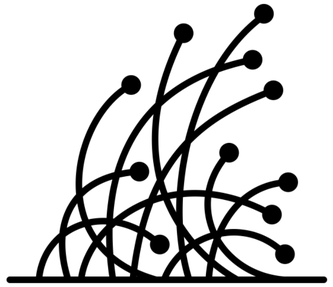


Variant calling and filtering

SNPs and indels

Dr Johanna Rhodes

Learning outcomes

- **Calling single nucleotide polymorphisms (SNPs) and insertions/deletions (indels)**
  - tools
  - considerations
- **Understanding vcf format**
- **Why we need to filter variants**
- **How to filter variants**
  - obtain high-confidence SNPs
  - commands
- **Quality control of filtered SNPs**
- **Mapping SNPs to genes**

Identifying variation within genomes

- Identifying variants is important for evolution studies, interring resistance mutations and in population genomics studies
    - Reads aligned to a reference genome enables identification of differences between individuals
- SNPs are the most abundant variants, along with insertion/deletions
- Widely used software for variant calling includes GATK and Freebayes
    - Can call variants individually or across multiple samples
    - Determine genotypes e.g. at each position, determine whether variant is homozygous reference, heterozygous or homozygous alternate
- Creates variant call format (vcf) file

Variant call format (VCF)

The details

```
##fileformat=VCFv4.3
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS      ID         REF    ALT    QUAL  FILTER   INFO                              FORMAT       NA00001         NA00002
NA00003
20     14370    rs6054257  G      A      29    PASS     NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ  0|0:48:1:51,51
1|0:48:8:51,51   1/1:43:5:.,.
20     17330    .          T      A      3     q10      NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ  0|0:49:3:58,50
0|1:3:5:65,3     0/0:41:3
20     1110696  rs6040355  A      G,T    67    PASS     NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ  1|2:21:6:23,27
2|1:2:0:18,2     2/2:35:4
20     1230237  .          T      .      47    PASS     NS=3;DP=13;AA=T                   GT:GQ:DP:HQ  0|0:54:7:56,60
0|0:48:4:51,51   0/0:61:2
20     1234567  microsat1  GTC    G,GTCT 50    PASS     NS=3;DP=9;AA=G                    GT:GQ:DP     0/1:35:4        0/2:17:2
1/1:40:3
```

Calling high-quality variants

Commands using GATK

```
gatk HaplotypeCaller –R reference.fa –I isolate_name.post_recal_reads.bam –O
isolate_name.raw_variants_recal.vcf -ERC GVCF –-pcr-indel-model NONE –ploidy 1
–stand-call-conf 30 –mbq 20 –A QualByDepth

gatk GenotypeGVCFs –R reference.fa -V isolate_name.raw_variants_recal.vcf -O
isolate_name.genotyped_variants_recal.vcf
```

Calling variants
Considerations

- Repetitive regions
  - Most fungi have 1-25% repetitive DNA content and genome size is correlated with number of transposon families hosted within the genome
  - Difficult to call SNPs in repetitive regions
    - lower quality scores in repetitive regions, so variant calls may be false positives
  - RepeatMasker to mask regions of repeats in the reference genome
    - Include in HaplotypeCaller using the -XL parameter
    - Covered tomorrow

Variant filtering

Filtering variants

What is the need?

- Initial variant calling is generally a very rough approximation, and will incorrectly identify many loci as SNPs or indels
- The INFO and FORMAT fields in the vcf contains a lot of information about each site in the genome, the reads aligned there, and the quality of variant calls.
- The variant filtration algorithm will use this information to filter out uninformative positions.

Filtering variants

Commands in GATK

- First, we separate the vcf into SNPs and indels (you can merge them back together after filtering if preferred)

```
gatk SelectVariants –R reference.fa –V
isolate_name.genotyped_variants_recal.vcf –O isolate_name.raw_snps_recal.vcf --
select-type-to-include SNP -select ‘vc.getGenotype(“WGS”).getAD().1*1.0 /
vc.getGenotype(“WGS”).getDP() > 0.90’
```

```
gatk SelectVariants –R reference.fa –V isolate_name.raw_variants_recal.vcf –O
isolate_name.raw_indels_recal.vcf --select-type-to-include INDEL
```

Filtering variants

Commands in GATK

- To gain a list of high confidence SNPs we filter on mapping quality, depth of coverage, Fisher strand bias and balance of alleles.
- Any SNP that fulfils any one of these criteria is labelled as ‘LowConf’. It is not removed
- We also include an additional filter on genotype quality
  - Note that we sort of did a filtering step earlier when selecting SNPs, where we filtered out SNPs that weren’t present in at least 90% of mapped reads.

```
gatk VariantFiltration -R reference.fa -V isolate_name.raw_snps_recal.vcf -O
isolate_name.filtered_snps_final.vcf -filter "QD < 2.0" --filter-name "LowConf" -filter
"FS > 60.0" --filter-name "LowConf" -filter "MQ < 40.0" --filter-name "LowConf" -filter
"MQRankSum < -12.5" --filter-name "LowConf" -filter "ReadPosRankSum < -8.0" --filter-
name "LowConf" -filter "SOR > 4.0" --filter-name "LowConf" -filter "DP < 5" --filter-
name "LowConf" -G-filter "GQ < 50" -G-filter-name "FILTER_GQ-50"
```

Quality control

- Using the two files, ‘isolate_name.genotyped_variants_recal.vcf’ and ‘isolate_name.filtered_snps_final.vcf’, you want to count the number of lines in each, excluding the header
- `grep -v “#” isolate_name.genotyped_variants_recal.vcf | wc -l`
- `grep -v “#” isolate_name.filtered_snps_final.vcf | grep -v “LowConf” | grep -v “FILTER_GQ-50” | wc -l`
- The difference between these two numbers will give you an idea of quality
  - High % filtered out indicates something weird is going on - cross-check with coverage and mapping statistics
    - Hybrid/diploid
    - Not the same species as reference
    - Poor quality sequencing
  - Usually range can be 1-10% filtered out

Other considerations

Making pipelines

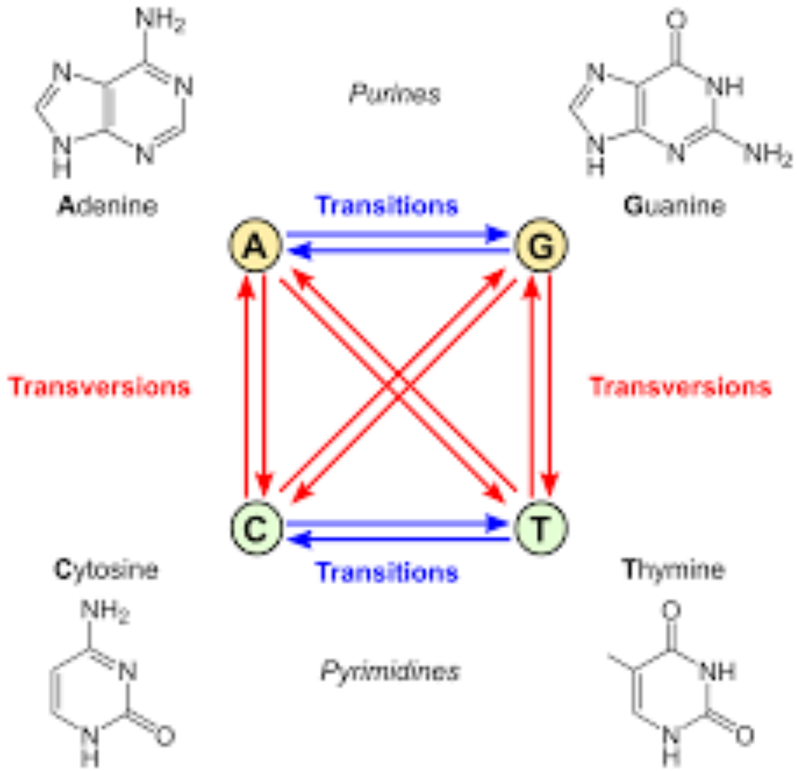
- Running these analyses (from raw read QC to filtering SNPs) one command at a time/one isolate at a time is inefficient
  - Time-consuming
  - Could receive many isolates in one go to analyse
- Automating the process as a ‘pipeline’
  - All commands in a script
  - High Performance Computing (HPC)
    - job submission —> running pipeline in parallel
    - may have more processing power available than standard laptop/computer, which is useful for big/highly complex genomes
    - consider how to transfer your data - clients such as Cyberduck provide a user friendly GUI to scp

Mapping SNPs to genes

Nucleotide changes

Transitions and transversions

- Nucleotide substitutions can take two forms:
  - transitions
    - purine-purine or pyrimidine-pyrimidine interchanges
  - transversions
    - purine-pyrimidine interchanges
- Transitions (G>A|C>T or A>G|T>C) occur more frequently in Ascomycota fungi
- Transition:transversion ratio indicative of certain biological processes



Mutations
In coding regions

- Synonymous (sSNP)
  - A SNP is described as synonymous if there is no change in the AA encoded
    - ‘silent’/evolutionary neutral
- Non-synonymous (nsSNP)
  - Change in the AA encoded
    - sometimes called ‘missense’
- Stop mutation (STP)
  - AA change encodes an AA that is a stop codon, resulting in premature truncation of protein
  - sometimes called ‘nonsense’
- Read-through mutation (RTH)
  - AA change results in stop codon changing to other AA and protein extended

Mutations

In non-coding regions

- Mutations in UTR regions and intergenic regions

Mapping SNPs to genes

snpEff

- Cingolani *et al.* 2012 PMID: 22728672
- Genomic variant annotations and functions effect prediction
- Has ~38k genomes pre-loaded, or can make custom database for new species/genomes
- Annotate the final vcf
- `java -jar snpEff.jar genome_ref isolate_name.final_SNPs.vcf > isolate_name.snps.annotated`
- Produces an annotated vcf with an additional ‘INFO’ column, and a html report

Mapping SNPs to genes

snpEff

- html results report

Number of effects by functional class

Type (alphabetical order)	Count	Percent
MISSENSE	7,115	34.968%
NONSENSE	46	0.226%
SILENT	13,186	64.806%

Missense / Silent ratio: 0.5396

Number of effects by type and region

Type

Type (alphabetical order)	Count	Percent
downstream_gene_variant	103,013	38.927%
intergenic_region	27,313	10.321%
intron_variant	247	0.093%
missense_variant	7,105	2.685%
non_coding_transcript_exon_variant	1	0%
splice_acceptor_variant	1	0%
splice_donor_variant	1	0%
splice_region_variant	47	0.018%
start_lost	2	0.001%
stop_gained	46	0.017%
stop_lost	8	0.003%
stop_retained_variant	21	0.008%
synonymous_variant	13,165	4.975%
upstream_gene_variant	113,663	42.951%

Region

Type (alphabetical order)	Count	Percent
DOWNSTREAM	103,013	38.934%
EXON	20,325	7.682%
INTERGENIC	27,313	10.323%
INTRON	231	0.087%
SPLICE_SITE_ACCEPTOR	1	0%
SPLICE_SITE_DONOR	1	0%
SPLICE_SITE_REGION	37	0.014%
UPSTREAM	113,663	42.959%

Mapping SNPs to genes

Annotated vcf

- Lots of information
- Perhaps searching for AA that results in drug resistance?
  - Search for gene of interest on FungiDB/NCBI and use ID to search: