Analysis of Sequence Variation

GSND 5340Q, BMDA

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Section 1

Sequence Alignment

Sequence Alignment

- Provides a measure of relatedness
- Alignment quantified by similarity (% identity)
- Useful for any sequential data type:
  - DNA/RNA
  - Amino acids
  - Protein secondary structure
- High sequence similarity might imply:
  - Common evolutionary history
- Similar biological function

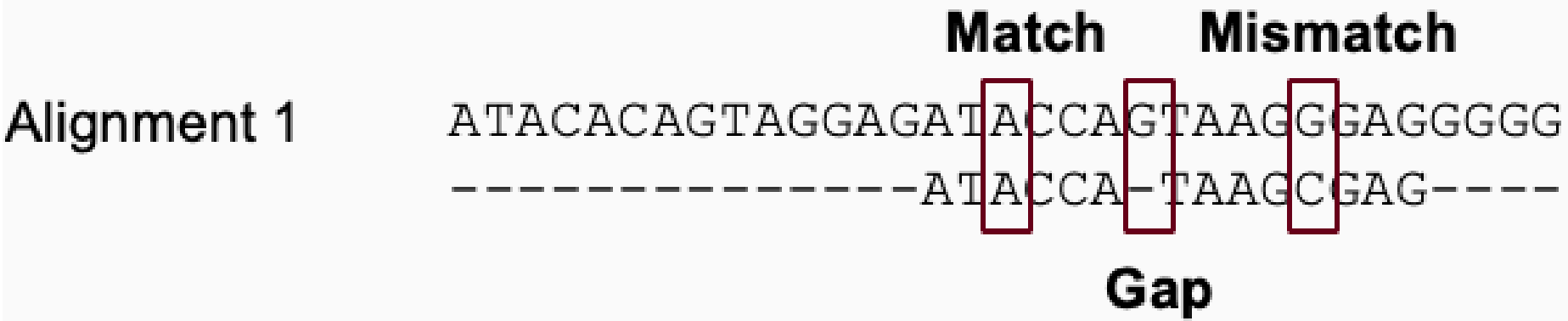
What Alignments Can Tell Us

- Homology - Orthologs, Paralogs
- Genomic identity/origin of a sequence/individual
- Genome/gene structure
- Genic structure (exons, introns, etc)
  - RNA 2D structure
  - Chromosome rearrangements/3D structure

DNA Sequence Alignment Example

Sequence 1 ATACACAGTAGGAGATACCAGTAAGGGAGGGGG

Sequence 2 ATACCATAAGCGAG



Scoring/Substitution Matrices

- Given alignment, how "good" is it?
- Higher score = better alignment
- Implicitly represent evolutionary patterns

	A	C	G	T	-
A	2	-3	-1	-3	-3
C	-3	2	-3	-1	-3
G	-1	-3	2	-3	-3
T	-3	-1	-3	2	-3
-	-3	-3	-3	-3	NA

```
ATACCAGTAAGGGAG
ATACCA-TAAGAGAG
```
Score = 22

```
ATACCAGTAAGG-GAG
ATACCA-TAAG-AGAG
```
Score = 19

```
ATACCA-GTAAGGGAG
A-TACCATAAGAGAG-
```
Score = -20

Sequence Alignment Algorithms

- **Global** alignments - beginning and end of both sequences must align
- **Local** alignments - one sequence may align anywhere within the other
- Multiplicity:
  - Pairwise alignments (2 sequences)
  - Multiple sequence alignment (3+ sequences)

Global Alignment

Both sequences are aligned from end to end

```
AAANTAIYYDPNPDMP A--
NTAI-YDPN--M-
```

Interior sequences are aligned as well as possible

```
AERAKDNLCRLEHTTLRKVTAAANTAIYYDPNPDMPVVAEDQEWVNVYYEM
A-----N------T-----------AI-YD--P------------N----M
```

However, sequences of vastly different length can produce meaningless alignments

Local Alignment

Alignment may begin and end at any position

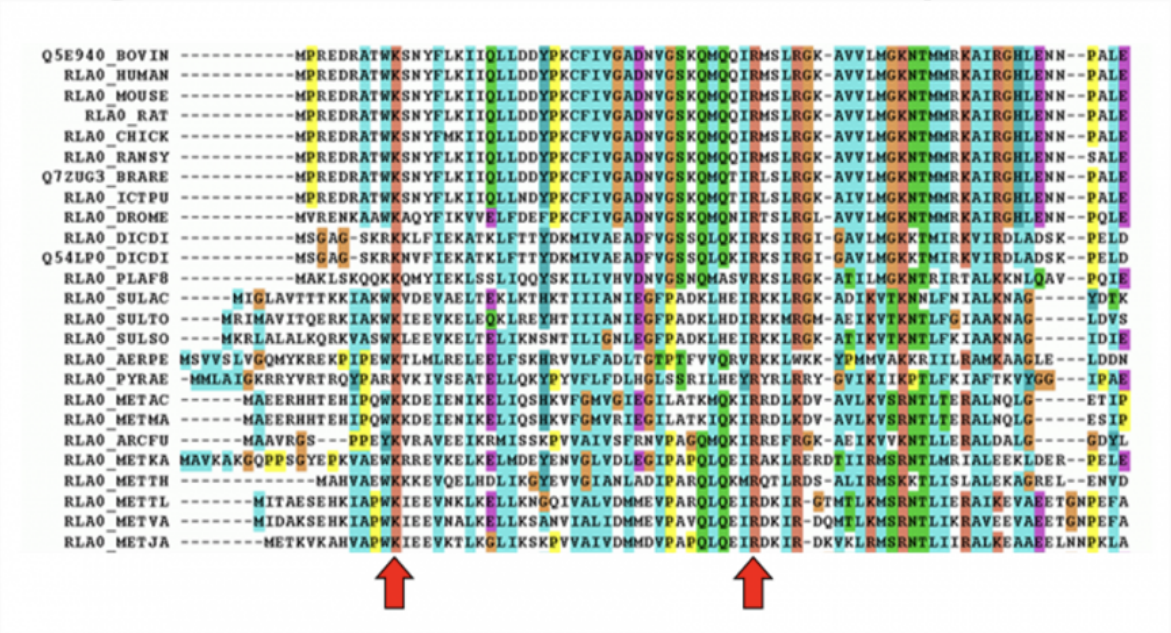
```
AAANTAIYYDPNPDMP -
 AANTAI-YDPN--M-
```

```
AERAKDNLCRLEHTTLRKVTAAANTAIYYDPNPDMPVVAEDQEWVNVYYEM
--------------------AANTAI-YDPN--M----------------
```

Local alignment may produce better alignments when sequence lengths differ greatly

Multiple Sequence Alignment

Like pairwise alignment, but with N sequences



Sequence consensus among many species suggests evolutionary pressure

Methods for Multiple Sequence Aligment (MSA)

1. **Progressive Alignment Algorithms**:
   - *ClustalW*: A widely used progressive alignment tool with a guide tree strategy.
   - *Clustal Omega*: An enhanced version of ClustalW with improved speed and accuracy.
2. **Iterative Alignment Algorithms**:
   - *MAFFT (Multiple Alignment using Fast Fourier Transform)*: Uses iterative refinement with consistency scores.
   - *MUSCLE (Multiple Sequence Comparison by Log-Expectation)*: Utilizes progressive alignment followed by iterative refinement.
3. **Hidden Markov Models (HMMs)**:
   - *HMMER*: Based on HMMs, used for alignment and homology detection.
   - *SAM (Sequence Alignment and Modeling System)*: Combines HMMs with profiles for database searches.

Methods for MSA (Continued)

4. **Probabilistic Alignment Methods**:
   - *ProbCons*: Generates a probabilistic alignment using a Bayesian framework.
   - *PRANK*: Considers sequence and alignment uncertainty in alignment generation.
5. **Structure-Based Alignment**:
   - *MUSTANG (Multiple Structural Alignment by Secondary Structures)*: Aligns based on protein structures considering sequence and structure.
   - *DALI (Distance Alignment Matrix Method)*: Aligns sequences based on structural similarity.

These methods vary in their approaches and are chosen based on factors such as alignment accuracy, computational efficiency, and the characteristics of the input sequences.

ClustalW: A Common MSA Tool

- ClustalW is one of the most widely used tools for multiple sequence alignment.
- It uses a progressive alignment approach.
- Available as standalone software or through a web server.

Example: Aligning TB genomes

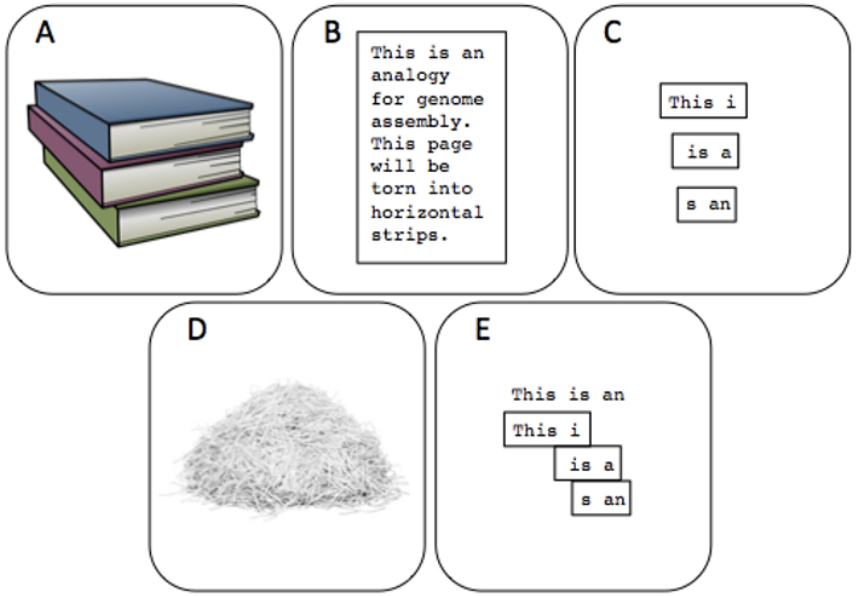
Download the following TB genomes:

- H37Rv
- Mycobacterium tuberculosis str. Erdman
- Combine into single FASTA, first 100 lines:

```
{ head -101 sequence.fasta; head -101 sequence-2.fasta; } \
> combined.fasta
```

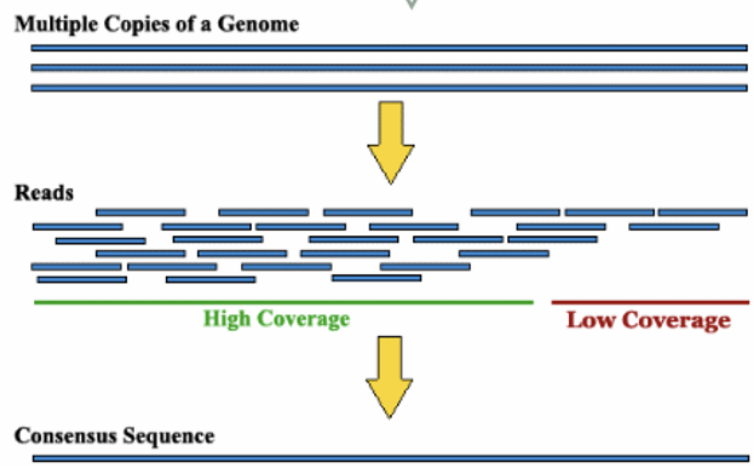
- Analyze using ClustalW

Example: Genome Assembly



If your genome was a book that had its sentences chopped into fragments, assembly is analogous to reconstructing all the sentences.

We need multiple copies of each book (genome) to arrive at a *consensus* text (DNA sequence) of the original



Example: Genome Assembly

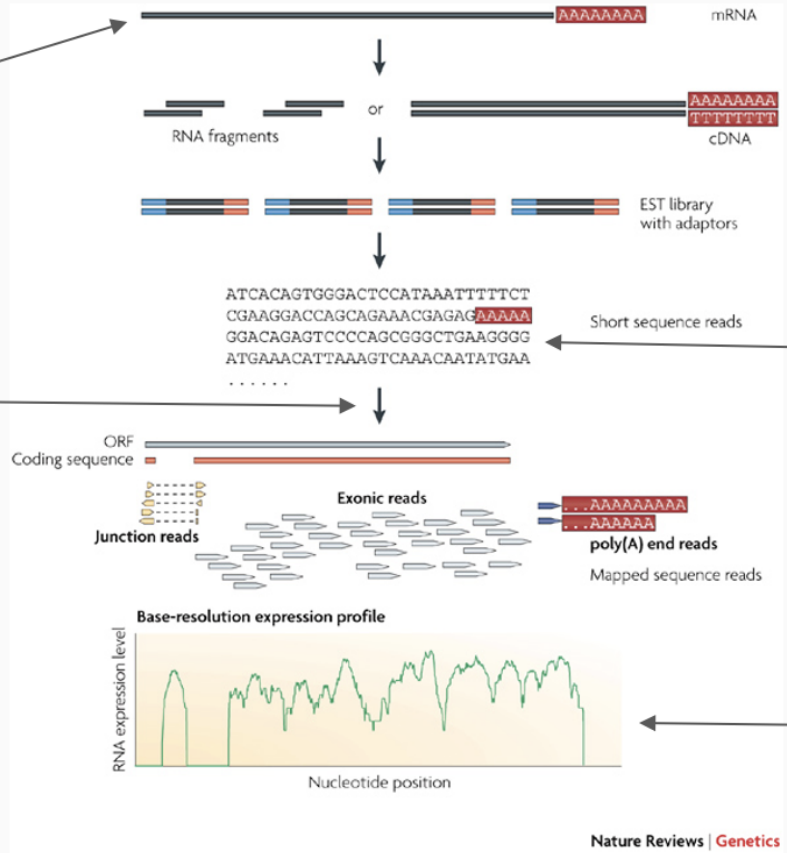
An error?
A polymorphism?
A different allele?
Incorrect alignment?

Greedy approach: take most frequent nucleotide at each aligned position

Example: mRNA-Seq Analysis

Start with a pool of mRNA molecules

Find all locations where sequences map in genome

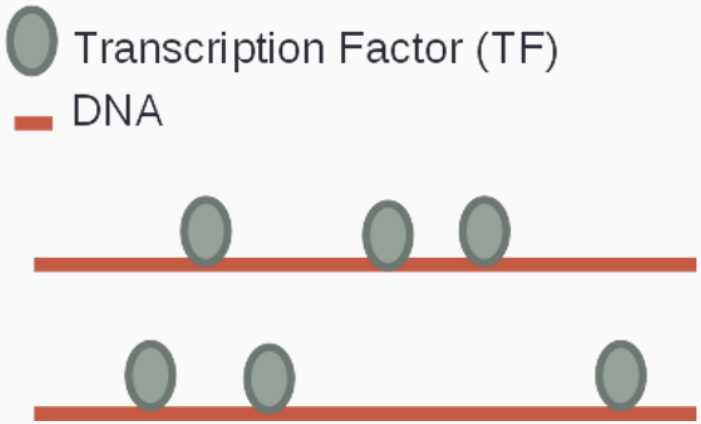


Millions of DNA sequences 30-150 nucleotides long

Count the number of sequences that map to individual regions (e.g. genes)

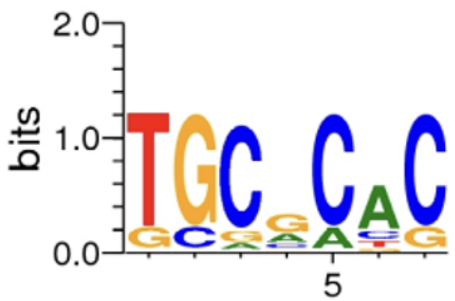
Example: DNA Binding Site Discovery

Identify genomic regions where a particular TF is bound across the entire genome



By extracting and aligning the DNA sequence corresponding to these binding events, we can identify which DNA sequences this TF tends to bind

...TGCTCAC...
...TGCACAC...
...GGCGCAC...
...TGAGCAC...
...TCGCCTC...
...TGCAACG...
...TCCCACG...
...GGTACTC...



Section 2

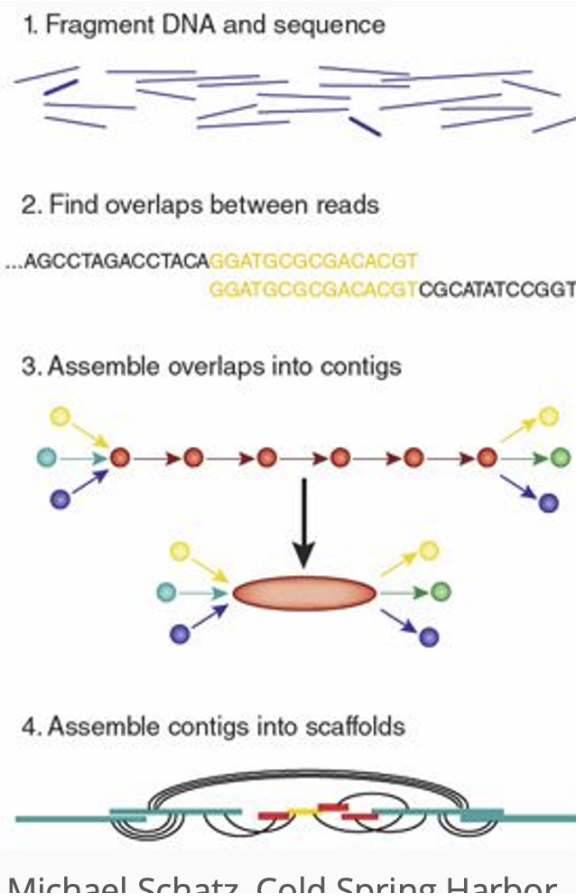
Whole Genome/Exome Sequencing

Whole Genome Sequencing

- **W**hole **G**enome **S**equencing (WGS)
- Generate enough reads to attain:
  - More than 95% coverage of source genome
  - More than 30x average depth
- Two strategies:
  - De novo: assemble reads into a new sequence
  - Re-sequence: refine an existing reference sequence

De novo assembly

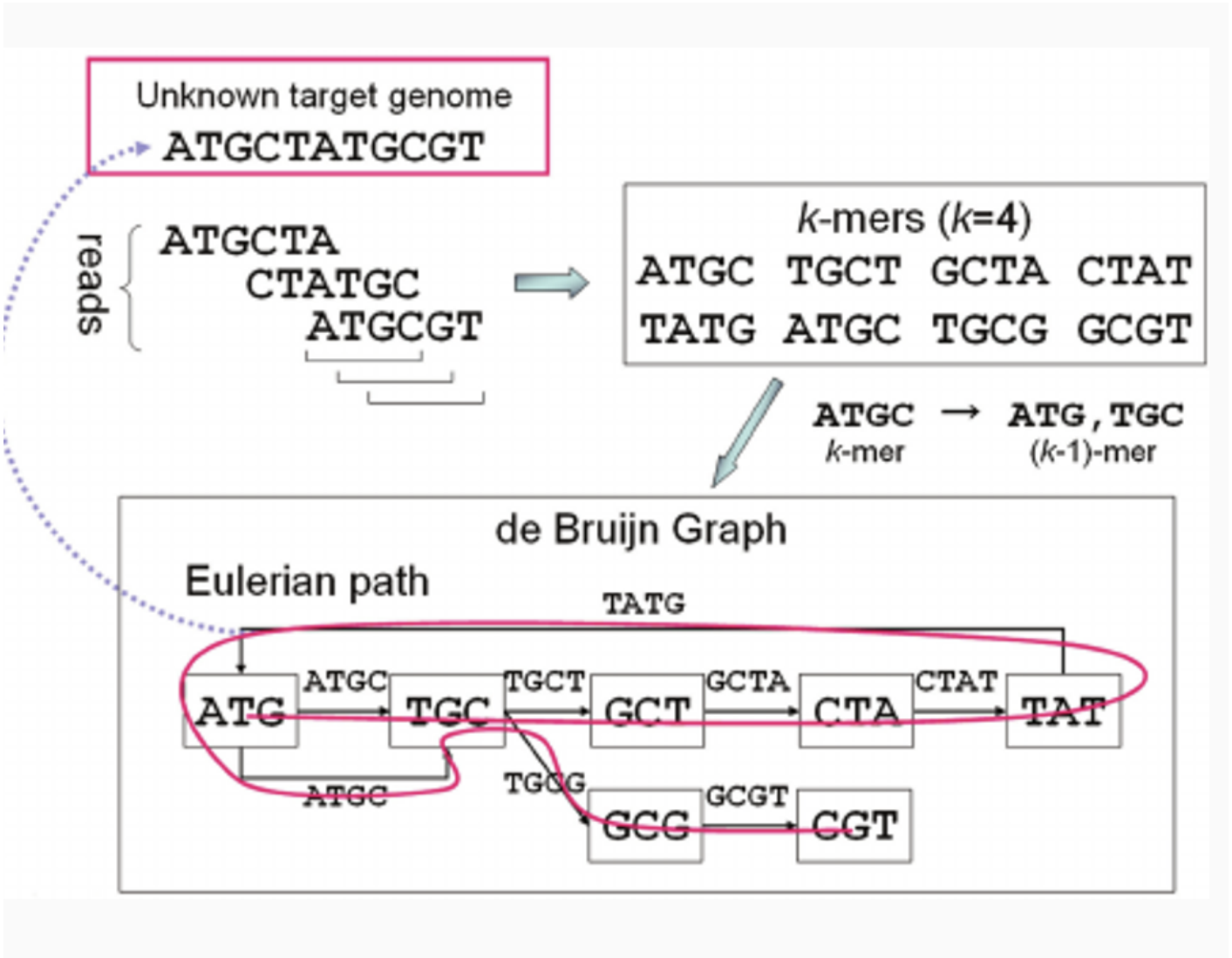
- **Genome Assembly** - Create new reference 'from scratch'
- Examine reads for overlapping sequence
- **Contig** - longer assembled sequence from short reads
- **Scaffold** - assembled contigs
- **Chromosome** - assembled scaffolds
- Assembly from short reads is hard



De novo assembly: graph-based

- Greedy assembly creates linear sequences
- Vulnerable to finding local optima
- Graph representation considers all sequence content simultaneously
- Graph data structure can encode variability (e.g. insertions, SNPs)
- Computationally much more expensive
- de Brujin Graphs and Overlap Layout Consensus

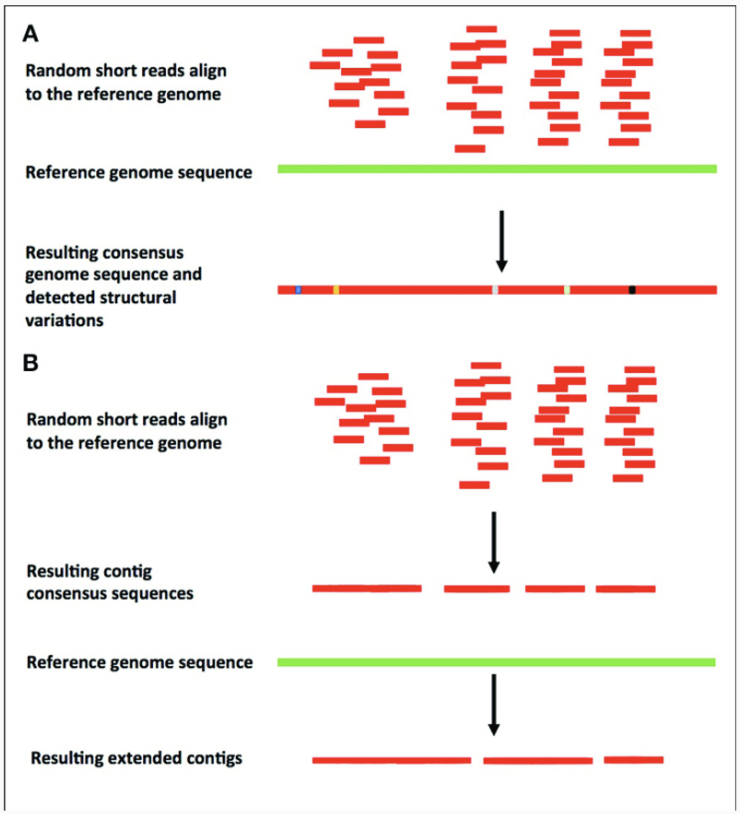
De novo assembly: graph-based

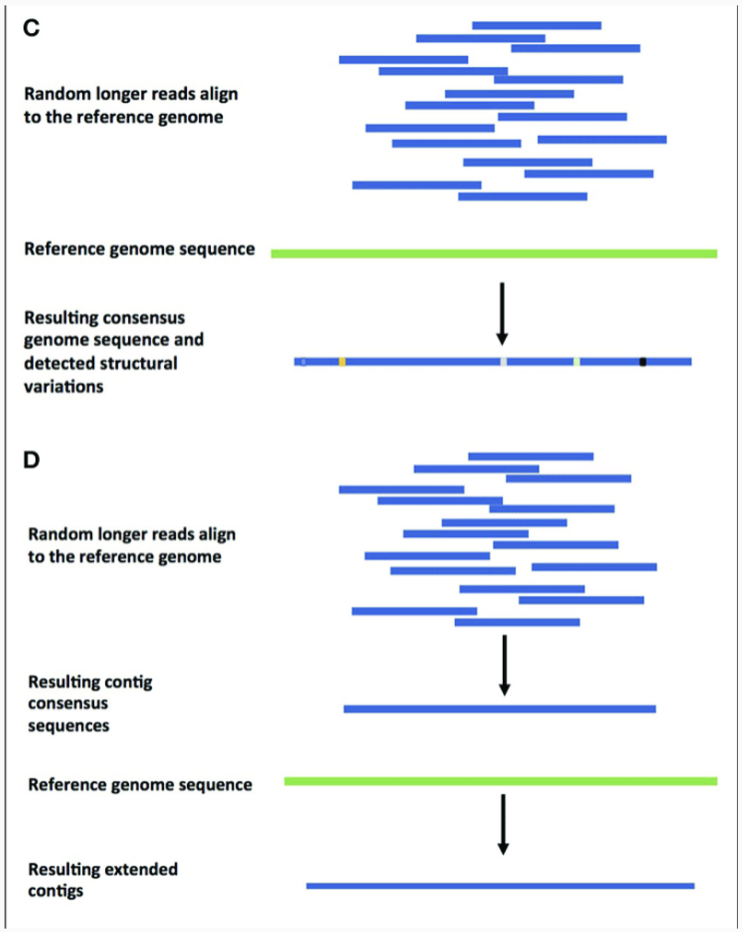


Reference guided genome assembly

- Refine existing reference with new sequence
- Can discover:
  - New structural variants
  - Novel insertions/alternate haplotypes or scaffolds
  - Polymorphisms
- Faster, easier than de novo assembly
- More sensitive to existing biases in reference

Reference guided genome assembly





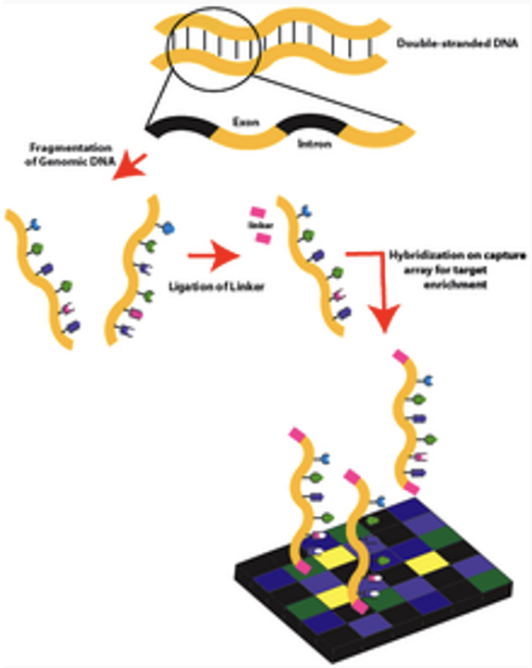
Kyriakidou, Maria, Helen H. Tai, Noelle L. Anglin, David Ellis, and Martina V. Strömvik. 2018. "Current Strategies of Polyploid Plant Genome Sequence Assembly." *Frontiers in Plant Science* 9 (November): 1660.

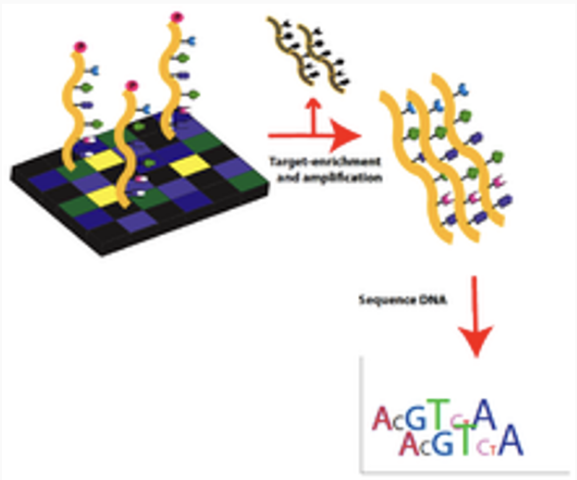
Whole Exome Sequencing

- **W**hole **E**xome **S**equencing (WES)
- Exons 1%-2% of human genome sequence
- Pre-select reads that map to exons
- Sequence to much greater depth than WGS
- Identify coding variants

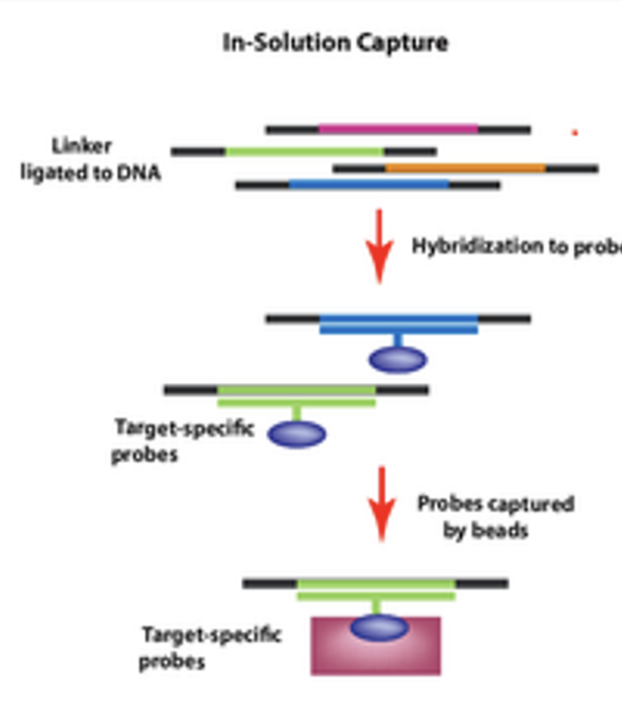
Exome Sequence Selection

Array-based capture





In-solution



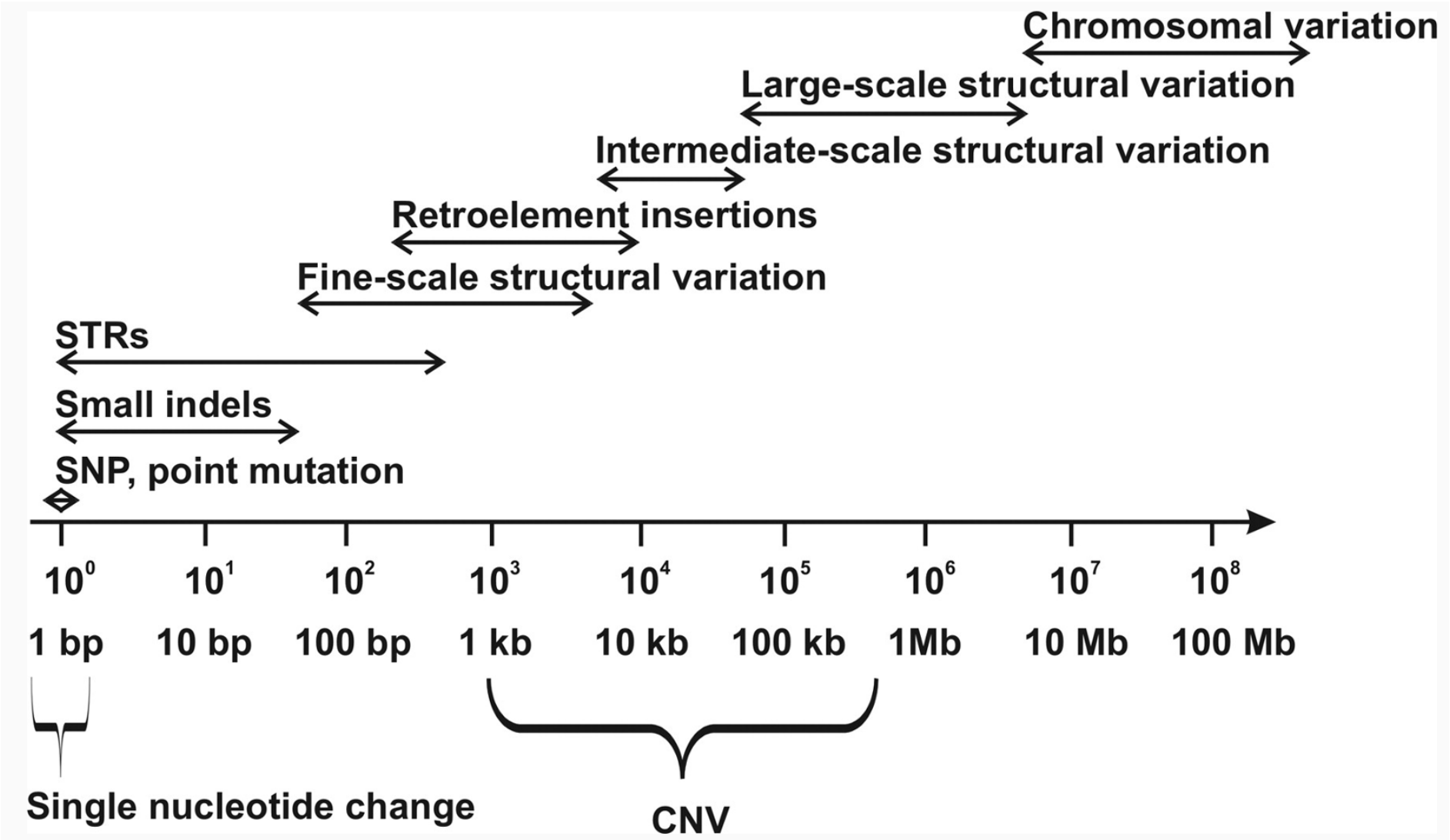
Section 3

SNP and Variant Calling

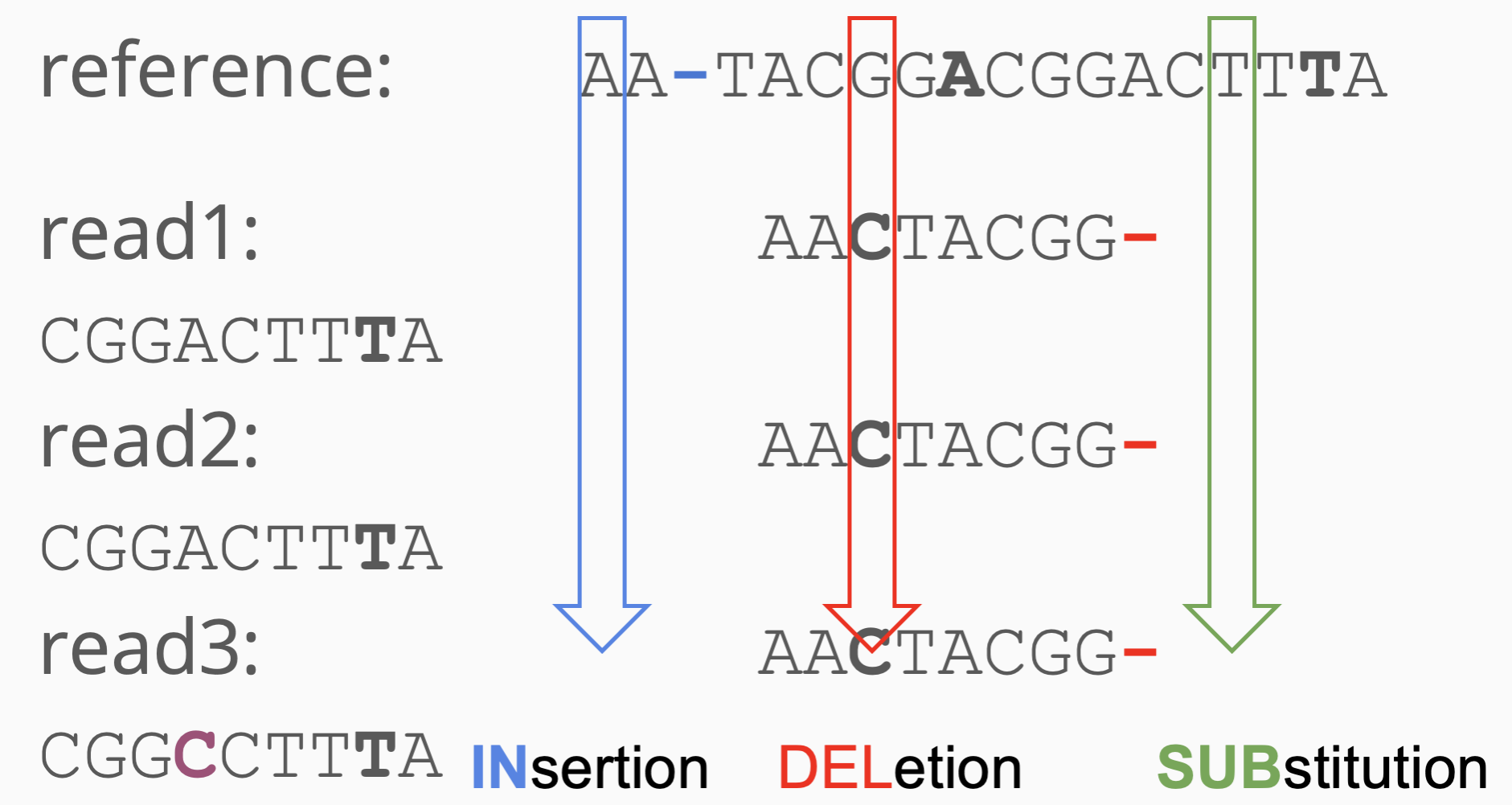
Genomic Variants

- Individual genomes from same species vary
- WGS/WES compared with reference can identify differences
- **Variant**: sequence that varies within species
- Two general types:
  - Small: <50 bp, single nucleotide polymorphisms (SNPs), indels
  - Large: >50 bp, copy number variations, duplications, deletions, translocations, inversions

Genomic Variants



Point mutations



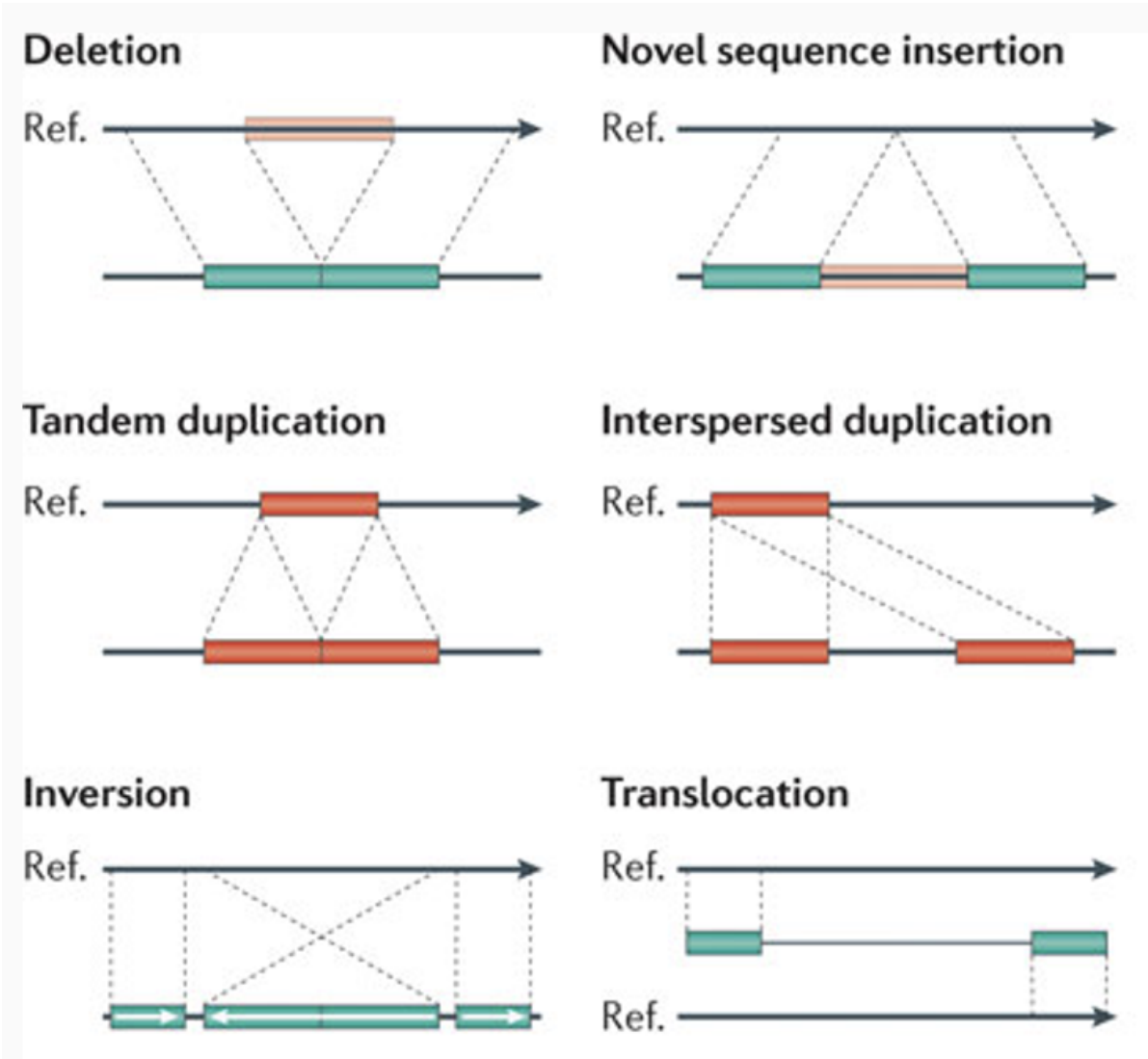
Single Nucleotide Polymorphisms (SNPs)

- Most commonly studied type of variant
- Types of single nucleotide alterations:
  - **Variant** (SNV) - any single mutation
  - **Polymorphism** (SNP) - SNV observed in significant frequency in population (e.g. $>1\%$)
- Typically base changes, e.g. A to C
- SNPs (usually) indicate shared ancestry
- May suggest disease mechanism

Structural variation

- Deletions - sequence missing
- Insertions:
  - Novel - new sequence added
  - Mobile-element - copied/moved from elsewhere in genome
- Duplications:
  - Tandem - consecutive
  - Interspersed - non-consecutive
- Inversion - segment is reversed
- Translocation - segment moves

Structural variation



Genotyping

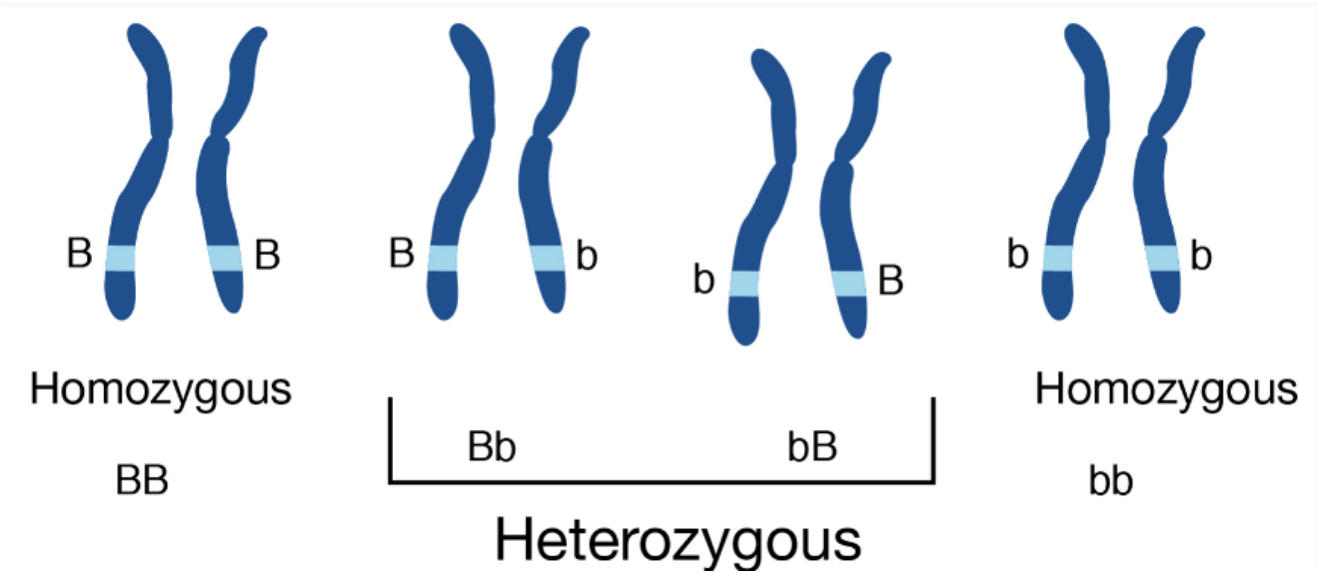
- **Genotype**: an individual's variant(s)
- **Phenotype**: an individual's physical form
- **De novo** variant calling/detection: given genomic reads and a reference, find all the variants
- **Genotyping**: examine individual for *a priori* variants at known locations
  - Can use arrays (i.e. SNP Chip) or WGS/WES

Human Genomic Variants

- Humans have diploid genome
  - i.e. 2 copies of each gene
- dbSNP - NCBI repository for SNPs
  - As of June 2021 (Build 155)
  - 3.3B submitted variants
  - 1B annotated human SNPs Every human has on average:
  - A variant every 1kb
  - 2-3 million SNPs

Human Genomic Terminology

- **Allele**: sequence containing a variant
- **Homozygous variant**: both same allele
  - i.e. either same variant or same as reference
- **Heterozygous variant**: different alleles
  - i.e. one is variant, one is reference/different variant



https://www.genome.gov/genetics-glossary/heterozygous

Human Genomic Terminology

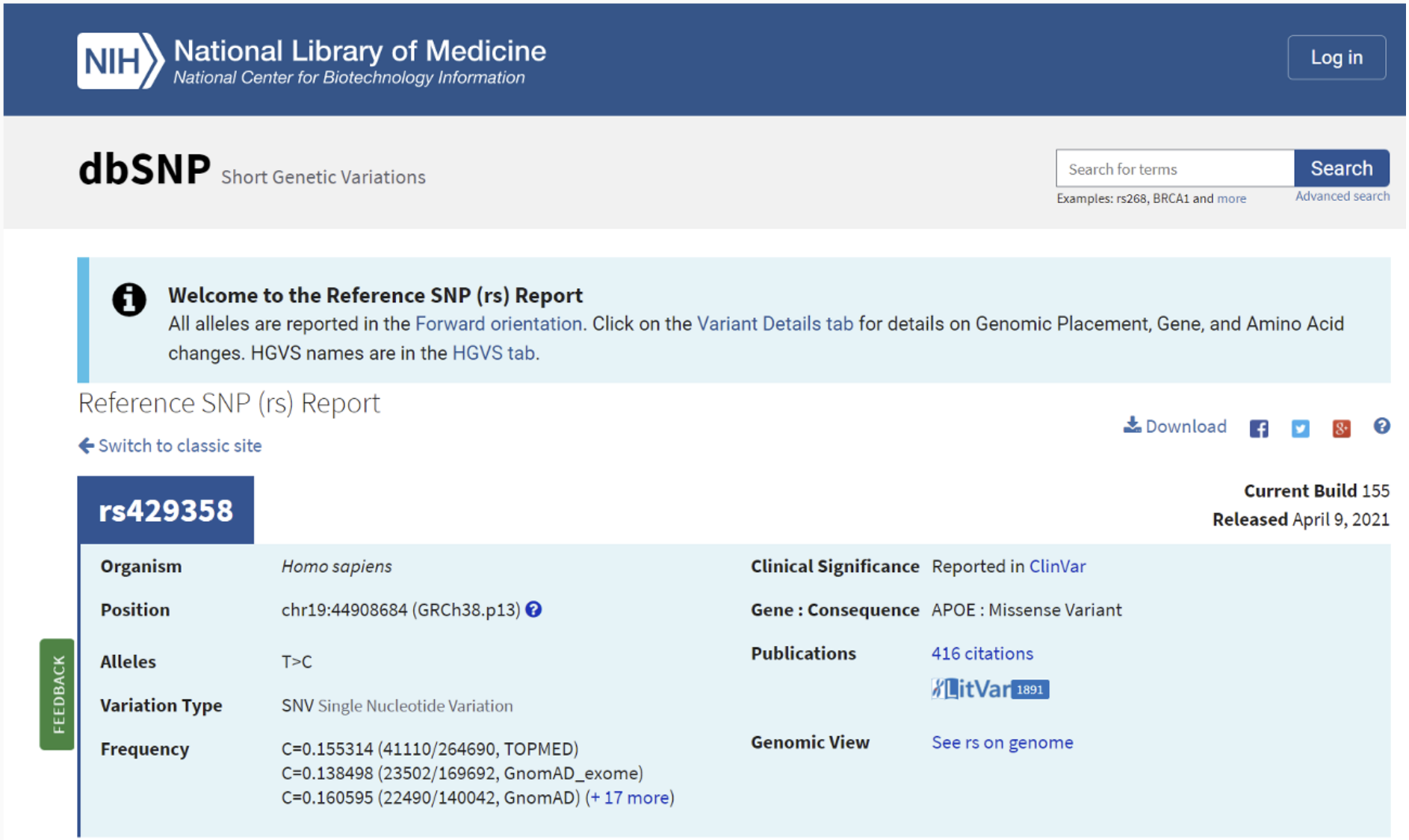
For coding (exonic) variants:

- **Synonymous or sense**: variant does not change amino acid sequence
- **Non-synonymous or mis-sense**: variant causes amino acid change
- **Non-sense**: causes early termination of protein by introducing stop codon
- **Frameshift**: insertion or deletion causes complete recoding of downstream proteins

Genomic Variant Terminology

- **Germline**: Inherited from parents
  - e.g. blue eyes, familial disease risk
- **Somatic**: Acquired during life
  - e.g. tumor vs normal tissue
- **Allele frequency**: how common is a given variant in some population, e.g.:
  - 1% of human population
  - 30% within people with some disease

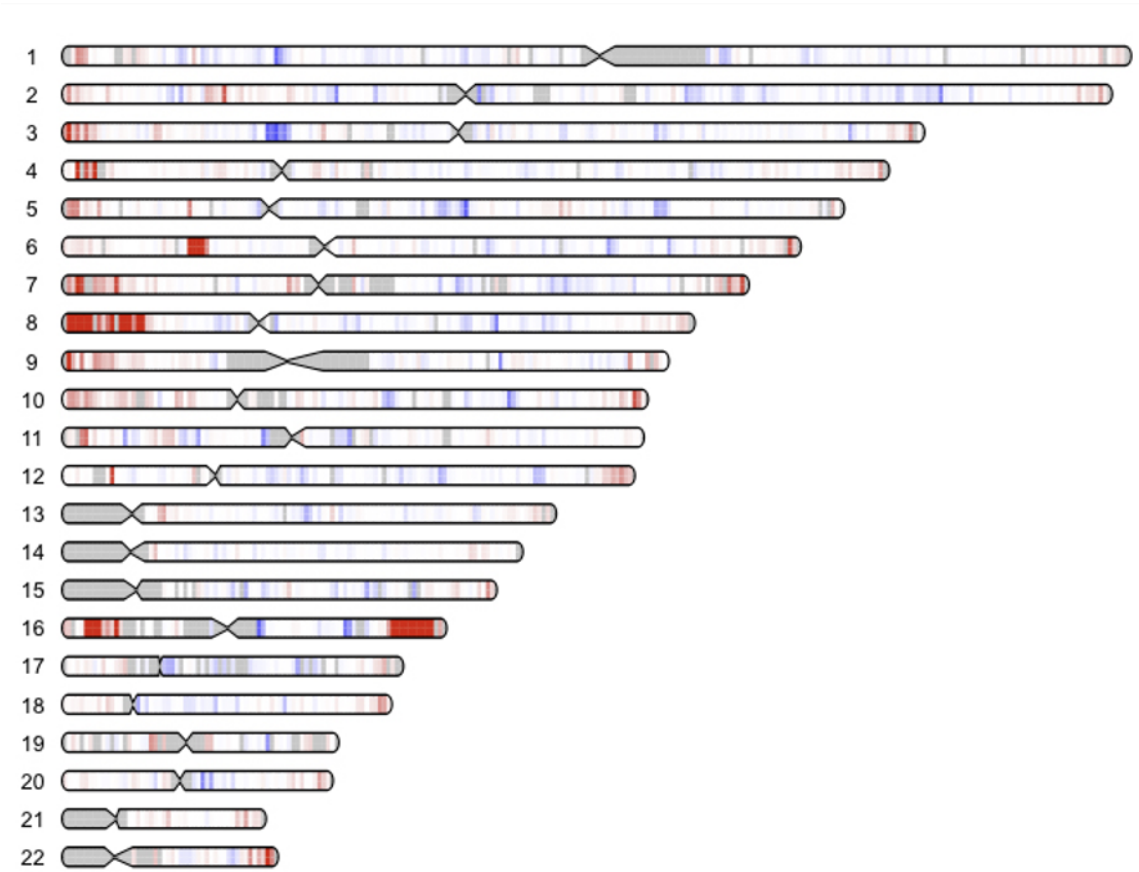
dbSNP - NCBI SNP Database

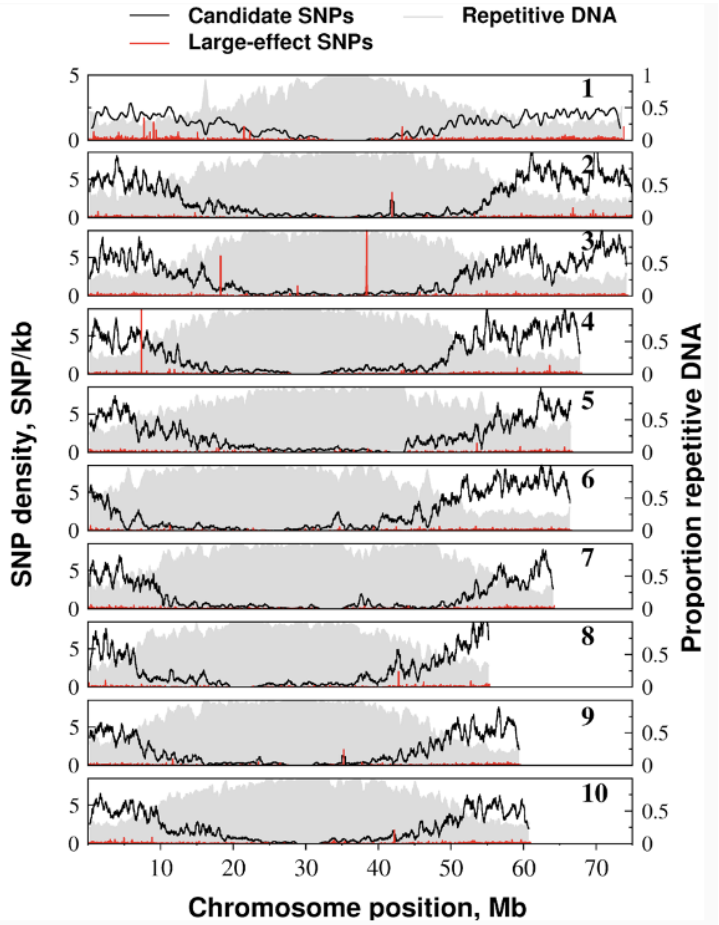


Variants Smaller Than A Read

- Finding SNPs, indels almost a solved problem
- SNPs called are 95% accurate
  - i.e. with sufficient coverage
- Structural variants cause false positives
- Duplications, somatic mutations may cause 3 or more alleles to be observed

SNP and indel density is non-random





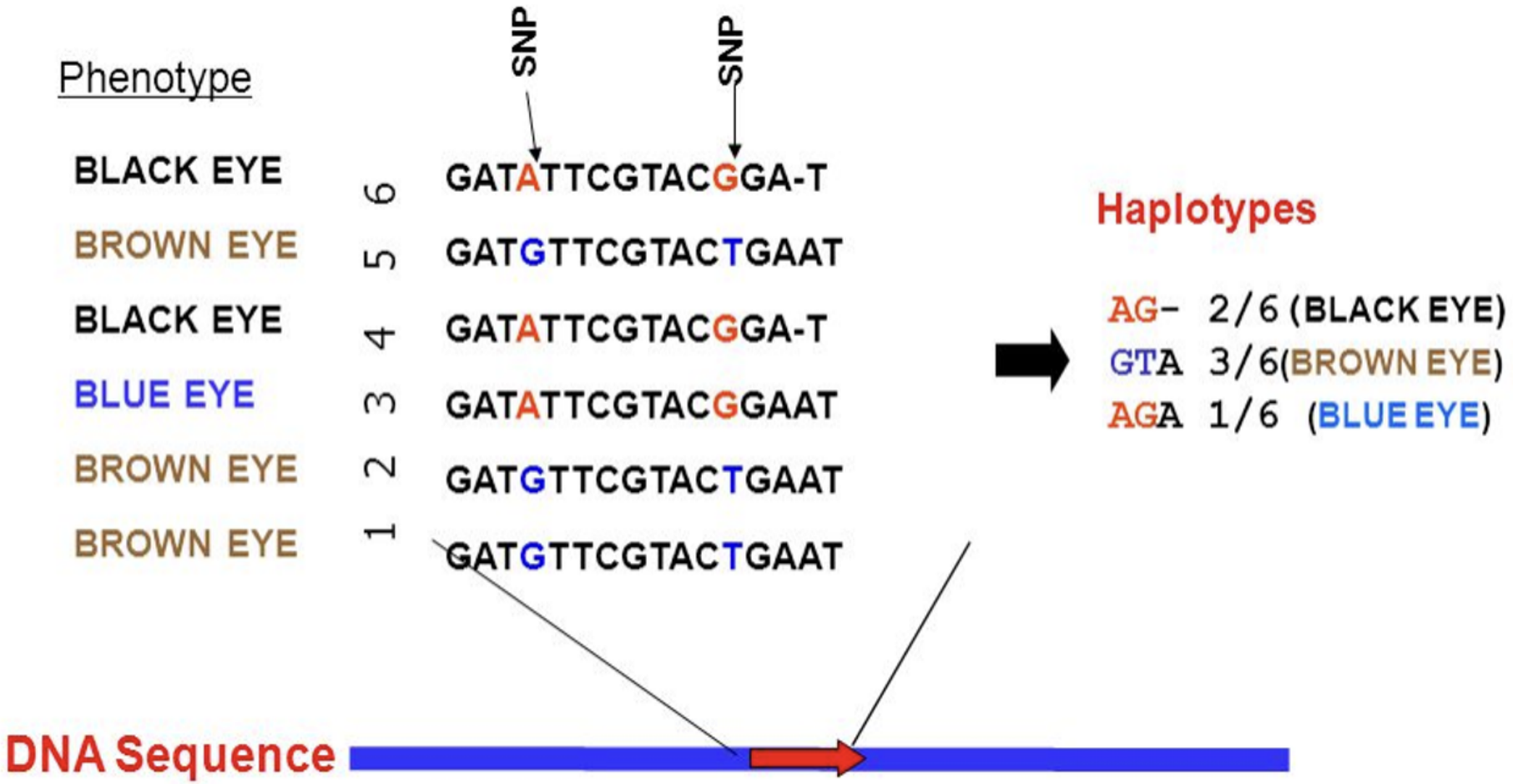
Variants Larger Than A Read

- **Structural Variation (SV)**
- Two types:
  - Balanced - Do not change amount of DNA
  - Copy Number Variants (CNV) - Change amount of DNA Scales:
  - Mini (hundreds of basepairs)
  - Macro (visible by a microscope) variants
  - Much harder to find (especially balanced) Non-random: SV 'hotspots'

Haplotyping

- DNA recombines in large blocks
- SNPs in a block move around together
- Looking at the common SNPs in a block, reveals the ancestry information
- **Linkage Disequilibrium (LD)**: adjacent SNPs co-occur more often than expected
  - i.e. 2 SNPs are in LD with each other

Haplotyping



Methods for SNP Calling

1. **GATK (Genome Analysis Toolkit)**:
   - Developed by the Broad Institute, GATK is a widely used toolkit for variant discovery in high-throughput sequencing data.
   - It employs a best practices pipeline for variant calling, including base quality score recalibration, indel realignment, and variant quality score recalibration.
   - GATK offers various tools such as HaplotypeCaller and UnifiedGenotyper for variant calling from both exome and whole genome sequencing data.
2. **Mutect2**:
   - Mutect2 is part of the GATK toolkit and is specifically designed for somatic mutation calling, particularly in cancer genomes.
   - It utilizes a probabilistic model to differentiate true somatic mutations from sequencing artifacts.
   - Mutect2 can be applied to both exome and whole genome sequencing data to identify somatic variants.

Methods for SNP Calling

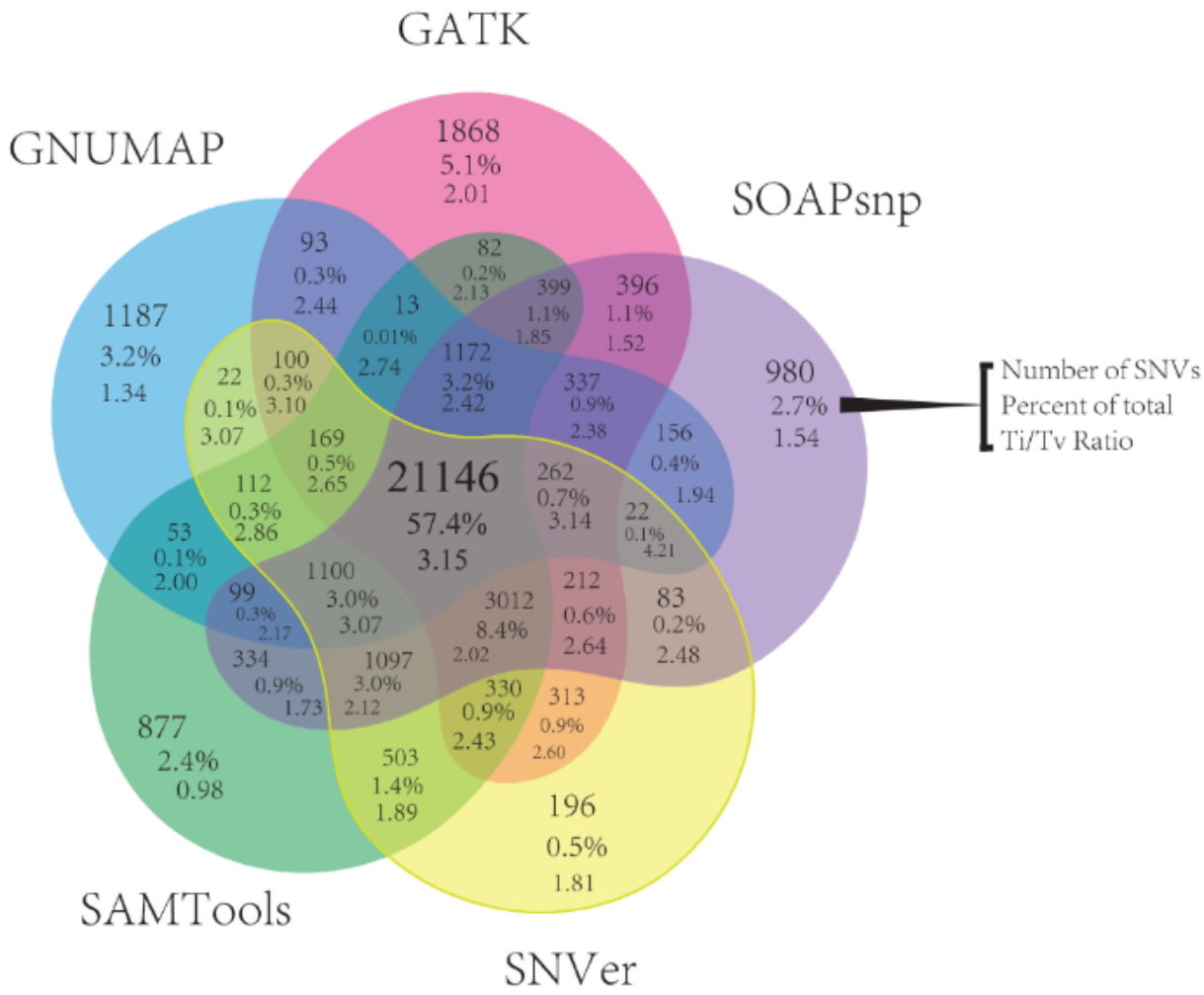
3. **Samtools**:
   - Samtools is a suite of programs for interacting with high-throughput sequencing data in the SAM/BAM format.
   - It includes the mpileup command for generating pileup data, and bcftools for variant calling from the pileup data.
   - Samtools is efficient and widely used for variant calling in both exome and whole genome sequencing datasets.
4. **FreeBayes**:
   - FreeBayes is a Bayesian genetic variant detector designed to detect SNPs, indels, and complex polymorphisms in high-throughput sequencing data.
   - It utilizes a haplotype-based approach to increase sensitivity and specificity, particularly in the context of population-scale sequencing data.
   - FreeBayes can be used for variant calling in both exome and whole genome sequencing studies.

Methods for SNP Calling

5. **VarScan**:
   - VarScan is a platform-independent tool for variant detection in massively parallel sequencing data.
   - It is optimized for calling germline variants, somatic mutations, and copy number alterations.
   - VarScan supports both exome and whole genome sequencing data and provides a range of options for variant calling and filtering.
6. **DeepVariant**:
   - DeepVariant is an end-to-end deep learning-based variant caller developed by Google.
   - It employs a convolutional neural network (CNN) architecture to call SNPs and indels with high accuracy.
   - DeepVariant is particularly effective in identifying complex variants and can be applied to both exome and whole genome sequencing data.

Inconsistencies Among SNP Callers

Low concordance of variant-calling pipelines (O'Rawe, *Genome Med*, 2013)



SAMtools Example

Multiple sample SNP calling:

```
## Load samtools
module load samtools

## mpileup
samtools mpileup -f genomefile.fa \
  myalignments.sorted.bam > myalignments.vcf
```

GATK Example

```
module load samtools
# Index reference
samtools faidx chrX_5MB.fa

module load gatk
# make sequence dictionary
gatk CreateSequenceDictionary -R chrX_5MB.fasta
# make .vcf file!
gatk HaplotypeCaller \
    -R chrX_5MB.fa -I proband_short_bwa.sorted.bam \
    -O proband_short.vcf
```

VCF files

8 fixed columns: #CHROM, POS, ID, REF, ALT, QUAL, FILTER, INFO, FORMAT
Additional columns, one for each sample, with sample ID

(a) **VCF example**

Header

```
##fileformat=VCFv4.1
##fileDate=20110413
##source=VCFtools
##reference=file:///refs/human_NCBI36.fasta
##contig=<ID=1,length=249250621,md5=1b22b98cdeb4a9304cb5d48026a85128,species="Homo Sapiens">
##contig=<ID=X,length=155270560,md5=7e0e2e580297b7764e31dbc80c2540dd,species="Homo Sapiens">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##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">
##ALT=<ID=DEL,Description="Deletion">
##INFO=<ID=SVTYPE,Number=1,Type=String,Description="Type of structural variant">
##INFO=<ID=END,Number=1,Type=Integer,Description="End position of the variant">
```

Body

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	SAMPLE1	SAMPLE2
1	1	.	ACG	A,AT	40	PASS	.	GT:DP	1/1:13	2/2:29
1	2	.	C	T,CT	.	PASS	H2;AA=T	GT	0|1	2/2
1	5	rs12	A	G	67	PASS	.	GT:DP	1|0:16	2/2:20
X	100	.	T	<DEL>	.	PASS	SVTYPE=DEL;END=299	GT:GQ:DP	1:12:.	0/0:20:36

Danecek et al. Bioinformatics 2011

Complete Variant Calling Pipeline (Outdated!)

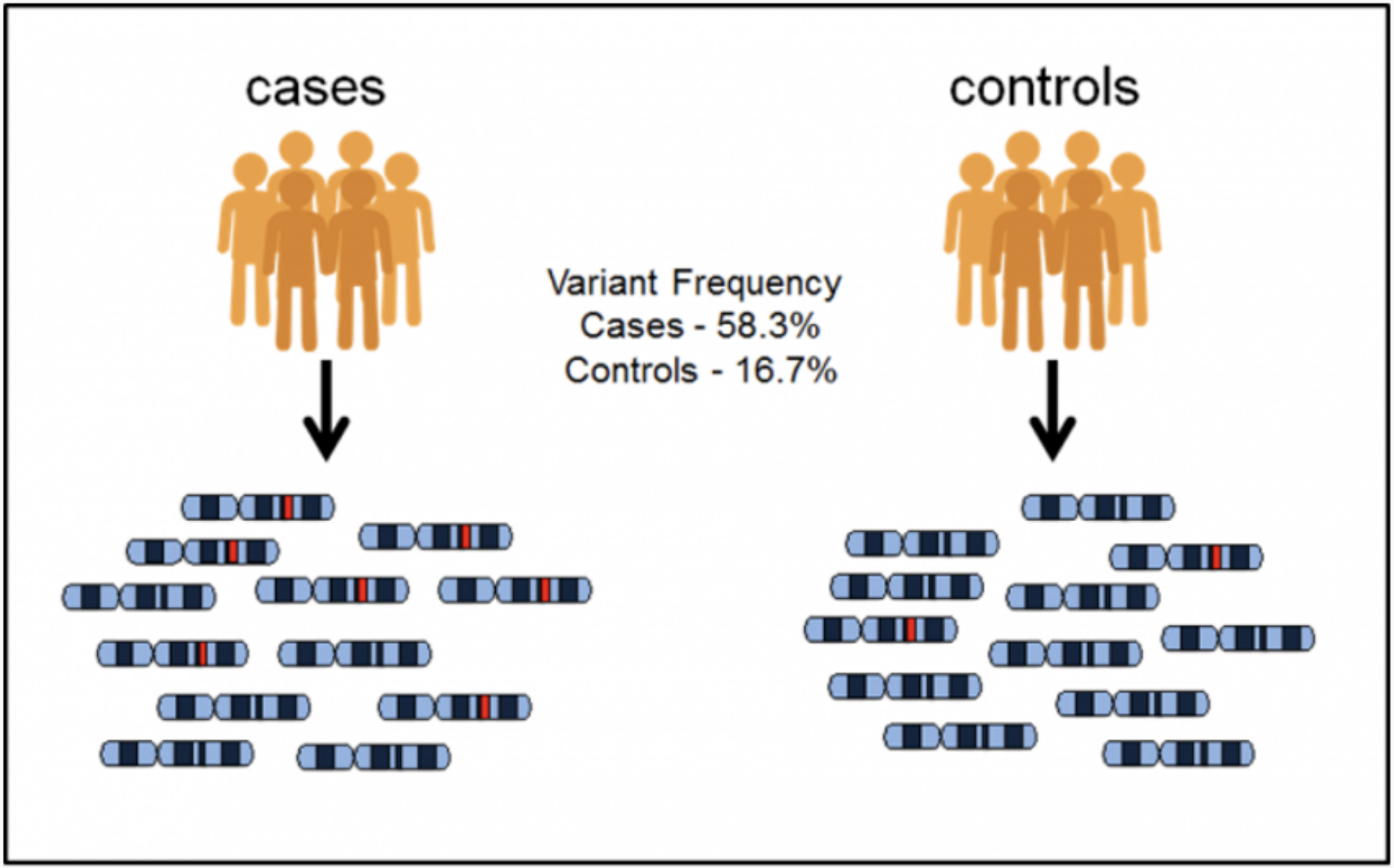
Our analysis pipeline consisted of the following:

- Align the FASTQ files to genome
- Convert SAM file to BAM, add read group info
- Filter the reads based on quality (BAMTools)
- Samtools to sort and index, and use Picard to mark duplicates
- GATK calibration, realignment, variant calling (HaplotypeCaller, Mutect2)
- Filter the called variants (GATK filtersnps and filterindels).
- Annotation of SNPs (snpEff, condel)
- Filter by frequency (thousand genomes, TCGA, etc.)
- Downstream analysis (rare variants, pedigree, pathway level, etc)

Genome Wide Association Studies (GWAS)

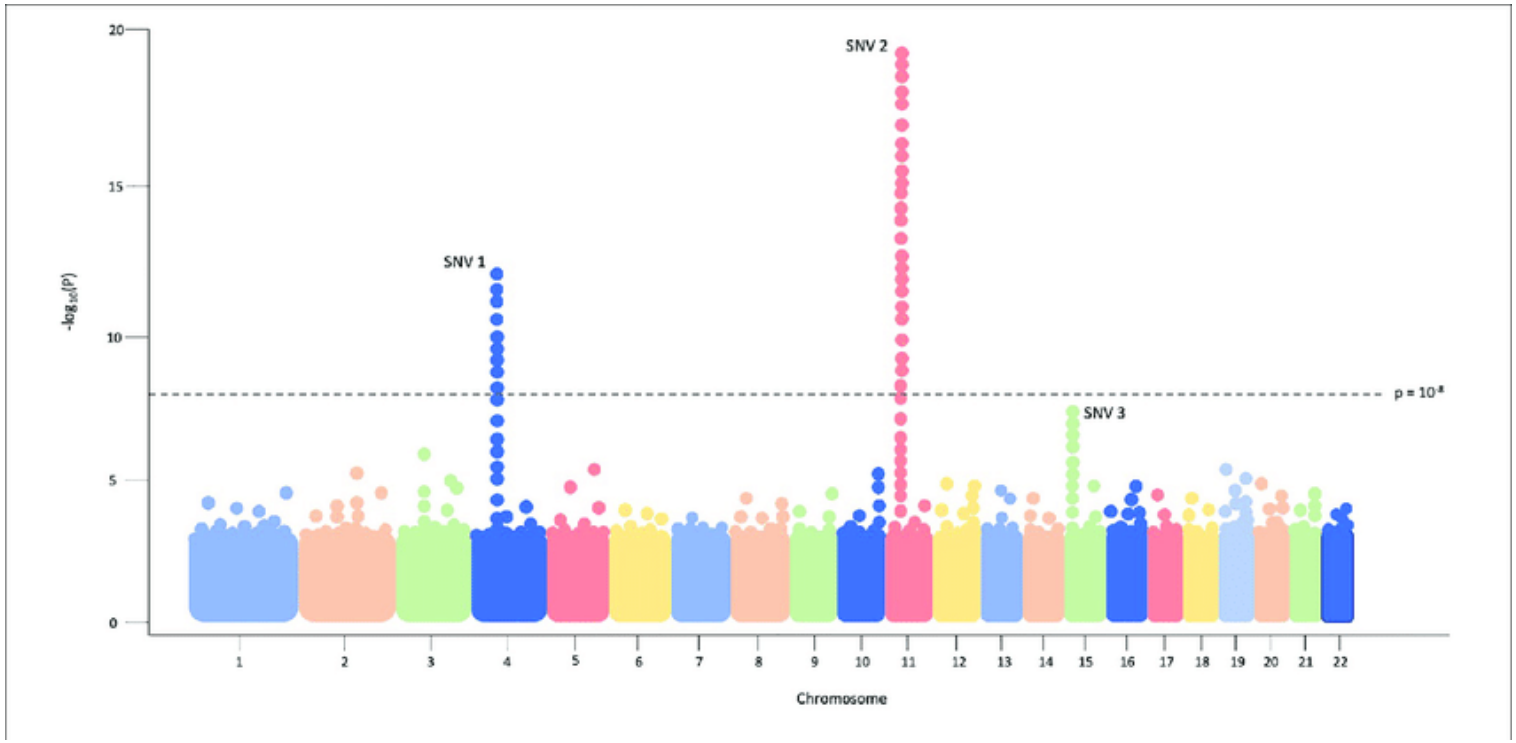
- Which SNPs are associated with a variable of interest?
  - e.g. disease, height Does the frequency of any SNP differ between groups? Associated SNPs have:
- Effect size - e.g. amount of increased risk
- p-value - precision of effect
- **Risk allele**: allele associated with increased or decreased probability of having a disease

Genome Wide Association Studies (GWAS)



Manhattan Plot

- In a **Manhattan plot**, each dot represents a genetic marker (SNP) on the x-axis, and its -log10(p-value) on the y-axis.
- The horizontal lines represent the significance threshold.
- Peaks represent regions where genetic variants are significantly associated with the trait or disease.



Genome-Wide Significance Threshold

- The genome-wide significance threshold is a threshold used to determine if an association between a genetic variant and a trait is statistically significant.
- It takes into account the multiple comparisons problem arising from testing thousands or millions of genetic markers across the genome.

Bonferroni Correction

- One method to establish the genome-wide significance threshold is the Bonferroni correction.
- The Bonferroni-corrected threshold is calculated by dividing the desired significance level (e.g., 0.05) by the number of tests performed.
- The most commonly accepted threshold is

$$p < 5 \times 10^{-8},$$

based on a Bonferroni correction for all independent common SNPs across the human genome

False Discovery Rate (FDR)

- Another method to control for multiple comparisons is the False Discovery Rate (FDR).
- FDR controls the proportion of false positives among all significant results.
- It is less conservative than the Bonferroni correction and allows for a higher number of false positives while still controlling the overall error rate.

Downstream Annotation and Analysis (Outdated!)

Downstream Annotation Tools (old list):

- snpEff (http://snpeff.sourceforge.net/)
- Condel (http://bg.upf.edu/condel/home)
- SIFT http://sift.jcvi.org/
- Polyphen 2 http://genetics.bwh.harvard.edu/pph2/
- http://mutationassessor.org/
- Ensembl variant effect predictor (http://www.ensembl.org/info/docs/variation/vep/index.html)
- Thousand Genomes variant frequency (e.g. 1% threshold) and Exome Sequence Project variant frequency (e.g. 1%).

Session info

```
sessionInfo()
```

```
## R version 4.4.2 (2024-10-31)
## Platform: aarch64-apple-darwin20
## Running under: macOS Sequoia 15.4.1
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.4-arm64/Resources/lib/libRlapack.dylib;  LAPACK version 3
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## time zone: America/Denver
## tzcode source: internal
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] compiler_4.4.2    fastmap_1.2.0     cli_3.6.5         tools_4.4.2
##  [5] htmltools_0.5.8.1 rstudioapi_0.17.1 yaml_2.3.10       rmarkdown_2.29
##  [9] knitr_1.50        xfun_0.52         digest_0.6.37     rlang_1.1.6
## [13] evaluate_1.0.3
```