Variant Calling - SNPs and short indels

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HTS workflow

Library preparation

- DNA extraction
- fragmentation
- adapter ligation
- amplification

Sequencing

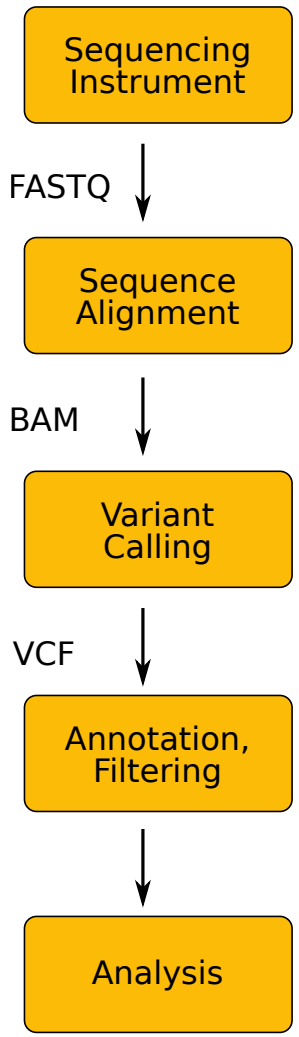
- base calling
- de-multiplexing

Data processing

- read mapping
- variant calling
- variant filtering

Analysis

- Variant annotation
- ...



Variant types

SNPs/SNVs . . . Single Nucleotide Polymorphism/Variation

```
ACGTTTAGCAT
ACGTTCAGCAT
```

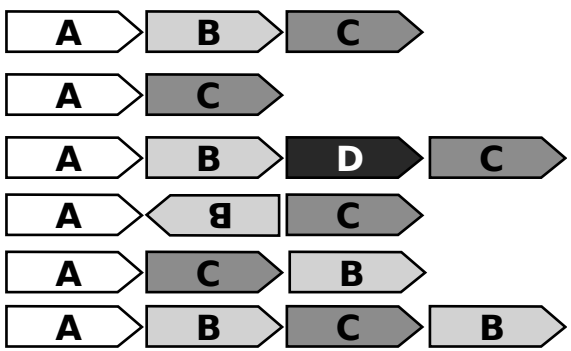
MNPs . . . Multi-Nucleotide Polymorphism

```
ACGTCCAGCAT
ACGTTTAGCAT
```

Indels . . . short insertions and deletions

```
ACGTTTAGCA-TT
ACGTT-AGCAGTT
```

SVs . . . Structural Variation



Some terminology

The goal is to determine the genotype at each position in the genome

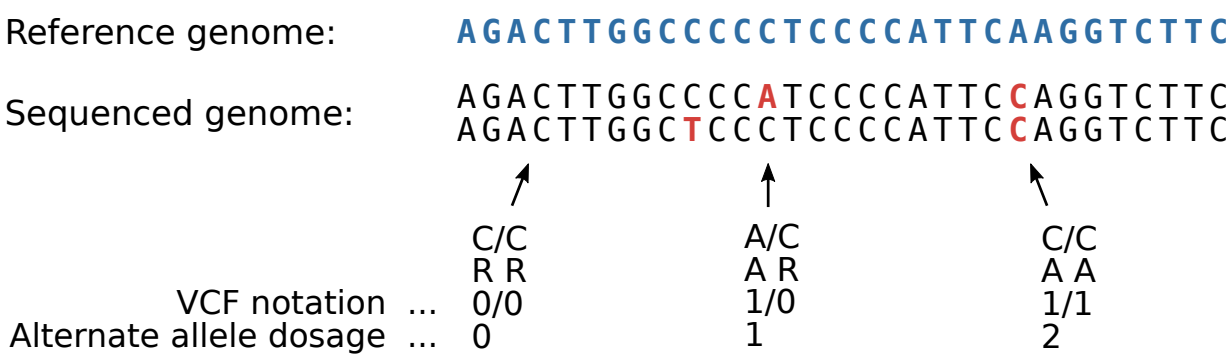
Genotype

- in the broad sense . . . genetic makeup of an organism
- in the narrow sense . . . the combination of alleles at a position

Reference and alternate alleles - R and A

Diploid organism

- two chromosomal copies, three possible genotypes
  - RR .. homozygous reference genotype
  - RA .. heterozygous
  - AA .. homozygous alternate



Germline vs somatic mutation

Germline mutation

- heritable variation in the germ cells

Somatic mutation

- variation in non-germline tissue, tumors...

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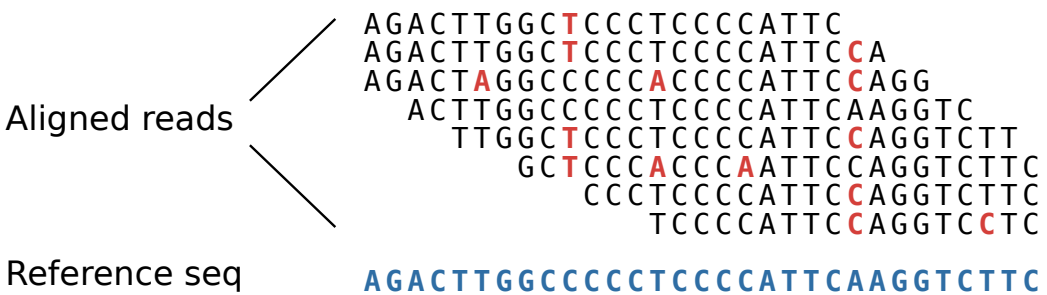
Germline variant calling

- expect the following fractions of alternate alleles in the pileup:
  - 0.0 for RR genotype (plus sequencing errors)
  - 1.0 for AA (plus sequencing errors)
  - 0.5 for RA (random variation of binomial sampling)

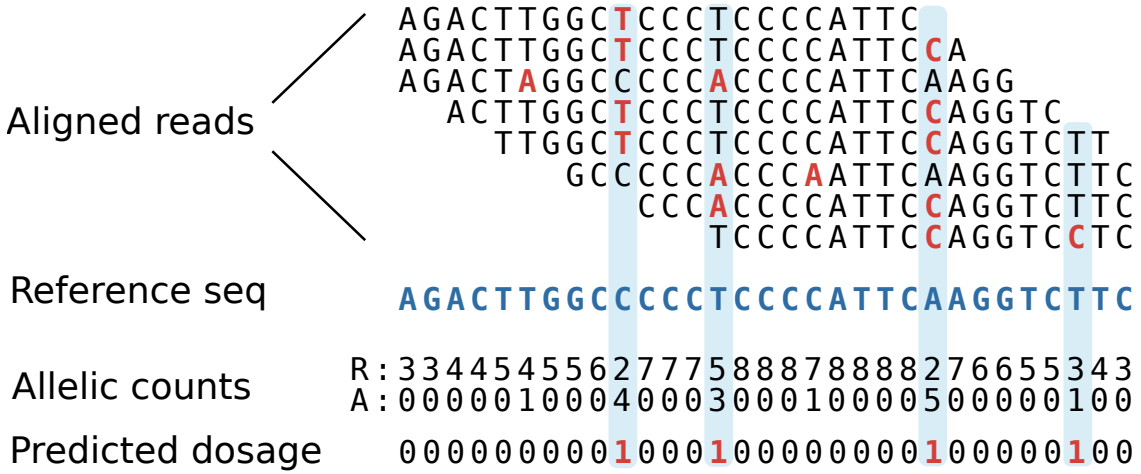
Somatic

- any fraction of alt AF possible - subclonal variation, admixture of normal cells in tumor sample



Naive variant calling

Use fixed allele frequency threshold to determine the genotype



alt AF	genotype
$[0, 0.2)$	RR .. homozygous reference
$[0.2, 0.8]$	RA .. herezogyous
$(0.8, 1]$	AA .. homozygous variant

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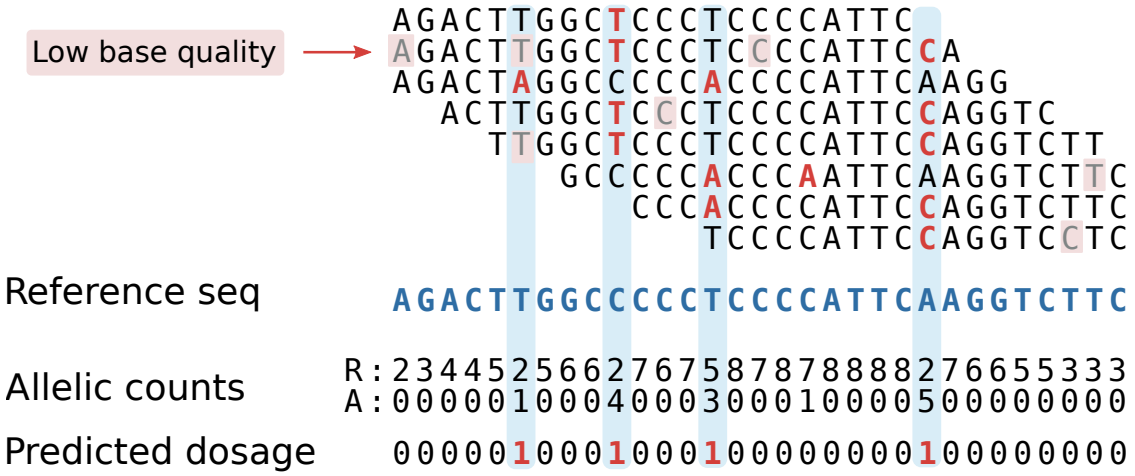
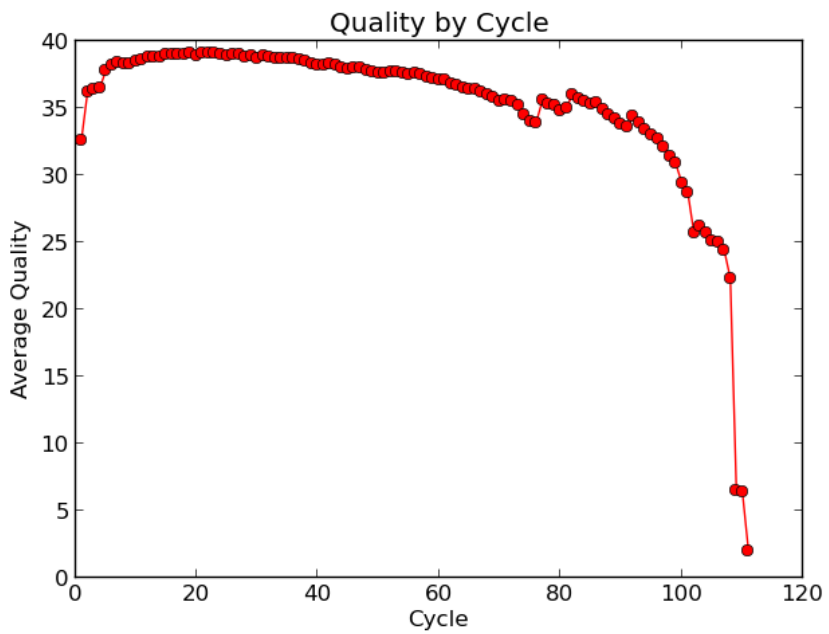


1) Filter base calls by quality
   e.g. ignore bases Q<20

Phred quality score

$$Q = -10 \log_{10} P_{\text{err}}$$

Quality	Error probability	Accuracy
10 (Q10)	1 in 10	90%
20 (Q20)	1 in 100	99%
30 (Q30)	1 in 1000	99.9%
40 (Q40)	1 in 10000	99.99%



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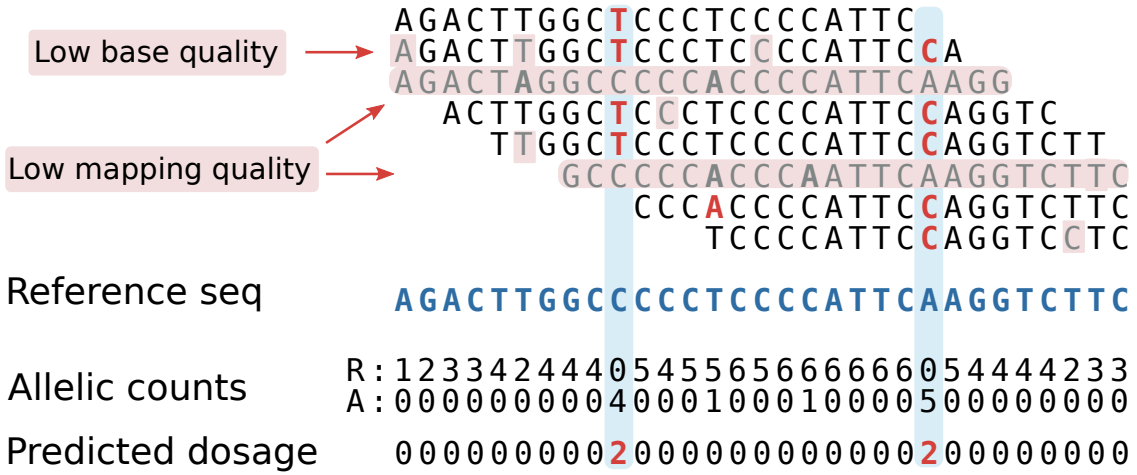

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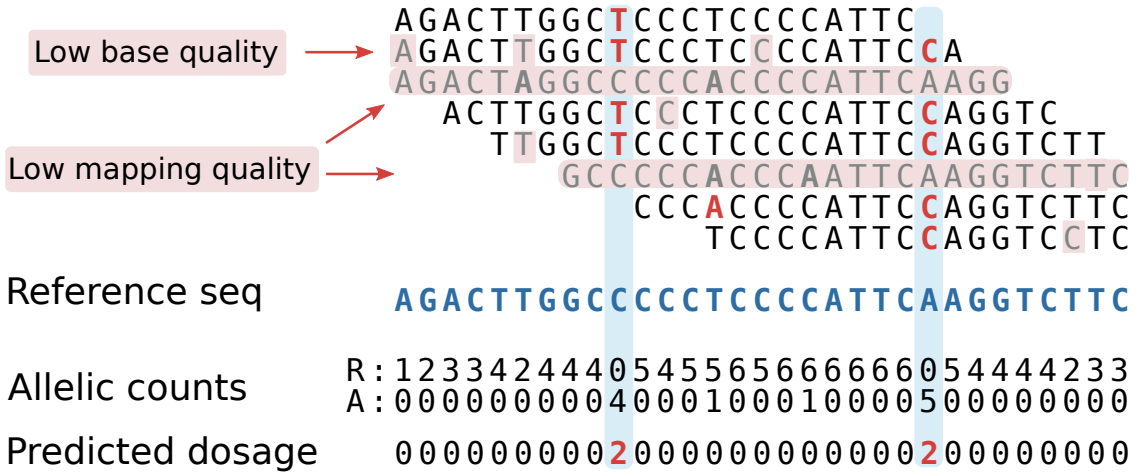

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Problems:

- undercalls hets in low-coverage data
- throws away information due to hard quality thresholds
- gives no measure of confidence

Real life calling models

More sophisticated models apply a statistical framework

Likelihood

Prior

$$P(G|D) = \frac{P(D|G)\,P(G)}{P(D)}$$

Posterior

Normalization

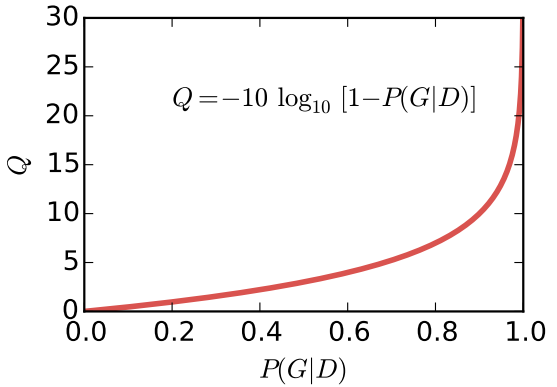
to determine:

1. the most likely genotype $g \in \{\text{RR}, \text{RA}, \text{AA}\}$ given the observed data D

$$g = \underset{G}{\operatorname{argmax}}\, P(G|D)$$

2. and the genotype quality

$$Q = -10 \log_{10}[1 - P(G|D)]$$



Important terms you may encounter

Genotype likelihoods

- which of the three genotypes RR, RA, AA is the data most consistent with?
- calculated from the alignments, the basis for calling
- takes into account:
  - base calling errors
  - mapping errors
  - statistical fluctuations of random sampling
  - local indel realignment (base alignment quality, BAQ)

Prior probability

- how likely it is to encounter a variant base in the genome?
- some assumptions are made
  - allele frequencies are in Hardy-Weinberg equilibrium

    $P(\text{RA}) = 2f(1-f)$, $P(\text{RR}) = (1-f)^2$, $P(\text{AA}) = f^2$
- can take into account genetic diversity in a population

$$P(G|D) = \frac{P(D|G)\,P(G)}{P(D)}$$

Variant calling example

Inputs

- alignment file
- reference sequence

Outputs

- VCF or BCF file

Example

```
bcftools mpileup -f ref.fa aln.bam | bcftools call -mv
```

Tips

`bcftools mpileup`

- increase/decrease the required number (`-m`) and the fraction (`-F`) of supporting reads for indel calling
- the `-Q` option controls the minimum required base quality (30)
- BAQ realignment is applied by default and can be disabled with `-B`
- streaming the uncompressed binary BCF (`-Ou`) is much faster than the default text VCF

`bcftools call`

- decrease/increase the prior probability (`-P`) to decrease/increase sensitivity

General advice

- take time to understand the options
- play with the parameters, see how the calls change

Factors to consider in calling

Many calls are not real, a **filtering** step **is necessary**

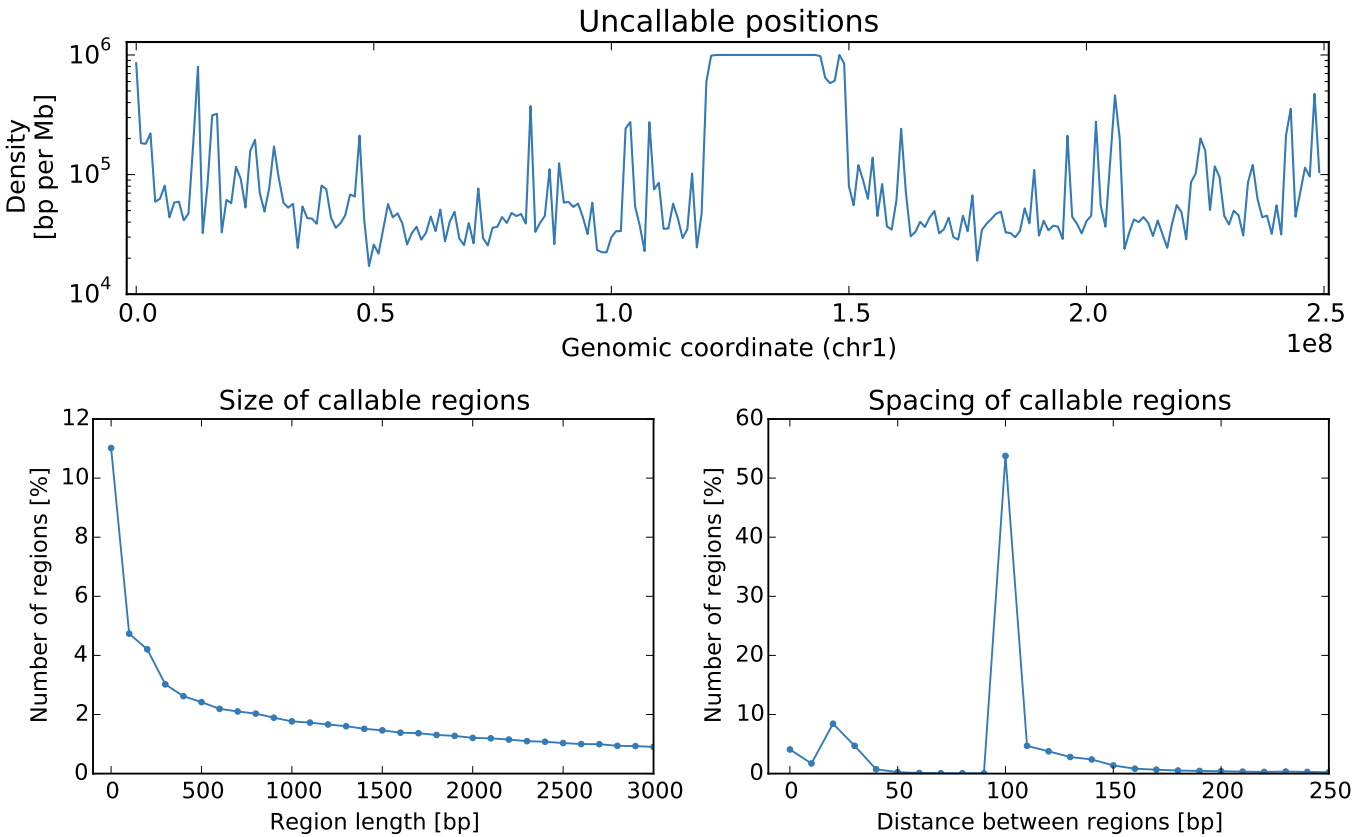
False calls can have many causes

- contamination
- PCR errors
- sequencing errors
  - homopolymer runs
- mapping errors
  - repetitive sequence
  - structural variation
- alignment errors
  - false SNPs in proximity of indels
  - ambiguous indel alignment

Callable genome

Large parts of the genome are still inaccessible

- the Genome in a Bottle high-confidence regions:
  - cover 89% of the reference genome
  - are short intervals scattered across the genome



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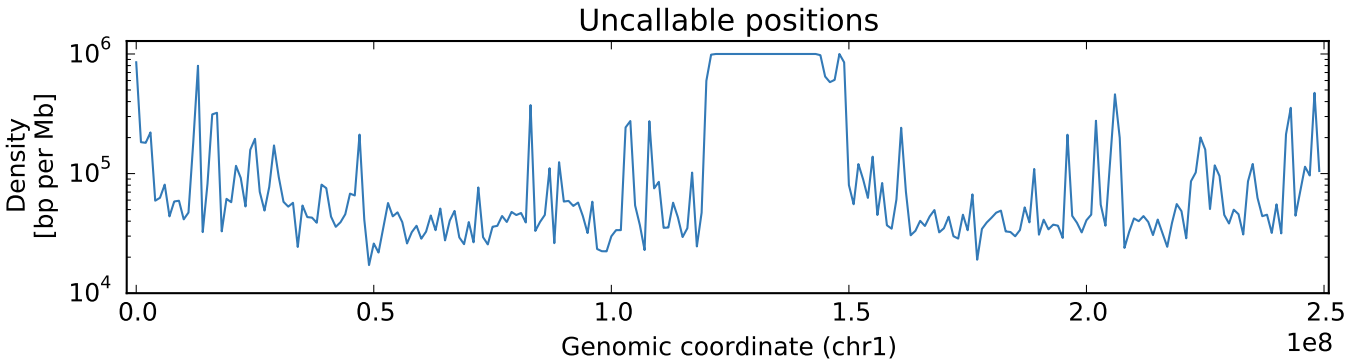
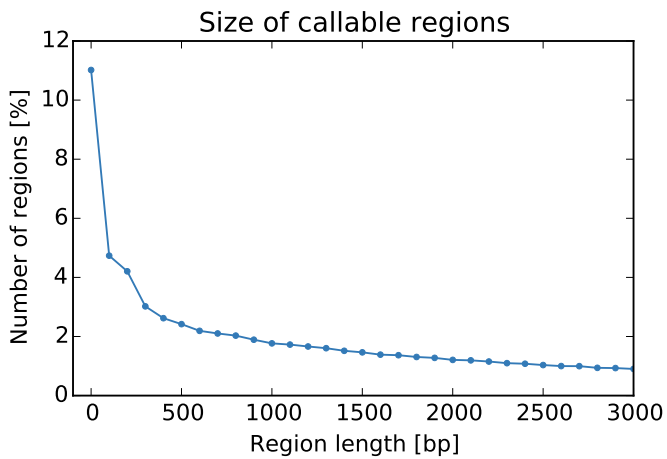
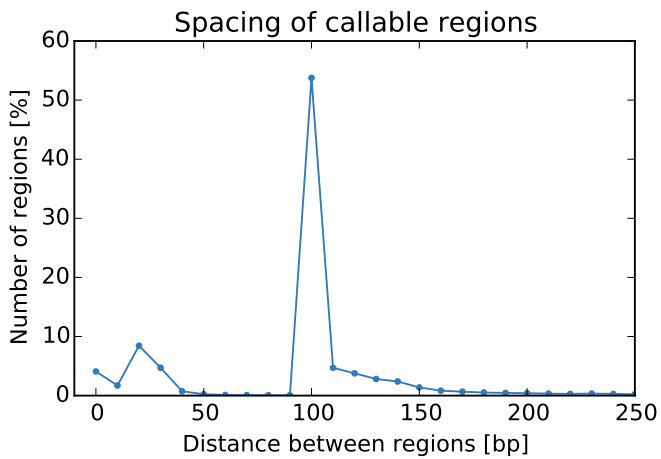
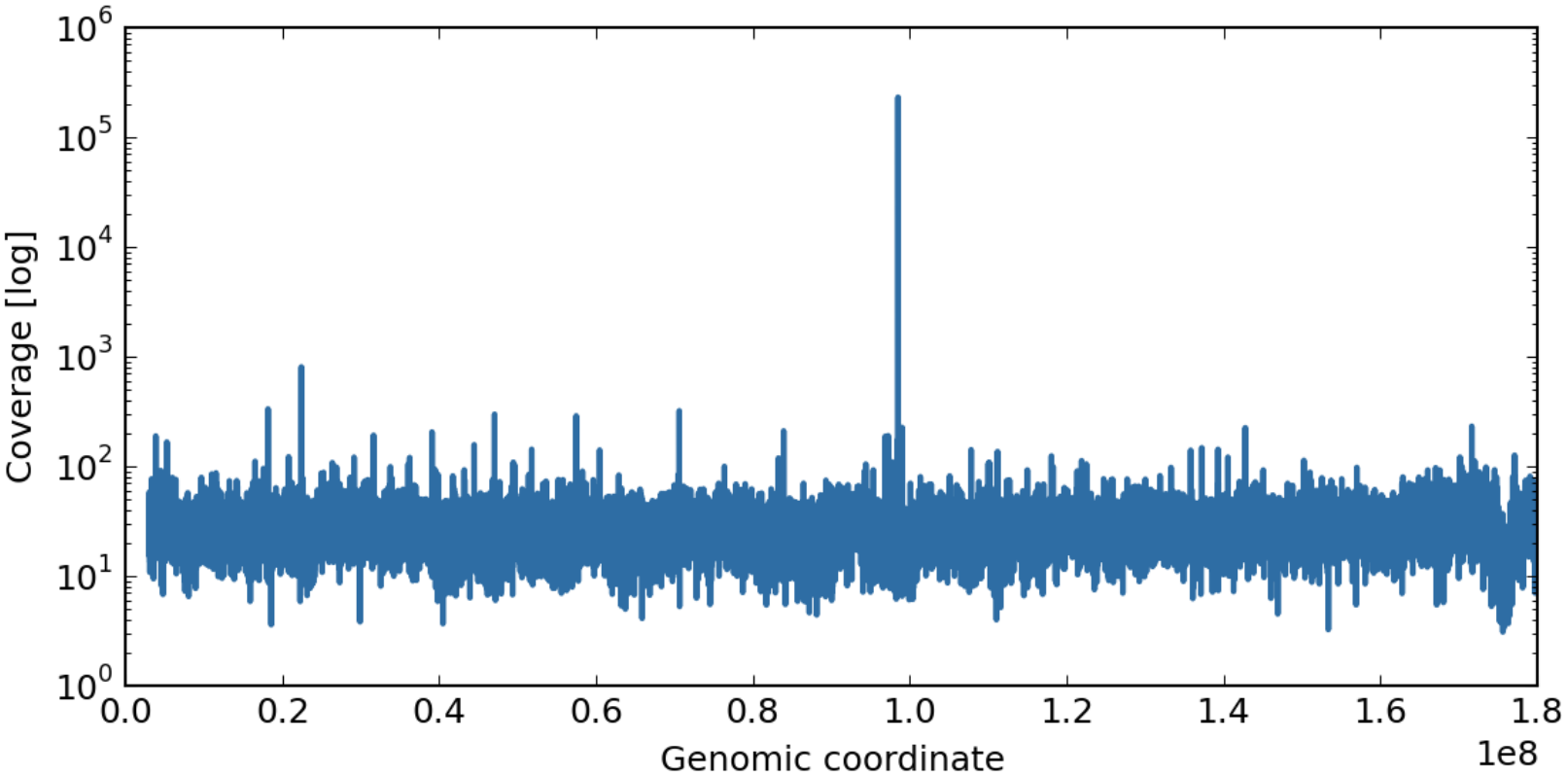






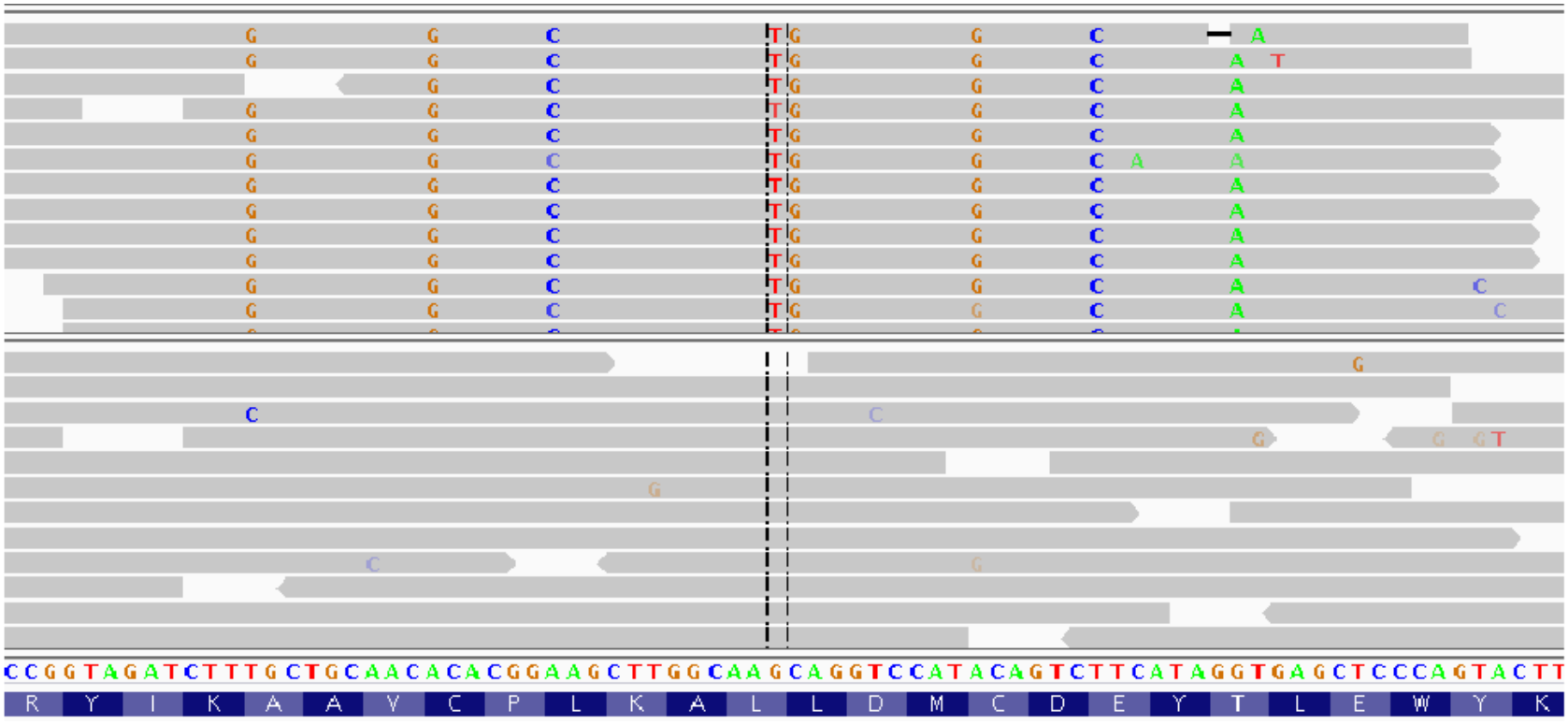
If possible, include only "nice" regions: for many analyses (e.g. population genetics studies) difficult regions can be ignored

Maximum depth



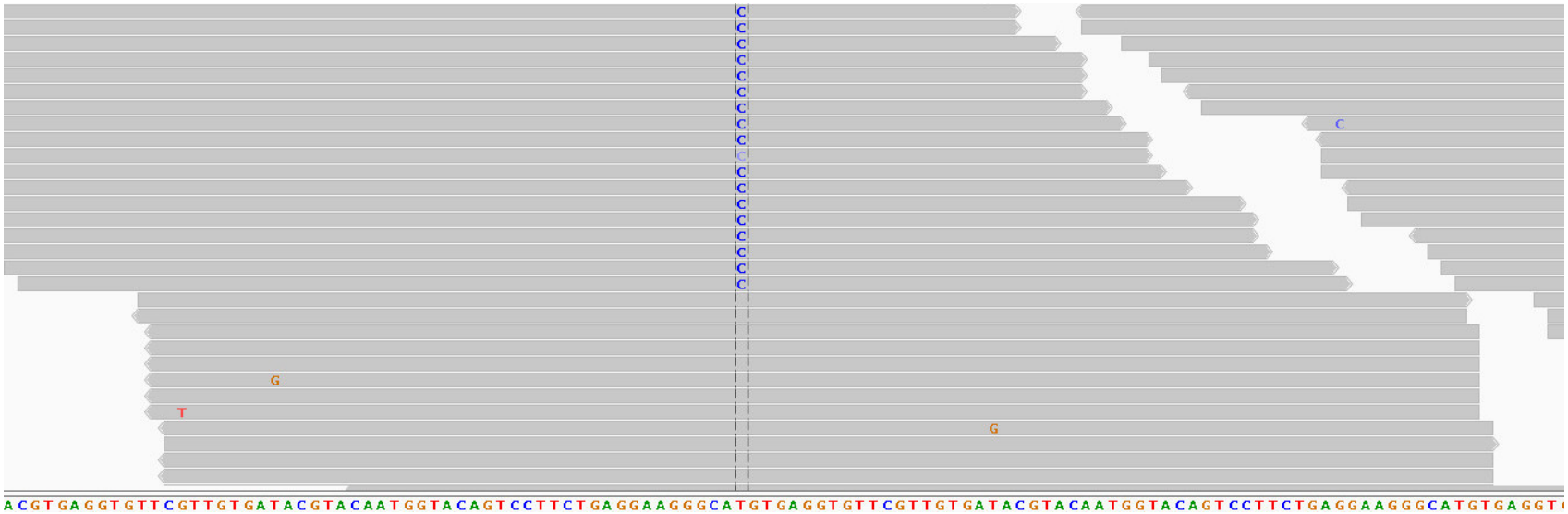
Q: Why is the sequencing depth thousandfold the average in some regions?

Mapping errors



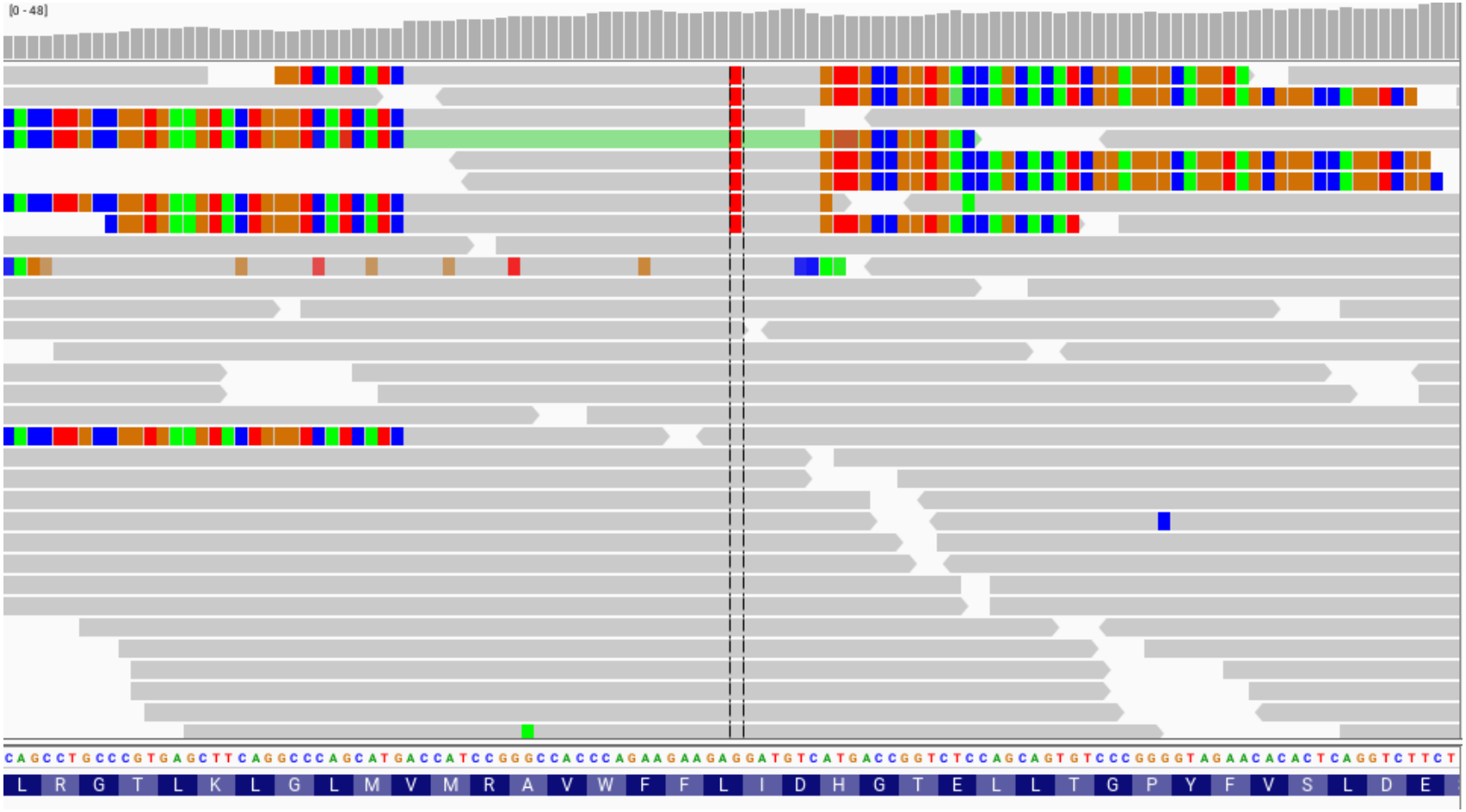
Q: RNA-seq (top) and DNA data (bottom) from the same sample has been mapped onto the reference genome. Can you explain the novel SNVs?

Strand bias



Q: Is this a valid call?

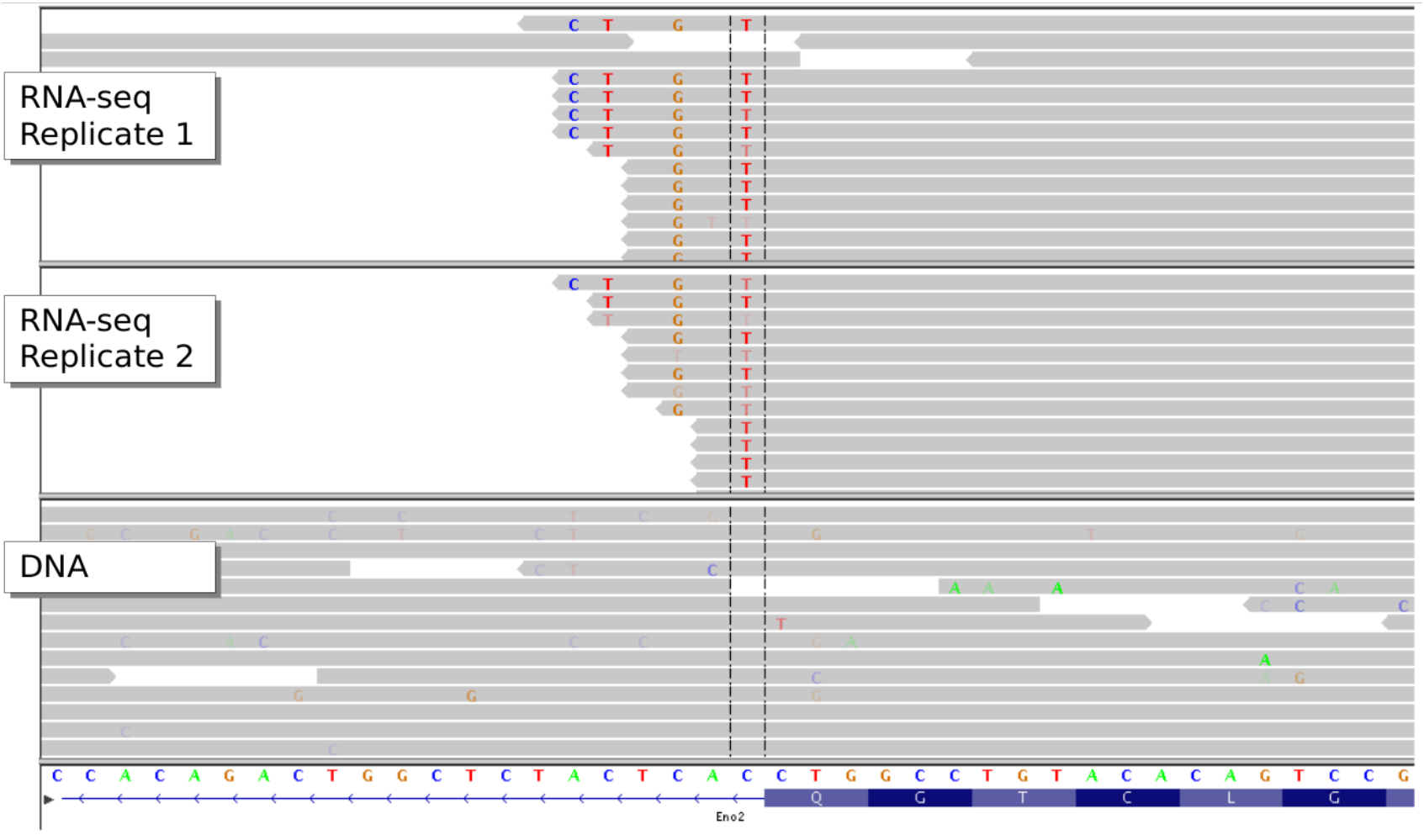
Change the display in IGV to reveal artefacts



Display soft-clipped bases...

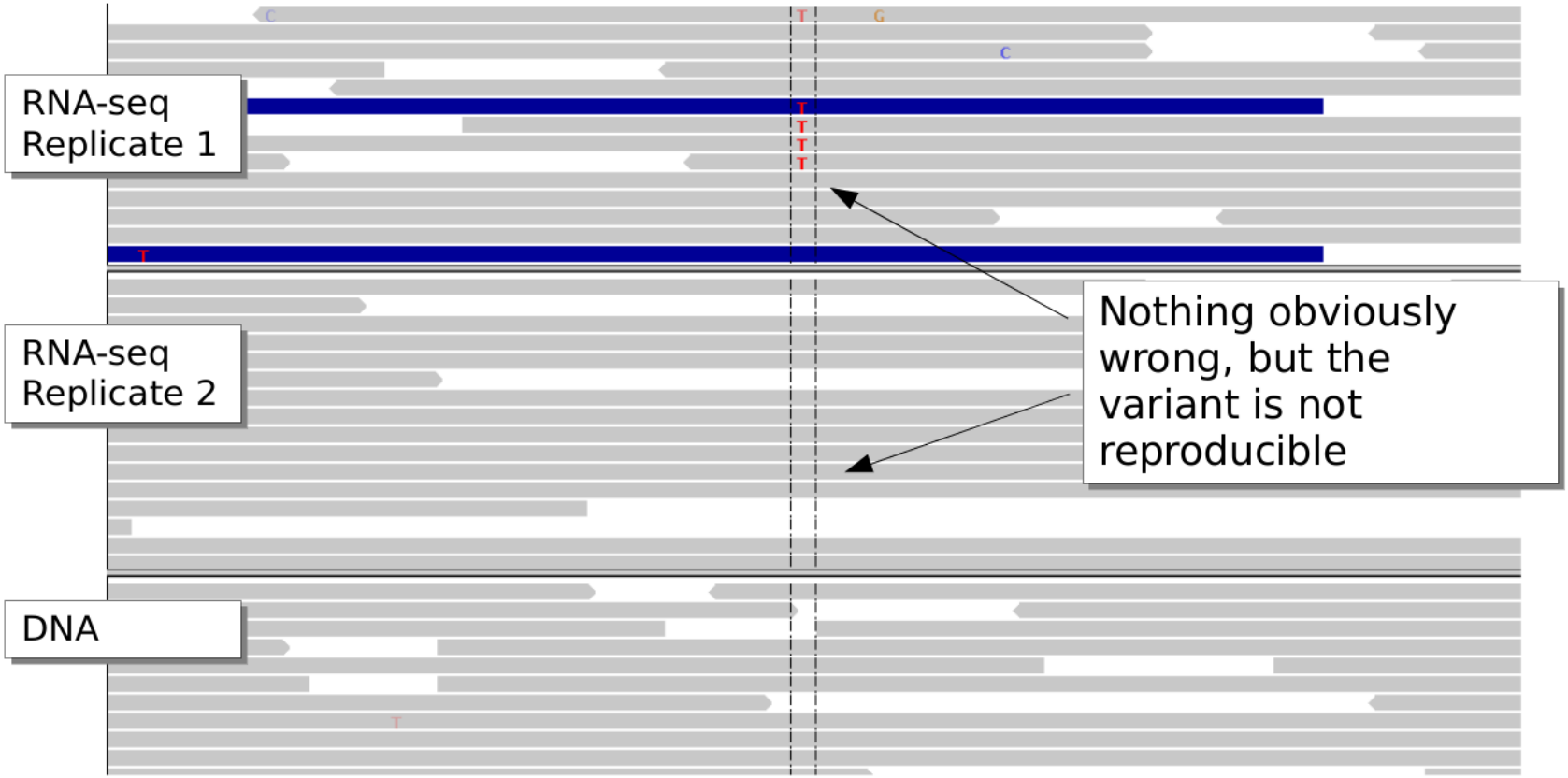
Too many soft-clipped reads in a region suggest mapping errors, beware!

Variant distance bias



Q: Can you explain what happened here?

Reproducibility



Mind the biological variability. If possible, validate and replicate.

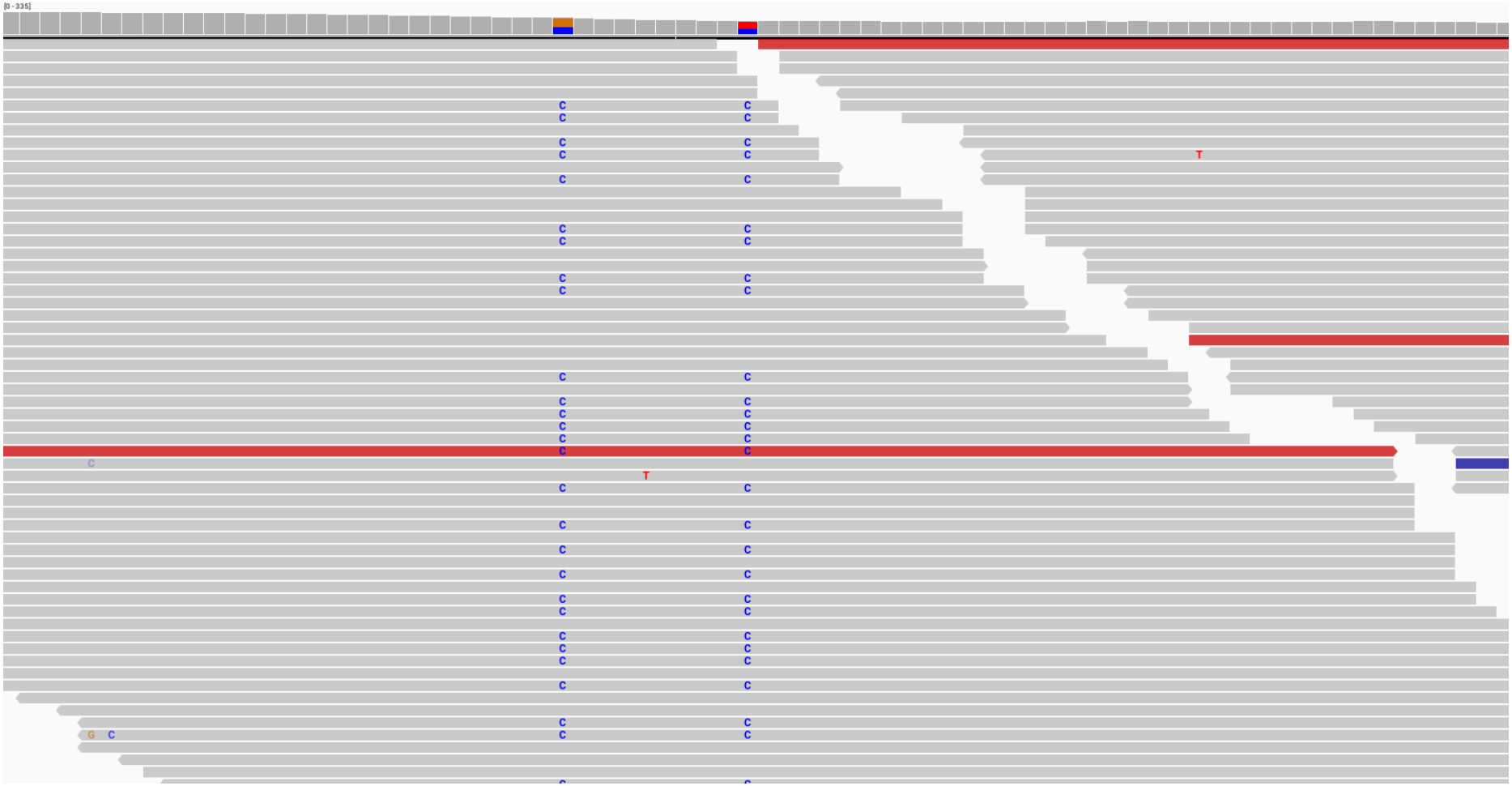
False SNPs caused by incorrect alignment

Pairwise alignemnt artefacts can lead to false SNPs

- multiple sequence alignment is better, but very expensive
- instead: base alignment quality (BAQ) to lower quality of misaligned bases

Q: How many SNPs are real?

What good SNPs look like?



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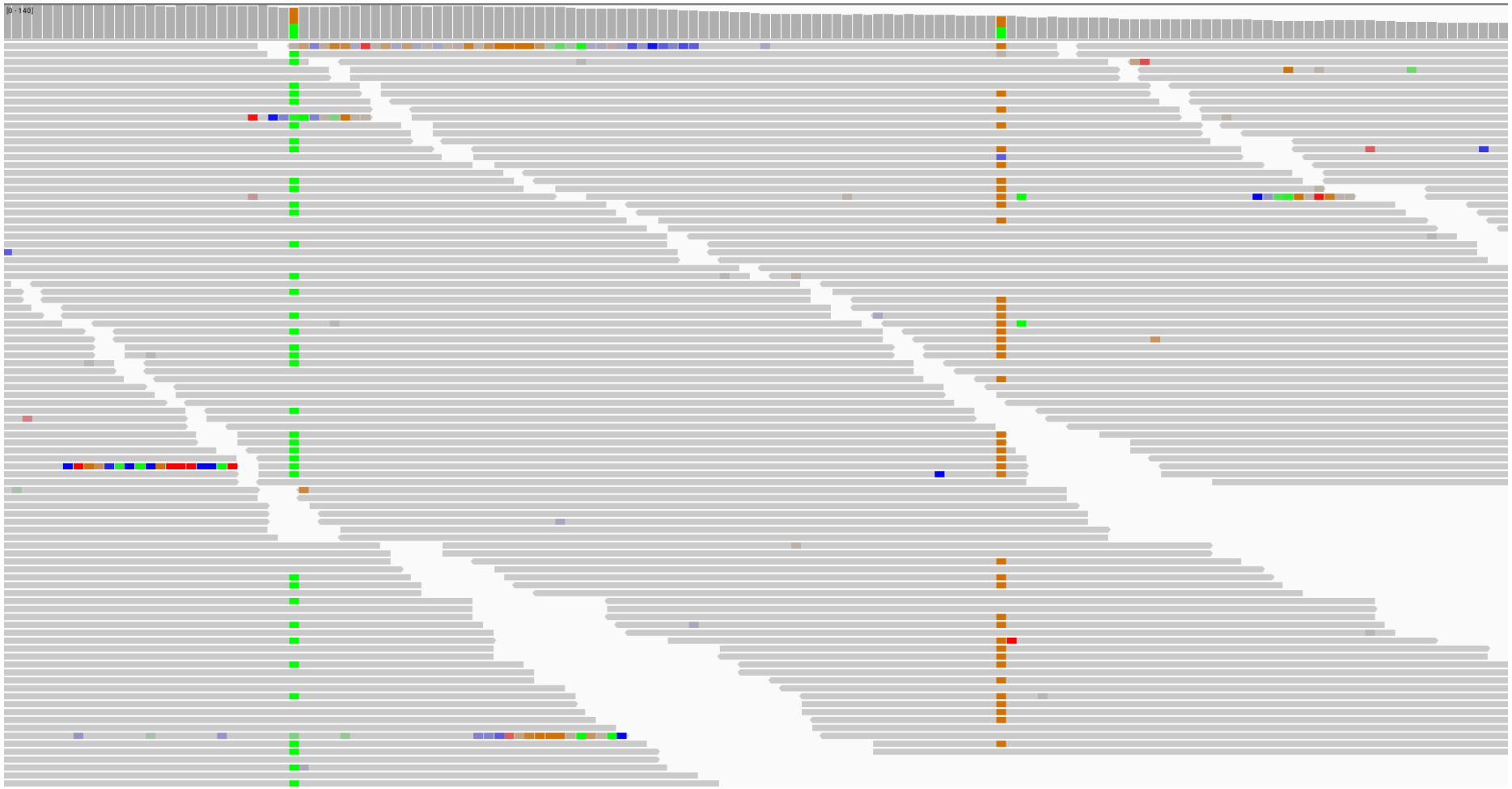


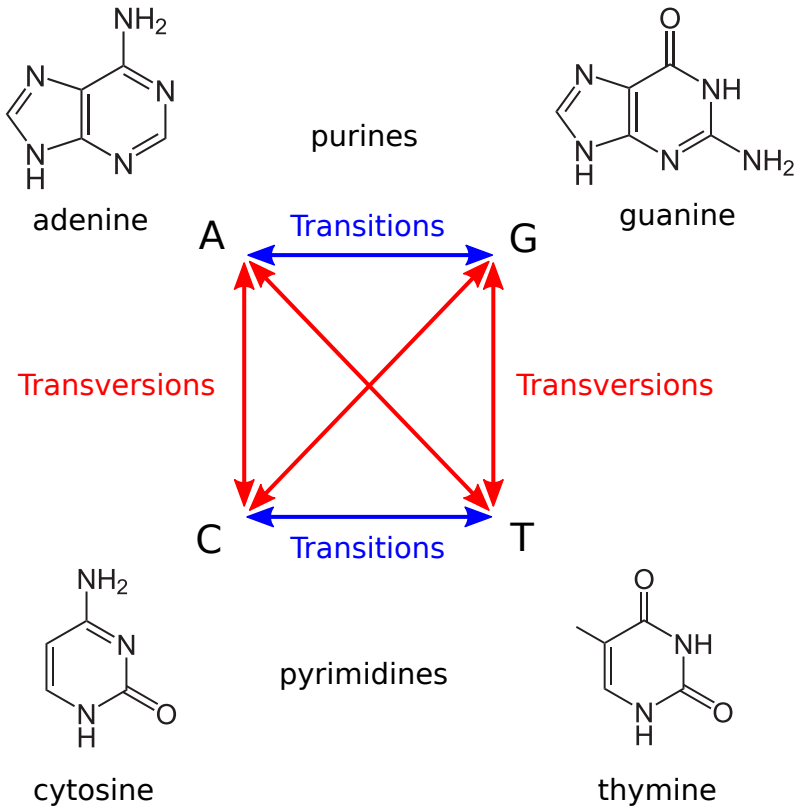
What good SNPs look like?

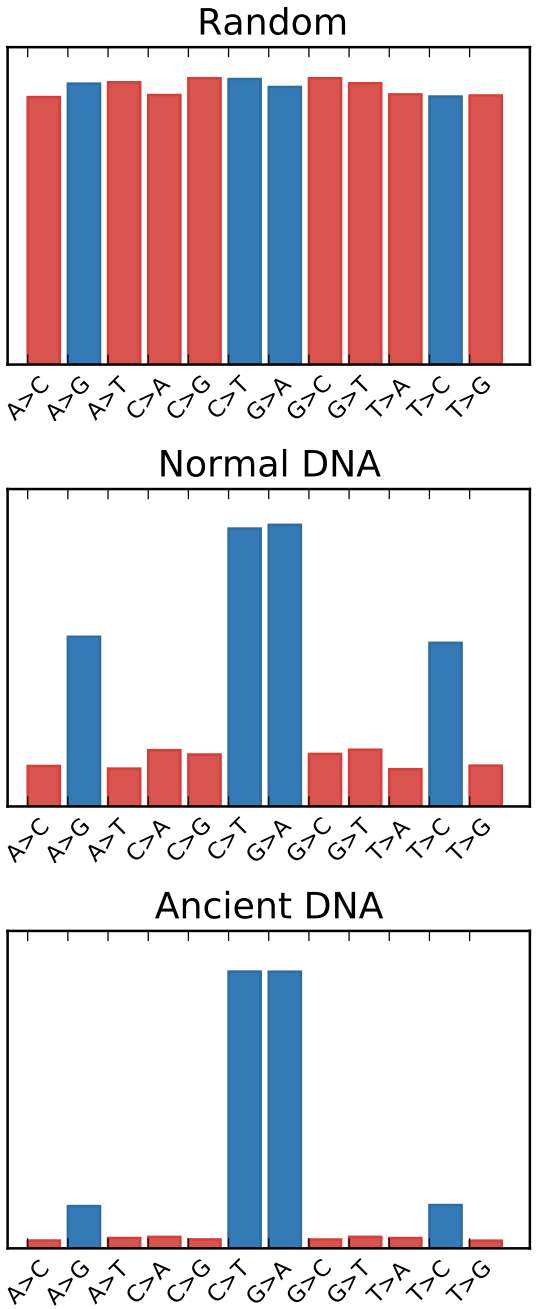
Q: Is this call real? There are many reads with MQ=0.

How to estimate the quality of called SNPs?

Transitions vs transversions ratio, known as ts/tv

- transitions are 2-3× more likely than transversions





Indel calling challenges

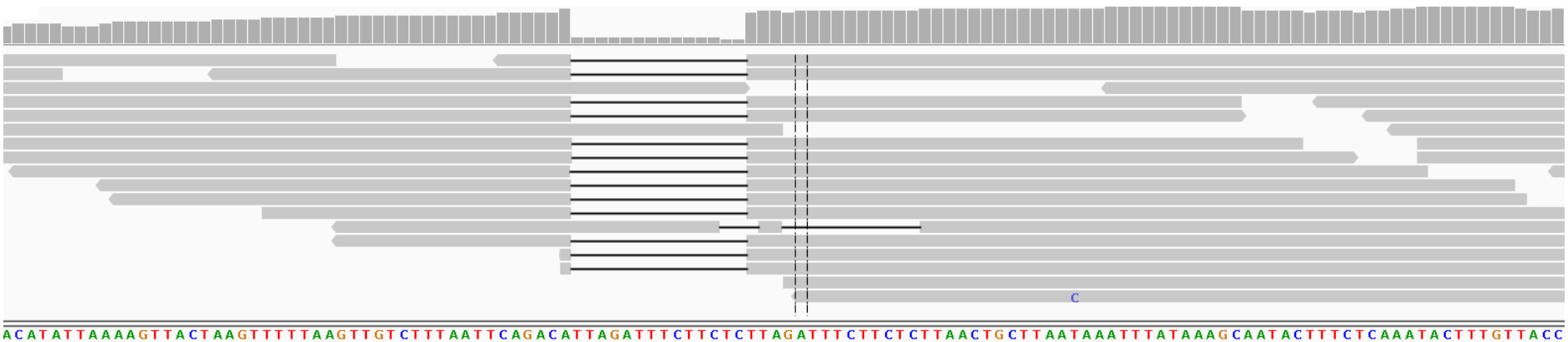
The sequencing error rate is elevated in microsatellites

Low reproducibility across callers

- 37.1% agreement between HapCaller, SOAPindel and Scalpel
  Narzisi et al. (2014) Nat Methods, 11(10):1033

Reads with indels are more difficult to map and align

- the aligner can prefer multiple mismatches rather than a gap
- indel representation can be ambiguous




```
CTTTAATTCAGACATTAGATTTCTTCTC
CTTTAATTCAGACATTAGATTTCTTCTCTTA
CTTTAATTCAGACA---------------TTAGATTTCTTCTCTTAACTGCTT
CTTTAATTCAGACATTAGATTTCTTC---TA----------TTAACTGCTT

CTTTAATTCAGACATTAGATTTCTTCTCTTAGATTTCTTCTCTTAACTGCTT
```

Future of variant calling

Current approaches

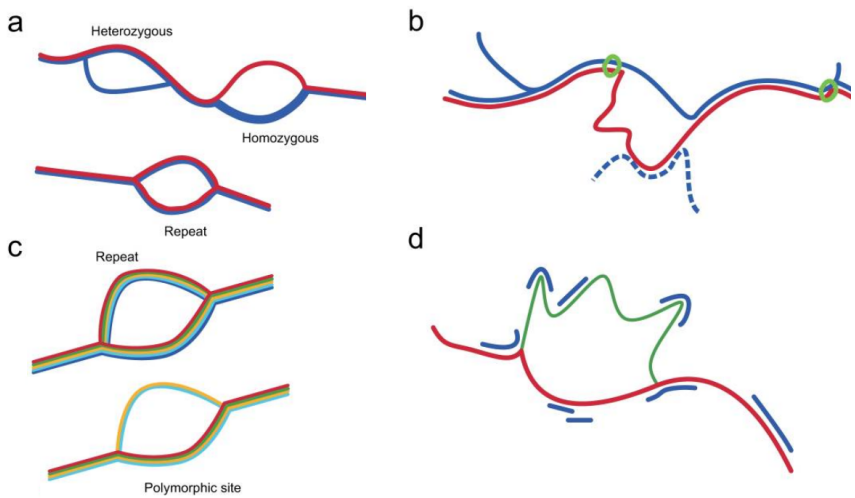
- rely heavily on the supplied alignment, but aligners see one read at a time
- largely site based, do not examine local haplotype and linked sites

Local *de novo* assembly based variant callers

- call SNPs, indels, MNPs and small SV simultaneously
- can remove alignment artefacts
- eg GATK haplotype caller, Scalpel, Octopus

Variation graphs

- align to a graph rather than a linear sequence



Iqbal et al. (2012) Nat Gen 44(2):226

Functional annotation

VCF can store arbitrary INFO tags (per site) and FORMAT tags (per sample)

- describe genomic context of the variant (e.g. coding, intronic, UTR)
- predict functional consequence (e.g. synonymous, missense, start lost)

Several tools for annotating a VCF, only few are haplotype-aware

BCFtools/csq	http://github.com/samtools/bcftools
VEP Haplosaurus	http://github.com/willmclaren/ensembl-vep