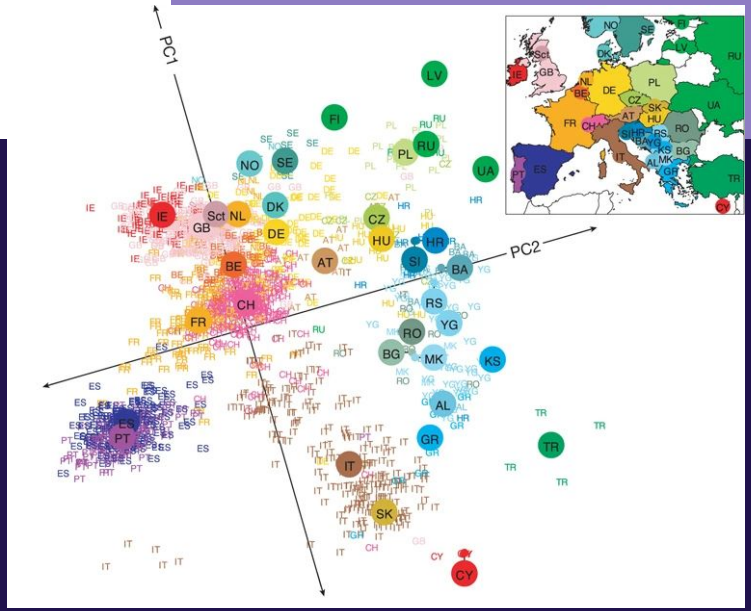
Physalia

Courses

Introduction to population genomics



26-30/05/2025

Physalia course

Thibault Leroy, Yann Bourgeois

Thibault LEROY
Habilitated, Dr.
Permanent researcher at INRAE
Toulouse, France

Bee GEES
BEE GENOMICS
TOULOUSE - FRANCE

GenPhySE
Génétique, Physiologie et Systèmes d'Elevage

Main interests:

- Footprints of natural and artificial selection

- Local adaptation to changing environments

- Population structure, gene flow, (adaptive) introgression

- Evolution of genomic variation within and between species

- Deleterious mutations /conservation Biology

- Speciation / Hybrid zones

- Mutation rates and spectra

- Methods in population genomics (demographic inferences, genome scans/GWAS, …)

- Metagenomics

Honey bees
(since 2023)

Wheat
(Postdoc, 2022)

Roses
(Postdoc, 2021-2022)

Populus/
Tillandsia
(Postdoc, 2019-2021)

Passerine birds
(Postdoc, 2018-2019)

Oaks
(Postdoc, 2014-2018)

Venturia
(PhD, 2009-2012)

Interests:
'Molecular ecology'
Conservation genomics
Phylodynamics
Transposable elements dynamics

Université de Toulouse

UNI BASEL

جامعة نيويورك ابوظبي
NYU ABU DHABI

UNIVERSITY OF PORTSMOUTH

IRD
Institut de Recherche pour le Développement
FRANCE

2009 2013 2016 2020 2022

Motivations for this Physalia course:

- Introduce population genomics methods
- Adapted to beginners with the idea of following a learning-by-doing strategy

- A lecture of a maximum of 2-hour per day
- Accessible for diverse levels, allowing you to grasp the essentials or explore further

3 levels: Basics Intermediate Advanced

- Practical: autonomous (PDF) + instructors providing support on Slack (troubleshooting)
- 2 x 15 minute discussion altogether (near 5pm and 6:45pm)

- This physalia course has the objective to introduce the topics: you are invited to contact us to ask additional questions after the course, when you needed for your research

Results of the little survey (23 participants)

Your biological models:

- Animals (16/23), including invertebrates (6/16)
- Plants (4/23)
- Fungi (1/23)
- Bacteria (2/23)

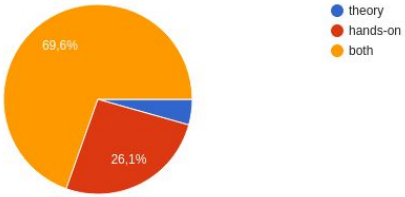
Your main expectation(s) for this course:

- selection scans/GEA (13/23) ↗
- demographic inferences (11/23) =
- GWAS (9/23) ↗
- population structure (7/23) ↘
- basic bioinformatics (7/23) =
- gene flow / introgression (5/23) ↗
- landscape genomics (5/23) ↘

  (not covered: “recombination”, “microbe-host coevolution”, “fastsimcoals, ABCtoolbox”, “focus on RADseq”, …)

Results of the little survey (23 participants)

Are you more interested in the theoretical or the hands-on parts of the course?



Your prior knowledge in population genomics / analyses?

Extremely large diversity of answers…ranging from complete beginners to already quite advanced profiles!
-> A true challenge for us! Importance to consider the color code in our slides:

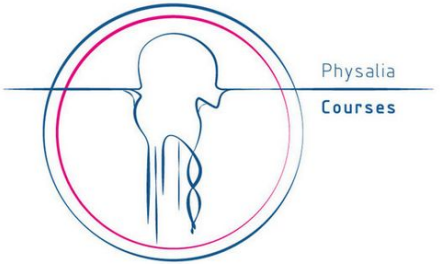
Basics Intermediate Advanced

Prior knowledge in computing and data analysis?

Do you have any experience with computing clusters?

- Yes (14/23)
- No (9/23) *-> Before the 1st practical: introduction of a few bash commands such as ssh, scp, cd, cp, mv, etc. and 'for' loops*

Program



INTRODUCTION TO POPULATION GENOMICS

Monday (2-7 pm) - Basic bioinformatics - Main Instructor : Thibault - Support: Yann

Tuesday (2-7 pm) - Population structure and introgression - Main Instructor : Yann - Support: Thibault

Wednesday (2-7 pm) - Demographic modeling methods - Main Instructor : Thibault - Support: Yann

Thursday (2-7 pm) - Genome-scans for association and selection - Main Instructor : Yann - Support: Thibault

Friday (2-7 pm) - Landscape genomics - Co-Instructors : Yann & Thibault

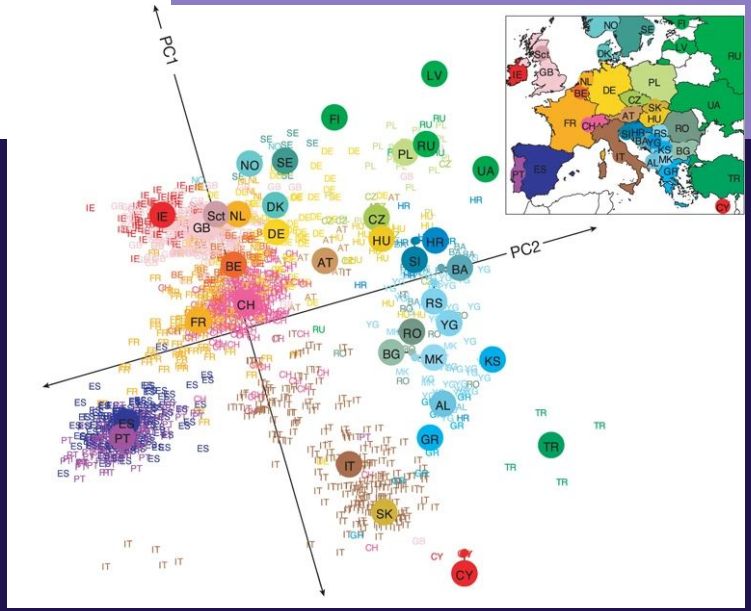
Physalia
Courses

Basic bioinformatics

26/05/2025

Physalia course

Thibault Leroy, Yann Bourgeois



Goals for today's lecture

- Pros and cons of using different strategies regarding the sequencing
- Understand the rationale of the bioinformatic analysis from raw data to variant calling
- Describe the common and specific parts of the pipelines depending on the strategies

Imagine that you work on the evolution/adaptation of unicorns…

Population 1: unicorns

Population 2: “uni**no**corns”

Imagine that you work on the evolution/adaptation of unicorns…

Population 1: unicorns

Population 2: “uni**no**corns”

<u>You may be interested by many questions:</u>

Are populations of unicorns and uninocorns consistent with a single global population or do they exhibit population structure (*e.g.* due to non-random mating)?

What are the evolutionary history of unicorns /uninocorns?

Are corns adaptive ? deleterious ?

How these populations vary through space?

If you have such questions in mind, you are at the right Physalia course !

Imagine that you work on the evolution/adaptation of unicorns…

Population 1: unicorns

Population 2: “uni**no**corns”

Which sequencing strategy can you use to answer these different questions?

Imagine that you work on the evolution/adaptation of unicorns…

Population 1: unicorns

Population 2: “uni**no**corns”

Which sequencing strategy can you use to answer these different questions?

Individual sequencing, moderate coverage (20-50X / individual)

Individual sequencing, low coverage (< 10X / individual)

Pooling of individual and sequencing (poolseq), moderate to high coverage (>50X)

Short reads (*e.g.* Illumina)
Long reads (*e.g.* PacBio/Nanopore)

Pros and cons of each strategy

Strategy	Individual / moderate coverage	Individual / low coverage	Pool-seq / moderate coverage
Accuracy (individual level)	+++ (Genotype calls)	+ (Genotype likelihood)	- - - (No information at the individual level)
Accuracy (population level) *e.g.* allele frequencies	+++	++ to +++	+ to +++ (depending on the number of individuals <-> pipetting biases)
Affordability Potential sequencing costs (prize are indicative and assume access to an internal facility, *e.g.* in my lab in Toulouse)	+ library 30€/sample (60*30€=1800 €) + Illumina 30X seq eq NovaSeq (1800X*, 5000€) **~7000€**	++ library 30€/sample (60*30€=1800 €) + Illumina seq eq NovaSeq (600X*, 1700€) **~3500€**	+++ libraries 1 per pool (2*60 €=120€) + Illumina seq (eq NovaSeq, 100X*, 350 €) **<500€**

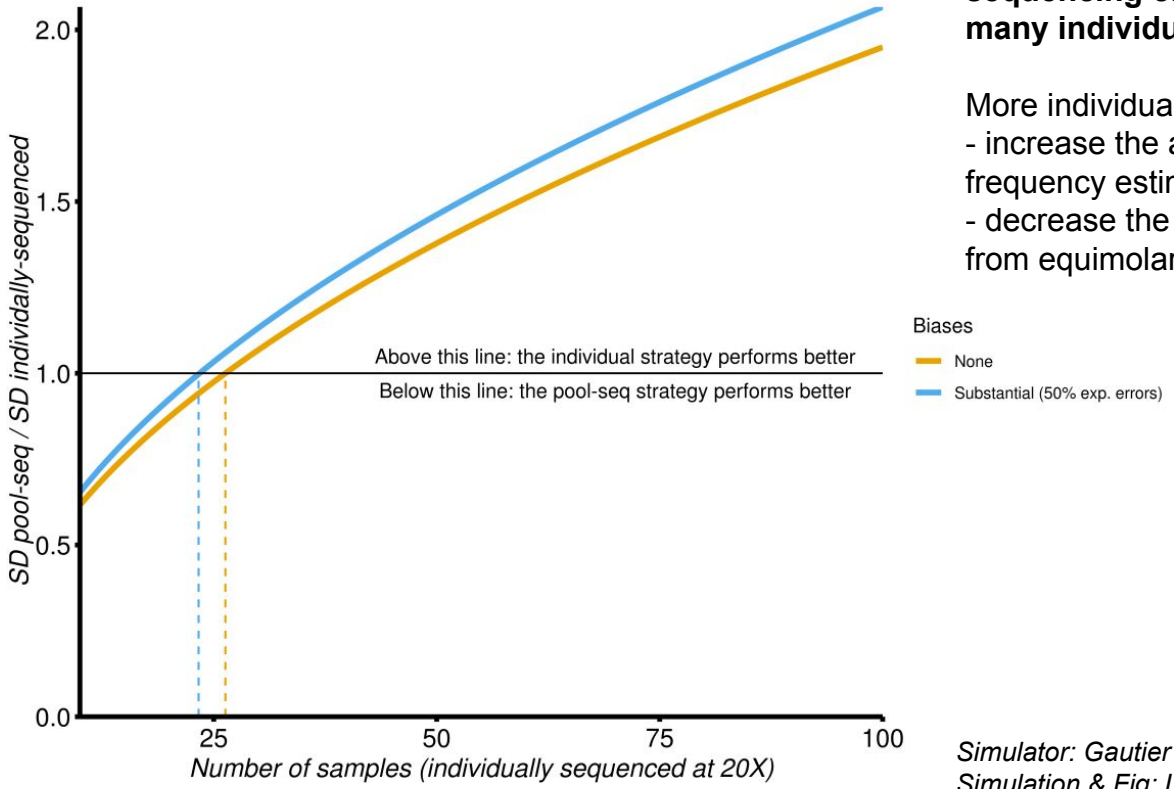
** assuming that unicorns/uninocorns have a genome size of ~250 Mb*

Poolseq can be an interesting strategy for population-level investigation

A pool-seq strategy of 50 individuals sequenced at a mean pool coverage of 100X

vs.

an individual-based genotyping strategy with a growing number of individually sequenced at 20X



Pool-seq is interesting if sequencing can be performed on many individuals per population!

More individuals included in pools:
- increase the accuracy of allele frequency estimation
- decrease the impacts of deviation from equimolarity (*e.g.* pipetting bias)

Simulator: Gautier et al. 2013 Mol Ecol
Simulation & Fig: Leroy & Rougemont 2020

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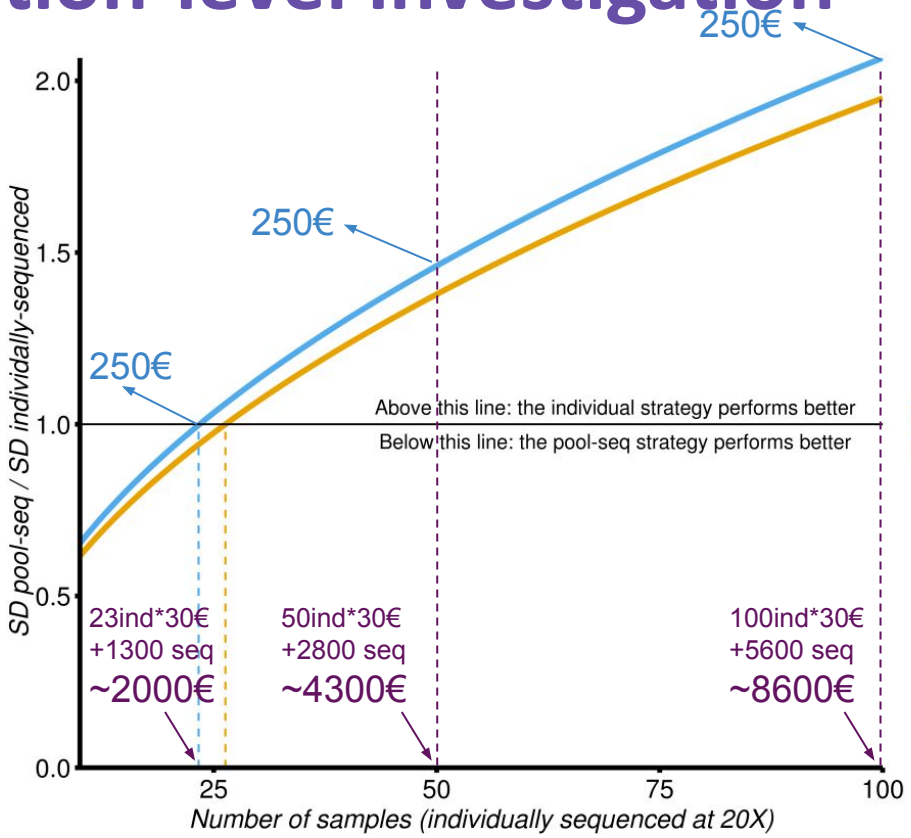

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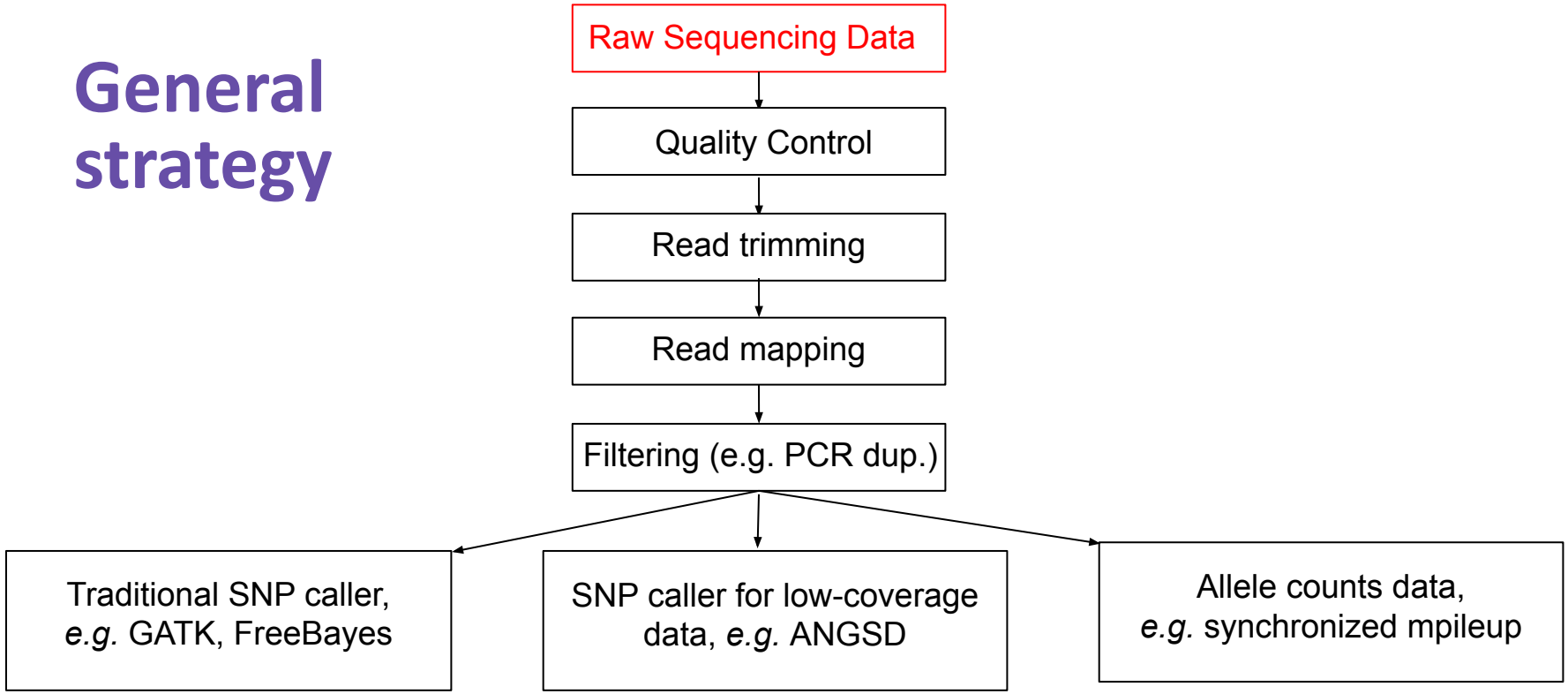
General strategy



VCF = Variant Calling Format

```
Chr1   6   G   A […] GT:DP  0/0:12  0/1:16  0/0:14 […]
Chr1   11  C   G […] GT:DP  0/0:14  1/1:19  0/1:15 […]
…
```

Genotype likelihood (GL)

```
Chr1  42  -2.0,-0.1,-3.0  -1.5,-1.2,-0.2  -0.5,-1.0,-3.5 […]
Chr1  78   1.0,-2.2,-0.3  -0.3,-1.5,-1.8  -3.2,-0.5,-0.7 […]
…
```

Allele counts (popoolation: A:T:C:G:N:*)<

```
Chr1  1  T  0:42:0:0:0:0   0:50:0:0:0:0
Chr1  2  G  7:0:0:35:0:0   0:0:0:50:0:0
Chr1  3  A  42:0:0:0:0:0   50:0:0:0:0:0
Chr1  4  C  0:0:20:22:0:0  0:0:26:24:0:0
….
```

Raw Sequencing Data

fasta vs. fastq

Fasta

> Sequence1
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC

Fastq

@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC
+
BBBBBBBBBBFFGGFGGGGGGGGHHHHHHHHHHHGGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH

Precise header @SEQ_ID:RUN_ID:FLOWCELL_ID:LANE:SAMPLE:READ:INDEX = High traceability !
Here: @SEQ_ID:RUN_ID:FLOWCELL_ID:LANE:TILE:X:Y:READ:FILTER_FLAG:CTRL_NB:INDEX

The most important information are on lines 2 and 4!
-> The 4th line corresponds to the quality values for the corresponding bases in 2nd line, in the exact same order !

fastq: rationale

Raw Sequencing Data

Fastq

@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA

GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC

+

BBBBBBBBBBFFGGFGGGGGGGGHHHHHHHHHHHGGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH

Base quality score (4th line)

```
SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
................................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
.................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN.....................................
EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE
PPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPP
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

```
S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 41)
    with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
    (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
N - Nanopore      Phred+33,  Duplex reads typically (0, 50)
E - ElemBio AVITI Phred+33,  raw reads typically (0, 55)
P - PacBio        Phred+33,  HiFi reads typically (0, 93)
```

fastq: rationale

Raw Sequencing Data

Fastq

```
@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC
+
BBBBBBBBBFFGGFGGGGGGHHHHHHHHHHGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH
```

```
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

What does it mean?

Phred Score	Probability of an incorrect base call	Accuracy	Associated character
0	1 in 1	0%	!
10	1 in 10	90%	+
20	1 in 100	99%	5
30	1 in 1000	99.9%	?
40	1 in 10000	99.99%	I

fastq: rationale

Raw Sequencing Data

Fastq

```
@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC
+
BBBBBBBBBFFGGFGGGGGGGHHHHHHHHHHGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH
```

```
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

What does it mean?

Associated character	Phred score	Probability of an incorrect base call	Accuracy
B			
F			
G			
H			

fastq: rationale

Raw Sequencing Data

Fastq

```
@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC
+
BBBBBBBBBFFGGFGGGGGGGHHHHHHHHHHGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH
```

```
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

What does it mean?

Associated character	Phred score	Probability of an incorrect base call	Accuracy
B	33		
F	37		
G	38		
H	39		

fastq: rationale

Raw Sequencing Data

Fastq

```
@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATAAAAC
+
BBBBBBBBBFFGGFGGGGGGGHHHHHHHHHHGGGGHHHHHHHHHHGGGHHHHFGHHFHHHH
LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

What does it mean?

Associated character	Phred score	Probability of an incorrect base call	Accuracy
B	33	0.0005	~99.95%
F	37	0.0002	~99.98%
G	38	0.0002	~99.98%
H	39	0.0001	~99.99%

One of the first things we are interested in is:
How many sequences are present in my fastq file?

Fastq.gz files:

There's no need to uncompress the FASTQ files, as most software can directly read `.fastq.gz` files! This helps save space on the computing cluster.

In a command-line Unix terminal:
To read a text file / FASTQ file
more `myfile.fastq`
less `myfile.fastq`

To read a compressed FASTQ file
zmore `myfile.fastq.gz`
zless `myfile.fastq.gz`

Raw Sequencing Data

One of the first things we are interested in is:
How many sequences are present in my fastq file?

Fastq.gz files:

zmore `file.fastq.gz` | grep “@” | wc -l
R1: 696057 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R1.fastq.gz | grep "@" | wc -l)
R2: 755300 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R2.fastq.gz | grep "@" | wc -l)

zmore `FILE.FASTQ.GZ` | grep “^@” | wc -l
R1: 624811 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R1.fastq.gz | grep "^@" | wc -l)
R2: 627027 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R2.fastq.gz | grep "^@" | wc -l)

zmore `FILE.FASTQ.GZ` | wc -l -> count the number of lines in a fastq.gz file and then divide this number by 4
zmore `FILE.FASTQ.GZ` | awk '{line++}END{print line/4}' -> single-line command with awk
R1: 624170 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R1.fastq.gz | awk '{s++}END{print s/4}')
R2: 624170 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R2.fastq.gz | awk '{s++}END{print s/4}')

Raw Sequencing Data

One of the first things we are interested in is:
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Fastq.gz files:

zmore `file.fastq.gz` | grep “@” | wc -l
R1: 696057 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00
R2: 755300 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00

zmore `FILE.FASTQ.GZ` | grep “^@” | wc -l
R1: 624811 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00
R2: 627027 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00

zmore `FILE.FASTQ.GZ` | wc -l -> count the number of lines in a fas
zmore `FILE.FASTQ.GZ` | awk '{line++}END{print line/4}'
R1: 624170 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00
R2: 624170 (zmore GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L00

Count the number of “@” using grep

```
@M07406:112:000000000-L7TK9:1:1101:7541:47631:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATA
+
A@ABBBBBDDFFGGFGGGGGGGGHHHHHHHHHHGGGGGHHHHHHHHHGGGHHHHFGFEEC@A
```

Count the number of line starting with “@” using grep

```
@M07406:112:000000000-L7TK9:1:1101:7541:4763 1:N:0:TTATAACC+GATATCGA
GATGCGGAATGAACTGGGATTCATAACTGCCCCCTGTTAACATTTCGTAAAAGTTGGTCATA
+
A@ABBBBBDDFFGGFGGGGGGGGHHHHHHHHHHGGGGGHHHHHHHHHGGGHHHHFGFEECCA@
```

Yes, but…

```
@M18478:112:000000000-L7TK9:1:1101:7541:1546 1:N:0:TTATAACC+GATATCGA
TATGCCGAAAAAACTGGGATTCATAACTGCACGCTGCCAACATTTCGTATAAGTTGCCAGAT
+
@@BCBBBBDDFFGGFGGGGGGGGHHHHHHHHHHGGGGGHHHHHHHHHGGGHHHHFGFEECCA@
```

Count the number of blocks of 4 lines in a fastq
The best strategy to use!

QC

FastQC, a convenient tool!

Raw Sequencing Data

↓

Quality Control

FastQC Report

GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L

Summary

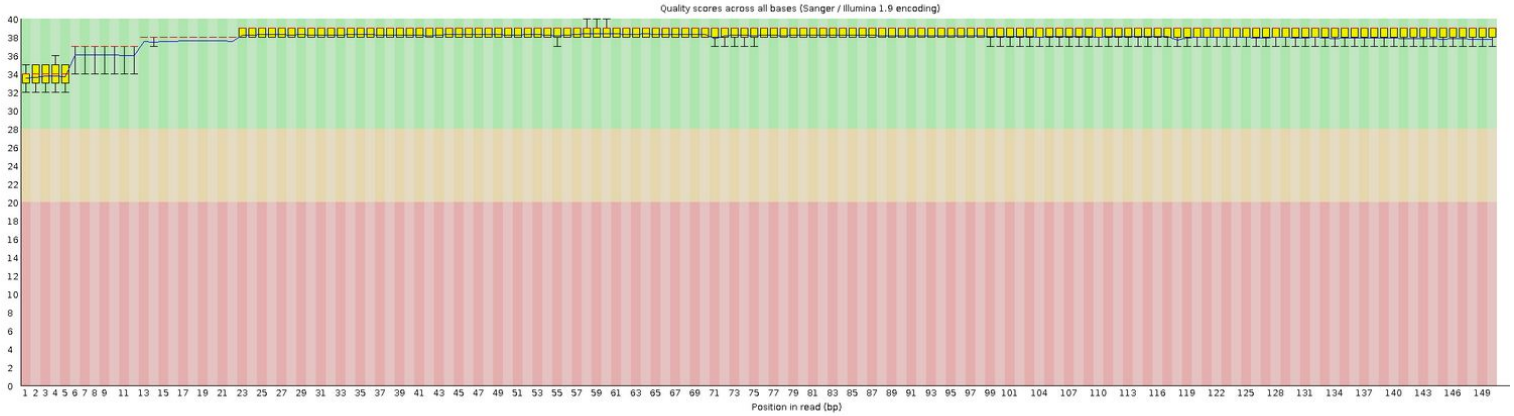
- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content
- Kmer Content

Basic Statistics

Measure	Value
Filename	GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	624170
Sequences flagged as poor quality	0
Sequence length	150
%GC	36

⟸ Total sequences: Of course, FastQC also provides this information!

Per base sequence quality



QC

FastQC, a convenient tool!

Raw Sequencing Data

↓

Quality Control

FastQC Report

GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L

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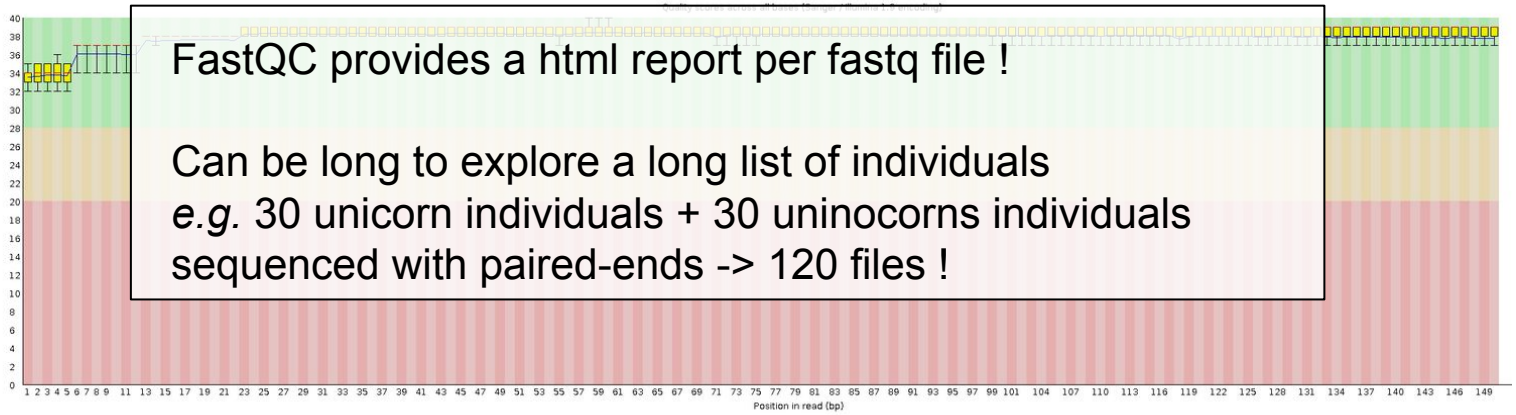
Per base sequence quality

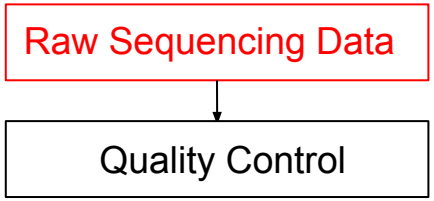


FastQC provides a html report per fastq file !

Can be long to explore a long list of individuals
e.g. 30 unicorn individuals + 30 uninocorns individuals sequenced with paired-ends -> 120 files !

QC

MultiQC, an even more convenient tool!



multiqc
v1.20.dev0 (5349c41)

- General Stats
- FastQC
  - Sequence Counts
  - Sequence Quality Histograms
  - Per Sequence Quality Scores
  - Per Base Sequence Content
  - Per Sequence GC Content
  - Per Base N Content
  - Sequence Length Distribution
  - Sequence Duplication Levels
  - Overrepresented sequences by sample
  - Top overrepresented sequences
  - Adapter Content
  - Status Checks
- Software Versions

multiqc

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2024-01-22, 16:30 CET based on data in: `/home/tleroy/Mallaurie/RawData/fastqc_Run1`

Welcome! Not sure where to start? Watch a tutorial video *(6:06)* don't show again

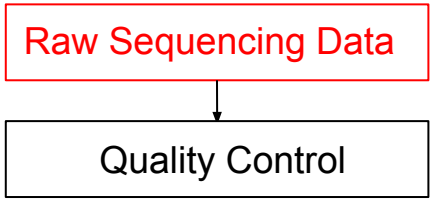
General Statistics

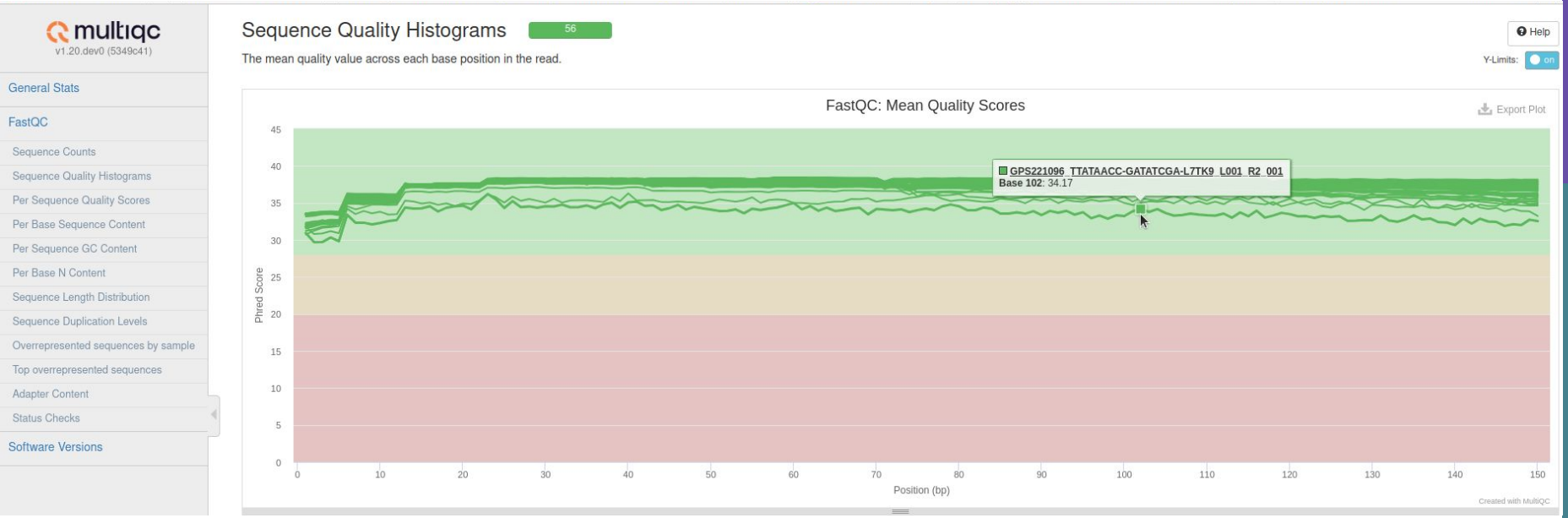
Copy table | Configure Columns | Plot | Showing 56/56 rows and 3/6 columns.

Sample Name	% Dups	% GC	M Seqs
GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R1_001	4.0%	36%	0.6
GPS220996_AGTTCAGG-CCAACAGA-L7TK9_L001_R2_001	3.1%	36%	0.6
GPS220999_ACTAAGAT-AACCGCGG-L7TK9_L001_R1_001	3.4%	36%	0.8
GPS220999_ACTAAGAT-AACCGCGG-L7TK9_L001_R2_001	3.0%	35%	0.8
GPS221002_CGGCGTGA-GCGCCTGT-L7TK9_L001_R1_001	1.5%	39%	0.3
GPS221002_CGGCGTGA-GCGCCTGT-L7TK9_L001_R2_001	1.2%	39%	0.3
GPS221004_TTGGACTC-GGAAGCAG-L7TK9_L001_R1_001	7.7%	43%	0.7
GPS221004_TTGGACTC-GGAAGCAG-L7TK9_L001_R2_001	7.2%	43%	0.7
GPS221008_AACGTTCC-GGAGTACT-L7TK9_L001_R1_001	1.0%	35%	1.0
GPS221008_AACGTTCC-GGAGTACT-L7TK9_L001_R2_001	0.7%	35%	1.0
GPS221013_GCTTGTCA-GAACATAC-L7TK9_L001_R1_001	2.7%	37%	0.6
GPS221013_GCTTGTCA-GAACATAC-L7TK9_L001_R2_001	2.4%	37%	0.6

QC

MultiQC, an even more convenient tool!





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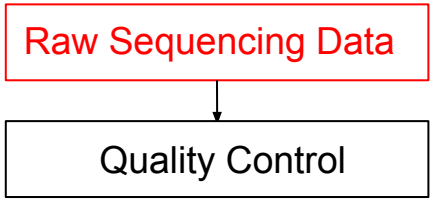
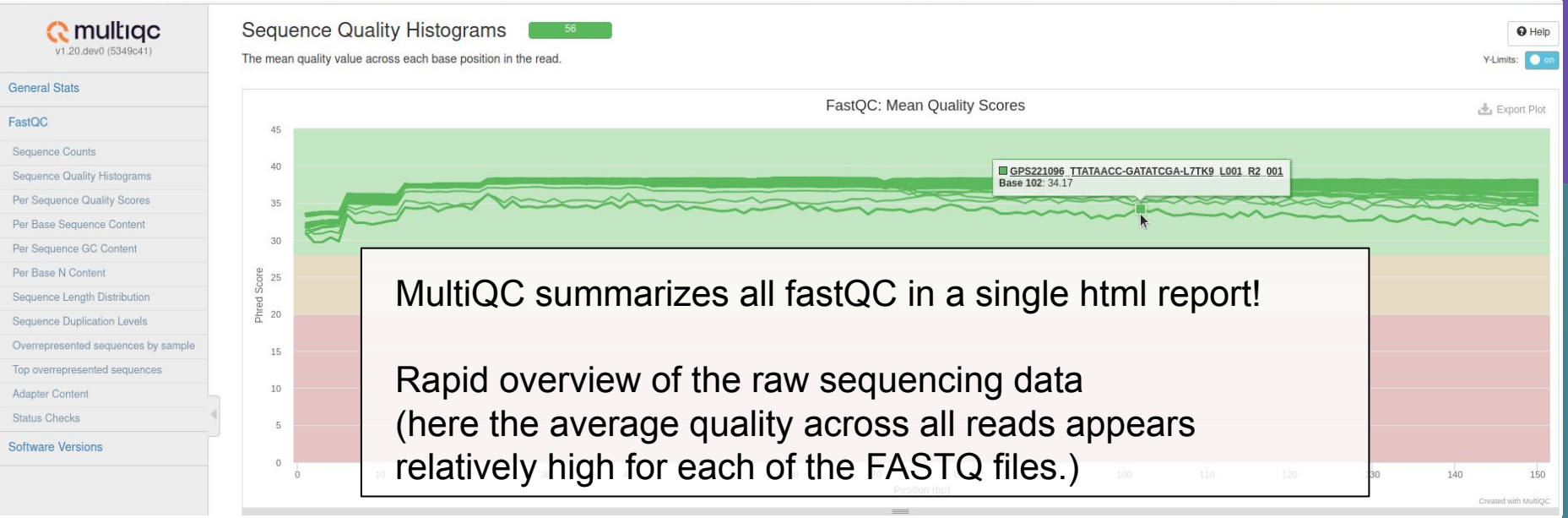
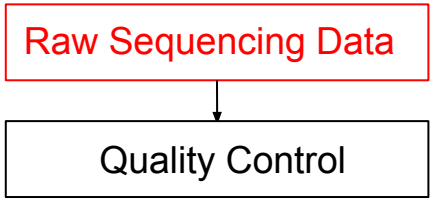




Short reads



Some general information about short read (typically Illumina) sequencing:

- Error probability is relatively low: nowadays ~ 0.1% per base

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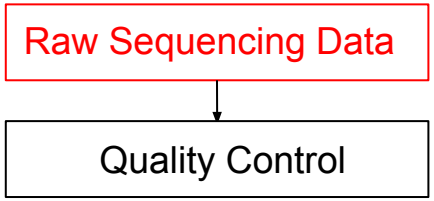

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NovaSeq 6000	2017	(2*)250	~99.7%
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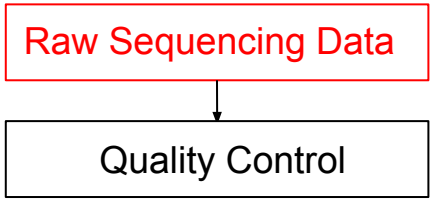

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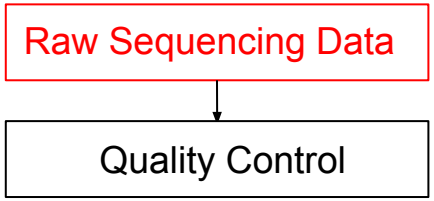
Short reads



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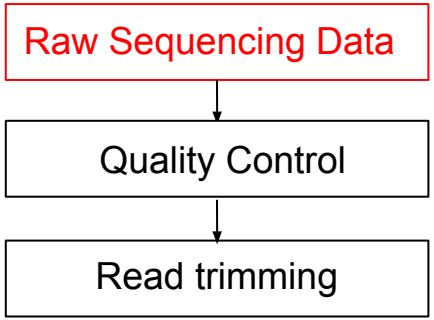
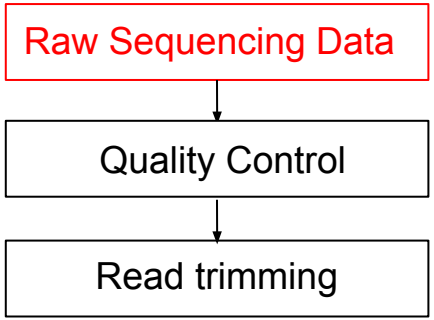
→ **Even with consistently high read quality and progressively lower error rates, read trimming low-quality bases is still recommended to improve overall accuracy and reliability of the data**

Trimming reads



To trim or not to trim, that is the question! → Even with increasing read quality, it remains encouraged for WGS data

Trimming reads



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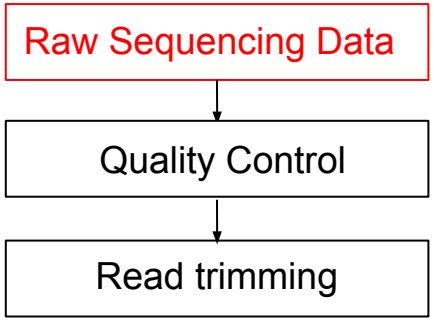
Trimming reads has the objectives:

- Removing adapters

- Removing low quality bases

- Excluding short reads after quality trimming

Trimming reads



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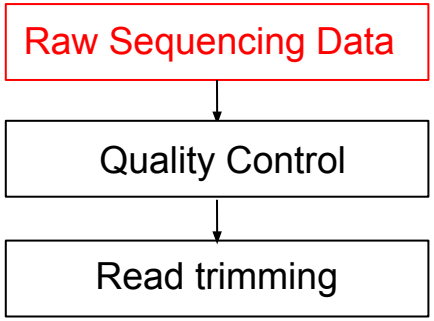
Trimmomatic is a particularly popular tool, trimming both Single-End (SE) or Paired-End (PE) data

```
trimmomatic PE -threads 4 InfileForward.fastq InfileReverse.fastq  \
        TrimmedOutfileForward_paired.fastq TrimmedOutfileForward_unpaired.fastq \
        TrimmedOutfileReverse_paired.fastq TrimmedOutfileReverse_unpaired.fastq \
        ILLUMINACLIP:Illumina_adapters.fa MINLEN:50 SLIDINGWINDOW:4:20
```

Trimming reads



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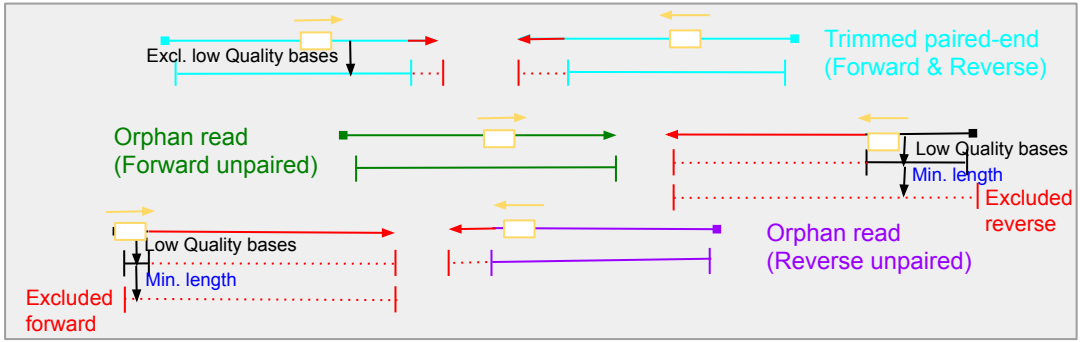

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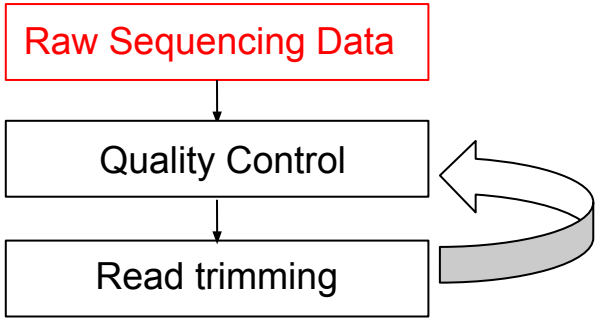
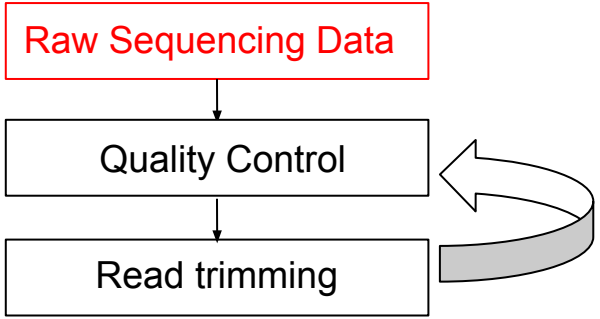
ILLUMINACLIP:Illumina_adapters.fa MINLEN:50 SLIDINGWINDOW:4:20

Trimming reads



Possibility to control the quality of the trimming by performing another round of QC

Trimming reads



Possibility to control the quality of the trimming by performing another round of QC

FastQC raw data (before)

R1
n=624170 reads

Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content

R2
n=624170 reads

Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
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FastQC trimmed data (after trimming)

R1 (paired)
n=491646 reads

Summary

- Basic Statistics
- Per base sequence quality
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- Per base sequence content
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R1 (unpaired)
n=131760 reads

Summary

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- Per base sequence quality
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- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
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R2 (unpaired)
n=396 reads

Summary

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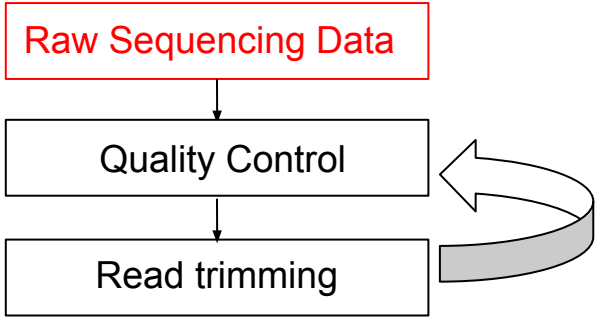
We indeed observe the expected improvement of the quality after trimming !

Trimming reads



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R2
n=624170 reads

Summary

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FastQC trimmed data (after trimming)

R1 (paired)
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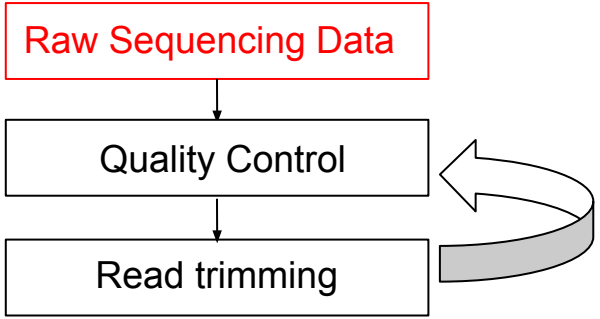
Note that FastQC tags provide useful indicators and should be treated as warnings. Achieving 'all green' status is not mandatory! Sometimes, warnings are even expected!

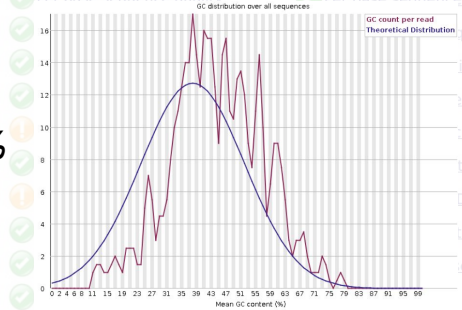
Trimming reads



Possibility to control the quality of the trimming by performing another round of QC

F med data (after trimming)

- Overall, the quality of R1 reads (first pass) is higher than that of R2 reads (second pass), leading to a higher probability of unpaired R1 compared to unpaired R2 reads
- In addition, here the fastq comes for a metagenomic project (ie not a single individual), which has impacts on the warnings
- And of course, the number of sequences matters…
  - *Drawing a distribution of GC% based on only 396 reads*
  - *=> The last red tag (unpaired R2) is understandable!*



R1 (unpaired) *n=131760 reads*	R2 (unpaired) *n=396 reads*
Summary	Summary
Basic Statistics	Basic Statistics
Per base sequence quality	Per base sequence quality
Per tile sequence quality	Per tile sequence quality
Per sequence quality scores	Per sequence quality scores
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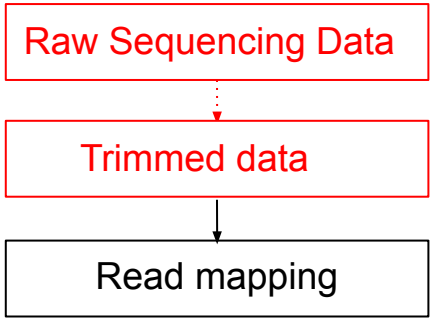
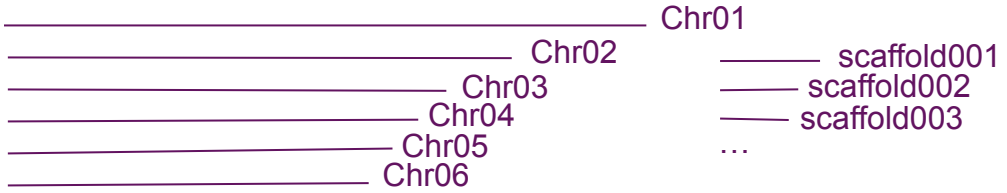
Mapping reads



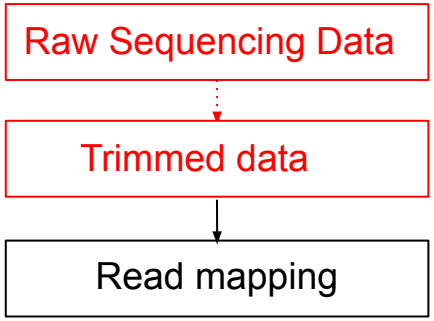
Mapping reads against a reference genome (make sure to choose the right version !) :

Trimmed reads



Reference genome

Mapping reads



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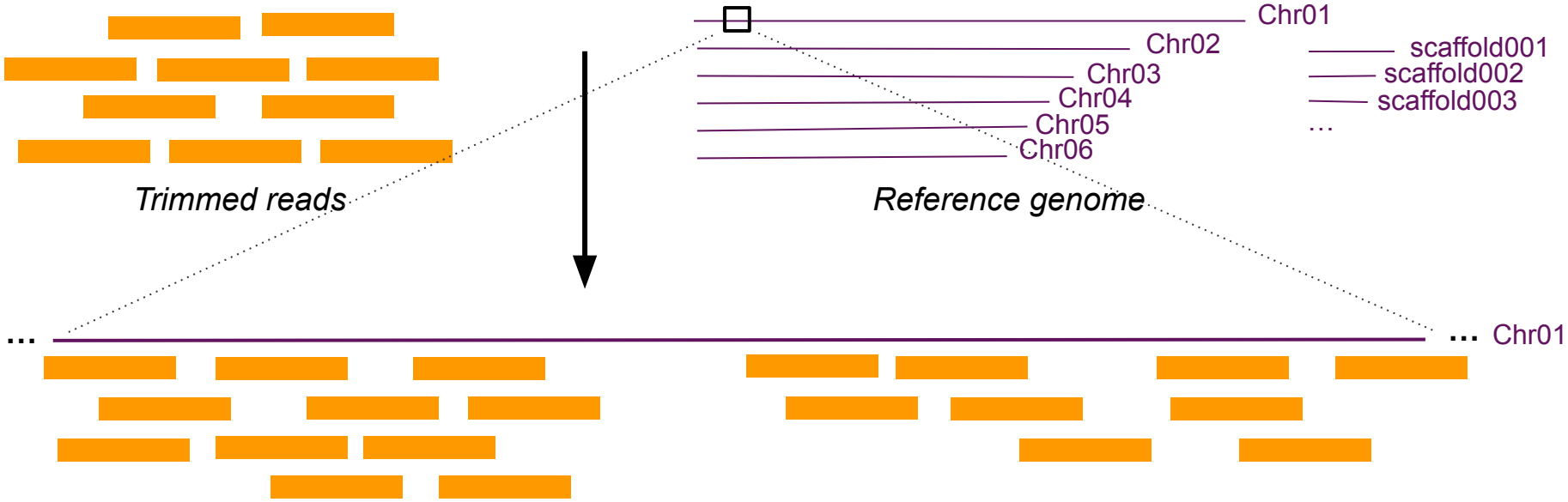

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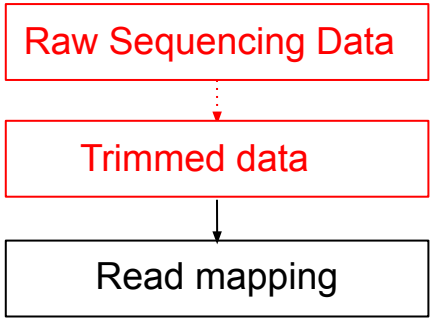

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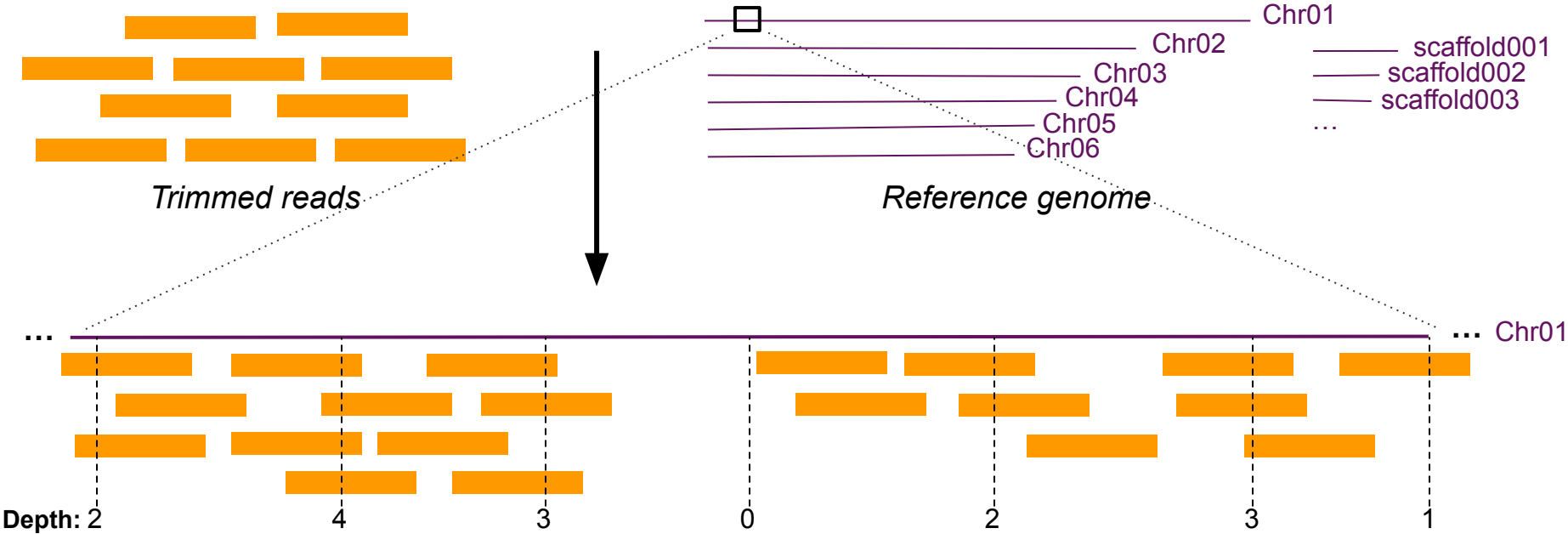

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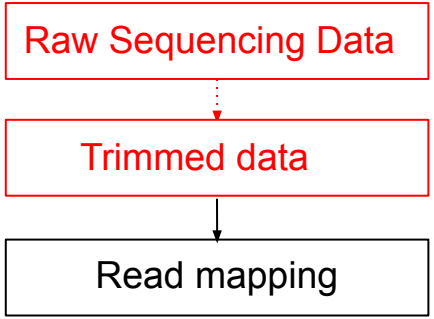

Mappers :

Properly speaking, a mapper is not an aligner!

Read mapping: *locating the approximate position of a read in a reference genome. The goal of read mappers is to identify the general location of reads on the reference genome, without necessarily requiring precise base-by-base alignment.*
Read alignment: *determining the exact sequence correspondence between each base of the read and the reference. This step is more computationally intensive but is expected to improve base-level precision.*

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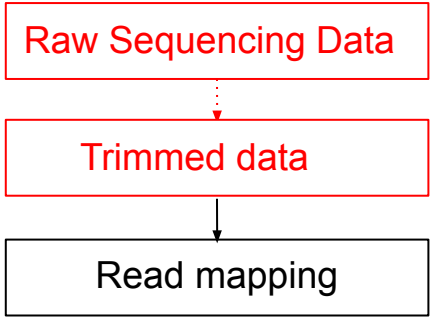

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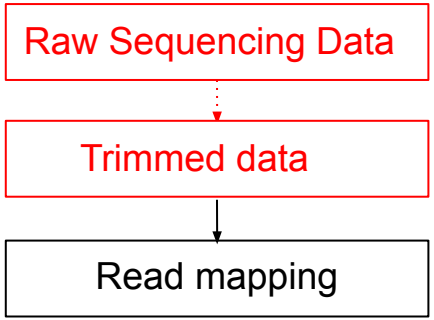
Mapping on a reference requires to have such a reference for the focal species or a (very) closely-related one

Read mapping corresponds to a balance between speed and accuracy: faster algorithms find approximate positions (mapping), while slower, precise methods (alignment) match each base accurately.
Software often allows fine-tuning of detection, *e.g.* --very-fast vs. --very-sensitive modes in Bowtie2

Mapping reads



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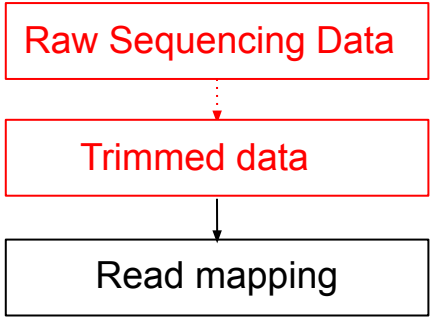
Most popular tools:

- Bowtie and BWA (bowtie2 and bwa-mem2) for WGS data
- STAR and HISAT2 for RNAseq data for RNAseq data (splice-aware algorithms)

Mapping reads



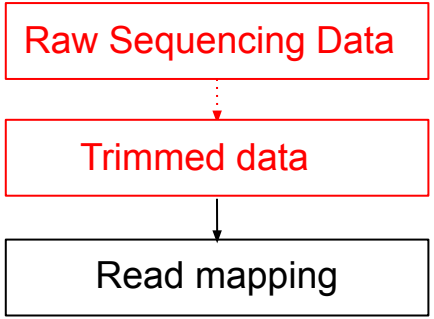
Different format used for the outputs: SAM (text-based) / **BAM (binary)** / CRAM (compressed)

Summarizing the results of the mapping (Samtools flagstat) :

1116143 + 0 in total (QC-passed reads + QC-failed reads)
1115448 + 0 primary → 1115448=trimmed paired-end reads (983292) + unpaired R1 (131760)+ unpaired R2 (396)
[…]
298709 + 0 mapped (26.76% : N/A)
[…]
298014 + 0 primary mapped (26.72% : N/A)
[…]
292198 + 0 properly paired (29.72% : N/A)
[…]

294038 + 0 with itself and mate mapped
[…]
2925 + 0 singletons (0.30% : N/A)
1560 + 0 with mate mapped to a different chr
413 + 0 with mate mapped to a different chr (mapQ>=5)

Mapping reads



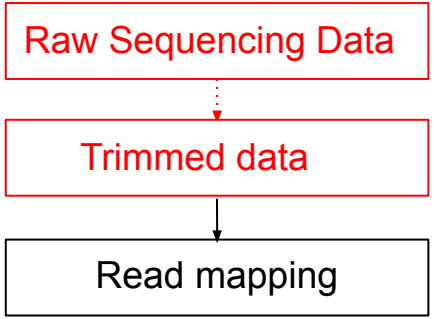
Different format used for the outputs: SAM (text-based) / **BAM (binary)** / CRAM (compressed)

Summarizing the results of the mapping (Samtools flagstat) :

1116143 + 0 in total (QC-passed reads + QC-failed reads)
1115448 + 0 primary → 1115448=trimmed paired-end reads (983292) + unpaired R1 (131760)+ unpaired R2 (396)
[…]
298709 + 0 mapped (26.76% : N/A) → 298709 / 1115448 ~ 26.8%
[…]
298014 + 0 primary mapped (26.72% : N/A) → 298014 / 1115448 ~ 26.7% (only primary, not secondary mapped)
[…]
292198 + 0 properly paired (29.72% : N/A) → 292198 / 983292 ~ 29.7% (PE reads on the same chr, different orientation, relatively short distance between the two PE reads)
[…]

294038 + 0 with itself and mate mapped
[…]
2925 + 0 singletons (0.30% : N/A)
1560 + 0 with mate mapped to a different chr
413 + 0 with mate mapped to a different chr (mapQ>=5)

Mapping reads



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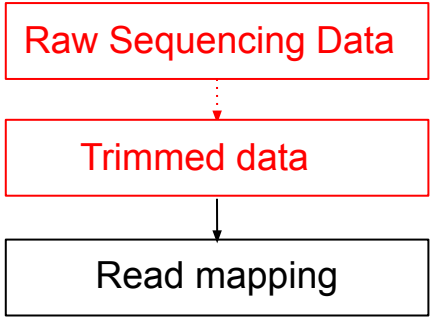
[…]
294038 + 0 with itself and mate mapped → 294038 / 983292 (PE reads mapped, even on different chr, distance, …)
[…]
2925 + 0 singletons (0.30% : N/A) → 2925 / 983292 ~ 0.3% (only one of the PE reads is mapped)
1560 + 0 with mate mapped to a different chr → 1560 / 983292 ~ 0.2% (PE reads map on different chr)
413 + 0 with mate mapped to a different chr (mapQ>=5) → 413 / 983292 ~ 0.04% (PE reads map on different chr, each with a “low but decent” mapping quality)

Mapping reads



Mapping qualities (MAPQ)

MAPQ is a score indicating the confidence in the mapping of a read to the genome (expressed in $-10\log_{10}$ probability that the mapping position is wrong).
-> MAPQ is calculated based on the likelihood function, providing values ranging from 0 to 60 (for bwa mem, note that the scale varies from one software to another)

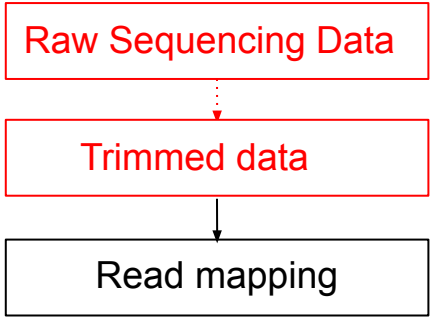
Confidence:
MAPQ10: 90%
MAPQ20: 99%
MAPQ30: 99,9%
MAPQ40: 99,99%

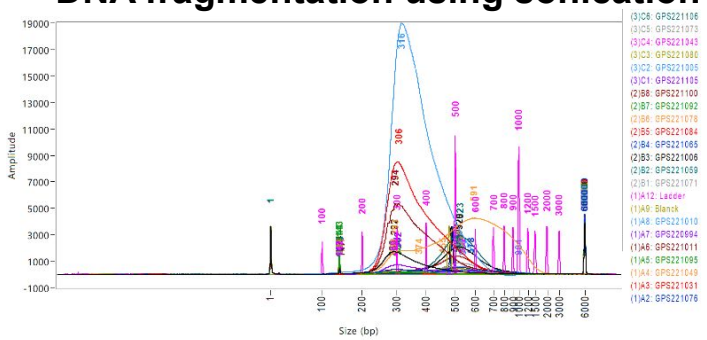
A **low MAPQ** (close to 0) suggests that the read may not be mapped correctly, either because it maps to multiple locations or the mapping is ambiguous.

A **high MAPQ** (close to 60 in bwa mem) suggests that the mapping is very confident and reliable.

Filtering low-confidence reads (e.g. MAPQ <5, <20, …) is generally performed since these mapping are more error-prone, excluding them therefore improve downstream analysis such as SNP calling

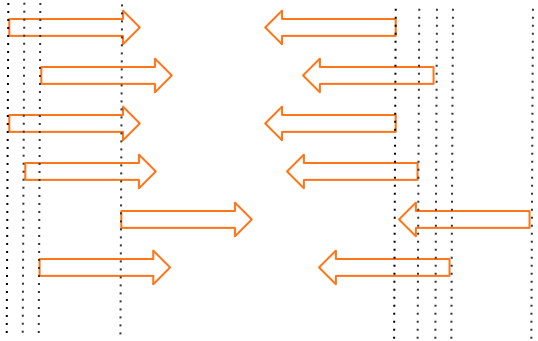
Mapping reads



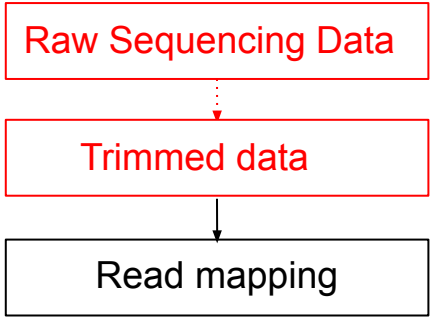


Detecting PCR duplicates (or not)

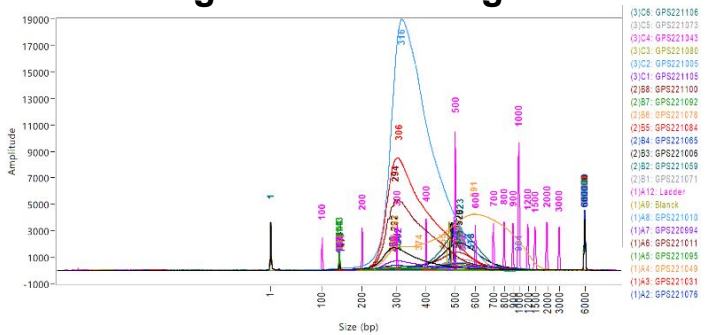
… … Chr01



Mapping reads



DNA fragmentation using sonication



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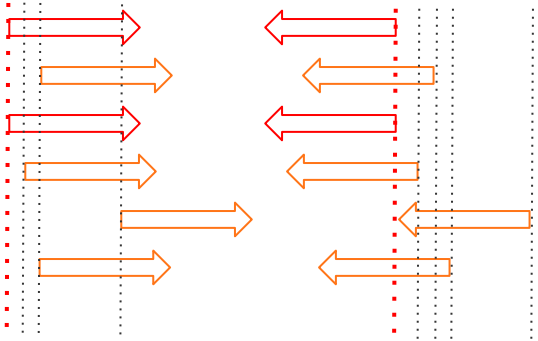
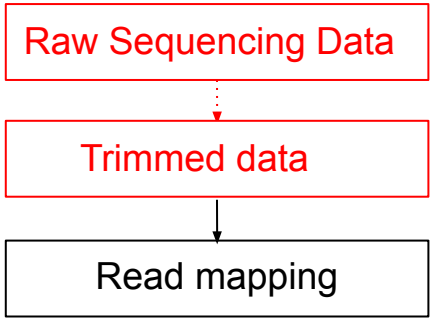


With PE data, it is expected to be quite infrequent to have reads starting and ending at the same locations

Mapping reads



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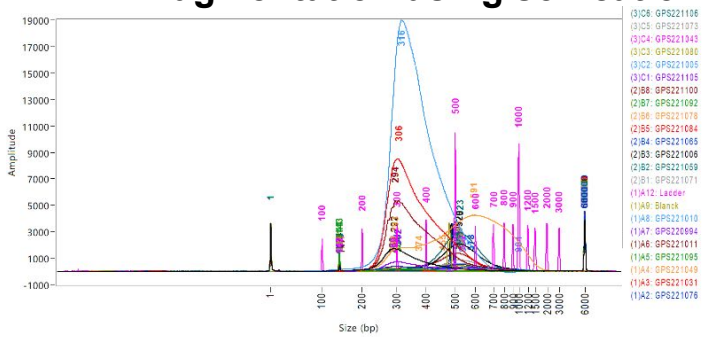

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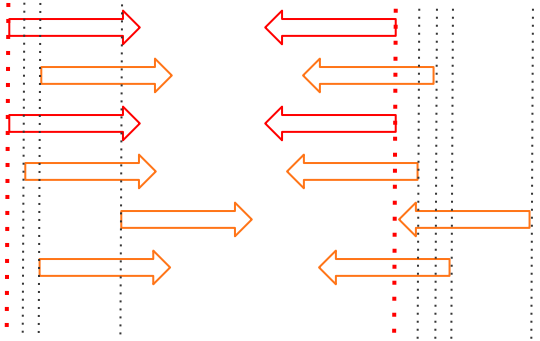

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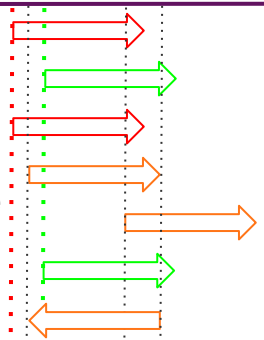
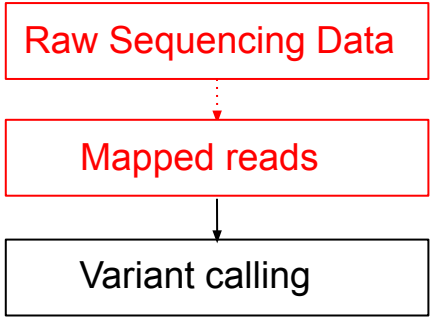


With SE data, it is more challenging to assess duplicates! A risk of overestimating the number of duplicates. A choice to be conservative or not!

With restriction enzymes (e.g. RADseq data), the proportion of false PCR duplicates can be high, as reads originate from the same restriction site. A difference between single-digest vs. double-digest RAD.

Popular tools: Picard (picard Markduplicates) and Samtools (markdup) -> Mark (not remove) duplicates!

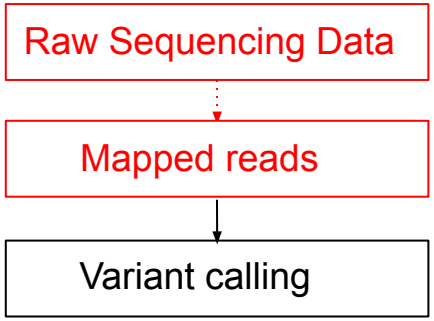
Variant calling



Briefly, variant callers are tools that detect genetic variants such as **single nucleotide polymorphisms (SNPs)**, **insertions**, **deletions**, and **structural variants** from mapped reads

SNP callers aim to identify the most likely genotypes, i.e., determining whether the data are more consistent with a homozygous or heterozygous site, while excluding rare alleles that might arise from sequencing errors

Variant calling



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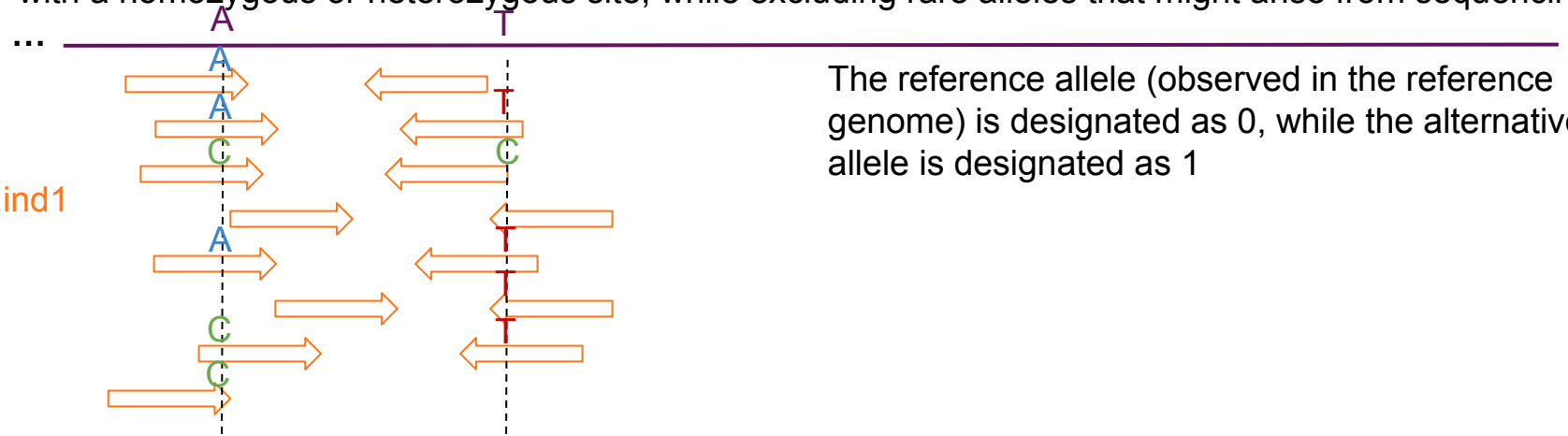
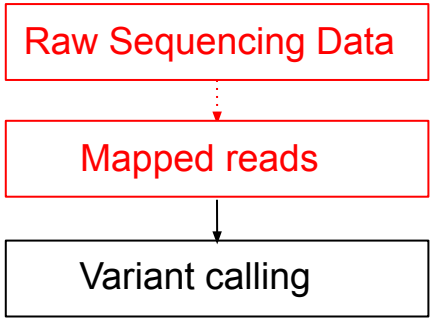


The reference allele (observed in the reference genome) is designated as 0, while the alternative allele is designated as 1

Variant calling



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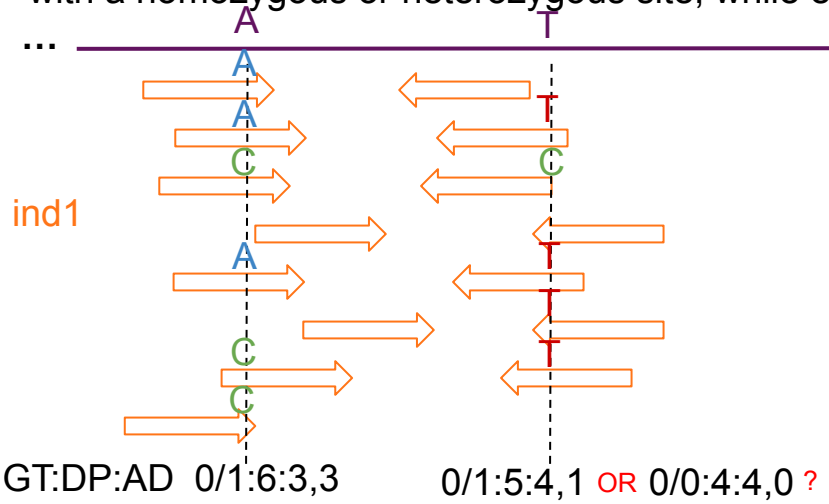

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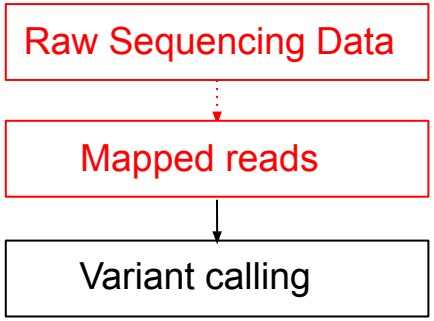
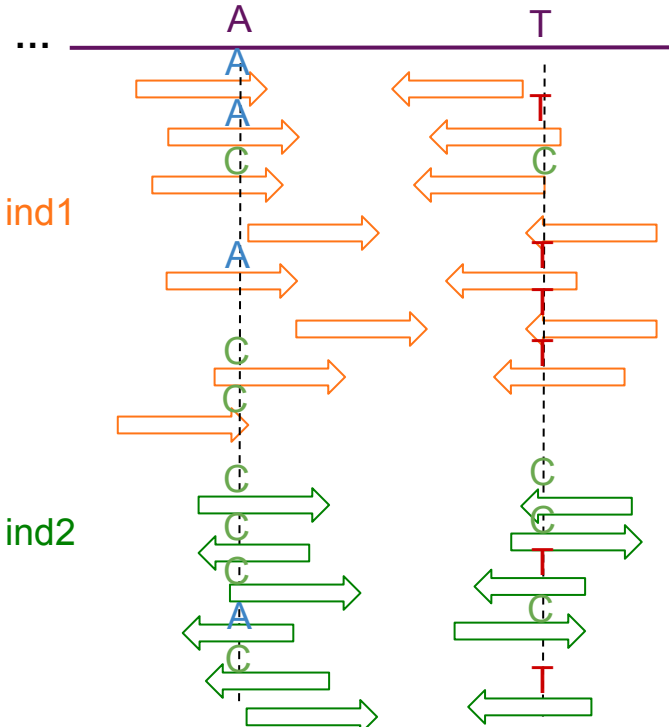
Variant callers assess base quality, mapping quality, and read depth to make accurate predictions of variants.
-> Variant calling is complex, with a difficult balance between identifying true positives and limiting the number of false positives

Variant calling



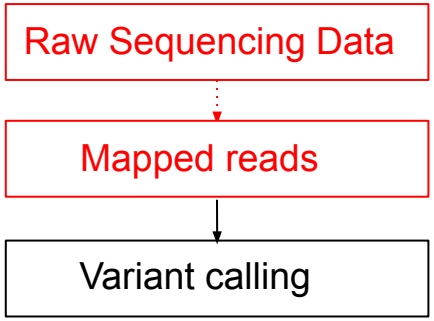


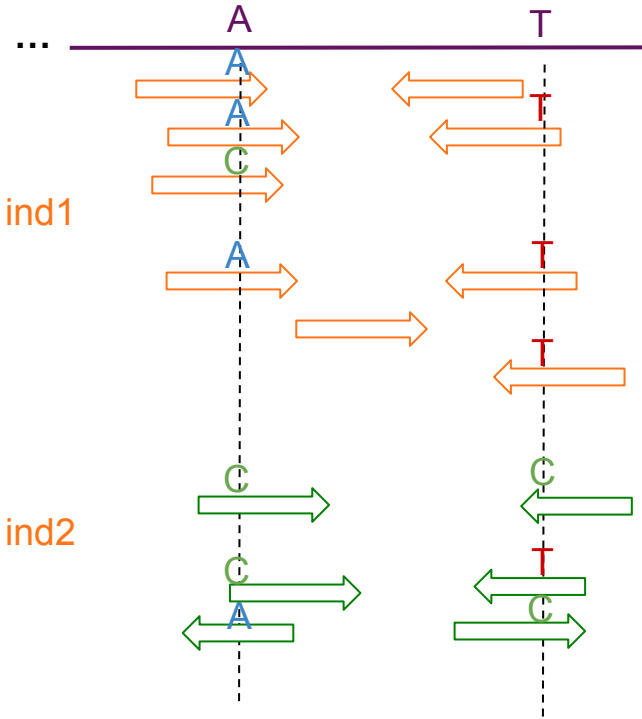
Multi-sample variant calling. By analyzing all individuals together, variant callers can:

- **Increase sensitivity**: Detect low-frequency variants that might be missed in single-sample analyses
- **Improve accuracy**: Use allele frequency data to distinguish true variants from sequencing errors
- **Enable joint genotyping**: Call genotypes across all samples consistently, which helps in downstream analyses like population genetics and association studies

Most popular tools are **GATK**, **FreeBayes** and **Samtools**

Variant calling





Here, the data appears to be associated with low coverage (< 10x)

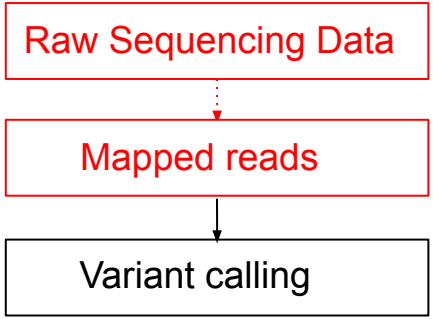
With fewer reads, the probability of accurately determining homozygous or heterozygous genotypes decreases, often leading to more uncertain genotype calls

A more accurate strategy is to compute the probability of each genotype (*i.e.* 0/0, 0/1, 1/1)

PosA	GT:GL	.:-1.5,-0.2,-2.0	.:-0.2,-1.2,-5.1
PosT	GT:GL	.:-1.2,-0.3,-1.2	.:-1.2,-0.3,-1.2

ANGSD is a popular tool, specialized for genotype likelihood calculations, especially in low coverage or population studies

Variant calling



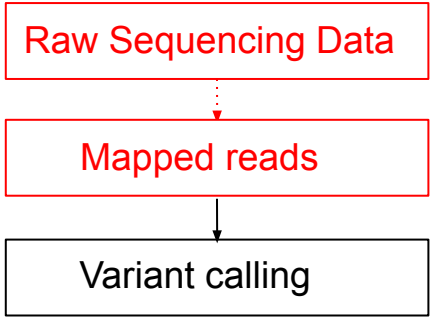
VCF (Variant Calling Format) file

```
##fileformat=VCFv4.2
##FILTER=<ID=PASS,Description="All filters passed">
##ALT=<ID=NON_REF,Description="Represents any possible alternative allele at this location">
##FILTER=<ID=LowQual,Description="Low quality">
##FORMAT=<ID=AD,Number=R,Type=Integer,Description="Allelic depths for the ref and alt alleles in the order listed">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth (reads with MQ=255 or with bad mates are filtered)">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=MIN_DP,Number=1,Type=Integer,Description="Minimum DP observed within the GVCF block">
##FORMAT=<ID=PGT,Number=1,Type=String,Description="Physical phasing haplotype information, describing how the alternate alleles are phased in relation to one another">
##FORMAT=<ID=PID,Number=1,Type=String,Description="Physical phasing ID information, where each unique ID within a given sample (but not across samples) connects records within a phasing group">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##FORMAT=<ID=PS,Number=1,Type=Integer,Description="Phasing set (typically the position of the first variant in the set)">
##FORMAT=<ID=RGQ,Number=1,Type=Integer,Description="Unconditional reference genotype confidence, encoded as a phred quality -10*log10 p(genotype call is wrong)">
##FORMAT=<ID=SB,Number=4,Type=Integer,Description="Per-sample component statistics which comprise the Fisher's Exact Test to detect strand bias.">
…
##GATKCommandLine=[...]
#CHROM POS     ID        REF     ALT     QUAL     FILTER   INFO     FORMAT IND01   IND02   IND03 ….
```

The header of a VCF provides extremely important information, explaining how to read the file, the commands used, etc… Read it!

The most important line is the last one starting with a "#", which provides the column names, including the names of individuals (from column 10)

Variant calling



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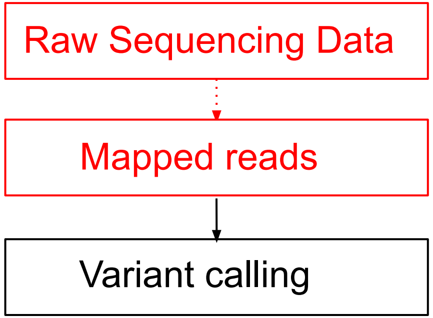
…
##GATKCommandLine=[...]
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT IND01 IND02 IND03 ….
Chr01 5671 . T C 332.46 . AC=2;AF=0.001196;DP=13376 GT:AD:DP:GQ:PL 1/1:1,12:13:2:363,2,0 ./.:0,0:0:.:0,0,0 …
Chr01 5698 . T C 3633.8 . AC=8;AF=0.004779;DP=12793 GT:AD:DP:GQ:PL 0/0:9,0:9:24:.:.:0,24,360 0/0:19,0:19:48:0,48,720 …
…
```

Two first variants are C/T variants, with a T allele in the reference genome (0) and a C as an alternative one (1)

Variant calling



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Chr01  5671   .    T     C     332.46   .       AC=2;AF=0.001196;DP=13376   GT:AD:DP:GQ:PL  1/1:1,12:13:2:363,2,0     ./.:0,0:0:.:0,0,0  …
Chr01  5698   .    T     C     3633.8   .       AC=8;AF=0.004779;DP=12793   GT:AD:DP:GQ:PL  0/0:9,0:9:24:.:.:0,24,360  0/0:19,0:19:48:0,48,720  …
…
NC_037638.1   41082   .   C   G,T   …
…
NC_037638.1   48060   .   G   AT    …
…
```
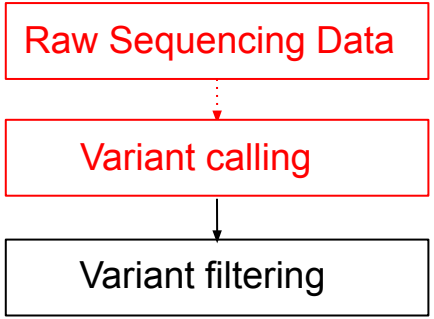

← Multiple alt alleles: 0/2 genotype =>C/T; 1/2 genotype =>G/T etc

← INDEL 0/1 genotype = G/AT

Variant filtering



Selecting most reliable variants

Quality by Depth (QD): ensures that variants have sufficient supporting read depth, filtering out low-quality variants with insufficient read
Mapping Quality (MQ): filtering out variants potentially associated with mapping errors
Base Quality (BQ): average base quality of the reads supporting the variant, filtering out variants with low base quality
Minimum Allele Frequency (AF): filtering out variants with low allele frequency (but could be an issue for some popgen analyses)
Depth of Coverage (DP): ensures that the variant is supported by a large number of reads
Variant type (SNPs *vs.* indels): calling INDELs is more challenging than SNPs. Using SNP variants only can be a good strategy
Missing rate: filtering out variants with excessive missing data is a standard to reduce false positives (true also at the sample level)
…

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	IND01	IND02	IND03 ….
Chr01	5671	.	T	C	332.46	.	AC=2;AF=0.001196;DP=13376	GT:AD:DP:GQ:PL	1/1:1,12:13:2:363,2,0		./.:0,0:0::0,0,0 …
Chr01	5698	.	T	C	3633.8	.	AC=8;AF=0.004779;DP=12793	GT:AD:DP:GQ:PL	0/0:9,0:9:24:.:.:0,24,360		0/0:19,0:19:48:0,48,720 …
…											

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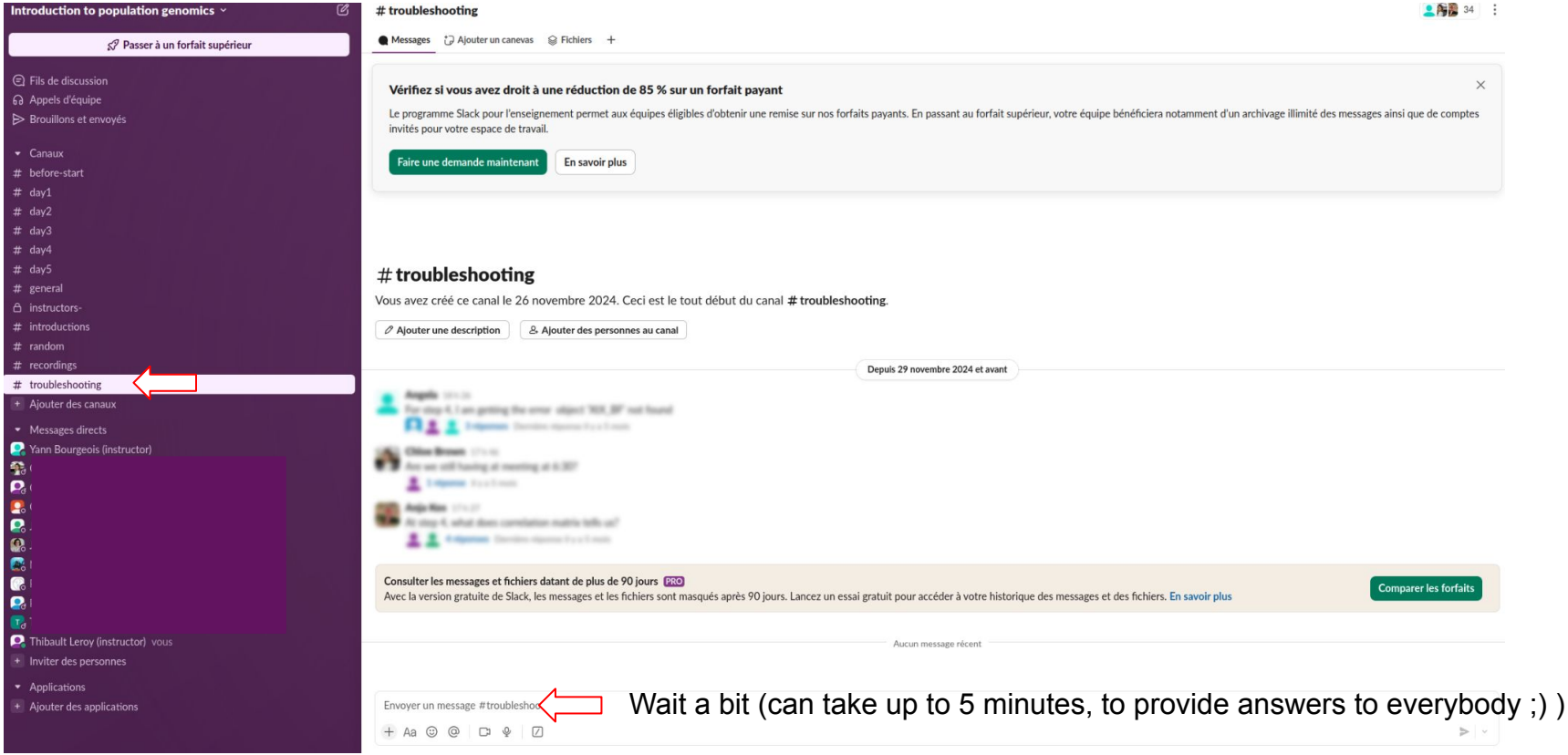
Chr01	5698	.	T	C	3633.8	PASS	AC=8;AF=0.004779;DP=12793	GT:AD:DP:GQ:PL	0/0:9,0:9:24:.:.:0,24,360		0/0:19,0:19:48:0,48,720 …
…											

Ask your question on Slack !

Don't hesitate, we're here to help !



Wait a bit (can take up to 5 minutes, to provide answers to everybody ;))

Question 1

Read trimming is important to:

A - Remove low-quality bases that could mislead alignment tools

B - Increase the original read length to improve alignment

C - Eliminate adapter sequences that may interfere with mapping

D - Merge paired-end reads into a single high-quality read file

E - Randomly delete parts of your data for fun

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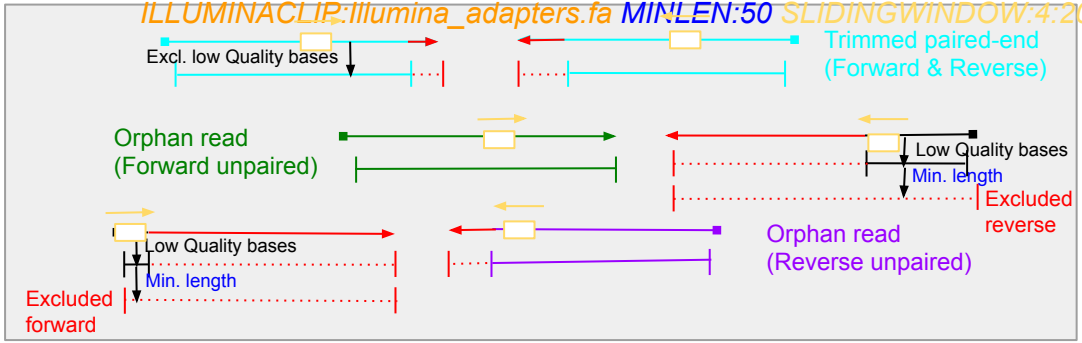
```
trimmomatic PE -threads 4 InfileForward.fastq InfileReverse.fastq  \
    TrimmedOutfileForward_paired.fastq TrimmedOutfileForward_unpaired.fastq \
    TrimmedOutfileReverse_paired.fastq TrimmedOutfileReverse_unpaired.fastq \
    ILLUMINACLIP:Illumina_adapters.fa MINLEN:50 SLIDINGWINDOW:4:20
```



Question 2

Read mapping and deduplication of sequencing reads are performed primarily to:

A - Align sequencing reads to a reference genome to determine their origin

B - Sort sequencing reads by length to improve downstream efficiency

C - Delete low-quality reads before alignment

D - Remove PCR duplicates that can bias quantification

E - Reduce false positives in variant calling due to PCR duplicates

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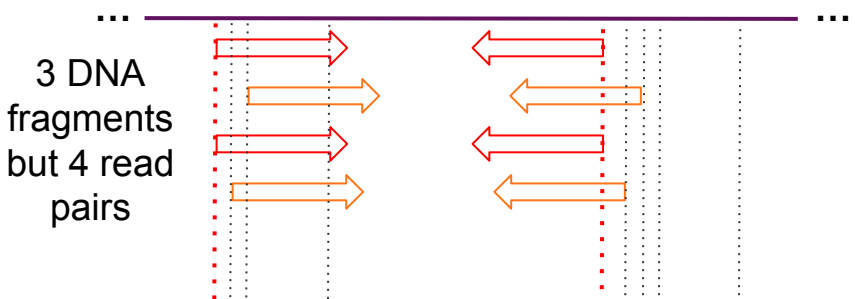

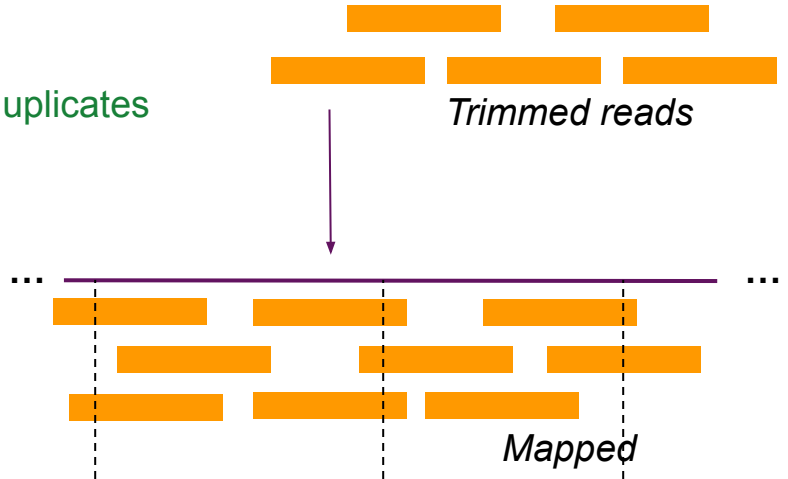

Question 3

What could you tell me about: "Chr1 6 G A […] 0/1:-323.03,-99.29,-802.53 " ?

A - Given the allele counts, this is probably a pool-seq dataset

B - The result is shown in a Variant Calling Format (VCF)

C - A single polymorphic site from one individual is presented

D - This result was obtained using a SNP caller that outputs genotype likelihoods

E - The values are not consistent with the genotype shown

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Physalia

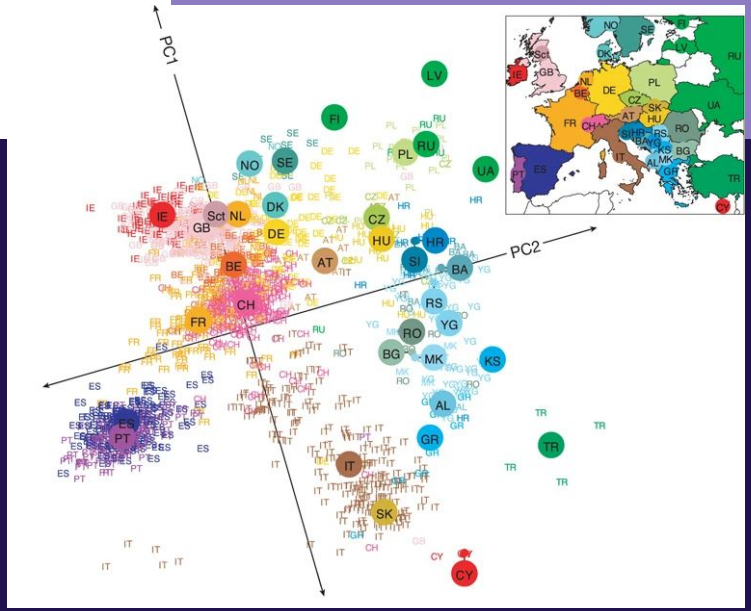
Courses

Basic bioinformatics

25/11/2024

Physalia course

Thibault Leroy, Yann Bourgeois



Physalia

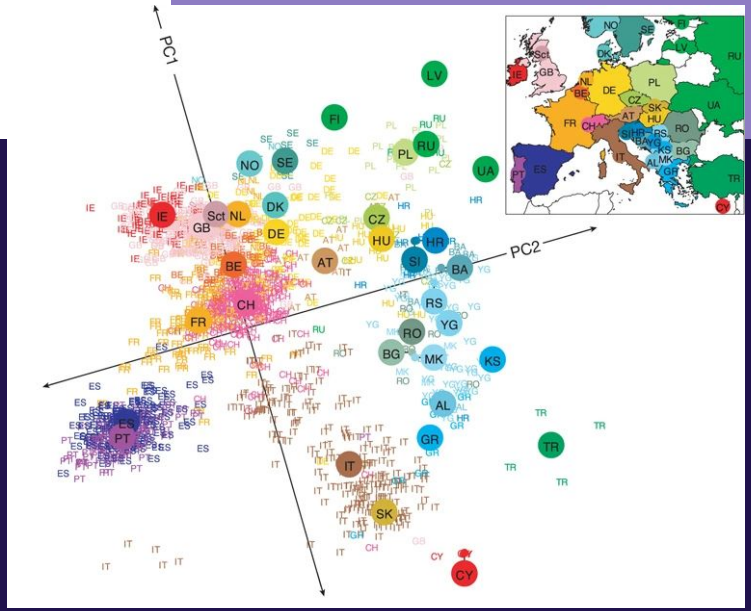
Courses

Basic bioinformatics Tutorial: Wrap-up (part 1)

25/11/2024

Physalia course

Thibault Leroy, Yann Bourgeois



Step 1: Evaluating the reference genome

/home/ubuntu/Share/software/assemblathon2-analysis/assemblathon_stats.pl Qrob_PM1N.fa > Qrob_PM1N.fa.assemblathon.txt

```
---------------- Information for assembly 'Qrob_PM1N.fa' ----------------


                         Number of scaffolds        550
                     Total size of scaffolds  814368469
                            Longest scaffold  115639695
                           Shortest scaffold       2095
                 Number of scaffolds > 1K nt        550 100.0%
                Number of scaffolds > 10K nt        454  82.5%
               Number of scaffolds > 100K nt        206  37.5%
                 Number of scaffolds > 1M nt         31   5.6%
                Number of scaffolds > 10M nt         12   2.2%
                          Mean scaffold size    1480670
                        Median scaffold size      50332
                         N50 scaffold length   55068941
                          L50 scaffold count          6
                                 scaffold %A      31.23
                                 scaffold %C      17.29
                                 scaffold %G      17.30
                                 scaffold %T      31.22
                                 scaffold %N       2.96
                         scaffold %non-ACGTN       0.00
             Number of scaffold non-ACGTN nt          0

            Percentage of assembly in scaffolded contigs      99.7%
          Percentage of assembly in unscaffolded contigs       0.3%
                  Average number of contigs per scaffold       40.0
Average length of break (>25 Ns) between contigs in scaffold       1121

                           Number of contigs      21977
              Number of contigs in scaffolds      21823
          Number of contigs not in scaffolds        154
                       Total size of contigs  790325162
                              Longest contig     621829
                             Shortest contig          0
                   Number of contigs > 1K nt      21180  96.4%
                  Number of contigs > 10K nt      14521  66.1%
                 Number of contigs > 100K nt       1807   8.2%
                   Number of contigs > 1M nt          0   0.0%
                  Number of contigs > 10M nt          0   0.0%
                            Mean contig size      35961
                          Median contig size      20487
                           N50 contig length      71598
                            L50 contig count       3283
                                   contig %A      32.18
                                   contig %C      17.82
                                   contig %G      17.83
                                   contig %T      32.17
                                   contig %N       0.00
                           contig %non-ACGTN       0.00
               Number of contig non-ACGTN nt          0
```

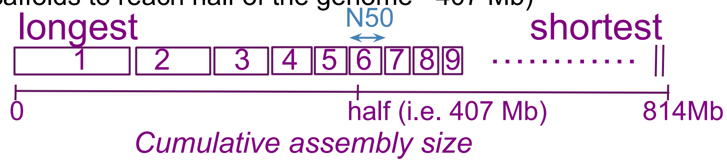
Assembly size 814 Mb (a bit more than the expected size of ~750 Mb)
longest scaffold: 115 Mb
2095 scaffolds

12 scaffolds of > 10 Mb (= 12 chromosomes)

N50: 55 Mb (6 longest scaffolds to reach half of the genome ~407 Mb)

GC content ~ 34.6%

N content ~ 3.0%



Based on the Assemblathon results, how long is the assembled genome?

-> 814.4 Mb

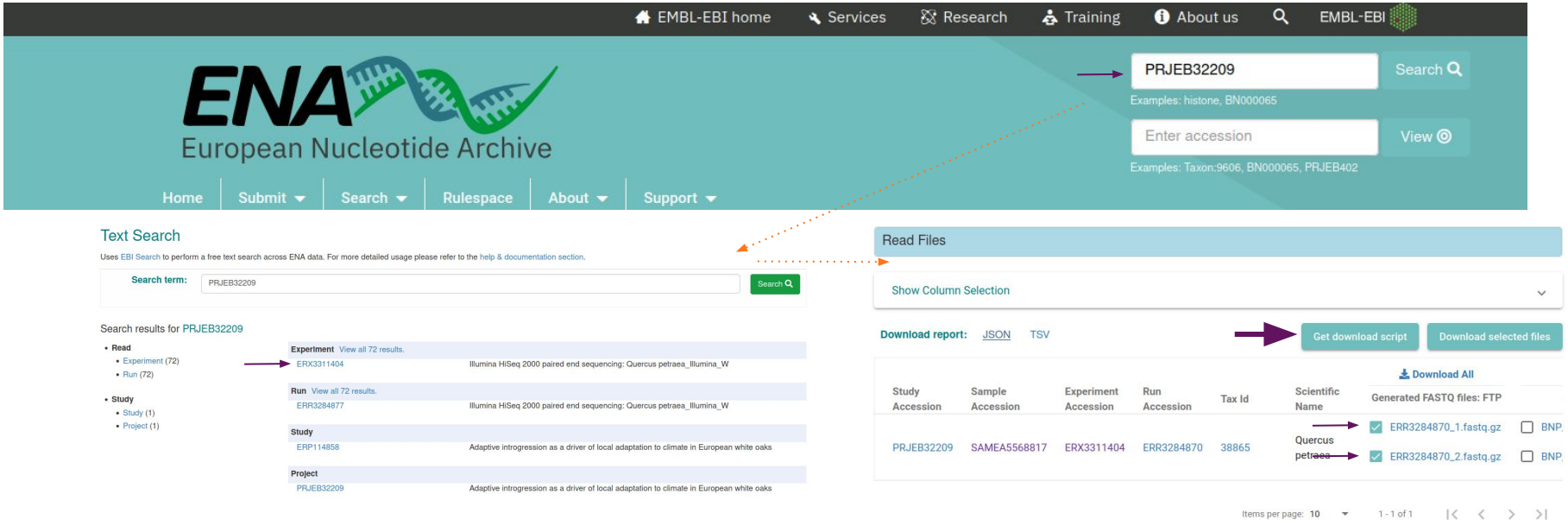
Does its total length exceed the expected genome size for this species (0.75 Gb)?

-> **Yes**, some duplicated regions of the genome in the assembly?

Additionally, is this assembly resolved at the chromosome level?

-> **Yes**, almost (12 long scaffolds), but still fragmented

Step 2: Downloading sequencing data from public repositories like the SRA (Sequence Read Archive)



Direct access to the right ftp link to download the data (direct from a computing cluster)

wget -nc ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR328/003/ERR3284873/ERR3284873_1.fastq.gz
wget -nc ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR328/003/ERR3284873/ERR3284873_2.fastq.gz

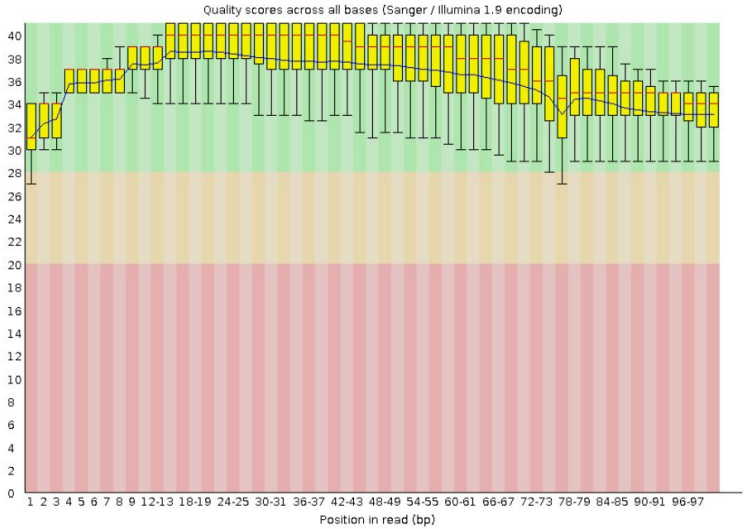
Step 3: Quality control of raw sequencing data

fastqc

Basic Statistics

Measure	Value
Filename	ERR3284869_1.subset.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	19306
Total Bases	1.8 Mbp
Sequences flagged as poor quality	0
Sequence length	30-101
%GC	33

Per base sequence quality



multiqc

multiqc

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2024-11-18, 07:35 CET based on data in:

- `/work/genphyse/cytogen/Thibault/beegenomics_2023disk/beegenomics/PuceOak/Puce_oak/analysis_physalia/ERR3284869_1.fastq.gz.subset4kreads`

Welcome! Not sure where to start? Watch a tutorial video *(6:06)* don't show again

General Statistics

Copy table | Configure columns | Scatter plot | Violin plot | Showing 6/6 rows and 3/6 columns. | Export as CSV

High percentage of duplicates (Kmer)

Sample Name	% Dups	% GC	M Seqs
ERR3284869_1.subset	37.7%	33%	0.0 M
ERR3284869_2.subset	36.7%	33%	0.0 M
ERR3284873_1.subset	34.7%	33%	0.0 M
ERR3284873_2.subset	34.7%	33%	0.0 M
ERR3284898_1.subset	29.2%	33%	0.0 M
ERR3284898_2.subset	29.0%	33%	0.0 M

FastQC

Step 3: Quality control of raw sequencing data

multiqc *subset* (multiqc .)
multiqc subset
"*" transformed in " " in the pdf :(

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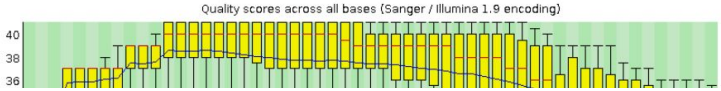

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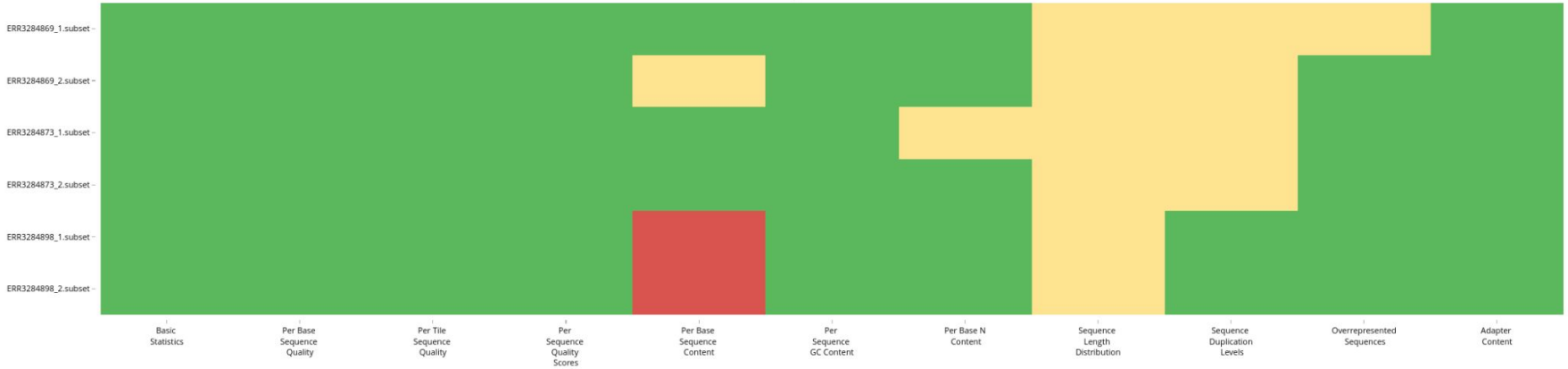
General Statistics

FastQC: Status Checks
11 samples



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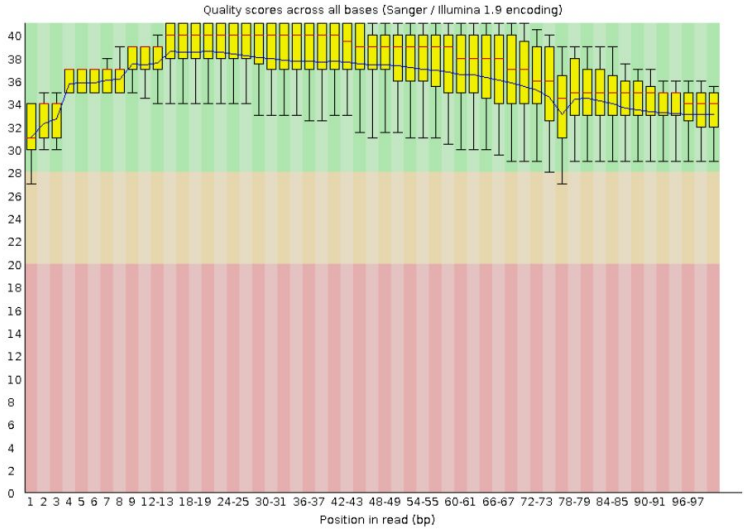

Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content

:iqc

egate results from bioinformatics analyses across
single report.

, 07:35 CET based on data in:

n/Thibault/beegenomics_2023disk/beegenomics/PuceOak/Puce_oak/
284869_1.fastq.gz.subset4kreads

o start? Watch a tutorial video (6:06) don't show again ×

CS

mns Scatter plot Violin plot Showing 6/6 rows and 3/6 Export as CSV

	% Dups	% GC	M Seqs
	37.7%	33%	0.0 M
	36.7%	33%	0.0 M
	34.7%	33%	0.0 M
	34.7%	33%	0.0 M
	29.2%	33%	0.0 M
	29.0%	33%	0.0 M

Step 4: Read trimming

"

```
for j in ERR3284869 ERR3284873 ERR3284898; do
        acc=$(echo "./$j")
        outacc=$(echo "../Trimming/$j")
        trimmomatic PE -threads 1 -phred33 "$acc"_1.subset.fastq.gz "$acc"_2.subset.fastq.gz
        "$outacc"_1.cleaned.subset.fastq.gz "$outacc"_1.cleaned_unpaired.subset.fastq.gz
        "$outacc"_2.cleaned.subset.fastq.gz "$outacc"_2.cleaned_unpaired.subset.fastq.gz
        ILLUMINACLIP:/home/ubuntu/src/conda/envs/Workshop_TL_YB_Calling2/share/trimmomatic-0.39-2/adaptersTruSeq3-PE-2.fa:2:30:10
        LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50
done
```

Remaining PE reads
Remaining SE reads R1 (R2 lost)
Remaining SE reads R2 (R1 lost)

General Statistics

Copy table | Configure Columns | Showing 12 rows.

Sample Name	% Dups	% GC	Length	M Seqs
ERR3284869_1.cleaned.subset	36.8%	33%	98	0.0
ERR3284869_1.cleaned_unpaired.subset	41.3%	34%	95	0.0
ERR3284869_2.cleaned.subset	36.1%	33%	97	0.0
ERR3284869_2.cleaned_unpaired.subset	35.1%	32%	92	0.0
ERR3284873_1.cleaned.subset	34.5%	33%	95	0.0
ERR3284873_1.cleaned_unpaired.subset	33.8%	33%	88	0.0
ERR3284873_2.cleaned.subset	34.7%	33%	97	0.0
ERR3284873_2.cleaned_unpaired.subset	32.0%	33%	93	0.0
ERR3284898_1.cleaned.subset	29.1%	33%	99	0.0
ERR3284898_1.cleaned_unpaired.subset	24.7%	35%	94	0.0
ERR3284898_2.cleaned.subset	28.9%	33%	98	0.0
ERR3284898_2.cleaned_unpaired.subset	27.6%	33%	95	0.0

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        "$outacc"_2.cleaned.subset.fastq.gz "$outacc"_2.cleaned_unpaired.subset.fastq.gz
        ILLUMINACLIP:/home/ubuntu/src/conda/envs/Workshop_TL_YB_Calling2/share/trimmomatic-0.39-2/adaptersTruSeq3-PE-2.fa:2:30:10
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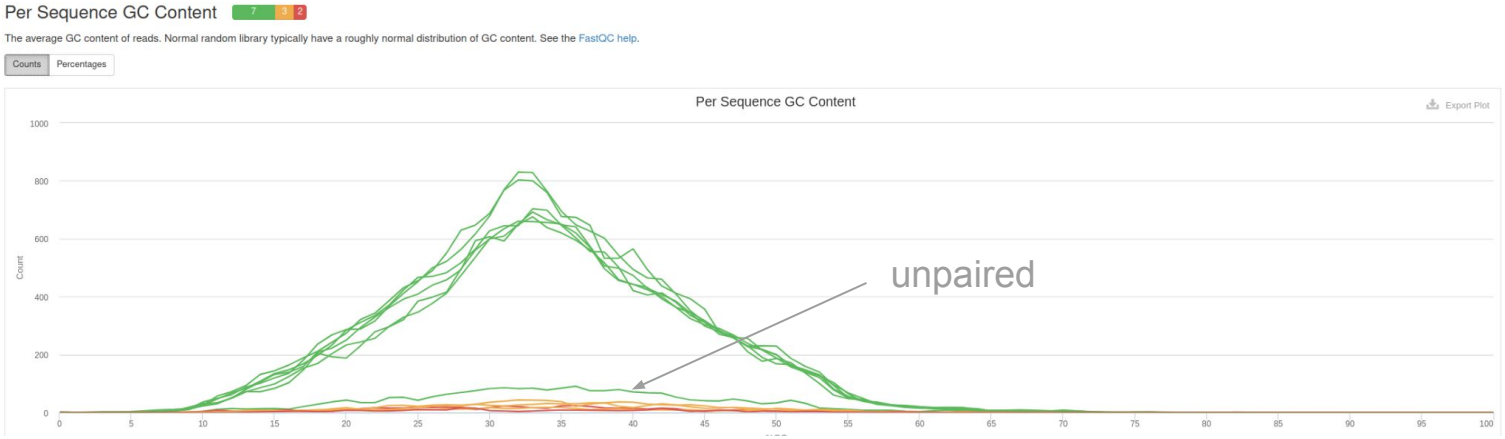

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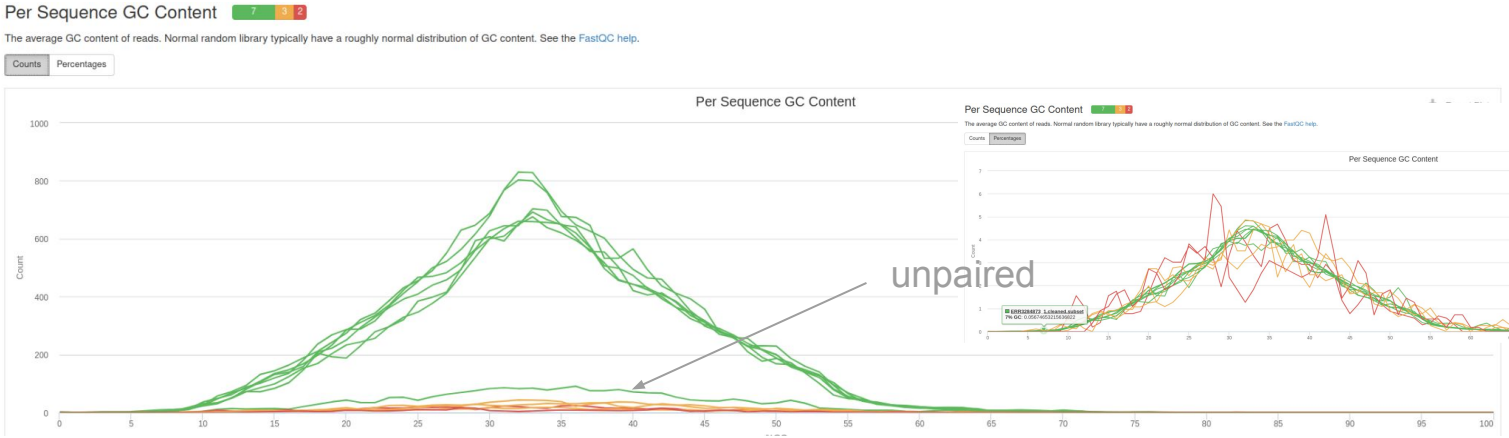

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done
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Remaining PE reads
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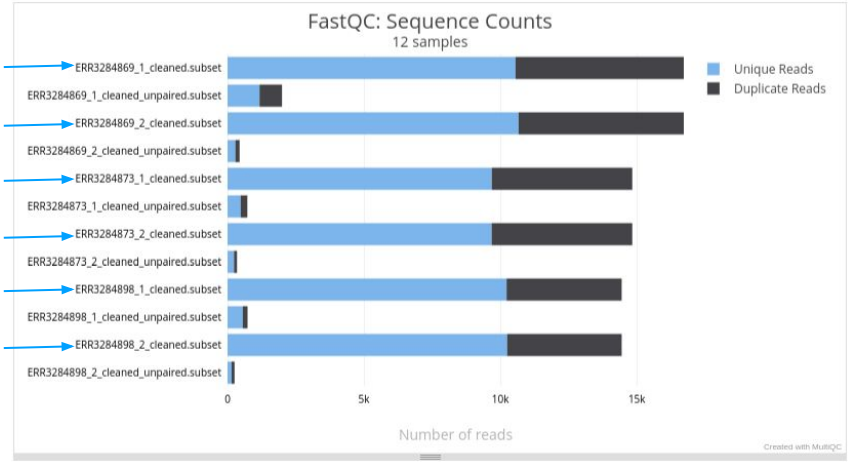
pool1
- Remaining PE reads R1
- Remaining PE reads R2

pool2
- Remaining PE reads R1
- Remaining PE reads R2

pool3
- Remaining PE reads R1
- Remaining PE reads R2



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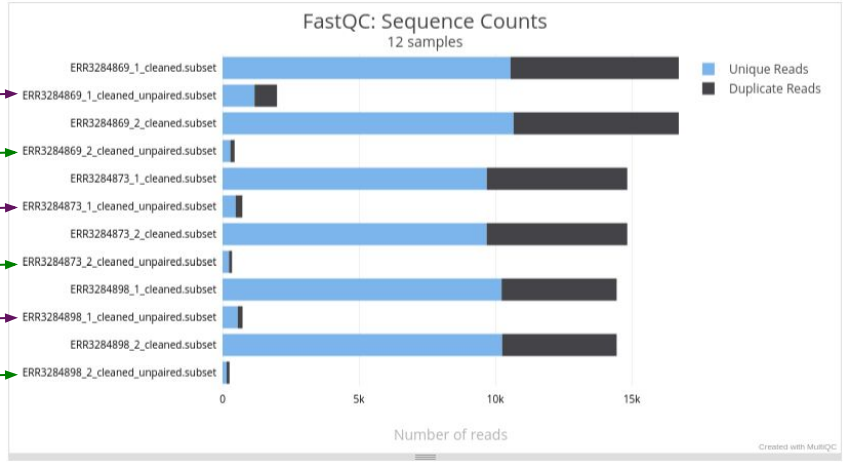
pool2
- Remaining SE reads R1 (R2 lost)
- Remaining SE reads R2 (R1 lost)

pool3
- Remaining SE reads R1 (R2 lost)
- Remaining SE reads R2 (R1 lost)



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done
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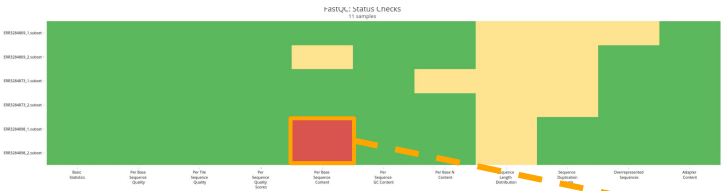
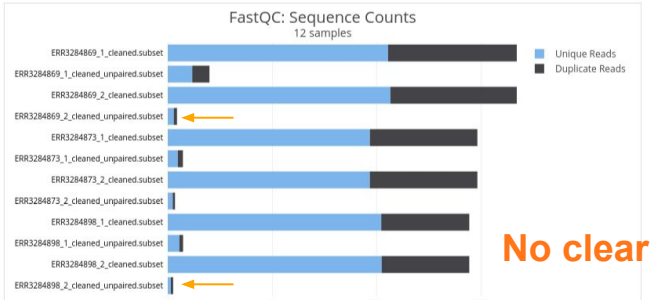
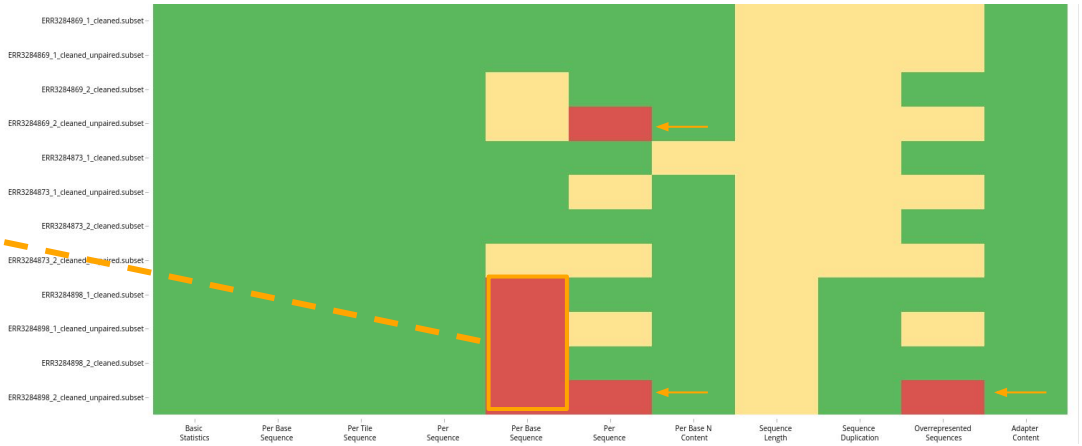
multiqc before trimming





multiqc after trimming

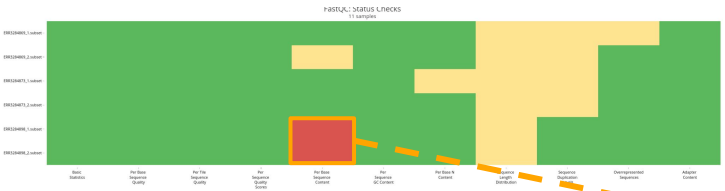


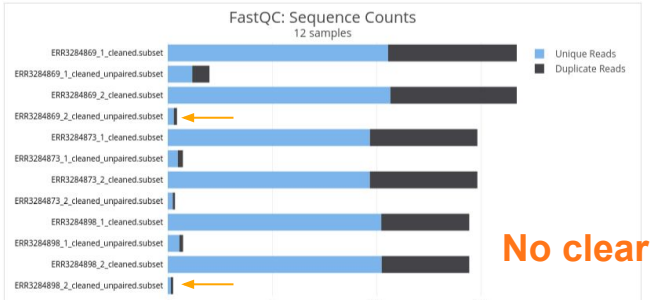
No clear improvements, some additional red tags, for some unpaired R2!

Step 4: Read trimming

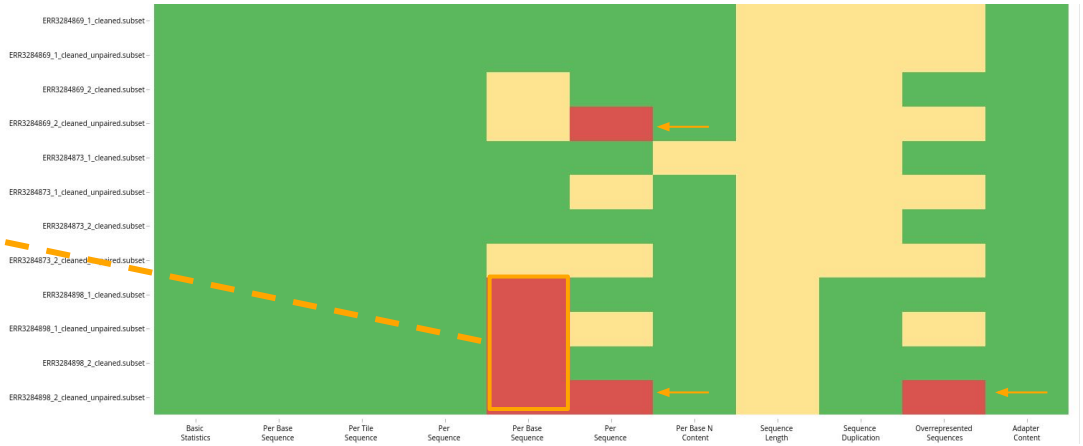
```
for j in ERR3284869 ERR3284873 ERR3284898; do
        acc=$(echo "./$j")
        outacc=$(echo "../Trimming/$j")
        trimmomatic PE -threads 1 -phred33 "$acc"_1.subset.fastq.gz "$acc"_2.subset.fastq.gz
        "$outacc"_1.cleaned.subset.fastq.gz "$outacc"_1.cleaned_unpaired.subset.fastq.gz
        "$outacc"_2.cleaned.subset.fastq.gz "$outacc"_2.cleaned_unpaired.subset.fastq.gz
        ILLUMINACLIP:/home/ubuntu/src/conda/envs/Workshop_TL_YB_Calling2/share/trimmomatic-0.39-2/adaptersTruSeq3-PE-2.fa:2:30:10
        LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50
done
```

multiqc before trimming





multiqc after trimming



No clear improvements, some additional red tags, for some unpaired R2!

Step 5: Mapping reads against a reference genome

```
cd ~/Day1_bioinfo/data-oak/Trimming
for j in ERR3284869 ERR3284873 ERR3284898; do
        acc=$(echo “$j”)
        outacc=$(echo “../Mapping/$j””_subset_trimmedPE”)
        bwa-mem2 mem ../$REFERENCE_GENOME “$acc”_1.cleaned.subset.fastq.gz
        “$acc”_2.cleaned.subset.fastq.gz | samtools view -Sb - > $outacc.bam
done
```

Step 6: Mapping reads against a reference genome

Flagstat

ERR3284869 (PE reads after trimming)

34162 + 0 in total (QC-passed reads + QC-failed reads)
33438 + 0 primary
0 + 0 secondary
724 + 0 supplementary
0 + 0 duplicates
0 + 0 primary duplicates
33760 + 0 mapped (98.82% : N/A)
33036 + 0 primary mapped (98.80% : N/A)
33438 + 0 paired in sequencing
16719 + 0 read1
16719 + 0 read2
29108 + 0 properly paired (87.05% : N/A)
32730 + 0 with itself and mate mapped
306 + 0 singletons (0.92% : N/A)
2740 + 0 with mate mapped to a different chr
1413 + 0 with mate mapped to a different chr (mapQ>=5)

Step 7: Removing PCR duplicates

```
for filemetrics in *.duplication_metrics.txt; do
        duplicates=$(grep "Library" $filemetrics)
        echo "$filemetrics        $duplicates"
done
```

filename	percentage duplicates
ERR3284869_withtrimming.trimmedPE.bam.duplication_metrics.txt	**0.072401**
ERR3284869_withtrimming.trimmedunpaired1.bam.duplication_metrics.txt	0.102675
ERR3284869_withtrimming.trimmedunpaired2.bam.duplication_metrics.txt	0.086877
ERR3284873_withtrimming.trimmedPE.bam.duplication_metrics.txt	**0.074513**
ERR3284873_withtrimming.trimmedunpaired1.bam.duplication_metrics.txt	0.069848
ERR3284873_withtrimming.trimmedunpaired2.bam.duplication_metrics.txt	0.07938
ERR3284898_withtrimming.trimmedPE.bam.duplication_metrics.txt	**0.084082**
ERR3284898_withtrimming.trimmedunpaired1.bam.duplication_metrics.txt	0.062445
ERR3284898_withtrimming.trimmedunpaired2.bam.duplication_metrics.txt	0.071926

MultiQC
(simpler approach, generally provide strongly overestimated values)

Sample Name	% Dups
ERR3284869_1.cleaned.subset	36.8%
ERR3284869_1.cleaned_unpaired.subset	41.3%
ERR3284869_2.cleaned.subset	36.1%
ERR3284869_2.cleaned_unpaired.subset	35.1%
ERR3284873_1.cleaned.subset	34.5%
ERR3284873_1.cleaned_unpaired.subset	33.8%
ERR3284873_2.cleaned.subset	34.7%
ERR3284873_2.cleaned_unpaired.subset	32.0%
ERR3284898_1.cleaned.subset	29.1%
ERR3284898_1.cleaned_unpaired.subset	24.7%
ERR3284898_2.cleaned.subset	28.9%
ERR3284898_2.cleaned_unpaired.subset	27.6%

Step 8: Allele counts & mpileup files for pool-seq data

mpileup

```
Qrob_Chr01    1    T    0    *    *    1    ^8.      C        0    *    *
Qrob_Chr01    2    C    0    *    *    1    .        C        0    *    *
Qrob_Chr01    3    T    0    *    *    1    .        C        0    *    *
Qrob_Chr01    4    G    0    *    *    2    .^9.     F@       0    *    *
Qrob_Chr01    5    A    0    *    *    2    ..       F@       0    *    *
Qrob_Chr01    6    A    0    *    *    3    G.^8.    F@C      1    ^7.  @
Qrob_Chr01    7    G    0    *    *    3    A..      FFC      3    …    FF@
Qrob_Chr01    8    T    0    *    *    4    ...^8.   FD@@     3    …    EFB
Qrob_Chr01    9    A    0    *    *    3    ...      HEF      3    …    FHD
Qrob_Chr01    10   T    0    *    *    4    ....     HFF@     3    …    HHF
```

Synchronized mpileup

```
Qrob_Chr01    1    T    0:0:0:0:0:0 0:1:0:0:0:0 0:0:0:0:0:0
Qrob_Chr01    2    C    0:0:0:0:0:0 0:0:1:0:0:0 0:0:0:0:0:0
Qrob_Chr01    3    T    0:0:0:0:0:0 0:1:0:0:0:0 0:0:0:0:0:0
Qrob_Chr01    4    G    0:0:0:0:0:0 0:0:0:2:0:0 0:0:0:0:0:0
Qrob_Chr01    5    A    0:0:0:0:0:0 2:0:0:0:0:0 0:0:0:0:0:0
Qrob_Chr01    6    A    0:0:0:0:0:0 2:0:0:1:0:0 1:0:0:0:0:0
Qrob_Chr01    7    G    0:0:0:0:0:0 1:0:0:2:0:0 0:0:0:3:0:0
Qrob_Chr01    8    T    0:0:0:0:0:0 0:4:0:0:0:0 0:3:0:0:0:0
Qrob_Chr01    9    A    0:0:0:0:0:0 3:0:0:0:0:0 3:0:0:0:0:0
Qrob_Chr01    10   T    0:0:0:0:0:0 0:4:0:0:0:0 0:3:0:0:0:0
```

→ data that will be imported for Friday's proposal

Note: For each pool, the format is A-count:T-count:C-count:G-count:N-count:-count, which represents the number of reads supporting the alleles A, T, C, G, ambigious allele (N) and indels (*).*

Step 9: SNP calling and vcf files for calling genotypes (moderate coverage data)

```
>NC_052392.1 Phoenix dactylifera cultivar Barhee BC4 chromosome 1,
palm_55x_up_171113_PBpolish2nd_filt_p, whole genome shotgun sequence
tgatcacaaggtctgtaaactgcaaaggtccatctatggacaaaagcaagcatctcggagttggaacactcgtttcaacg
aggtaatcaaatcgtttgatttcatcaagaacgaagagaaaccatgtgtttataagaagggttagtgggagggctattgt
aaTCCTCGTATTATACATGGATGATATTCTTCCAATTGGGAATGgtgttcccatgctaacttaggtcaaggtatggttgt
ctaaggatttctccataaaagaccttggggaagcatcctatagtttggggattaaggtctatagagatagatctaagatg
atgcttgtcctgtcatagaatatgtacatagaggaagtgctgaagaggttcggcatggaaacctctaaaaggggaatTAT
TAcctcttaggcatgggataaatctccaagaagatgtgccccaagaCATCGGAAGAGATAcagtggatgagcaagatccc
ttatgcttcgggtatagggagcctcatgtataccatgctatgtacgtgacttgatatagtacatgctgtgagtgtcacaa
gcatatatcattcaaatccaggcaagaagcactggatagctgtgaagaacagtcttaagtacttgagaagaactaaggac
atgttcttggtctttgggagaagatcagagttgaaggtagaaggatgcacacatattgatgatagaaagtctacatcaga
atatgtgcaatggtggttcagtaaattggaagagttccaaacaaccgatcattgtagattctaccttagaagctgaatgt
taagccgtctctaagtgTGTAGTAGAAATCTTatggtttaagaagttcattgcagagttaggtgtaatgtcatcagatgc…
```

Step 9: SNP calling and vcf files for calling genotypes (moderate coverage data)

Assemblathon

Number of scaffolds	2391		
Total size of scaffolds	773189301	←	Assembly size 773 Mb
Longest scaffold	40814151		
Shortest scaffold	8352		
Number of scaffolds > 1K nt	2391	100.0%	
Number of scaffolds > 10K nt	2390	100.0%	
Number of scaffolds > 100K nt	1054	44.1%	
Number of scaffolds > 1M nt	53	2.2%	
Number of scaffolds > 10M nt	17	0.7%	← 17 scaffolds with scaffold >10 Mb
Mean scaffold size	323375		
Median scaffold size	86140		
N50 scaffold length	4728343	←	length of the 19th scaffold (the one reaching half of the total assembly)
L50 scaffold count	19	←	cumulative of the 19 longest scaffolds ~ half of the total assembly
scaffold %A	29.85		
scaffold %C	20.15		
scaffold %G	20.14		
scaffold %T	29.86		
scaffold %N	0.00		
scaffold %non-ACGTN	0.00		
Number of scaffold non-ACGTN nt	0		

Percentage of assembly in scaffolded contigs	49.9%
Percentage of assembly in unscaffolded contigs	50.1%
Average number of contigs per scaffold	1.1
Average length of break (>25 Ns) between contigs in scaffold	100

Step 9: SNP calling and vcf files for calling genotypes (moderate coverage data)

flagstat

e.g. subset_Dayri.RG.bam.flagstat.txt

265664 + 0 in total (QC-passed reads + QC-failed reads)
3128 + 0 secondary
0 + 0 supplementary
35838 + 0 duplicates
265321 + 0 mapped (99.87% : N/A)
262536 + 0 paired in sequencing
131379 + 0 read1
131157 + 0 read2
251687 + 0 properly paired (95.87% : N/A)
261850 + 0 with itself and mate mapped
343 + 0 singletons (0.13% : N/A)
6869 + 0 with mate mapped to a different chr
4041 + 0 with mate mapped to a different chr (mapQ>=5)

Take home message !
Even when it is not clearly indicated, it is essential to perform QC (reference genome, fastqc, mapping etc.) ;)

Step 9: SNP calling and vcf files for calling genotypes (moderate coverage data)

zmore subset_Dayri.g.vcf.gz | grep -v "##" | head

individual vcf (gvcf)

```
#CHROM  POS     ID      REF     ALT     QUAL    FILTER  INFO    FORMAT  Dayri
NC_052395.1     1       .       C       <NON_REF>.      .       END=23999855    GT:DP:GQ:MIN_DP:PL      0/0:0:0:0:0,0
NC_052395.1     23999856        .       A       <NON_REF>.      .       END=23999858    GT:DP:GQ:MIN_DP:PL      0/0:2:6:2:0,6,90
```

zmore datepalm.combine.NC_052395.1.g.vcf.gz | grep -v "##" | head

multiple individual vcf

```
#CHROM  POS     ID      REF     ALT     QUAL    FILTER  INFO    FORMAT  Dayri   Halawy  Hayany  Khisab  Medjool
NC_052395.1     1       .       C       <NON_REF>.      .       END=23999853    GT:DP:GQ:MIN_DP:PL      ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0
NC_052395.1     23999854        .       A       <NON_REF>.      .       .       GT:DP:GQ:MIN_DP:PL      ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:0:0:0:0,0,0 ./.:1:3:1:0,3,16
```

zmore datepalm.NC_052395.1.jointvcf.gz | grep -v "##" | head

joint calling

```
#CHROM  POS     ID      REF     ALT     QUAL    FILTER  INFO    FORMAT  Dayri   Halawy  Hayany  Khisab  Medjool
NC_052395.1     23999872        .       G       T       727.18  .
AC=6;AF=0.750;AN=8;BaseQRankSum=0.00;DP=24;ExcessHet=0.6695;FS=0.000;MLEAC=7;MLEAF=0.875;MQ=60.00;MQRankSum=0.00;QD=33.05;ReadPosRankSum=0.524;SOR=0.914      GT:AD:DP:GQ:PL  1/1:0,5:5:15:208,15,0
1/1:0,7:7:21:290,21,0   ./.:1,0:1:0:0,0,0       0/1:3,2:5:74:74,0,119   0/1:1,4:5:30:164,0,30
NC_052395.1     23999888        .       T       C       194.51  .
AC=1;AF=0.100;AN=10;BaseQRankSum=-7.120e-01;DP=43;ExcessHet=0.0000;FS=3.680;MLEAC=1;MLEAF=0.100;MQ=60.00;MQRankSum=0.00;QD=27.79;ReadPosRankSum=1.07;SOR=0.636      GT:AD:DP:GQ:PGT:PID:PL:PS
0/0:9,0:9:27:.:.:.:0,27,403     0/0:10,0:10:30:.:.:.:0,30,432   0/0:2,0:2:6:.:.:.:0,6,90        0/0:14,0:14:42:.:.:.:0,42,582   0|1:2,5:7:69:0|1:23999888_T_C:204,0,69:23999888
```

filtering SNPs

zmore datepalm.NC_052395.1.jointvcf.filtered.gz | grep -v "##" | grep "PASS" | head

```
NC_052395.1     23999872        .       G       T       727.18  PASS
AC=6;AF=0.750;AN=8;BaseQRankSum=0.00;DP=24;ExcessHet=0.6695;FS=0.000;MLEAC=7;MLEAF=0.875;MQ=60.00;MQRankSum=0.00;QD=33.05;ReadPosRankSum=0.524;SOR=0.914      GT:AD:DP:GQ:PL
1/1:0,5:5:15:208,15,0   1/1:0,7:7:21:290,21,0   ./.:1,0:1:0:0,0,0       0/1:3,2:5:74:74,0,119   0/1:1,4:5:30:164,0,30
NC_052395.1     23999888        .       T       C       194.51  PASS
AC=1;AF=0.100;AN=10;BaseQRankSum=-7.120e-01;DP=43;ExcessHet=0.0000;FS=3.680;MLEAC=1;MLEAF=0.100;MQ=60.00;MQRankSum=0.00;QD=27.79;ReadPosRankSum=1.07;SOR=0.636
GT:AD:DP:GQ:PGT:PID:PL:PS       0/0:9,0:9:27:.:.:.:0,27,403     0/0:10,0:10:30:.:.:.:0,30,432   0/0:2,0:2:6:.:.:.:0,6,90        0/0:14,0:14:42:.:.:.:0,42,582   0|1:2,5:7:69:0|1:23999888_T_C:204,0,69:23999888
```

zmore datepalm.NC_052395.1.jointvcf.filtered.gz | grep -v "##" | grep "PASS" | wc -l

3618 (3,618 filtered sites)

zmore datepalm.NC_052395.1.jointvcf.filtered.gz | grep -v "##" | grep "LowQual" | wc -l

657 (657 sites considered as Low-Quality based on the parameter set we used)

Step 10: Genotype likelihood (low coverage data)

Number of scaffolds	6457		
Total size of scaffolds	1799143587		← Assembly size 1.8 Gb
Longest scaffold	263920458		
Shortest scaffold	5000		
Number of scaffolds > 1K nt	6457	100.0%	
Number of scaffolds > 10K nt	3430	53.1%	
Number of scaffolds > 100K nt	852	13.2%	
Number of scaffolds > 1M nt	209	3.2%	
Number of scaffolds > 10M nt	8	0.1%	← 8 scaffolds with scaffold >10 Mb
Mean scaffold size	278635		
Median scaffold size	10682		
N50 scaffold length	150641573		← length of the 5h scaffold (the one reaching half of the total assembly)
L50 scaffold count	5		← cumulative of the 5 longest scaffolds ~ half of the total assembly
scaffold %A	28.22		
scaffold %C	19.06		
scaffold %G	19.07		
scaffold %T	28.22		
scaffold %N	5.44		
scaffold %non-ACGTN	0.00		
Number of scaffold non-ACGTN nt	0		

Percentage of assembly in scaffolded contigs	97.5%
Percentage of assembly in unscaffolded contigs	2.5%
Average number of contigs per scaffold	6.5
Average length of break (>25 Ns) between contigs in scaffold	2752

Step 10: Genotype likelihood (low coverage data)

flagstat

e.g. subset_Sample_AC27-3.srtd.bam.flagstat.txt

523479 + 0 in total (QC-passed reads + QC-failed reads)
1800 + 0 secondary
0 + 0 supplementary
0 + 0 duplicates
521725 + 0 mapped (99.66% : N/A)
521679 + 0 paired in sequencing
260829 + 0 read1
260850 + 0 read2
508465 + 0 properly paired (97.47% : N/A)
518171 + 0 with itself and mate mapped
1754 + 0 singletons (0.34% : N/A)
7691 + 0 with mate mapped to a different chr
2764 + 0 with mate mapped to a different chr (mapQ>=5)

Take home message !
Even when it is not clearly indicated, it is essential to perform QC (reference genome, fastqc, mapping etc.) ;)

Step 10: Genotype likelihood (low coverage data)

```
grep -v "#" urban_contryside_anole.vcf | head -30000 | tail -2
chr4    120457343    .    T    G    .    PASS    NS=23;DP=60;AF=2.62007e-06    DP:GL:PL:GP    2:0,-0.602068,-7.29999:0,6,73:0.800003,0.199997,4.00962e-08
0:0,0,0:0,0,0:0.333333,0.333333,0.333333 (...continued)
chr4    120457344    .    G    T    PASS    NS=26;DP=92;AF=0.0170235    DP:GL:PL:GP    4:0,-1.20414,-10.6139:0,12,106:0.941178,0.0588216,2.2899e-11
0:0,0,0:0,0,0:0.333333,0.333333,0.333333 (...continued)
```

Step 10: Genotype likelihood (low coverage data)

zmore urban_contryside_anole.vcf.gz

```
##fileformat=VCFv4.2(angsd version)
##FORMAT=<ID=GT,Number=1,Type=Integer,Description="Genotype">
##FORMAT=<ID=GP,Number=G,Type=Float,Description="Genotype Probabilities">
##FORMAT=<ID=PL,Number=G,Type=Float,Description="Phred-scaled Genotype Likelihoods">
##FORMAT=<ID=GL,Number=G,Type=Float,Description="scaled Genotype Likelihoods (loglikeratios to the most likely (in log10))">
#CHROM  POS     ID      REF     ALT     QUAL    FILTER  INFO    FORMAT  ind0    ind1    ind2    ind3    ind4    ind5    ind6    ind7    ind8
        ind9    ind10   ind11   ind12   ind13   ind14   ind15   ind16   ind17   ind18   ind19   ind20   ind21   ind22   ind23   ind24   ind25
        ind26   ind27   ind28   ind29   ind30ind31      ind32   ind33   ind34   ind35
chr4    119999851       .       C       A       .       PASS    .       GP:GL   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000
016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999992,0.000008,0.000000:0.000000,-0.301034,-3.20000
0       0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999992,0.000008,0.000000:0.000000,-0.301034,-3.200000   0.999984,0.000016,0.000000:0.0
00000,0.000000,0.000000 0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999992,0.000008,0.000000:0.000000,-0.301034,-2.600000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000
016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
0.999984,0.000016,0.000000:0.000000,0.
000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000   0.999984,0.000016,0.000000:0.000000,0.000000,0.000000
chr4    119999852       .       C       A       .       PASS    .       GP:GL   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000
010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999997,0.000003,0.000000:0.000000,-0.602068,-6.09976
5       0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999995,0.000005,0.000000:0.000000,-0.301034,-2.500000   0.999990,0.000010,0.000000:0.0
00000,0.000000,0.000000 0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999995,0.000005,0.000000:0.000000,-0.301034,-2.600000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000
010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
0.999990,0.000010,0.000000:0.000000,0.
000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000   0.999990,0.000010,0.000000:0.000000,0.000000,0.000000
```