8		
	Stm_6	19	2015	March	Hanh Phuc International Hospital,97, Nguyen Thi Minh Khai Street, Ben Nghe Ward, Quận 1		
3	Stm_7	19	2015	June	Bệnh viện quận 8, 82 Cao Lỗ, phường 4, Quận 8		
	Stm_8	19	2015	September	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
	Stm_9	19	2015	July	An Binh Hospital,146 An Bình, 7th Ward, Quận 5		
4	Stm_10	19	2015	October	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
	Stm_11	19	2015	July	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
	Stm_12	19	2015	July	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
5	Stm_13	19	2015	May	Hanh Phuc International Hospital,97, Nguyen Thi Minh Khai Street, Ben Nghe Ward, Quận 1		
	Stm_14	19	2015	April	Bệnh viện Nhân dân 115, 527 Sư Vạn Hạnh, 12th Ward, Quận 10, Ho Chi Minh City		
6	Stm_15	19	2015	March	Bệnh viện quận 8, 82 Cao Lỗ, phường 4, Quận 8		
	Stm_16	19	2015	April	Bệnh viện Nhân dân 115, 527 Sư Vạn Hạnh, 12th Ward, Quận 10, Ho Chi Minh City		
7	Stm_17	19	2015	January	Bệnh viện Nhân dân 115, 527 Sư Vạn Hạnh, 12th Ward, Quận 10, Ho Chi Minh City		
	Stm_18	19	2015	July	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
8	Stm_19	19	2015	July	An Binh Hospital,146 An Bình, 7th Ward, Quận 5		
	Stm_20	19	2015	July	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
9	Stm_21	19	2015	July	Bệnh viện Bệnh Nhiệt đới, 764 Võ Văn Kiệt, phường 1,Quận 5		
	Stm_22	19	2015	June	An Binh Hospital,146 An Bình, 7th Ward, Quận 5		
10	Stm_23	19	2015	June	An Binh Hospital,146 An Bình, 7th Ward, Quận 5		
	Stm_24	19	2015	June	An Binh Hospital,146 An Bình, 7th Ward, Quận 5		

GENERAL INFORMATION

Your isolate names

The isolate names you can see in the subfolders of your group folder. There are two files for every isolate `_1.fastq.gz` and `_2.fastq.gz`. These represent the forward and reverse reads of paired end sequencing for that isolate.

When you work with your own sequencing data after the course, other naming conventions will be used. As in the example above, it is likely this formats will include helpful pieces of information, so find out what your own sequencing data names mean when the time comes!

How to use this module

As in some previous modules, you will be provided with many of the commands you will need to perform the analysis. As you will be distributing the tasks between people in your group, each role has their own set of guiding pages and focuses on different skills. You will learn about the other roles while integrating the results of your individual analyses and have the opportunity to work through the other sections in your own time.

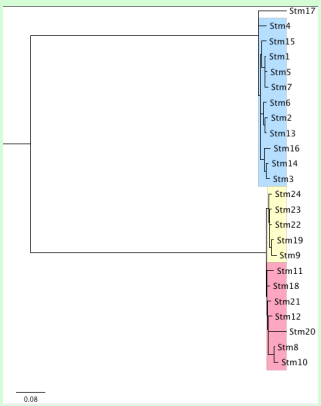
GENERAL INFORMATION

Team Presentation

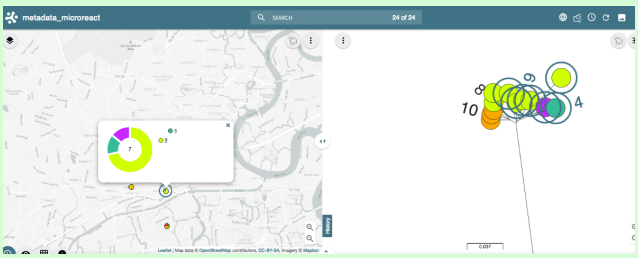
At the end of the task compile your findings and interpretations into a 5 minute presentation. All team members should contribute to making the presentation. Examples of some of the exciting key images you might produce are below, but don't be limited by these ideas - please be as creative as you like!

ANTIMICROBIAL RESISTANCE INVESTIGATORS

Presentation



TREE BUILDERS



GEOREFERENCERS

TREE BUILDERS

General Information

In this role you are responsible for the construction of a tree from whole genome sequencing data for your isolates. During this section you and your fellow tree maker will map the sequence data of your isolates to the *Salmonella enterica* Typhimurium reference genome SL1344. To save on time, you will only be mapping the fastq files to 2.5 million base pairs of the genome. Although this will take a long time, keep in mind that this step would ordinarily take many more hours of computation time. If there is more than one of you working on the mapping portion, you can work on half the samples in step 1. When you get to Step 2, combine your data and work on one computer together.

Bioinformatic processing of data into biologically-meaningful outputs involves the **conversion of data** into many different forms. Just like working in the laboratory, it's useful to to break this process down into individual steps and have a plan.

A rough guide of the steps for this task is below and in the following schematic. Check that you understand the principles of each one and then get started:

Step 1. Map and call SNPs for each isolate using commands introduced earlier in the course
Step 2. Create a whole genome sequence alignment

Step 3. Build a phylogenetic tree from the SNP data in your alignment

Step 4. Interpret your phylogeny and report the lineages to the geo-referencer

Step 1: Map and call SNPs for each isolate

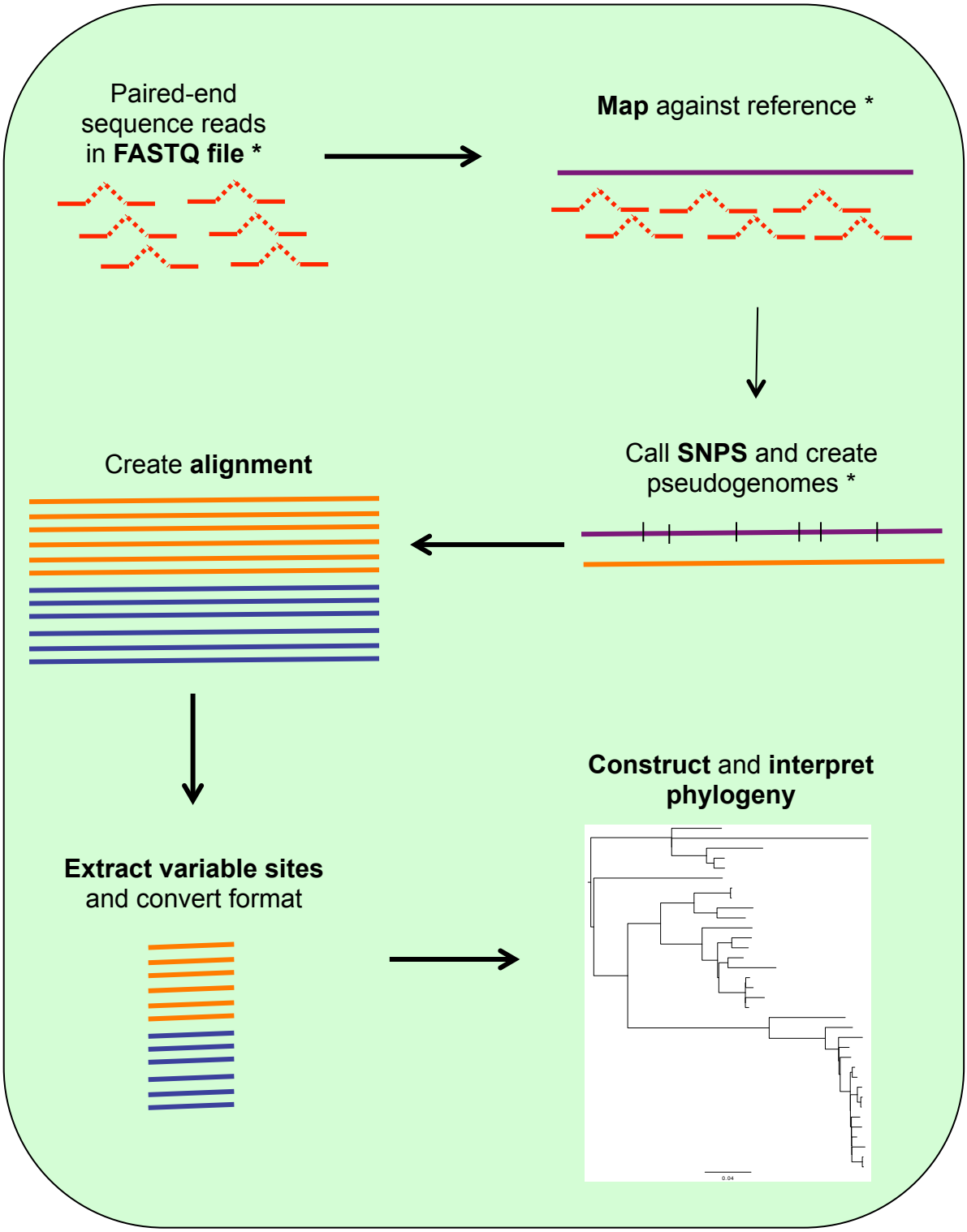
Your reference sequence for this is *Salmonella enterica* serovar Typhimurium strain SL1344, called S**almonella_enterica_serovar_Typhimurium_SL1344_2.5MB.fasta** in the task folder. You may want to create a local copy in your working directory by using the **cp** command.

Map the sequencing data for each isolate to the reference genome and obtain a pseudogenome (incorporating the isolate SNPs into the reference sequence). The required commands were covered in the **mapping and phylogeny** modules. If you struggle with the commands, ask the instructors for a command cheat sheet.

NOTE: Before you continue onto the next step, you must do some housekeeping. Refer to the mapping and phylogeny module, for which files you should remove.

TREE BUILDERS

Schematic of task workflow



***do for each isolate**

TREE BUILDERS

Step 2: Create a whole genome sequence alignment for your data

Now you have created pseudogenomes (.fasta **NOT** .fastq) for each of your samples, you can use this data to **create a sequence alignment** to build a phylogenetic tree. Using this mapping based approach we are able to avoid the computational power required to align millions of base pairs of DNA that would be needed with e.g. CLUSTAL or MUSCLE. Here, because all of the isolates were mapped to the same reference genome, they are already the same length, so they can just be pasted together to form an alignment. Then, you can combine them with **information from global reference isolates** that were created for you in the same way.

Due to time constrains we have mapped all the samples to the same reference. The file can be found in the **pseudogenome** folder. The pseudogenomes of all 24 strains were combined together using the **`cat`** command as below.

```
cat *_pseudogenome.fasta > All_pseudogenomes.fa
```

This produces a mutlifasta file **`'All_pseudogenomes.fa'`** that contains all 24 sequences. You can check all 24 sequences are present by opening it in seaview.

Here, the * acts as a wildcard symbol and a single file containing all of the pseudogenome sequences pasted one after the other is created. Both .fa and .fasta files are sequence files, but the extension is useful for distinguishing files with single (.fasta) and multiple (.fa) sequences

You should now have a file containing 24 taxa each 2.5MB long. Most of the sites in this alignment will be conserved and not provide useful information for phylogenetic inference, so we will shorten the alignment by **extracting the variable sites** using the program **snp-sites**

```
snp-sites -o All_snps.aln All.aln
```

TREE BUILDERS

Step 3: Build a phylogenetic tree from the SNP data in your genome alignment

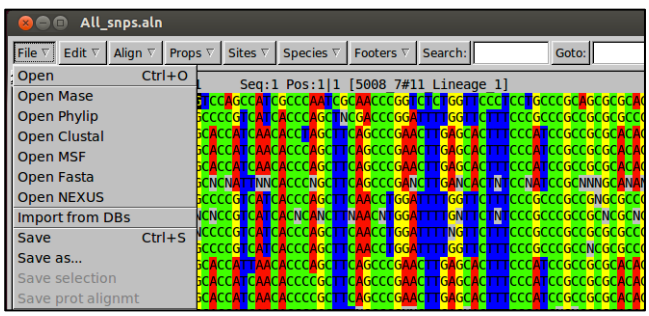
Now you will build a phylogenetic tree from the SNP alignment that you created in the last step. There are a lot of programs for building phylogenetic trees, and here we are going to use one called RAxML which evaluates trees based on maximum likelihood.

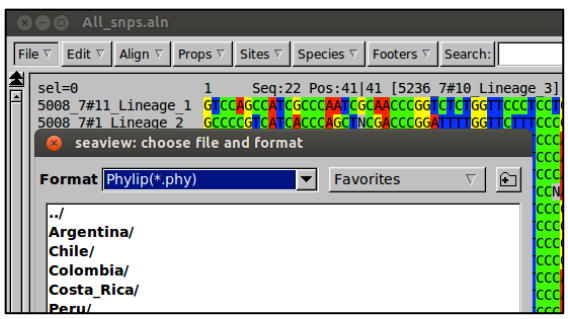
The reference is:

A. Stamatakis: "RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models". In *Bioinformatics*, 2006

Like all programs, RAxML has requirements for the format of input files. Your **All_snps.aln** file is multifasta format and RAxML requires **phylip format**, so open the file in **seaview** and save it as phylip format under the name **All_snps.phy** by typing

`seaview All_snps.aln` then doing **File > Save As > Format > Phylip(*.phy)**





Then, back at the command line, run **RAxML** by typing the following:

`raxmlHPC -m GTRGAMMA -p 12345 –n STm -s All_snps.phy`

Recall that with a single iteration of a maximum likelihood method you risk recovering a tree from a local maximum, which means it might not be the best one. This can be avoided by running multiple iterations with different starting points (we can't do that now because of time). The addition of multiple runs is done by adding the following flag to the command.

`-N 20` would run the program with 20 different starting trees (which is typically enough to find a problem if one exists)

TREE BUILDERS

Step 4: Interpret your phylogenetic tree

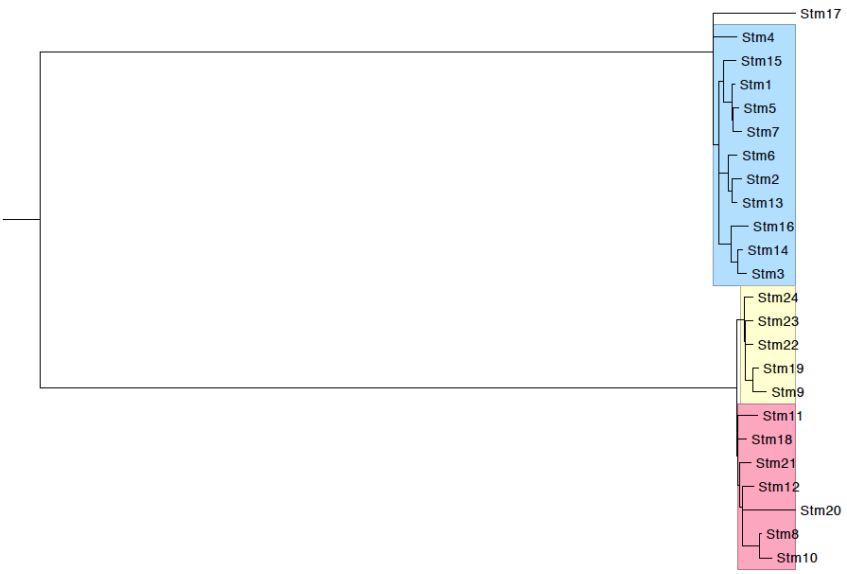
Open you final tree file (**`RAxML_result.STm`**) in FigTree and midpoint root it by selecting **Tree > Midpoint Root.**

You will now need to give this file that you have saved in FigTree to your georeferencers in your group. Go to **File > Export trees >** select **Newick** file format. Remember to save your file with a **.nwk** suffix so that you know what type of file it is.

Interpret your phylogenetic tree by first taking some time to make some general observations:

- Are there distinct clades resent in the isolates?
- Are there isolates that do not cluster with other isolates?

Then, using the relationships with the known lineages, define each of your isolates as belonging to lineage 1, 2, 3, 4 or Other and pass the information on to your georeferencer. A picture of your tree as well as your general observations about it should go into your team presentation. Take some time to make figure(s) you are happy with and create a pdf picture file by selecting **File > export PDF**



ANTIMICROBIAL RESISTANCE INVESTIGATORS

General Information

In this role, you are responsible for the investigation of antimicrobial resistance in isolates from your isolates. You will correlate the phenotypic metadata with the genetic information contained in the isolates using a local assembly approach with **ARIBA**. ARIBA, Antimicrobial Resistance Identifier by Assembly, is a freely available tool that can be installed from the ARIBA github repository. This tool required a FASTA input of reference sequences, which can be a mutli-fasta file or database of antibiotic resistance genes or non-coding sequences. This **database** will serve as one of your inputs and the other is **paired sequence reads**. ARIBA reports which of the reference sequences were found, plus detailed information on the quality of the assemblies and any variants between the sequencing reads and the reference sequences.

We have installed ARIBA in the virtual machine. You will download the CARD database (https://card.mcmaster.ca/home) for resistance detection for your samples, however other databases can be installed.
Further information and installation instructions are detailed in the github wiki page: https://github.com/sanger-pathogens/ariba/wiki. The data can then be visualised using Phandango, an interactive also freely available tool to visualise your outputs http://jameshadfield.github.io/phandango/.

Step 1. Run ARIBA

Step 2. Visualise outputs (**phandango.csv** and .**phandango.tre**) in Phandango

Step 3. Compare resistance gene present with metadata

Step 4. Summarise your findings in text and screen shots for the presentation

Step 1: Run ARIBA

On the command line, navigate to your group folder in the **Group_task_Georeferencing** folder. To run ARIBA you will need to download and format the database. Type:

```
ariba getref card out.card
```

Next you will need to format the reference database for ARIBA. Type:

```
ariba prepareref –f out.card.fa –m out.card.tsv out.card.prepareref
```

ANTIMICROBIAL RESISTANCE INVESTIGATORS

Next you will need to run local assemblies and call variants, type:

```
ariba run out.card.prepareref reads_1.fastq.gz reads_2.fastq.gz out.run
```

The command should take about 5 minutes per sample. Be patient and wait for the command prompt (denoted by a $ sign).

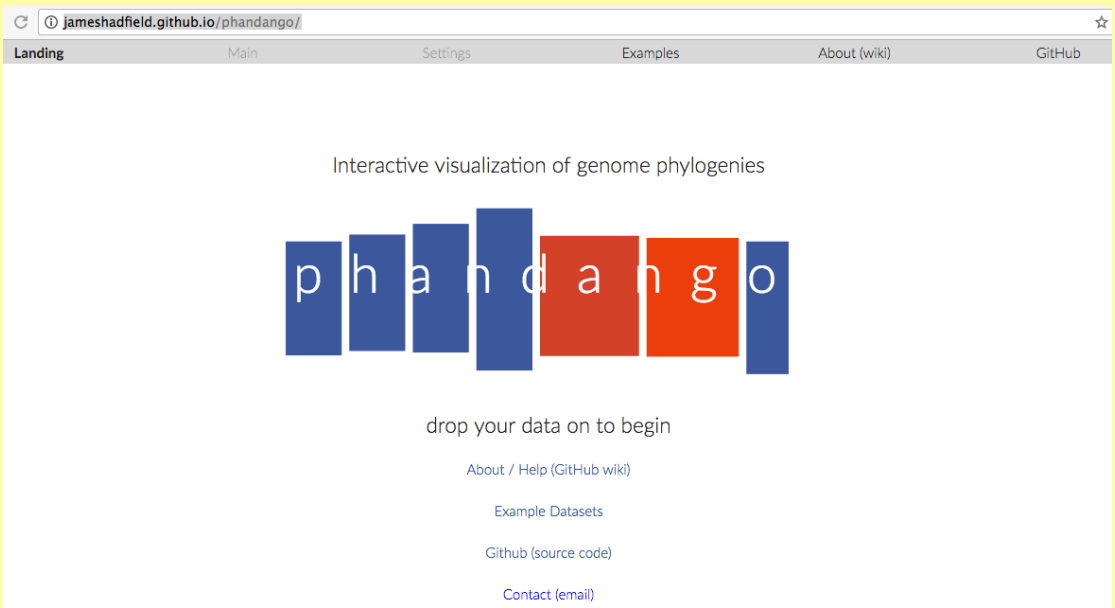
Next you will need to summarise the data from several runs. These are included in newly generated folders. You will combine the data in **report.tsv** files.

```
ariba summary out.summary out.run1/report1.tsv out.run2/report2.tsv out.run3/report3.tsv
```

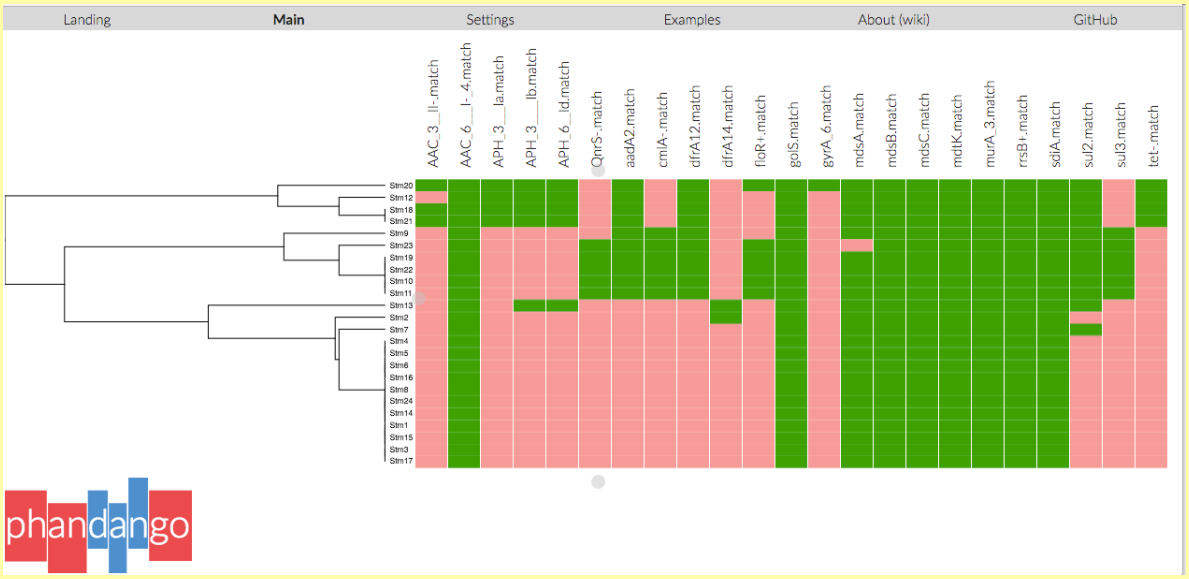
Three files will be generated, a **.csv** file with the summary of all the runs and two **.phandango** files. You will need to drag and drop the **`out.summary.phandango.tre`** and **`out.summary.phandango.csv`** into the Phandago window.

To understand the whole picture you will need to run ARIBA for **ALL 24 samples**. To save time, we have already done this for all 24 samples. Please use the files in the **ariba_reports** folder for subsequent steps. We suggest you run the analysis for a few samples to get an idea of the results.

Step 2. Visualise in Phandango

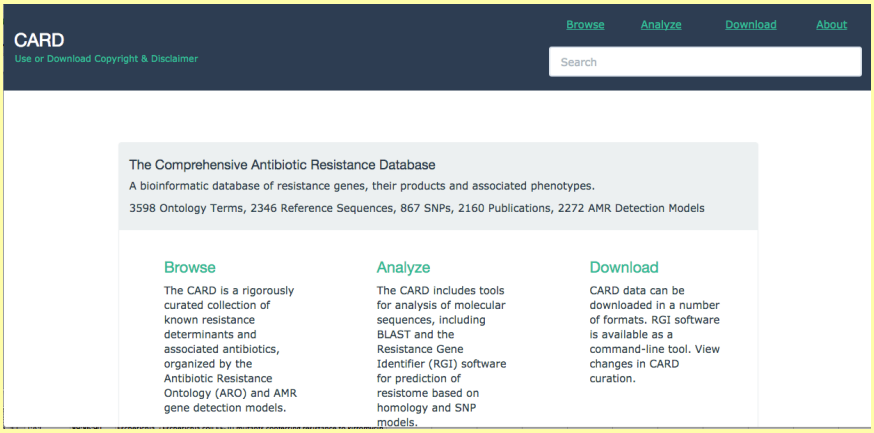


ANTIMICROBIAL RESISTANCE INVESTIGATORS



On the left hand side is a dendogram of the phylogenetic relationship of the resistance data and the strains. On the top panel are the matching resistance genes found. The green colour indicates positive match and salmon pink is a negative match.

Consult the CARD database (https://card.mcmaster.ca/home) for the resistance phenotype of the genes detected. Note that underscores (_) in the output data denotes prime (‘) or bracket, therefore AAC_3_-II is AAC(3)-II. The codes for these in a file names ‘**01.filter.check_metadata.tsv’** produced when you **prepared** your database (p. 11). Consult the report.tsv of the particular sample of interest for the gene names. You can open both .tsv files in excel.



Some general points to consider are:

- Does the presence of the gene correlate well with the phenotypic results?
- Is it the same in multiple isolates that share the resistance?
- Do you think it is vertically or horizontally transmitted?

ANTIMICROBIAL RESISTANCE INVESTIGATORS

Step 4: Summarise your findings for the presentation

Coordinate with your other team members to investigate the relationship of your resistance with where the isolates lie in the phylogenetic tree (that the Tree builders produced) and in the region of England and Wales (Georeferencer).

Consolidate your findings into some slides for the presentation and ensure the geo-referencer produces a map of the distribution of resistance to complement your work.

GEO-REFERENCERS AND REPORTERS

General Information

Geo-referencing information from pathogens can provide insight into the processes that drive their epidemiology. This can be used to infer whether single introductions of a pathogen have occurred followed by local evolution (as in the *S. sonnei* in Vietnam story described in the introductory talk and Holt *et al*, *Nature Genetics*, 2013) or whether it transmits frequently across borders. It can also indicate regions affected by antimicrobial resistance.

In this role, you are going to use the metadata provided and the tools **spatialepidemiology.net** and **Microreact.**

In this role, you will complete the following steps:

Step 1. Identify the global positioning coordinates (longitude and latitude) of the addresses where the isolates were collected

Step 2. Create a map of the metadata of the isolates

To start, open the metadata file for the strains is in the inbuilt spread sheet program on the virtual machine and note the address column, as well as the two empty global positioning columns.

In **Step 1** you will be locating isolates based on their **Address** and filling out the information in the Latitude and Longitude columns.

Eventually you will obtain phylogenetic relationships for your samples from the Tree Builders in your team, which you will use as the input for **Microreact**. For now **get started with Steps 1 and 2.**

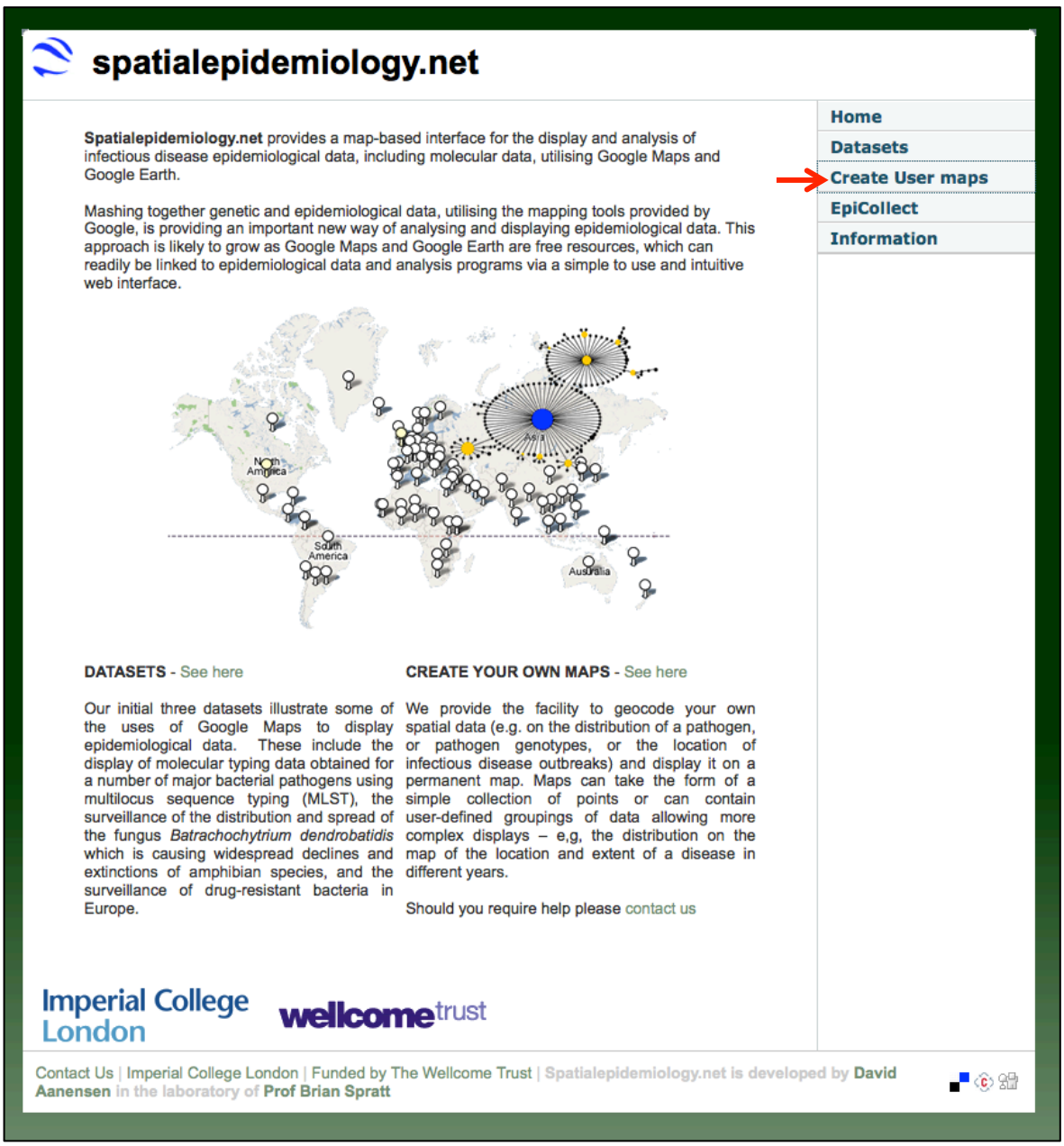
GEO-REFERENCERS AND REPORTERS

Step 1: Identify the longitude and latitude of the isolate addresses

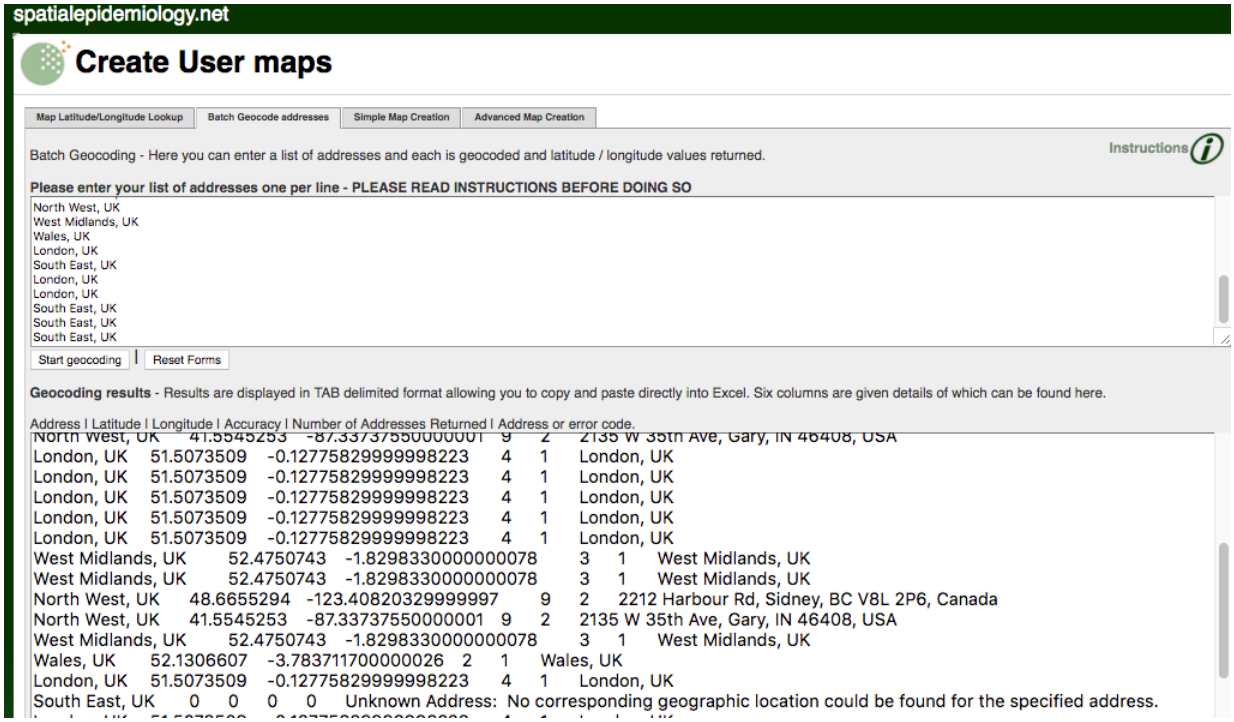
Open a browser window and navigate to www.spatialepidemiology.net to obtain latitude and longitude coordinates for your addresses.

Click on the **Create User Maps** option on the right hand column (red arrow)

Then click on the 2nd tab **Batch Geocode addresses** and copy and paste the address column from your metadata file into this field. Click **Start geocoding**.



GEO-REFERENCERS AND REPORTERS



As described on the website, this returns the address geocoding in six columns. The first three: **Address, Latitude and Longitude** are what we are after to update our metadata file.

You are also given a measure of address accuracy (for how specific the address is) and multiple addresses where a single one could not be specified.

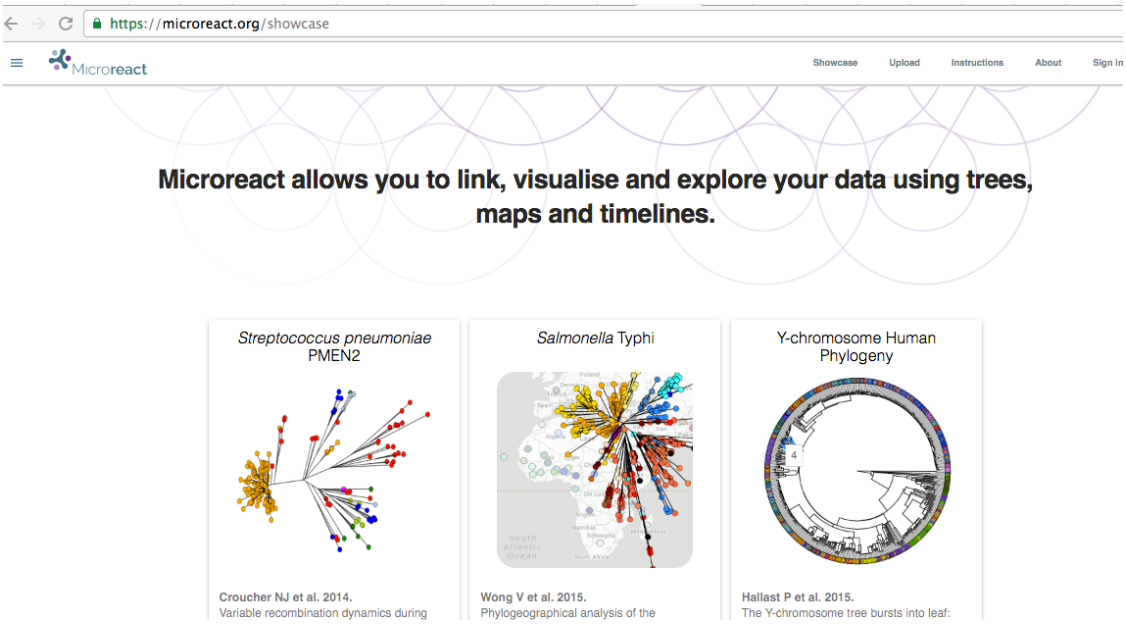
Copy and Paste this information directly from the field into the spread sheet program and **manually curate** (i.e. decided between the options for each one) the address until you have a single latitude and longitude for each isolate.

Then, update the latitude and longitude columns in your metadata file.

GEO-REFERENCERS AND REPORTERS

Step 2: Create a map of the metadata of the isolates
Although www.spatialepidemiology.net is a complete geo-referencing tool, we are going to use some of the added functionality available in **Microreact** to visualize and explore your trees and metadata.

Microreact enables you to visualize phylogenetic relationships of isolates linked to geographic locations. Dynamic visualization of the data with interactive map, tree and metadata windows. http://microreact.org



To prepare for the next step, save your updated metadata file with GPS locations as a **.csv** by doing **File > Save as > metadata.csv**

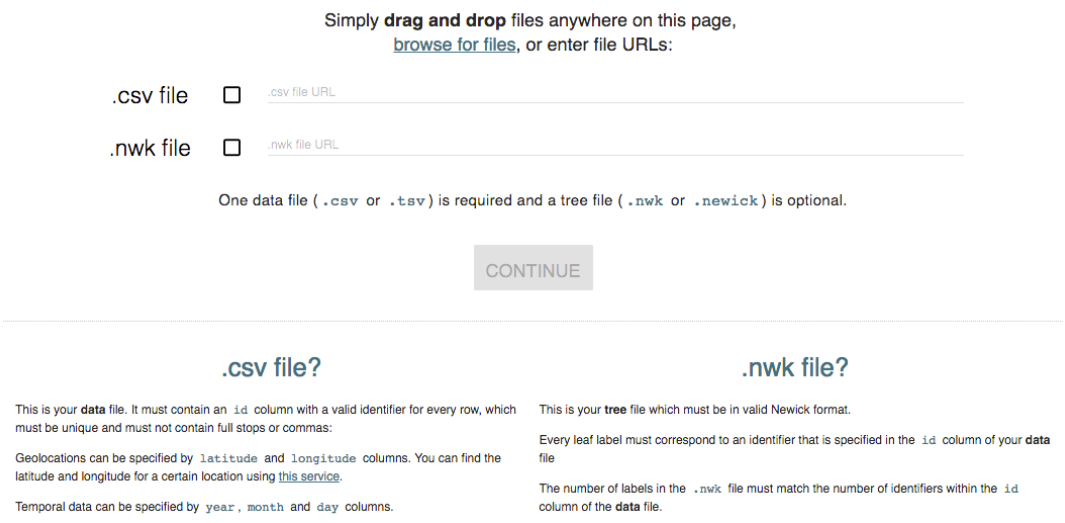
Read the instructions on how to set format your metadata file to visualise in microreact. This is vital for the next steps.

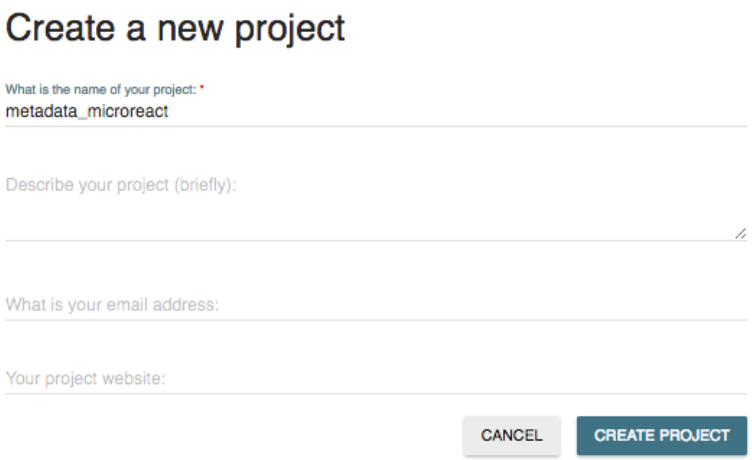
Note: You can obtain more HTML colour codes at http://htmlcolorcodes.com/

You will need a **NEWICK (.nwk)** file from the tree builders and the **.csv** metadata file you have just saved for the next step.

GEO-REFERENCERS AND REPORTERS

Drag the relevant **.csv** file and **.nwk** in the 'UPLOAD' section on the website.

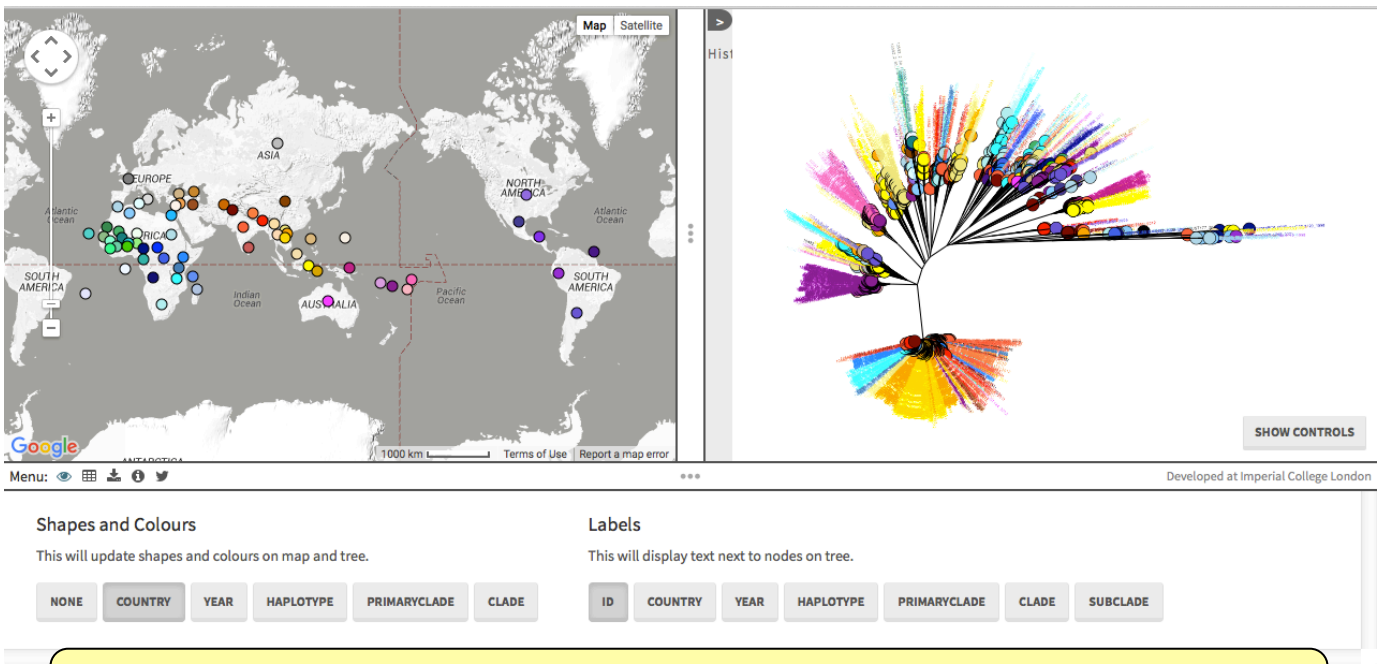




You can include a different name for your projects and a brief description if you would like.

Leave 'project website' section blank and your email is optional. Then 'create project'.

GEO-REFERENCERS AND REPORTERS



The resulting map and tree enables you to query your data.

Look at the distribution of your isolates across England and Wales when coloured by:

- **Clade** – how are the isolates distributed? Are there any patterns you can see to the distribution? What factors might be driving the distribution?
- **Antimicrobial resistances** – coordinate with your team antimicrobial resistance investigator for this – are there patterns to any of the resistances? And is this related to clades?

Take snap shots of the images and report your findings in the group presentation.
Some examples are below.

GEO-REFERENCERS AND REPORTERS

Other geo-tagging resources

Pathogens do not respect borders and global travel is increasingly frequent. For this reason the effective tracking and tracing of pathogens internationally is more important then ever. The analysis of your *S.* Typhimurium isolates tells us about how the pathogen behaves on a city-wide scale. To see if the epidemiology and resistance patterns you observed in your HCMC translate to the global scale, we need effective collaboration. For the geo-tagging recourses mentioned below, you can use either your own geographical data or data from the course for practice.

Other free geo-tagging resources.

WGSA: Is a web application for the processing, clustering and exploration of microbial genome assemblies. You can upload your assemblies and accompanying metadata to view assembly stats and view other metadata. https://www.wgsa.net/

EpiCollect: Is a freely available web and mobile app tool that is used for data collection (questionnaires), using multiple mobile phones and the data can be centrally viewed using Google maps/ tables and charts. www.epicollect.net

Phylocanvas: Metadata in binary format can be displayed next to the tree leaves by uploading a .csv file together with the tree file. http://phylocanvas.net

CartoDB: Much like the Google maps exercise you completed, CartoDB allows the user to map and analyze location data . This tool can take multiple file formats as input e.g. XLS, CSV and SQL amongst others. https://cartodb.com/

DISCLAIMER: All the locations and dates of the Salmonella isolates are fictitious and solely for educational purposes. No data was collected from Public Health England.

End of module…

ANY QUESTIONS?
Please feel free to ask at any time!