

BIOINF 3000 / BIOTECH 7005:

Bioinformatics and Systems Modelling

Week 10: Ancient DNA Practical

Yassine Souilmi (he/him/his)

yassine.souilmi@adelaide.edu.au

Shyamsundar Ravishankar (he/him/his)

shyamsundar.ravishankar@adelaide.edu.au

Introduction

In this practical, we are going to use unique aspects of ancient DNA (aDNA) sequencing data (fragmentation, damage, contamination) to understand the impact of laboratory procedures (type of library, damage repair) on damage patterns.

The data is real data from my group, and they have been generated by extracting aDNA from dingoes skeletal samples from around Australia (Souilmi et al. 2024). The dataset we will use in this practical includes an additional set of modern dingoes from (Zhang et al. 2020). The data represents modern and ancient dingo genetic diversity, New Guinea singing dogs, and a modern village dog from Bali (Figure 1).

After aDNA extraction, we built several types of sequencing libraries and damage repair, (which we will explore later). For a subset of the samples, we then performed in-solution enrichment of mitochondrial genome sequences using predesigned oligonucleotides as molecular ‘baits’. The libraries enriched for mitochondrial DNA fragments were then sent to a sequencing service provider.

You now have access to fastq files generated by Illumina machines. The samples were sequenced in 2 x 150 sequencing—i.e. the machine will sequence 100 nucleotides from the start of the DNA molecules, and 100 nucleotides from the end of the same molecules (Figure 2).

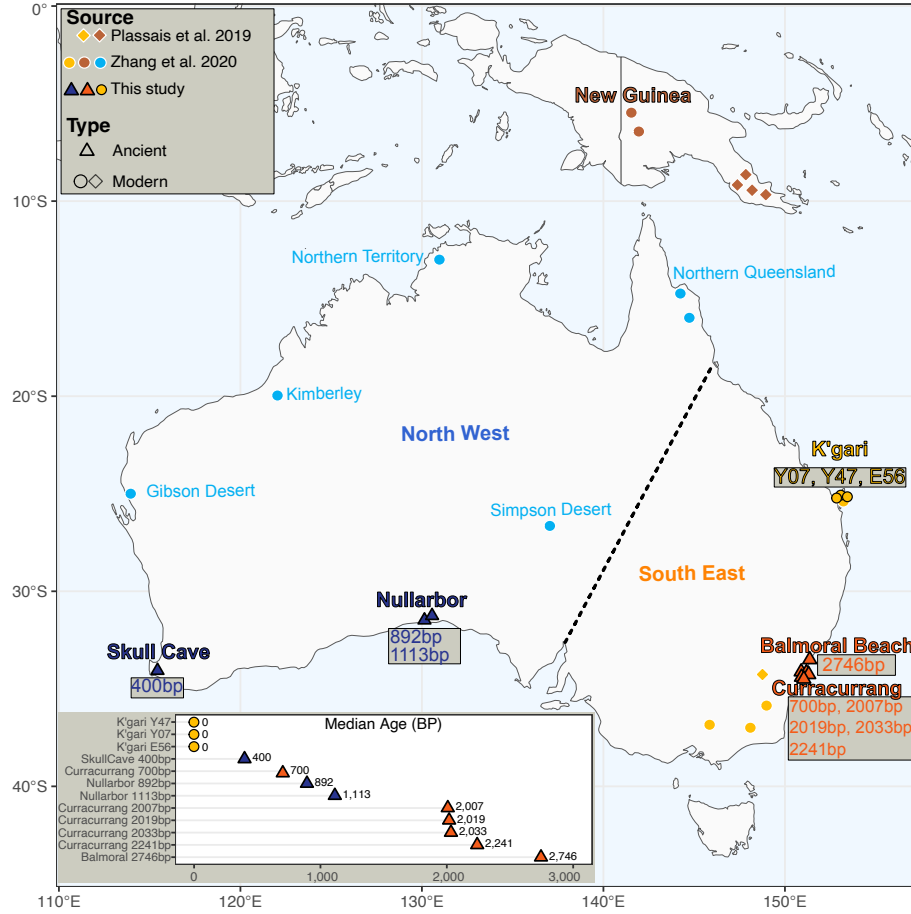


Figure 1: This is Fig. 1 of Souilmi et al. (2024) representing the geographic and temporal distribution of dingo samples. Approximate locations of the modern and ancient dingo samples with new genome-scale data presented in this study are shown on the map (ancient dingoes = blue and orange triangles, modern K'gari dingoes = bordered yellow circles). Yellow, blue, and red circles and diamonds (without black borders) represent the source localities for previously published data modern dingoes and New Guinea singing dogs. Broadly, dingoes are divided into two major populations in the “north west” (blue shades) and the “south east” (orange/yellow); the dotted line roughly indicates the transition between these two populations (based on data from mtDNA, Y chromosome haplotypes, genome-wide SNPs, morphometric data, and environmental barriers). (Inset) estimated age of samples (median; years B.P./cal. years B.P.) for which new genome-scale data are presented in this study.

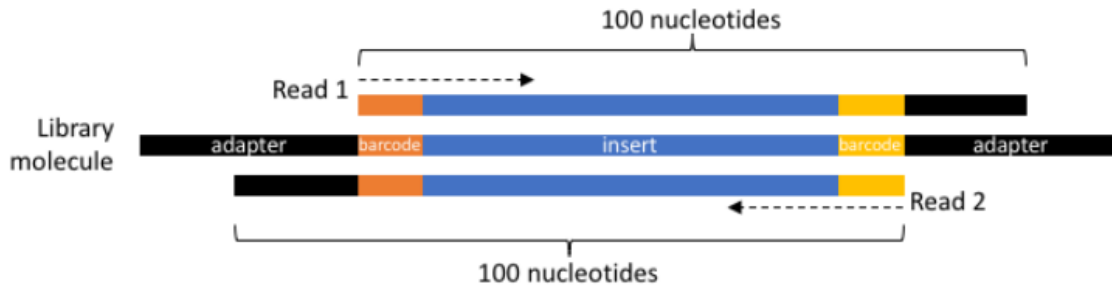


Figure 2: A sequencing library molecule is made of an insert (an ancient DNA fragment) flanked by sequencing adapters. In this particular experiment, each individual library is identified by a unique 5-mer barcode added between the adapters and the insert to allow for demultiplexing (i.e., to divide sequencing reads into separate files for each unique barcode combination). The library molecules are sequenced in two steps. First, sequencing starts from one end of the library molecules and goes for 150 nucleotides (Read 1). Second, sequencing starts from the other end and also goes for 150 nucleotides (Read 2). Because ancient DNA inserts are short, sequencing outputs (a.k.a “reads”) are likely to contain the insert as well as some barcode and/or adapter sequences.

0. Refresher on Loops in Bash

A `for` loop is a structure that allows you to repeat a set of commands for each item in a list. The syntax is as follows:

```
for item in list; do
    # do things to the item
done
```

Example:

Lets do some setting up first...

```
mkdir -p ~/Prac10/loops

cd ~/Prac10/loops

# First lets create the files
for i in $(seq 1 20) ; do
    echo $i > file${i}.txt
done
```

```
ls
```

Say we have 3 files `file1.txt`, `file2.txt` and `file3.txt`. We can print the files as:

```
for i in file1.txt file2.txt file3.txt ; do
    echo $i
done

# lets print the name of the file without .txt
for i in file1.txt file2.txt file3.txt ; do
    basename $i .txt
done

# can use *.txt instead of typing all files
for i in *.txt ; do
    basename $i .txt
done

# lets rename all the files from file1.txt to file1_newer_and_cooler.txt
for i in *.txt ; do
    name=$(basename $i .txt)
    new_name="${name}_newer_and_cooler.txt"
    mv ${i} ${new_name}
done

ls
```

In this prac, we will be using for loops to run the same command on multiple files/samples.

1. Data preparation

Below we describe some of the basic steps to process high throughput sequencing data in ancient DNA research. These steps were covered in Week 6 of this course under 'Alignment/NGS'. See below for a refresher and also learn about the extra steps needed to handle ancient DNA sequence data.

Setting up

Set up working directories, input data and software environment.

[illegible]

The first thing you do when you receive sequencing data is perform a quality control. The programs [fastp](#) (Chen et al. 2018) and [fastqc](#) (Andrews et al. 2012) are very easy to use and produces html reports that can be visualised in any internet browser. We have already run [fastp](#) for all the same. Let's look at the html reports for samples D01_W0235 and A19053. The data can be found in `data/fastp/`

1.3. Read collapsing

Typically, more than 95% of the read pairs (Read 1 and Read 2) can be collapsed in ancient DNA datasets because the short insert DNA end up being sequencing twice, during Read 1 and Reads 2.

We have also run this already in order to save time and get to the exciting parts of ancient DNA analysis. **AdapterRemoval** produces metrics that we can use **MultiQC** to summarise.

```
## Activate the bioinf environment. It has MultiQC
conda activate bioinf

cd ~/Prac10/data/adapterremoval/

# combine the reports using multiqc
multiqc .
```

open the `multiqc_report.html`.

Q3: What do you notice from the report?

Q4: What can you say about the ratio of collapsed reads?

Q5: What can you learn from the read-length?

2. Read Alignment

Now we will align the sequencing reads to the dog mitochondrial reference genome. We download the reference genome from ENSEMBL database (Birney and Team 2003), a genomic database containing several useful resources. For convenience, you can find the reference indexed inside `data/reference`.

2.1. Alignment

We use `bwa aln` alignment (Li and Durbin 2009) according to the parameters in Oliva et al. (2021). These parameters have been tuned to deliver optimal performance for aDNA samples. Again to save time, we have aligned the samples for you already (it is similar to alignment in Week 6). Additionally, these reads have been sorted using [samtools](#) (Li et al. 2009).

3. Removing Duplicate reads

When sequencing libraries are built from extracted DNA, PCR amplification is often used to increase the amount of DNA available for sequencing. This is especially critical in ancient DNA (aDNA) studies, where the DNA of interest is highly degraded and present in very low amounts. However, PCR amplification can introduce an issue: the same DNA fragment may be copied and sequenced multiple times.

These duplicate sequences artificially inflate the representation of certain alleles or regions, skewing downstream analyses. For instance, if the same fragment is sequenced multiple times,

it might appear that a particular allele is more common than it actually is. To avoid this bias, it is standard practice to detect and mark duplicated sequences in the data.

Duplicate marking works by identifying reads that map to the exact same location in the genome, meaning they have the same start coordinate. Since the sequencing process is random, it is highly unlikely for independent reads to start at the exact same position unless they originate from the same DNA molecule. Therefore, reads that share the same start point are flagged as duplicates and can be removed from further analysis to ensure more accurate results.

3.1 MarkDuplicates

We will use a tool called [MarkDuplicates](#) (Broad Institute, n.d.) which is part of the Picard suite of tools. Picard is a toolkit to manipulate mapped sequence data.

```
# Lets reactivate adna environment
conda activate adna

cd ~/Prac10/rmdups/

for bam in ../data/alignment/*bam; do
    sn=$(basename $bam .bam)
    picard MarkDuplicates INPUT=$bam \
        OUTPUT=${sn}_rmdup.bam REMOVE_DUPLICATES=TRUE \
        AS=TRUE METRICS_FILE="${sn}_rmdup.metrics" \
        VALIDATION_STRINGENCY=SILENT
    samtools index ${sn}_rmdup.bam
done
```

4. Read damage

We are interested in the characteristic post-mortem damage that can be detected in ancient DNA is the deamination of C into U, which will be amplified and sequenced as T. Deamination of C occurs primarily at the extremities of ancient DNA molecules, and the resulting 5' C-to-T substitutions (and complementary 3' G-to-A substitutions) can be summarised using the program mapDamage (Figure 3).

We will use the [mapDamage](#) package (Jónsson et al. 2013) to capture the misincorporation rate and location in within the reads.

Lets run mapdamage on a few samples and check the results.

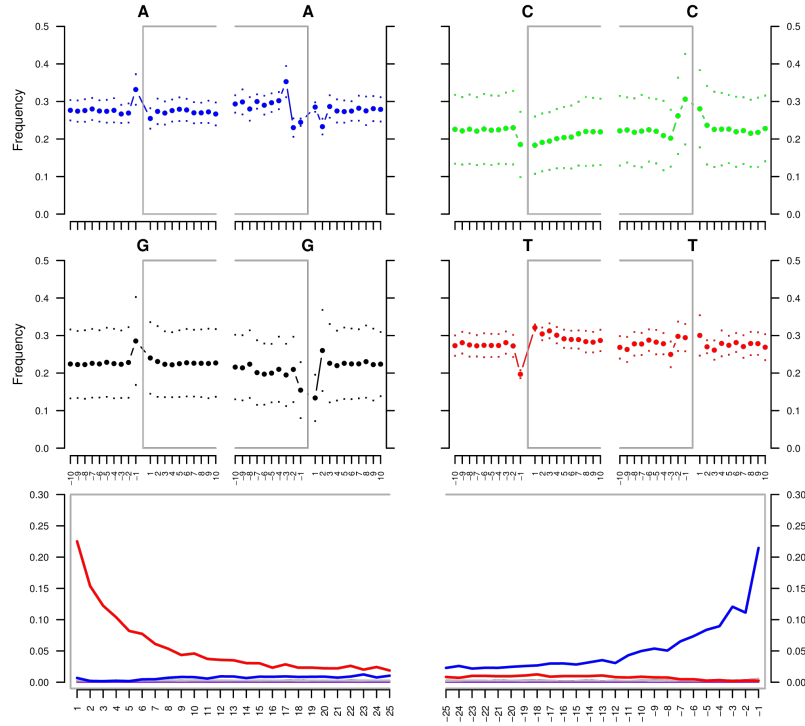


Figure 3: Fragment misincorporations as predicted by mapDamage. Notice the ‘smiley’-like pattern characteristic of aDNA resulting from excess C>T transitions in the 5’ end, and excess G>A transitions in the 3’.

```
cd ~/Prac10/mapdamage/
ref="/Prac10/data/reference/dog_mtDNA.fasta"

for bam in ../rmdups{D10,D11,D12,D13,Y47,BaliVD}*_rmdup.bam; do
    mapDamage -i $bam -r $ref --no-stats
done
```

- **Q6:** What samples do you think are ancient?
- **Q7:** What do you notice about the fragment length of the ancient and modern samples?

5. Trimming the damaged reads

To mitigate the impact of DNA damage, it's necessary to trim the damaged bases from the ends of reads before further processing. This ensures that ancient damage does not falsely influence variant calling or other analyses.

In this practical session, for the sake of simplicity and uniformity, we will trim two bases from both the 5' and 3' ends of every read, regardless of whether the sample is modern or ancient. However, in real-world scenarios, this step is typically only applied to aDNA samples where damage is observed, and the extent of trimming is determined by the damage patterns seen in MapDamage results.

We will be using a tool called `trimBam` from [BamUtil](#) (Jun et al. 2015)

- **Q8:** Based on the mapDamage plot how many bases should we trim?

```
cd ~/Prac10/trim_bam

for bam in ../rmdups/*_rmdup.bam; do
    bam trimBam $bam tmp.bam -L 2 -R 2
    samtools sort tmp.bam -o $(basename $bam _rmdup.bam).trimmed.bam
    samtools index $(basename $bam _rmdup.bam).trimmed.bam
done
```

6. Understanding Population Structure using the mitochondrial DNA

Mitochondrial DNA (mtDNA) is a uniparental marker that is maternally inherited (Figure 4). It is a valuable marker to understand population history of a species. The maternal inheritance preserves a direct lineage of maternal ancestry. mtDNA are small molecules that are found in high copy number within cells, this increases its chances of surviving in ancient samples, making it easier to reconstruct compared to nuclear DNA. They also do not recombine and so the entire mtDNA is inherited as a single unit. Its relatively high mutation rate provides

sufficient variability to distinguish between populations, track evolutionary changes, and infer migration patterns across generations. These characteristics make mtDNA a powerful marker for reconstructing ancient population dynamics.

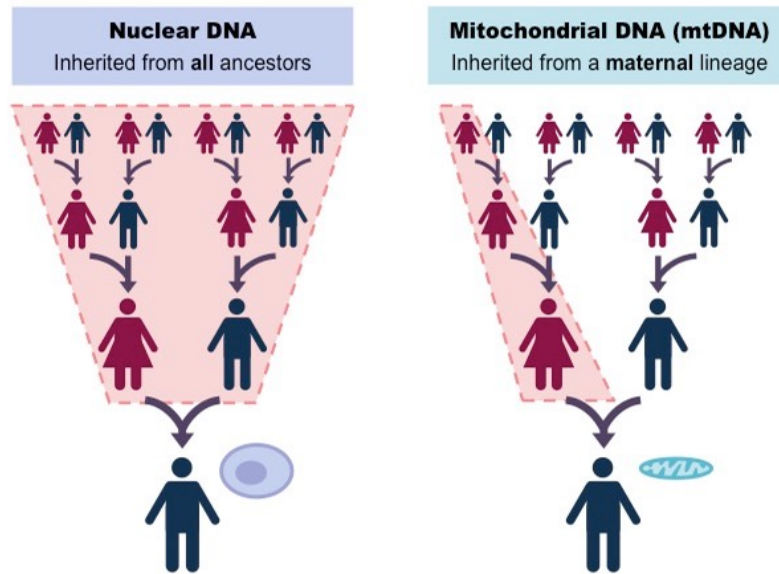


Figure 4: Nuclear DNA is inherited from both parents while mtDNA is only maternally inherited.

6.1 Create a consensus MT Genome

First, let's reconstruct the mitochondrial genome of our samples. We will use the [consensus](#) function from `samtools`. We will use the alignment file as input and get the entire mitochondrial genomes as a FASTA file. See doc to understand what all the options in the command below does.

```
cd ~/Prac10/fasta

for bam in ../trim_bam/*.trimmed.bam; do
  sn=$(basename $bam .trimmed.bam)

  samtools consensus -r chrM \
    -o ${sn}_consensus.fasta -a \
    --min-MQ 25 --min-BQ 30 -c 0.75 \
    -d 2 ${bam}
```

```
sed -i 's/chrM/'$sn'/' ${sn}_consensus.fasta
done
```

Merge all the genomes into a single FASTA file.

```
cd ~/Prac10/fasta

for fasta in *consensus.fasta ; do
    cat $fasta
done > concatenated.fasta
```

6.2 Perform Multiple Sequence Alignment

We then want to compare how the mitochondrial genomes across our samples compare to each other. For this we will use the tool [MUSCLE](#) (Edgar 2004) that can perform multiple sequence alignment (MSA).

```
# MUSCLE Multiple Alignment
muscle -in concatenated.fasta -out aligned.fasta
```

6.3 Convert MSA to Nexus

Finally, we will convert the MSA to Nexus format to make it compatible for tools to visualise the multiple alignment. Nexus is a useful format that can store information about the alignment as well as metadata about the samples such as geographical origin. We will use [seqmagick](#) (Yu 2024) for this.

```
# seqmagick conversion
seqmagick convert --alphabet dna aligned.fasta aligned.nex
```

6.4 Visualise Structure in PopART

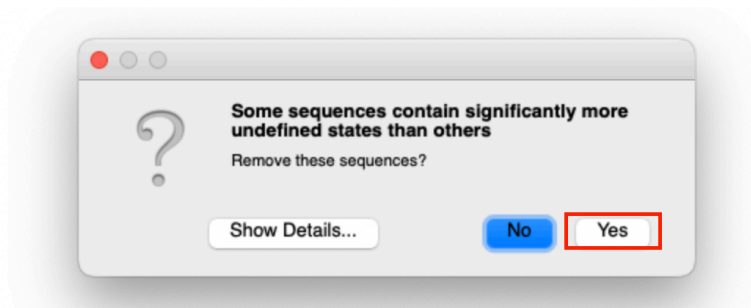
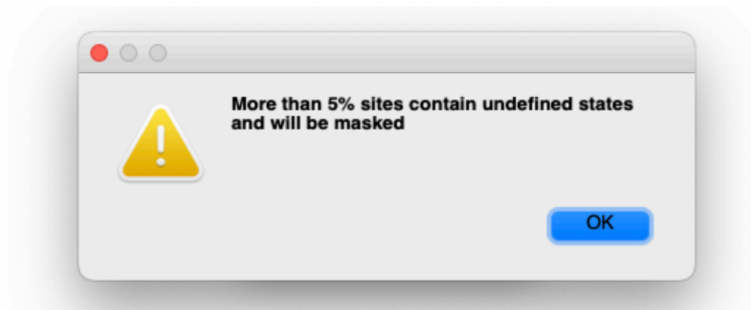
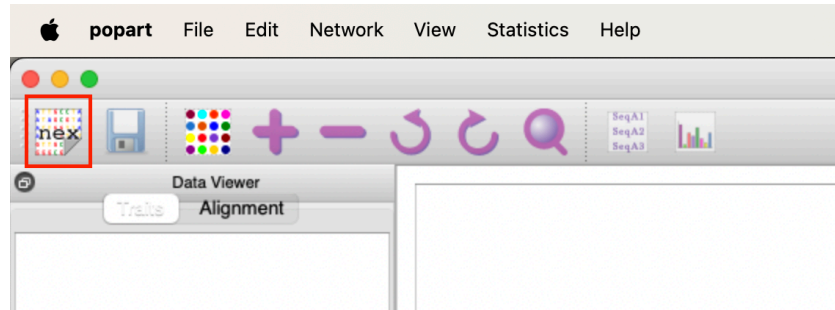
Before, visualising the multiple alignment we need to add metadata about our samples to the Nexus file.

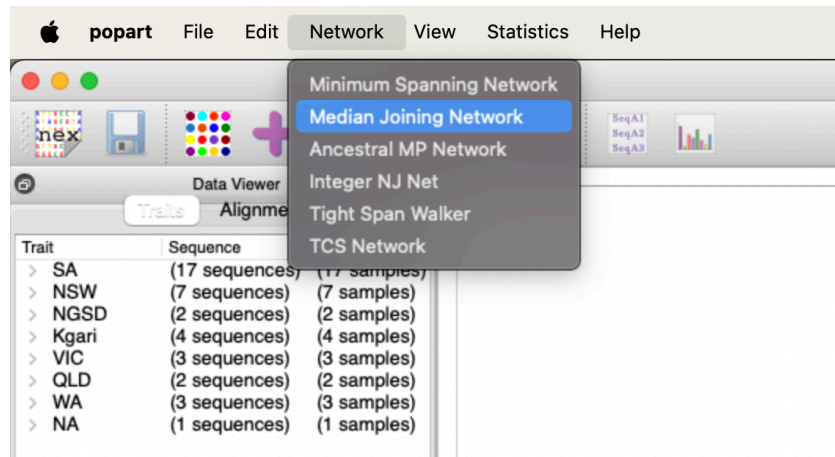
```
cat aligned.nex ~/Prac10/data/traits.nex > aligned_traits.nex
```

We can use [PopART \(Population Analysis with Reticulate Trees\)](#) (Leigh and Bryant 2015) to create a median-joining network (Bandelt, Forster, and Röhl 1999). This is a tool to visualise the differences in mitochondrial sequences. It clusters similar sequences together and calculates the number of differences between dissimilar sequences.

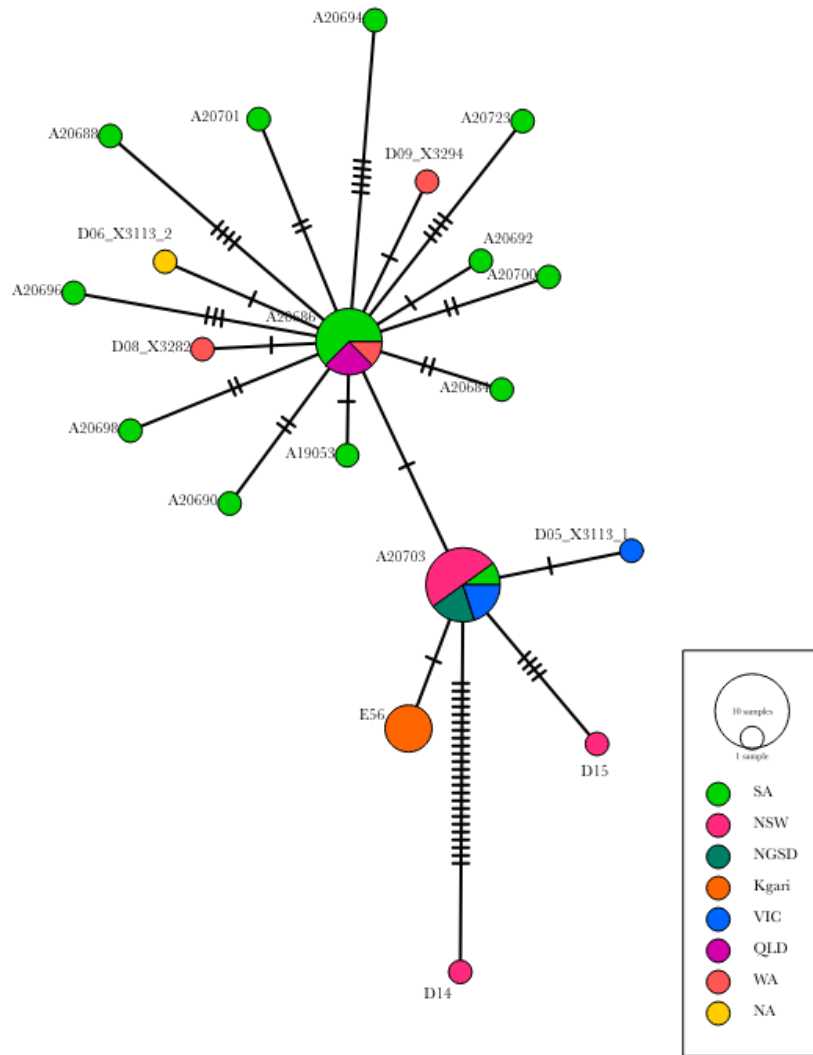
Unfortunately, PopART does not have a command line tool. You will have to run PopART on your local computer. It is free to use and can be downloaded from [here](#).

Once you've installed PopART, follow the instructions below.





We should be able to create something like this.



- **Q9:** What can you infer about the population structure of Dingoes?

References

- Andrews, Simon, Felix Krueger, Anne Segonds-Pichon, Laura Biggins, Christel Krueger, and Steven Wingett. 2012. "FastQC." Babraham, UK: Babraham Institute.
- Bandelt, H. J., P. Forster, and A. Röhl. 1999. "Median-Joining Networks for Inferring Intraspecific Phylogenies." *Molecular Biology and Evolution* 16 (1): 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>.
- Birney, E, and Ensembl Team. 2003. "Ensembl: a genome infrastructure." *Cold Spring Harbor Symposia on Quantitative Biology* 68 (0): 213–15. <https://doi.org/10.1101/sqb>.

2003.68.213.

- Broad Institute. n.d. “Picard Tools.” *Broad Institute, GitHub Repository*. <http://broadinstitute.github.io/picard/>.
- Chen, Shifu, Yanqing Zhou, Yaru Chen, and Jia Gu. 2018. “fastp: an ultra-fast all-in-one FASTQ preprocessor.” *Bioinformatics* 34 (17): i884–90. <https://doi.org/10.1093/bioinformatics/bty560>.
- Edgar, Robert C. 2004. “MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput.” *Nucleic Acids Research* 32 (5): 1792–97. <https://doi.org/10.1093/nar/gkh340>.
- Ewels, Philip, Måns Magnusson, Sverker Lundin, and Max Käller. 2016. “MultiQC: summarize analysis results for multiple tools and samples in a single report.” *Bioinformatics* 32 (19): 3047–48. <https://doi.org/10.1093/bioinformatics/btw354>.
- Jónsson, Hákon, Aurélien Ginolhac, Mikkel Schubert, Philip L. F. Johnson, and Ludovic Orlando. 2013. “mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters.” *Bioinformatics* 29 (13): 1682–84. <https://doi.org/10.1093/bioinformatics/btt193>.
- Jun, Goo, Mary Kate Wing, Gonçalo R. Abecasis, and Hyun Min Kang. 2015. “An Efficient and Scalable Analysis Framework for Variant Extraction and Refinement from Population Scale DNA Sequence Data.” *Genome Research*, April, gr.176552.114. <https://doi.org/10.1101/gr.176552.114>.
- Leigh, Jessica W., and David Bryant. 2015. “Popart: Full-Feature Software for Haplotype Network Construction.” *Methods in Ecology and Evolution* 6 (9): 1110–16. <https://doi.org/10.1111/2041-210X.12410>.
- Li, Heng, and Richard Durbin. 2009. “Fast and accurate short read alignment with Burrows–Wheeler transform.” *Bioinformatics* 25 (14): 1754–60. <https://doi.org/10.1093/bioinformatics/btp324>.
- Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. “The Sequence Alignment/Map format and SAMtools.” *Bioinformatics* 25 (16): 2078–79. <https://doi.org/10.1093/bioinformatics/btp352>.
- Lindgreen, Stinus. 2012. “AdapterRemoval: easy cleaning of next-generation sequencing reads.” *BMC Research Notes* 5 (1): 337. <https://doi.org/10.1186/1756-0500-5-337>.
- Oliva, Adrien, Raymond Tobler, Alan Cooper, Bastien Llamas, and Yassine Souilmi. 2021. “Systematic benchmark of ancient DNA read mapping.” *Briefings in Bioinformatics* 22 (5): bbab076. <https://doi.org/10.1093/bib/bbab076>.
- Souilmi, Yassine, Sally Wasef, Matthew P Williams, Gabriel Conroy, Ido Bar, Pere Bover, Jackson Dann, et al. 2024. “Ancient genomes reveal over two thousand years of dingo population structure.” *Proceedings of the National Academy of Sciences* 121 (30): e2407584121. <https://doi.org/10.1073/pnas.2407584121>.
- Yu, Guangchuang. 2024. *Seqmagick: Sequence Manipulation Utilities*. <https://github.com/yulab-smu/seqmagick>.
- Zhang, Shao-jie, Guo-Dong Wang, Pengcheng Ma, Liang-liang Zhang, Ting-Ting Yin, Yan-hu Liu, Newton O. Otecko, et al. 2020. “Genomic regions under selection in the feralization

of the dingoes.” *Nature Communications* 11 (1): 671. <https://doi.org/10.1038/s41467-020-14515-6>.