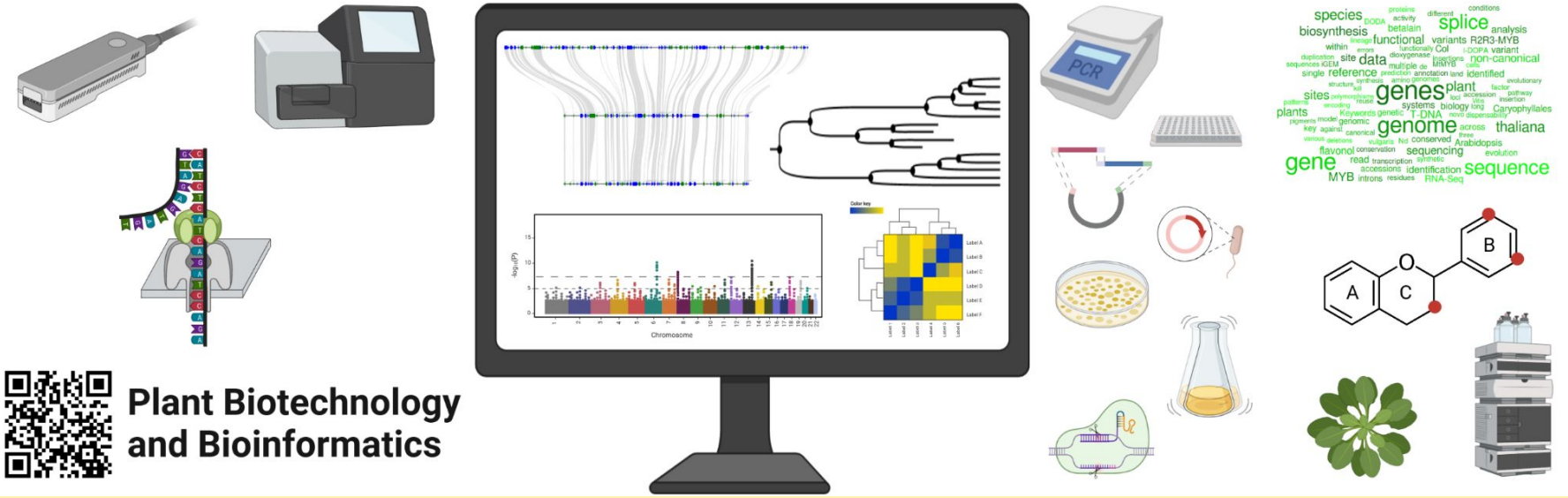
Technische Universität Braunschweig



Plant Biotechnology and Bioinformatics

Genomics #2 - Big Data Analytics in Life Sciences

Prof. Dr. Boas Pucker (Plant Biotechnology and Bioinformatics)

Outline - Genomics #2

- Structural & functional annotation
- Read mapping, variant calling, variant annotation
- Genome-Wide Association Studies (GWAS) / Mapping-By-Sequencing (MBS)
- Comparative genomics / synteny analysis
- Phylogenetic analyses

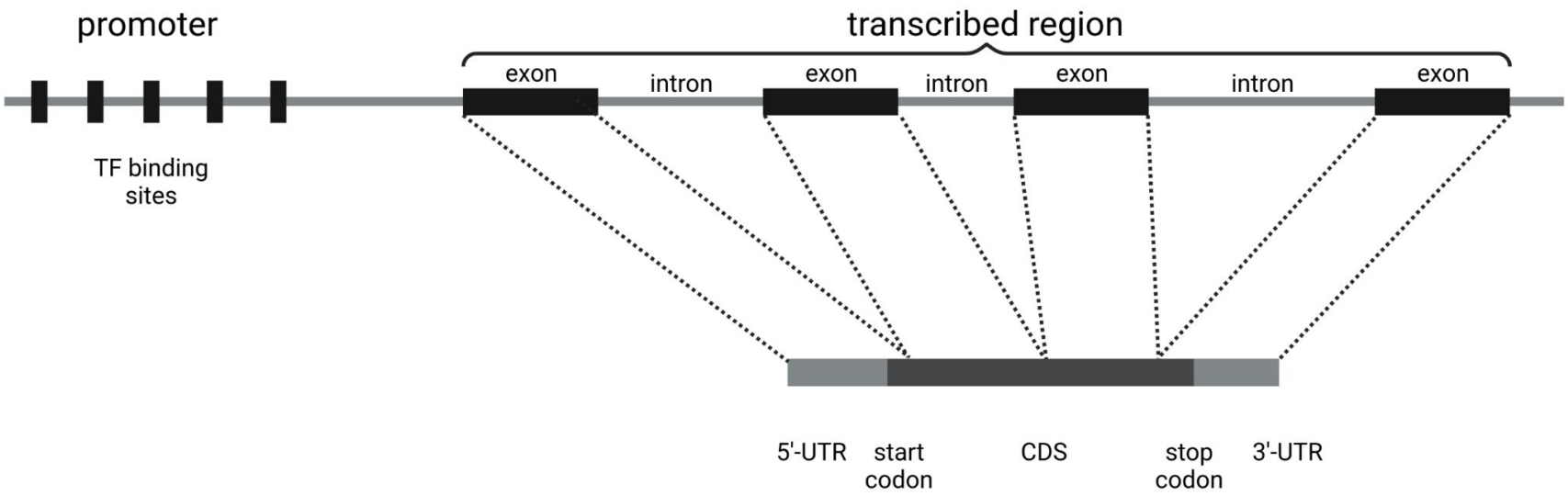
Genome sequence annotation

Finding genes in a genome sequence

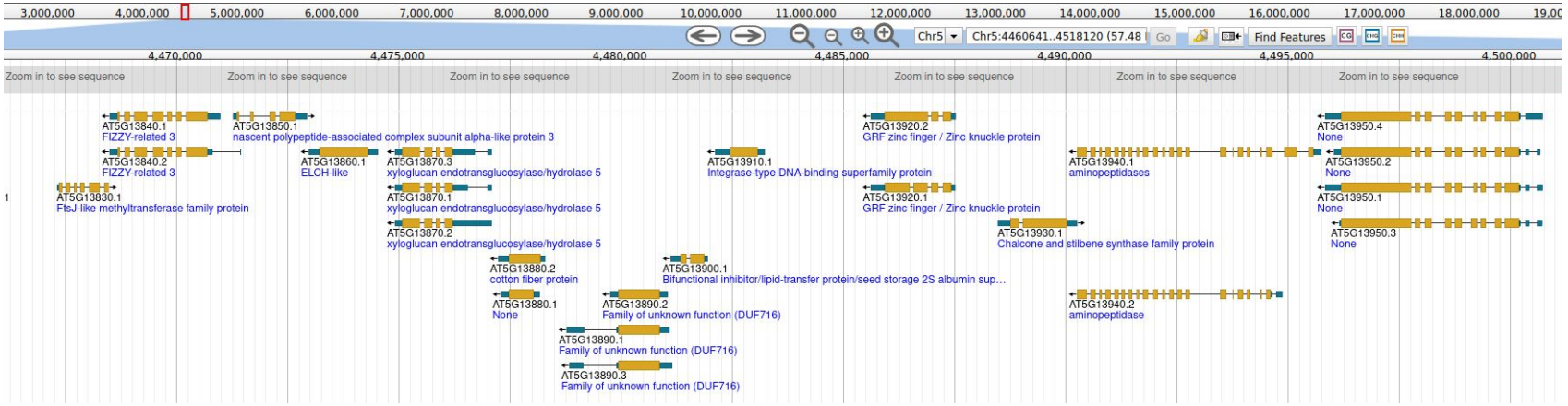
Siadjeu & Pucker et al., 2020: 10.3390/genes11030274

Plant gene structure

CDS = (Protein) Coding Sequence
ORF = Open Reading Frame
UTR = UnTranslated Region
TF = Transcription Factor

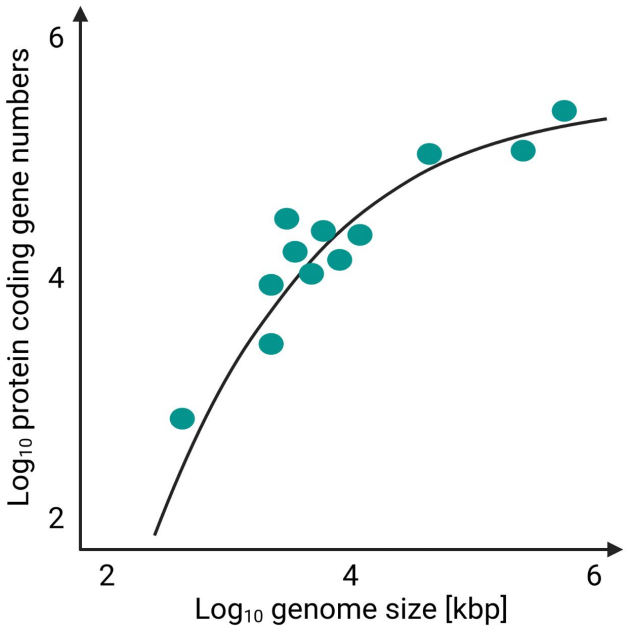


Finding genes in a genome sequence



Gene numbers

- Average number of genes in plants: 27200
- Gene number is not proportional to genome size

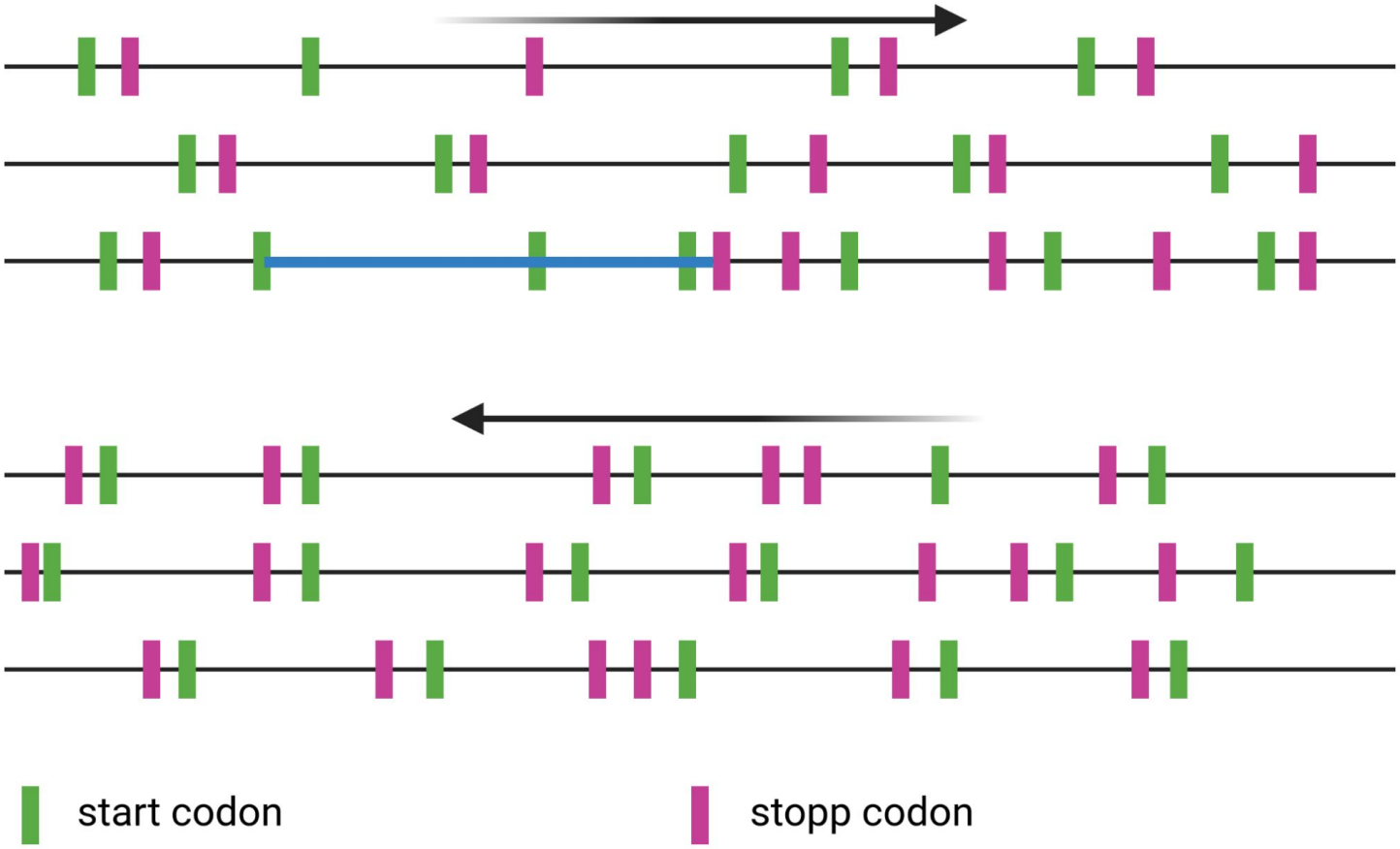


Pucker & Brockington, 2019: 10.1186/s12864-018-5360-z
Michael, 2014: 10.1093/bfgp/elu005

Repeat masking

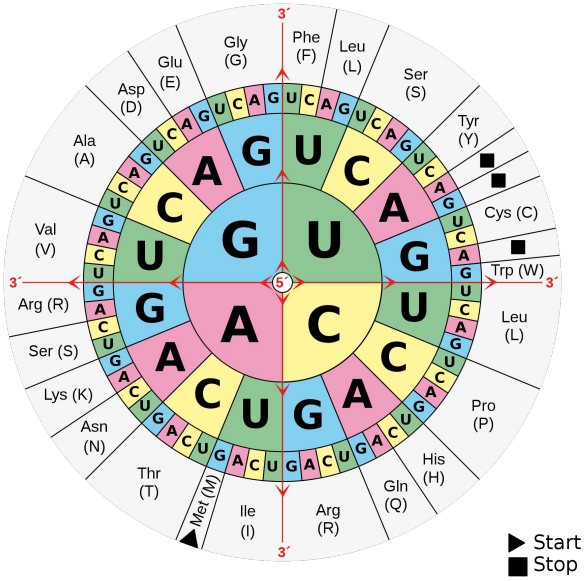
- Simple sequence repeats (SSR)
- Transposable elements (TE)
- Centromeric repeats (CEN)
- Telomeric repeats (TEL)

Finding ORFs



Codon usage

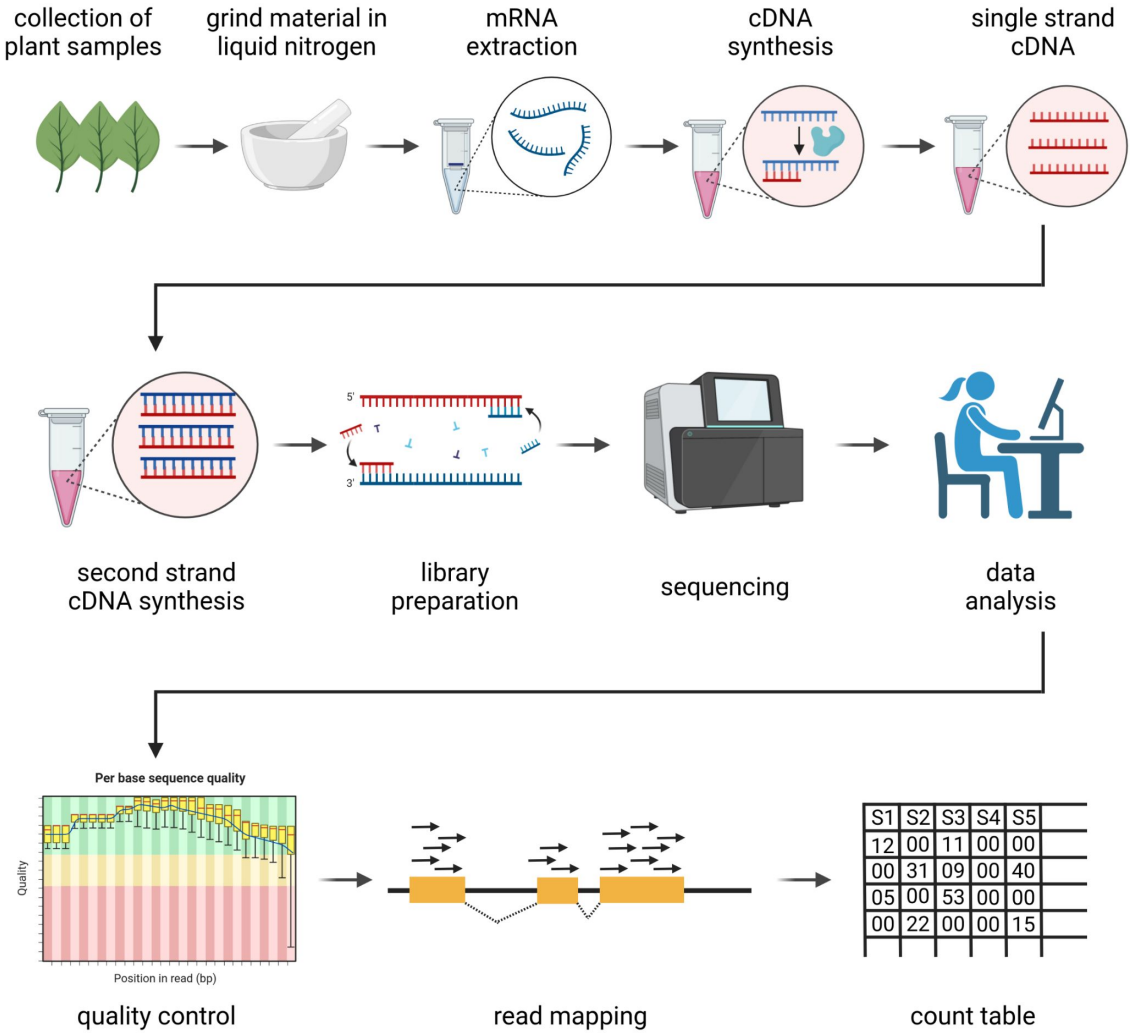
- Protein coding sequences have specific properties
- Codon usage is different between species i.e. different species prefer different codons for certain amino acids
- Rare codons slow down translation (useful between domains)
- Usage of dicodons (=hexamers)
- Codon usage can give additional CDS/ORF support



Parameters for *ab initio* gene prediction

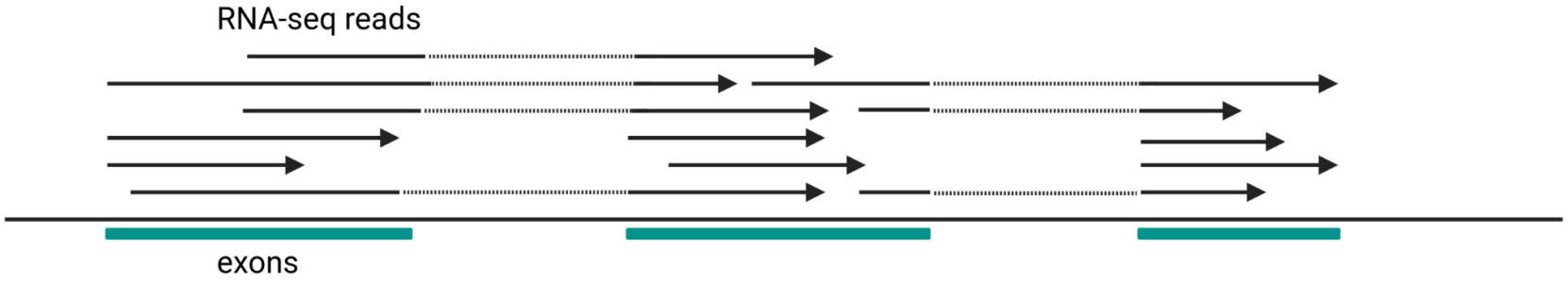
- Features: utr, exon, intron, intergenic region
- Composition of different features (dicodons)
- feature lengths
- Number of features per gene
- Possible splice sites

RNA-seq


collection of plant samples
grind material in liquid nitrogen
mRNA extraction
cDNA synthesis
single strand cDNA
second strand cDNA synthesis
library preparation
sequencing
data analysis
Per base sequence quality
Quality
Position in read (bp)
quality control
read mapping
S1 S2 S3 S4 S5
12 00 11 00 00
00 31 09 00 40
05 00 53 00 00
00 22 00 00 15
count table

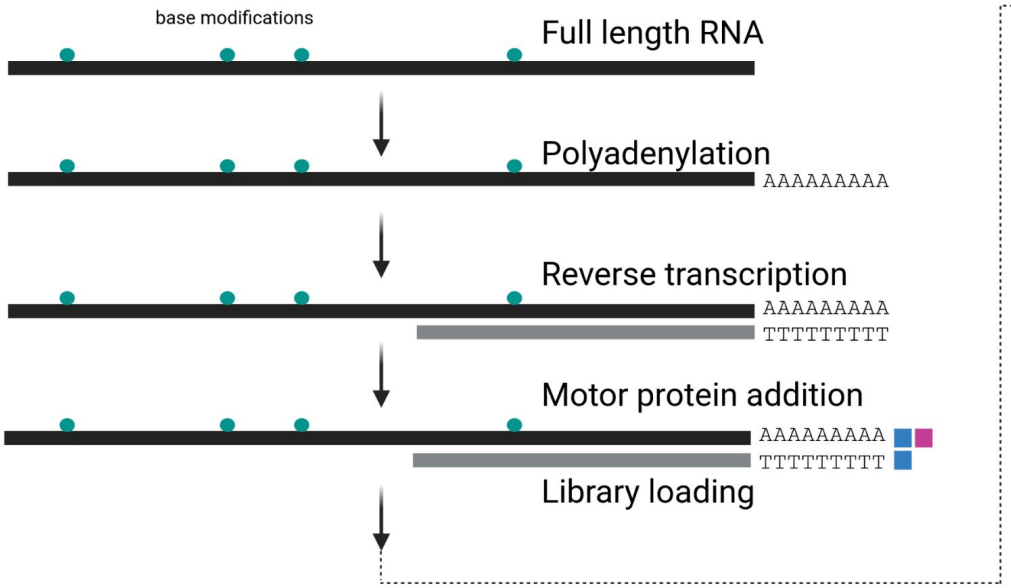
RNA-seq hints

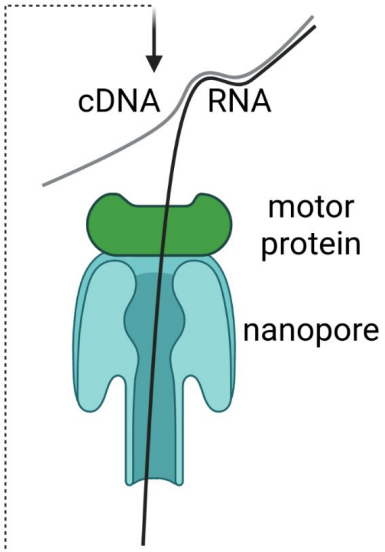
- Aligned RNA-seq reads indicate exon positions
- Splitting of reads indicates intron positions
- CDS can be identified as ORF within the covered regions



Full length transcript sequences

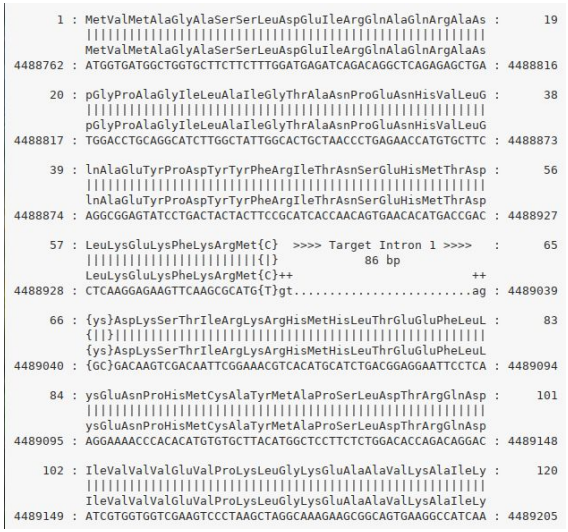
- Capturing full length transcripts for RNA-seq (Illumina)
- Full length cDNA sequencing (PacBio, ONT)
- Direct RNA sequencing or cDNA sequencing (ONT)





Proteins as hints (exonerate)

- Exonerate can align coding sequences/peptide sequences to DNA
- Intron positions are identified and intron information is given
- Exonerate can handle different types of splice sites



Annotation quality assessment

- BUSCO (Benchmarking Universal Single-Copy Orthologs)
  - Example: C:98%[S:85%,D13%],F:1.2%,M0.8%,n:1500
  - C = complete BUSCO genes
  - S = single copy BUSCO genes
  - D = duplicates BUSCO genes
  - F = fragmented BUSCO genes
  - M = missing BUSCO genes
  - n = number of BUSCO query sequences

- NLRome: assessment of high continuity genome sequences

Transposable elements (TEs)

- Transposons shape plant genomes (genome obesity)

- Systematics:
  - Class I (retrotransposons)
    - LTR: Copia, Gypsy, Bel-Pao, Retrovirus, ERV
    - DIRS: DIRS, Ngaro, VIPER
    - PLE: Penelope
    - LNE: R2, RTE, Jockey, L1, I
    - SINE: tRNA, 7SL, 5S
  - Class II (DNA transposons) - Subclass 1
    - TIR: Tc1-Mariner, hAT, Mutator, Merlin, Transib, P, PiggyBac, PIF-Harbinger, CACTA
    - Crypton
  - Class II (DNA transposons) - Subclass 2
    - Helitron
    - Maverick

Wicker et al., 2007: 10.1038/nrg2165

Annotation of TEs

- Benchmarking study of TE annotation tools: https://github.com/oushujun/EDTA

- RepeatMasker: https://www.repeatmasker.org/
  - Screens genomic sequence for TEs
  - Soft/hard masking of genomic sequence
  - Dfam and Repbase are important databases
    - Dfam: open collection of TE sequences (https://www.dfam.org/home)
    - Repbase: subscription-based collection (https://www.girinst.org/)
  - Different search engines can be used

- RepeatModeler2: http://www.repeatmasker.org/RepeatModeler
  - Pipeline for discovery of TEs

- Extensive *de novo* TE Annotator (EDTA): https://github.com/oushujun/EDTA
  - Complex pipeline for TE annotation

Ou et al., 2019: 10.1186/s13059-019-1905-y
Flynn et al., 2020: 10.1073/pnas.1921046117
Tarailo-Graovac & Chen, 2009: 10.1002/0471250953.bi0410s25

Generic Feature Format (GFF)

- SequenceID
- Source
- Feature type
- Start
- End
- Score
- Strand
- Phase
- Attributes (IDs)

```
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ctg000000_np2  AUGUSTUS  CDS           7686   8051   0.18  -  0  ID=g3.t1.CDS1;Parent=g3.t1;
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ctg000000_np2  AUGUSTUS  CDS           11163  11261  0.8   -  0  ID=g5.t1.CDS2;Parent=g5.t1;
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```

Sharing structural annotation

- Species specific databases:
  - TAIR: *Arabidopsis thaliana*
  - BananGenomeHub: *Musa acuminata*

- EMBL/EBI: European Nucleotide Archive of the European Bioinformatics Institute (https://www.ebi.ac.uk/ena/browser/home)

- PLAZA (https://bioinformatics.psb.ugent.be/plaza/)

- Phytozome (https://phytozome-next.jgi.doe.gov/)

Van Bel et al., 2022: 10.1093/nar/gkab1024

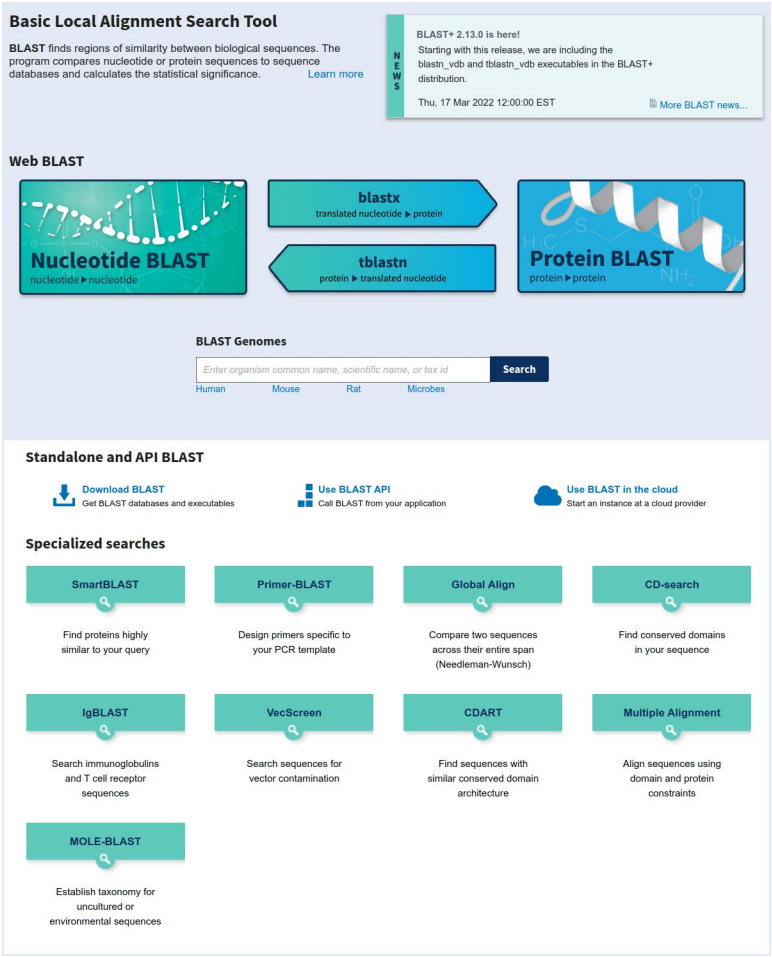
Functional annotation

- What is the function of a gene?
- Knockout experiments for all genes are time consuming and expensive
- Annotation transfer: orthologs are assumed to have the same function
- Tools:
  - BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi
  - Pfam: https://pfam.xfam.org/
  - InterProScan5: http://www.ebi.ac.uk/interpro/search/sequence/
  - Shoot: https://github.com/davidemms/SHOOT
  - KEGG: https://www.genome.jp/kegg/
  - GO: http://geneontology.org/
  - MetaCyc: https://metacyc.org/
  - KIPEs: https://github.com/bpucker/KIPEs
  - BRENDA: https://www.brenda-enzymes.org/
  - Mercator: https://plabipd.de/portal/mercator4

Altschul et al., 1990: 10.1016/S0022-2836(05)80360-2
Mistry et al., 2021: 10.1093/nar/gkaa913
Jones et al., 2014: 10.1093/bioinformatics/btu031
Karp et al., 2002: 10.1093/nar/30.1.59
Emms & Kelly, 2022: 10.1186/s13059-022-02652-8
Kanehisa & Goto, 2000: 10.1093/nar/28.1.27
Ashburner et al., 2000: 10.1038/75556
Pucker et al., 2020: 10.3390/plants9091103
Schomburg et al., 2002: 10.1093/nar/30.1.47
Schwacke et al., 2019: 10.1016/j.molp.2019.01.003

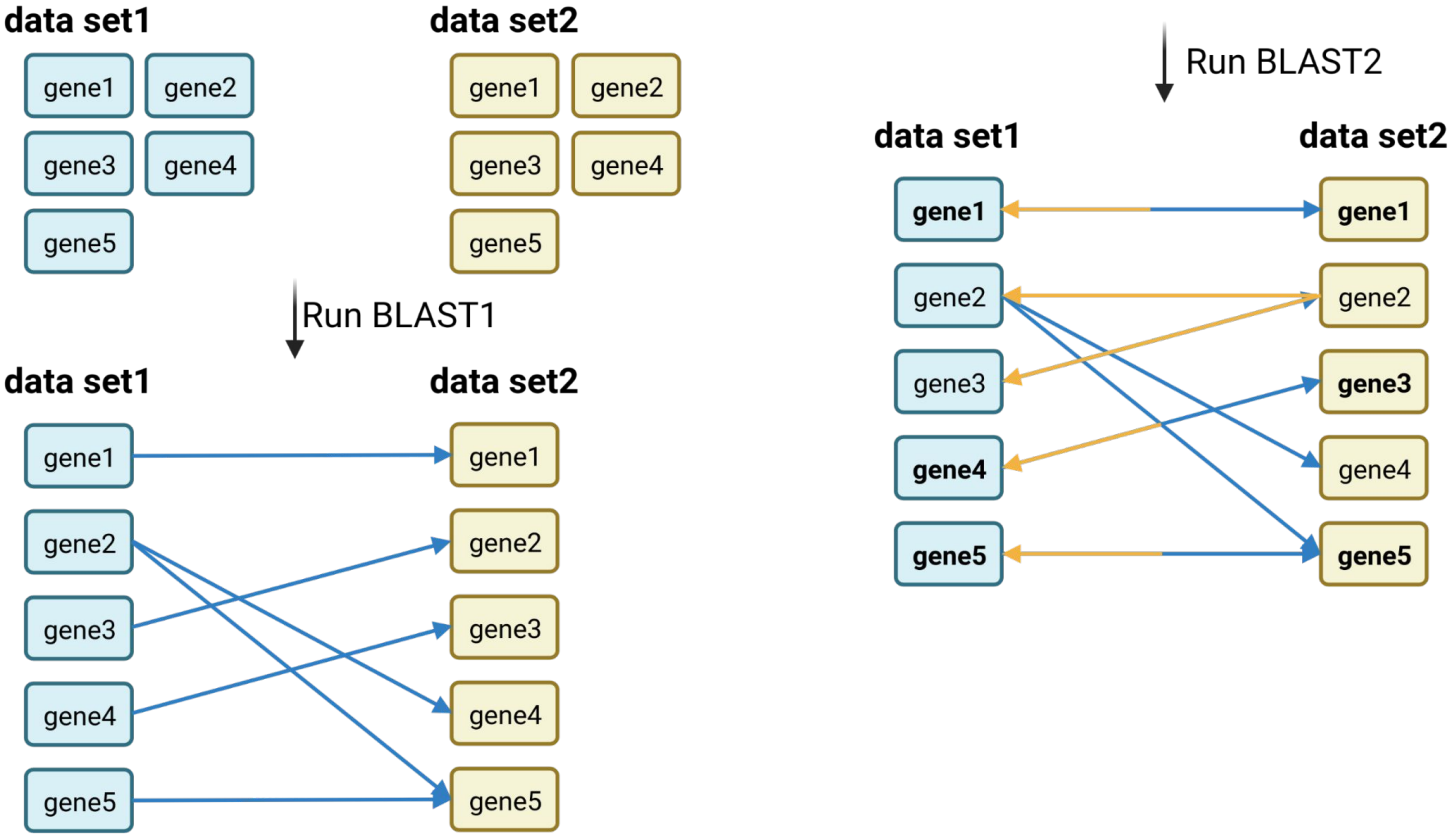
BLAST: Basic Local Alignment Search Tool

- Probably the most famous website of the NCBI
- Comparison of sequences against a large database
- Similar sequences are likely to have similar functions (ideally orthologs)
- Numerous variants of the initial BLASTn were developed



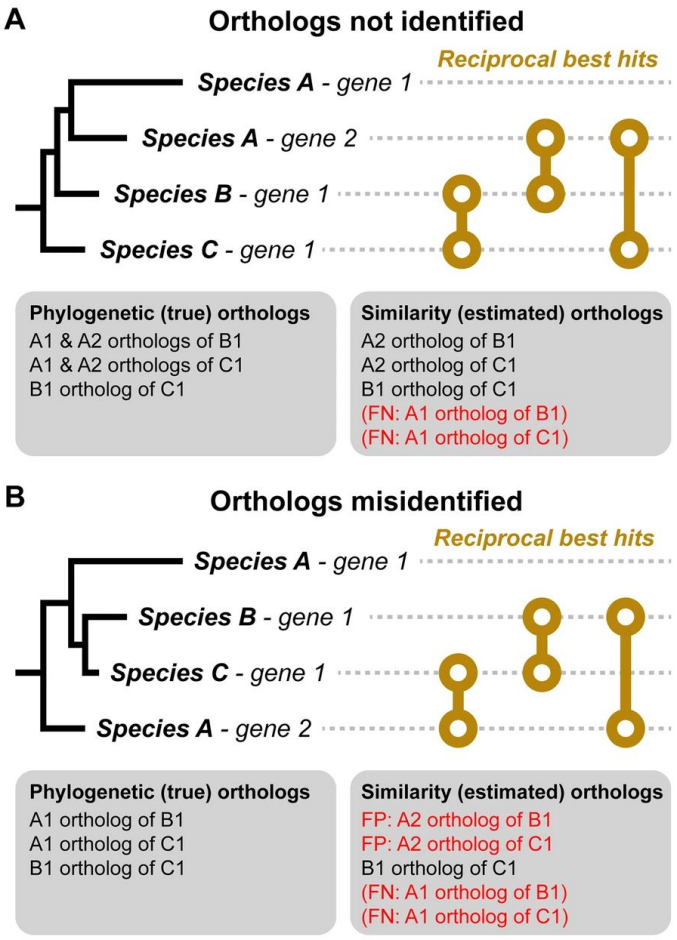
https://blast.ncbi.nlm.nih.gov/Blast.cgi
Altschul et al., 1990: https://doi.org/10.1016/s0022-2836(05)80360-2

Reciprocal Best BLAST Hits (RBHs)



OrthoFinder2

- Identification of orthologs often not possible
- Orthogroups are better reflection of reality
- Assumption: orthologs have the same function



https://doi.org/10.1186/s13059-019-1832-y

Pfam: Protein family database

- Assignment of protein functions based on Hidden Markov Models (HMMs)
- Sequences are screened based on HMM profile

Sequences are aligned

↓

Modelling of insertions/deletions

↓

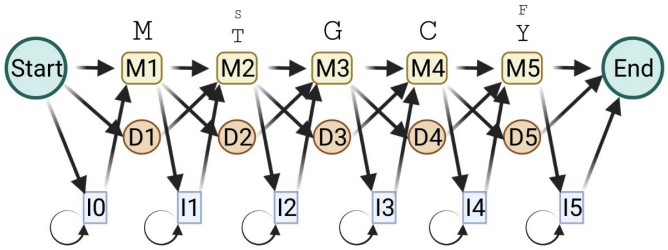
Amino acid frequency at each alignment position is encoded

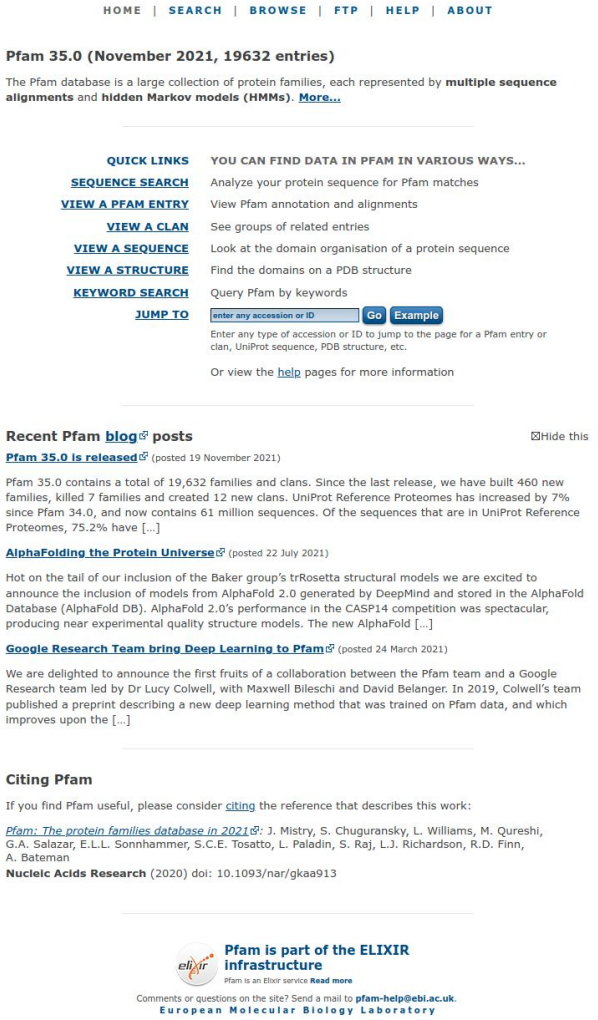
↓

HMM profile generated

```
seq1    MTGC-Y
seq2    MSGC-F
seq3    MTGC-Y
seq4    M-GCAY
        123456
```

2 = deletion
5 = insertion

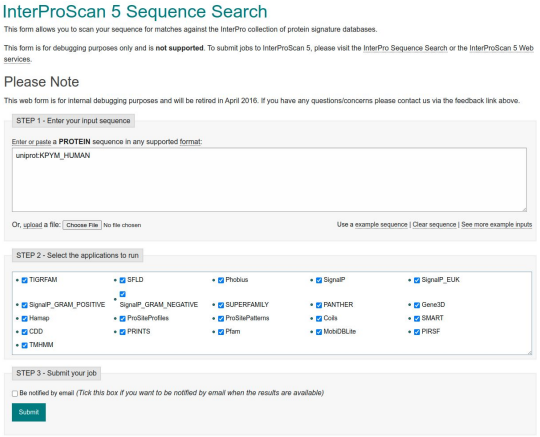




http://pfam.xfam.org/
Mistry et al., 2020: 10.1093/nar/gkaa913

InterProScan5

- Screen of protein sequences against collection of protein signatures (KEGG, Pfam, PANTHER)
- Allows the assignment of functional annotation terms
- Available as web service, but also as stand alone tool
- Search based on sequence similarity via DIAMOND



Results for job iprscan5-I20220320-102557-0980-87120812-p2m

Tool Output | Submission Details

Download in XML format | Download in TSV format | Download in GFF3 format | Download in SVG format | Download HTML tarball file | Download in JSON format

```
sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   Gene3D   G3DSA:3.40.47.10          -   1    241   4.8E-101        T   20-03-2022
sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   SUPERFAMILY   SSF53901          Thiolase-like   241   393   2.98E-51        T   20-03-20
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sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   Pfam   PF02797 Chalcone and stilbene synthases, C-terminal domain   244   394   1.5E-71
sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   PANTHER   PTHR11877:SF81   BNAA02G30320D PROTEIN   6   394   0.0   T   20-03-2022
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sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   PIRSF   PIRSF000451   PKS_III 7   394   0.0   T   20-03-2022   IPR011141
sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   Pfam   PF00195 Chalcone and stilbene synthases, N-terminal domain   10   233   2.9E-119
sp|P13114|CHSY_ARATH   ba46b0f06bfbf1c91d161f0f91310f43   395   ProSitePatterns   PS00441 Chalcone and stilbene synthases active site.   161   177   -
```

http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=iprscan5&sequence=uniprot:KPYM_HUMAN
Jones et al., 2014: 10.1093/bioinformatics/btu031

Shoot

- Initial search based on sequence similarity
- Phylogenetic relationships of sequences are considered based on a tree
- Universal tool, but computationally more intensive than a simple sequence similarity analysis

SHOOT.bio - the phylogenetic search engine

SHOOT is a phylogenetic alternative to BLAST. Instead of returning a list of similar sequences to a query sequence it returns a maximum likelihood phylogenetic tree with your query sequence embedded in it.

Try it out: https://shoot.bio/

Preprint: https://www.biorxiv.org/content/10.1101/2021.09.01.458564

Using the SHOOT command line tool

SHOOT allows you to search a protein sequence against a database of gene trees. It returns your gene grafted into the correct position within its corresponding gene tree.

Preparing a SHOOT phylogenetic database

0. Install dependencies:
   - Python libraries: ete3, sklearn, biopython
   - DIAMOND
   - MAFFT
   - EPA-ng & gappa (https://github.com/lczech/gappa)
   - Alternatively, IQ-TREE can be used instead of the combination EPA-ng + gappa
1. Run an OrthoFinder analysis on your chosen species, using the multiple sequence alginment option for tree inference, "-M msa".
   - Paper: Emms, D.M., Kelly, S. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol 20, 238 (2019). https://doi.org/10.1186/s13059-019-1832-y
   - GitHub: https://github.com/davidemms/OrthoFinder
   - Tutorials: https://davidemms.github.io/
2. Run `python create_shoot_db.py RESULTS_DIRECTORY` , replacing "RESULTS_DIRECTORY" with the path to the OrthoFinder results directory from step 1.
3. Resolve polytomies (only necessary if using EPA-ng): `python bifurcating_trees.py RESULTS_DIRECTORY`

The OrthoFinder RESULTS_DIRECTORY is now a SHOOT database.

Running SHOOT

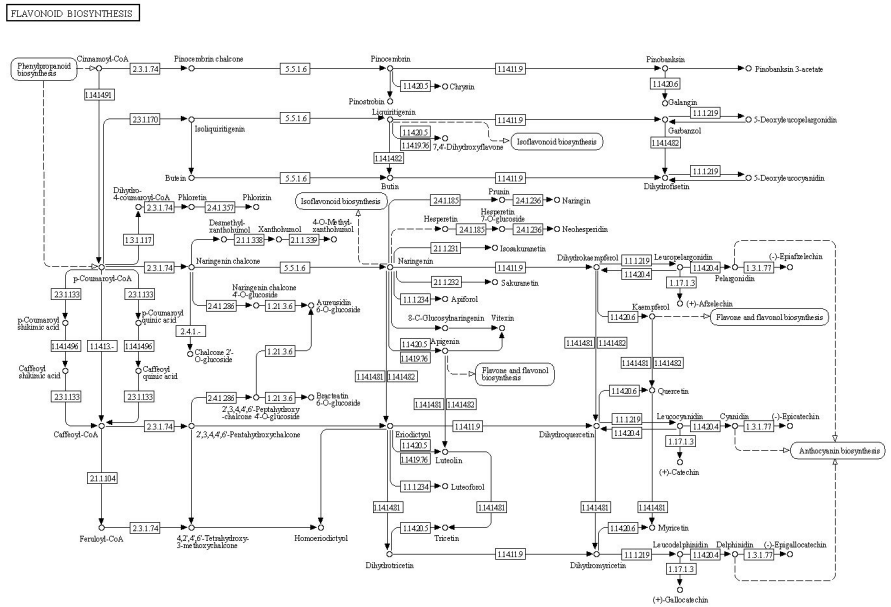
```
python shoot INPUT_FASTA SHOOT_DB
```

where INPUT_FASTA is a fasta file containing the amino acid sequence for the search and SHOOT_DB is the SHOOT database directory created using the steps above.

https://github.com/davidemms/SHOOT

KEGG: Kyoto Encyclopedia of Genes and Genomes

- Maps of pathways showing the individual reactions with catalyzing enzymes
- Information about genomes and genes
- Chemical details about enzymes, substrates, and products
- KEGG is financed through a subscription model (for FTP download), but website is freely accessible

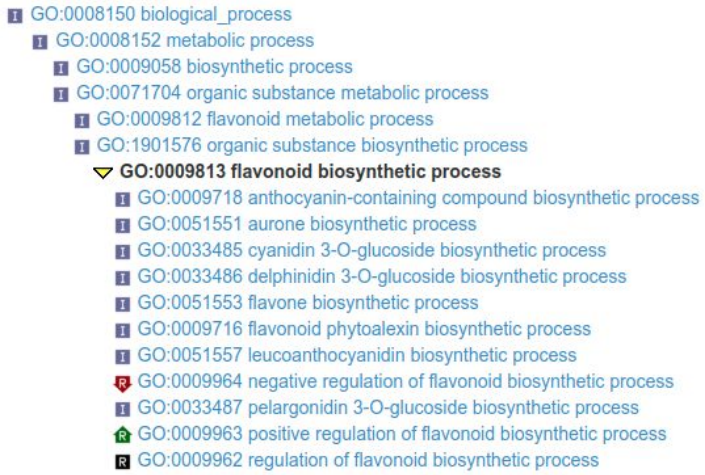


map00941 Flavonoid biosynthesis

Kanehisa & Goto, 2000: https://doi.org/10.1093/nar/28.1.27
Kanehisa et al., 2022: https://doi.org/10.1002/pro.4172
https://www.genome.jp/kegg/

Gene Ontology (GO)

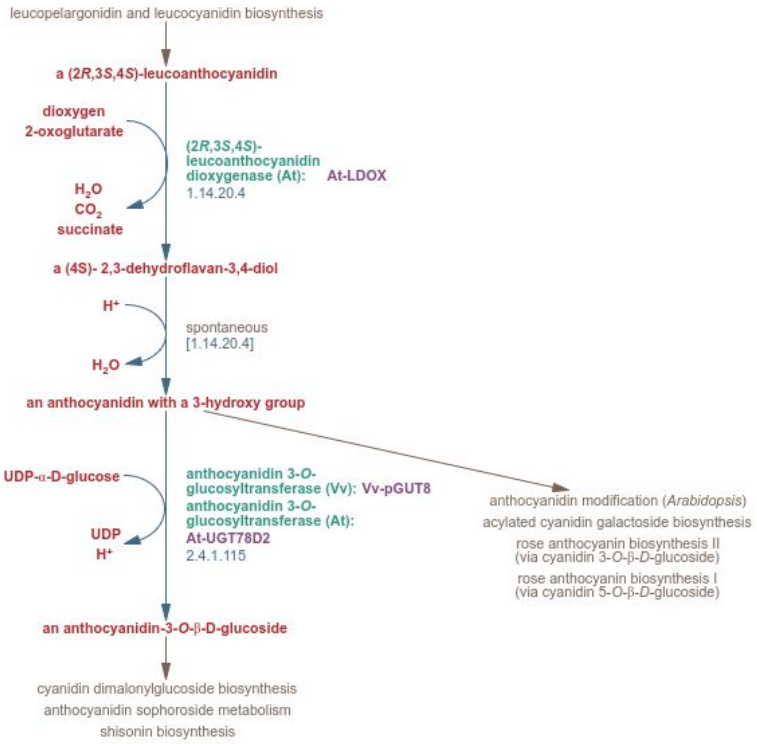
- Defined statements about the function of a gene (controlled vocabulary)
- Hierarchical structure
  - Example: ‘metabolic process’ > ‘biosynthetic process’ > … > ‘chalcone synthase’
- Supported by the Alliance of Genome Resources
- Connected with various other databases e.g. TAIR, FlyBase, Reactome, UniProt
- Machine readable to allow automatic processing
- Tools: Blast2GO and AmiGO
  - analyze the function of a sequence (web service and standalone)



http://amigo.geneontology.org/amigo/term/GO:0009813
The Gene Ontology Consortium, 2007: 10.1093/nar/gkm883

MetaCyc: Metabolite Encyclopedia

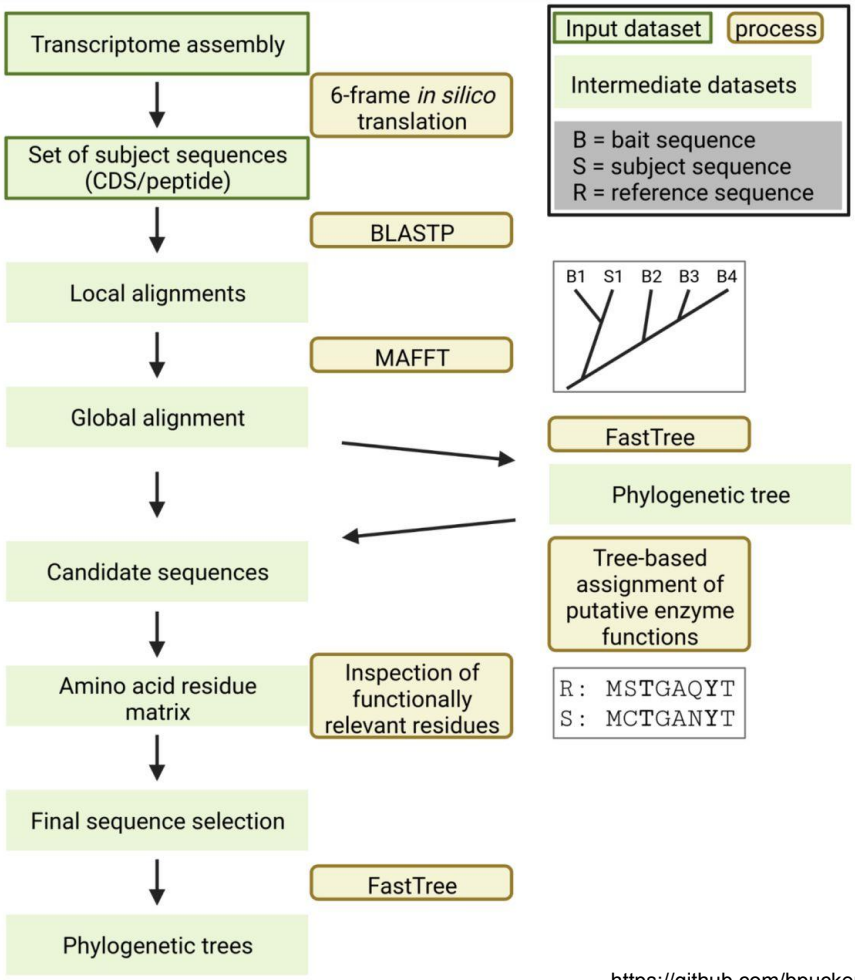
- Integrates genomic data with functional annotation
- Visualization of pathway databases
- Shows intermediates and enzymes of biosynthesis pathways
- MetaFlux: flux-balance analysis



https://metacyc.org/
Karp et al., 2015: https://doi.org/10.48550/arXiv.1510.03964
Figure: https://biocyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5125

KIPEs

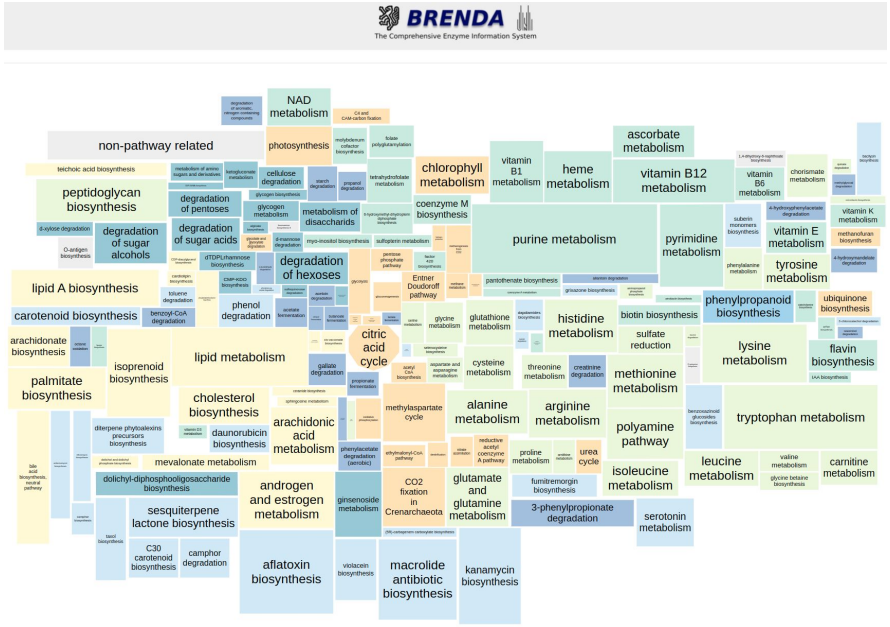
- KIPEs = Knowledge-based Identification of Pathway Enzymes
- Identification of genes involved in well-studied biosynthesis pathways
- Prediction about functionality of enzymes
- Requires existing knowledge from other species



https://github.com/bpucker/KIPEs
Pucker et al., 2020: 10.3390/plants9091103
Rempel et al., 2023: 10.1371/journal.pone.0294342

BRENDA: BRaunschweig ENzyme DAtabase

- Enzyme database hosted at TU Braunschweig (BRICS)
- Text and structure-based queries
- Visualization of pathways
- Manual curation of datasets
- Many details about enzyme properties (substrates, kinetics, mutants, …)



www.brenda-enzymes.org
Chang et al., 2021: 10.1093/nar/gkaa1025

Mercator - functional annotation of protein sequences

- Online tool for the annotation of all protein sequences in a submitted FASTA file

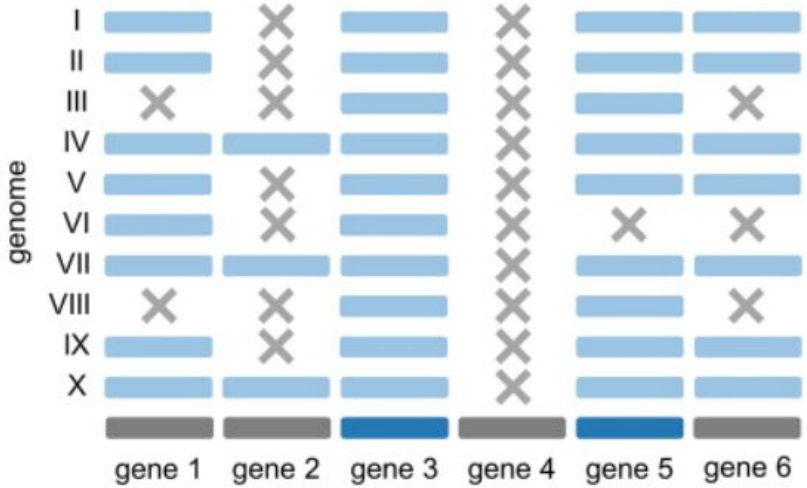
- FASTA format:
  - Header line starts with '>' followed by the sequence name
  - Header is followed by unrestricted number of sequence lines

```
>TRINITY_DN100013_c0_g1_i1
MIGPMPGMEGKLMLPGASPVGLEVVLSLVVASTPILSDTSLCTPSFYHFLLLLGPITSISNLIVRPFFLTLLSSITVIYGRLCFFAFDFYVY
>TRINITY_DN100016_c0_g1_i1
MPKKSSNIQQNNQNRIKRKGRPPKKKKYVQQIDSSDEDILSVRHASTRPRIISIRRNEIPMRPEIHIPPTSIPSFNNPNNIHQSSSDRQMPP
>TRINITY_DN100019_c0_g1_i1
MPPKAADKKPAAKAPATASKAPEKKDAGKKTAATGDKKKRTKARKETYSSYIYKVLKQVHPDTGISNRAMSILNSFVNDIFERVATEASKLA
>TRINITY_DN10001_c0_g1_i2
MAKVGNPIVDIETDGSVNEPESSEKNIEVSSSSTQAPESTNTTELLVNEKKAFSLATPAVRRVAREHNIDINNIKGTGKNGRITKEDILNYV
>TRINITY_DN100025_c0_g1_i1
MVENQDGCFFKPGWKEFVRSNDLEGGDFLVFNLVDKISYQVVIFDGTCCPKDLCPFSIMNPIFQHLRNKIFLSKKEEIKLKGNRKVHSVNEN
>TRINITY_DN10002_c0_g1_i1
MSDELNQVFQRCREQKRPVFVAFVTAGYPDSEETVDILLGLEAGGADIIELGIPFTDPMVDGKTIQDANNVALENKIDISKCLSYVSESRAK
```

https://plabipd.de/portal/mercator-sequence-annotation
Lohse et al., 2014: 10.1111/pce.12231
Haak et al., 2018: 10.3389/fmolb.2018.00062

Intraspecific variation

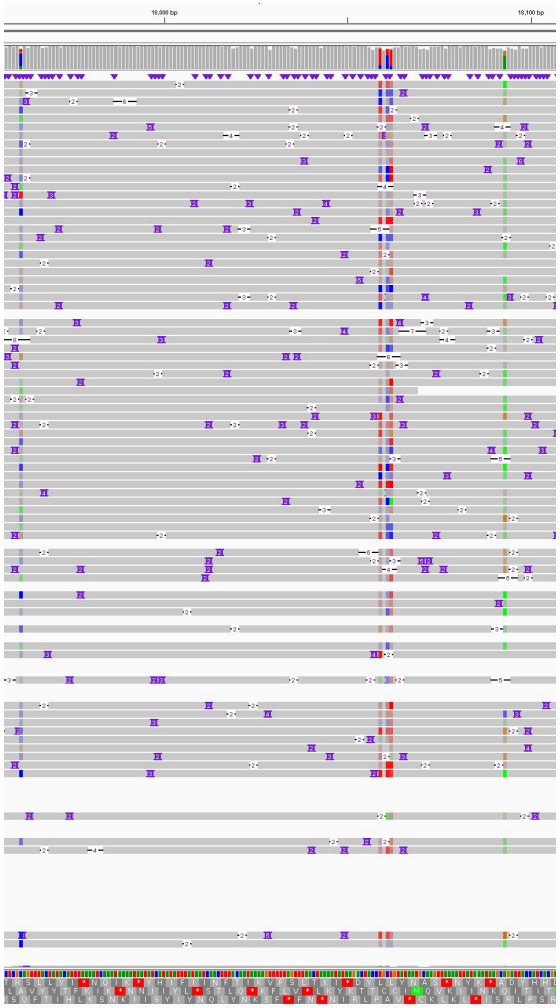
- Different accessions of the same species have genomic differences
- Genotype determines phenotype (traits)
- Specific traits are relevant for breeding or biotechnological/agronomical applications



Sielemann et al., 2021: 10.1186/s13007-021-00718-5

Differences between samples and reference

- Differences are identified against a reference genome sequence (classic approach)
- Reads of a sample are aligned against the reference (mapping)
- Differences are identified by tools (variant calling)



Read mapping

- Alignment of reads to a genome sequence
- Mapping needs to be fast at the cost of accuracy
- Manual inspection of read mappings via Integrated Genomics Viewer (IGV)

SAM/BAM

- SAM = Sequence Alignment/Map format
- BAM = Binary Alignment/Map format (binary version of SAM)
- Another way to store read information: contains information from FASTA and FASTQ file (reads mapped to reference)

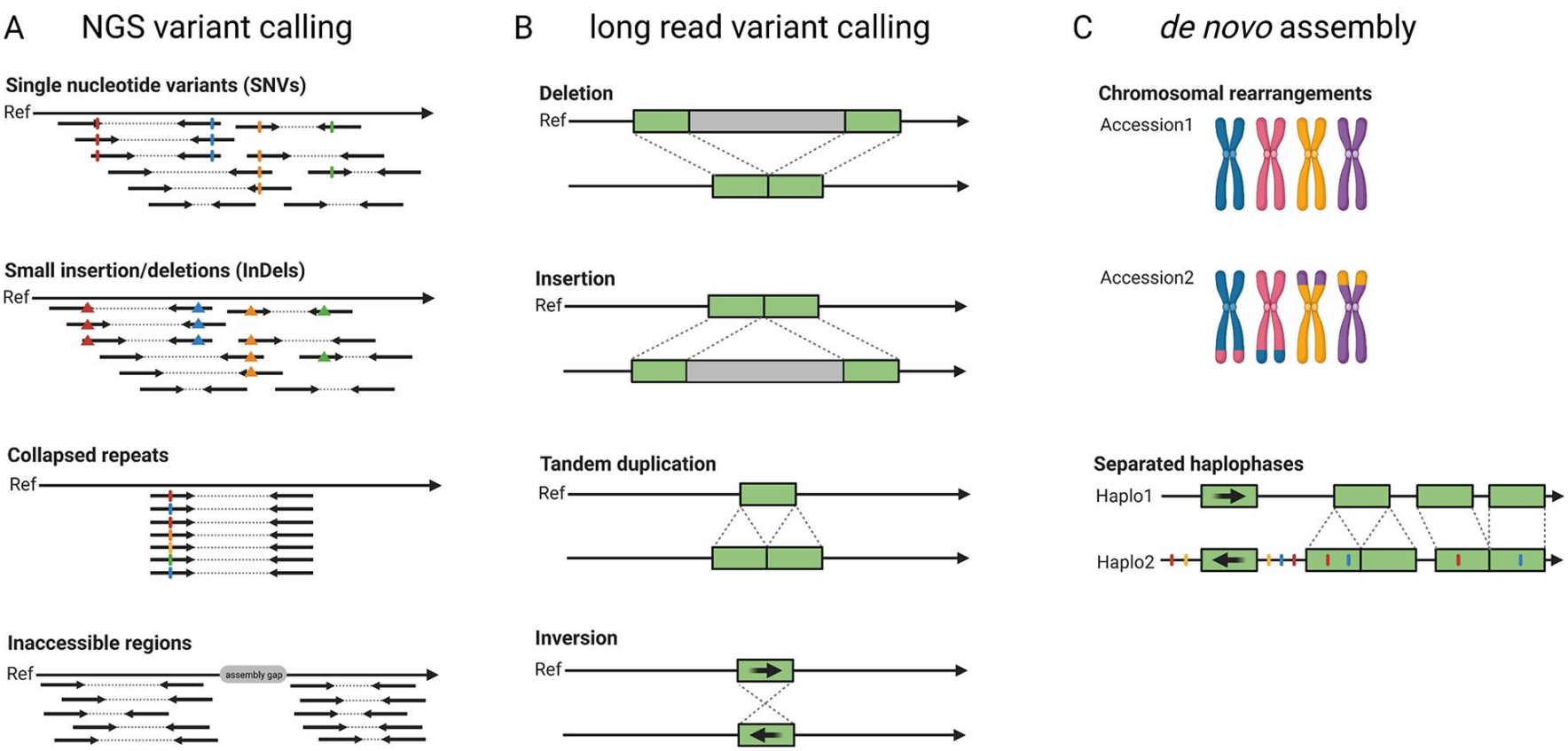
Variant calling

- Identification of sequence differences between reads and reference sequence
- Differences are listed in a specific file type: Variant Caller Format (VCF)
- ONT long reads are well suited for the identification of large structural variants

Types of sequence variants

- SNVs vs. SNPs
  - Single Nucleotide Variants = no information about their effect
  - Single Nucleotide Polymorphisms = no detrimental impact
- MNVs
  - Multiple Nucleotide Variants
- InDels
  - Insertions: additional bases in sample compared to reference
  - Deletions: loss of bases in sample compared to reference
- Inversions
  - Orientation of sequence differs between sample and reference
- Tandem duplications

Read mapping vs. de novo genome sequence assembly



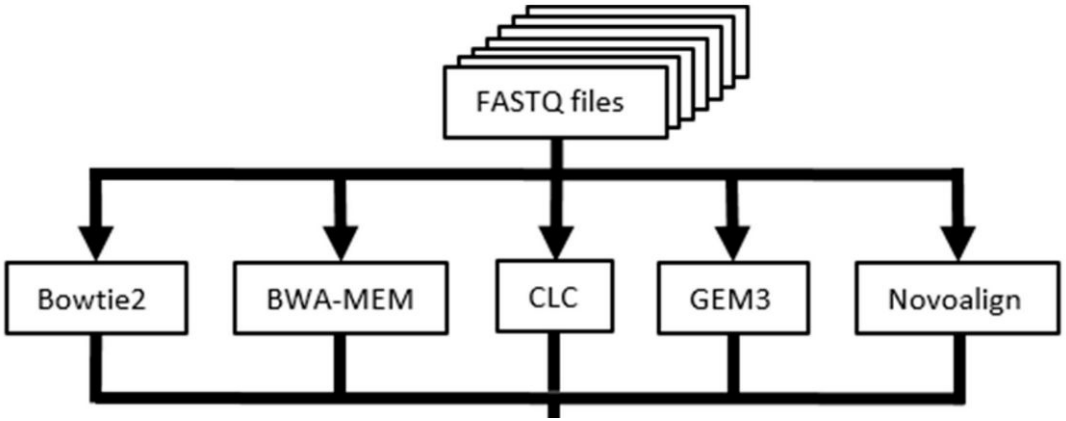
Pucker et al., 2022: 10.1017/qpb.2021.18

Challenges of read mappings

- Speed / computational costs
  - BLAST would be too slow
- Specific assignment of reads / repeats
  - (especially challenging in polyploid species)
- Splitting around InDels or across introns

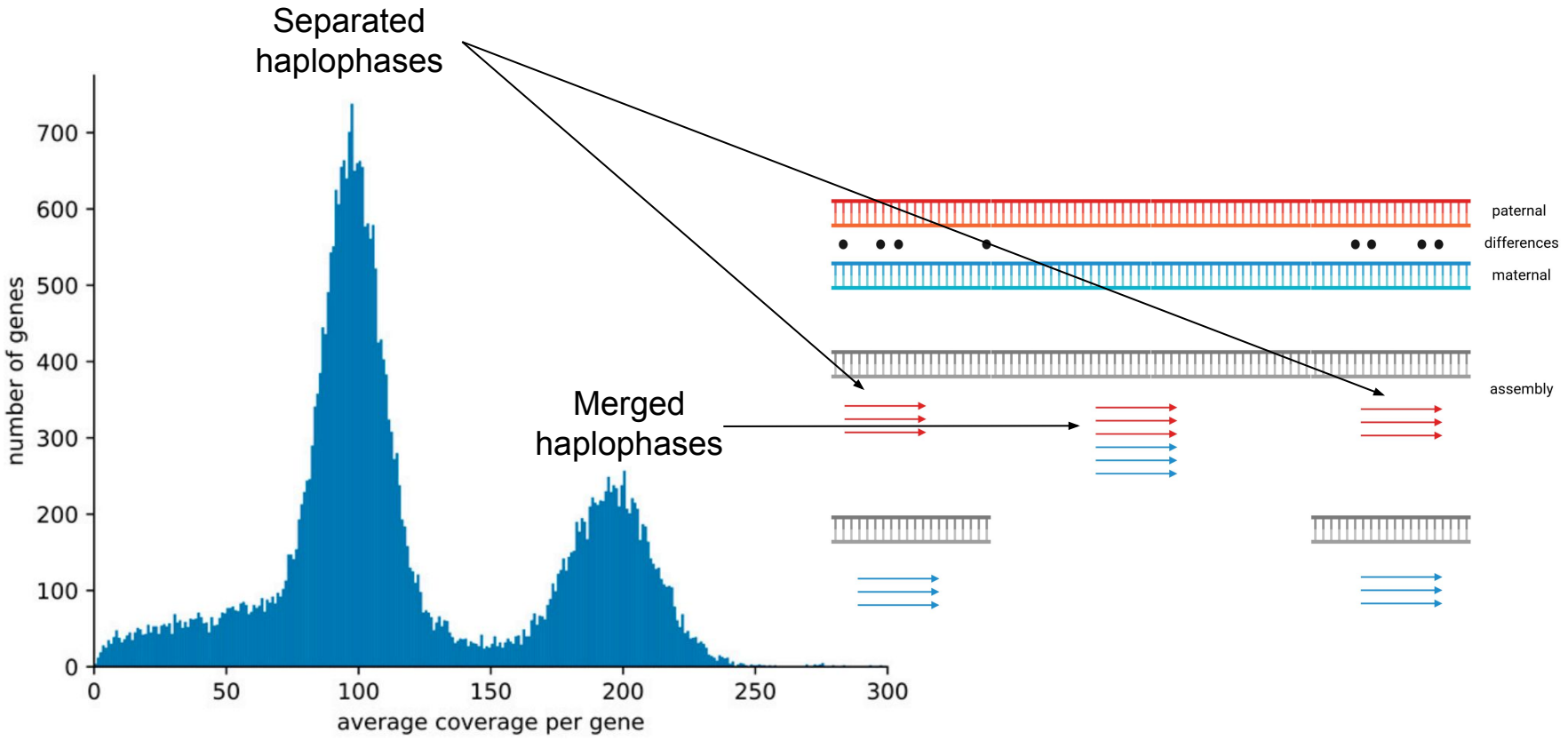
Short read mappings

- Large number of tools for mappings
- DNA seq read mappers: BWA MEM, bowtie2
- RNA-seq read mappers (split read): STAR, HiSAT2

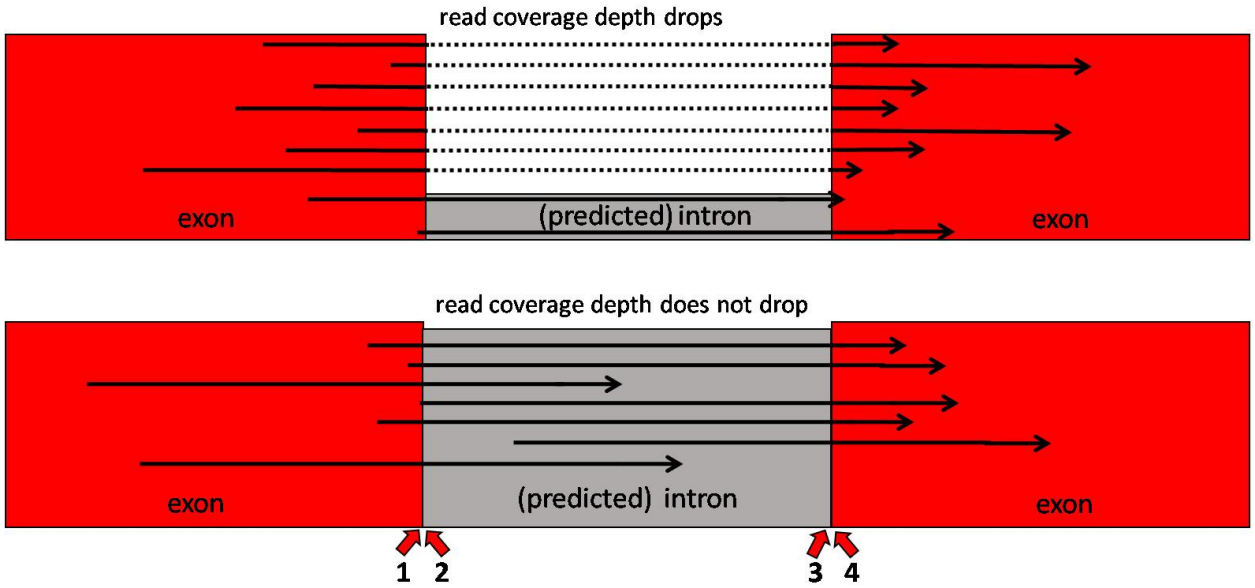


Li, 2013: 10.48550/arXiv.1303.3997
Langmead & Salzberg, 2012: 10.1038/nmeth.1923
Dobin et al., 2013: 10.1093/bioinformatics/bts635
Kim et al., 2015: 10.1038/nmeth.3317
Schilbert et al., 2020: 10.3390/plants9040439

Coverage analysis: haplotype phasing / ploidy



Coverage analysis: splice sites



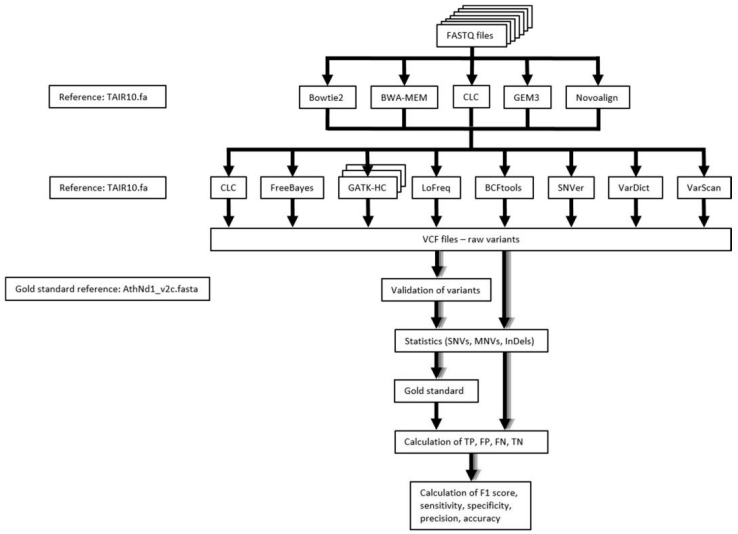
Pucker & Brockington, 2018: 10.1186/s12864-018-5360-z

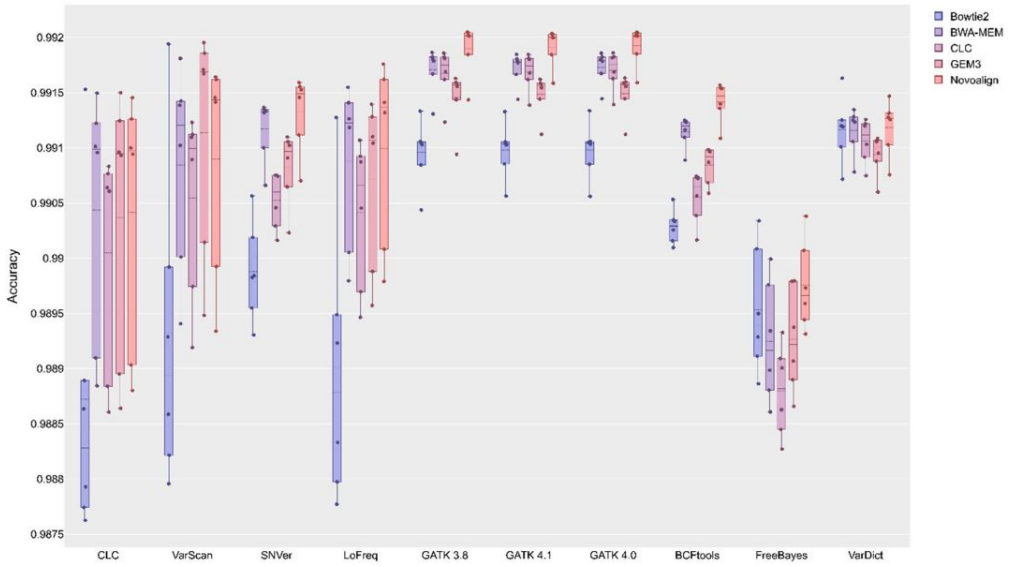
Short read variant callers

- Millions of differences between samples and reference
- Automatic detection with tools required
- Tools:
  - Genome Analysis Tool Kit (GATK): https://gatk.broadinstitute.org/hc/en-us
  - Samtools: http://www.htslib.org/
  - VarDict: https://anaconda.org/bioconda/vardict
  - VarScan: https://github.com/dkoboldt/varscan
  - SNVer: http://snver.sourceforge.net/
  - FreeBayes: https://github.com/freebayes/freebayes

McKenna et al., 2010: 10.1101/gr.107524.110
Li et al., 2009: 10.1093/bioinformatics/btp352
Lai et al., 2016: 10.1093/nar/gkw227
Koboldt et al., 2009: 10.1093/bioinformatics/btp373
Wei et al., 2011: 10.1093/nar/gkr599
Garrison & Marth, 2012: 10.48550/arXiv.1207.3907

Benchmarking of variant callers

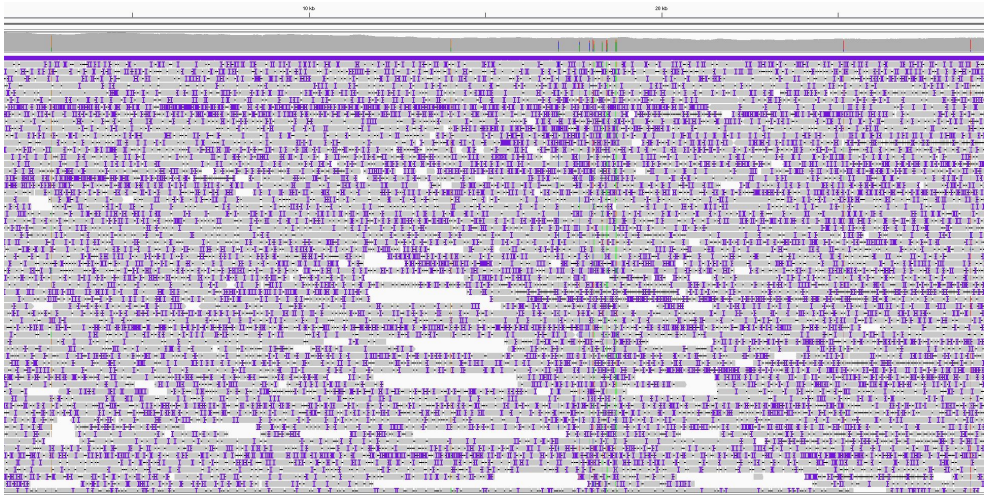




Schilbert et al., 2020: 10.3390/plants9040439

Long read mapping

- Noisy alignments due to high rate of sequencing errors
- InDels are particularly abundant (purple ticks)
- Rapid development of new tools
- Benchmarking studies are missing and would require frequent updates



Long read mapping tools

- Collection of long read tools: https://long-read-tools.org/
- Over 600 tools available
- Examples:
  - BWA-SW: http://bio-bwa.sourceforge.net/
  - Minimap2: https://github.com/lh3/minimap2
  - GraphMap: https://github.com/isovic/graphmap
  - NGMLR: https://github.com/philres/ngmlr
  - Winnowmap2: https://github.com/marbl/Winnowmap

Amarasinghe et al., 2021: 10.1093/gigascience/giab003
Li & Durbin, 2010: 10.1093/bioinformatics/btp698
Li, 2018: 10.1093/bioinformatics/bty191
Sovic, 2016: 10.1038/ncomms11307
Sedlazeck et al., 2018: 10.1038/s41592-018-0001-7
Jain et al., 2022: 10.1038/s41592-022-01457-8

Long read variant calling

- Rapid development of new tools and lack of benchmarking studies

- Tools:
  - SVIM2: https://github.com/eldariont/svim
  - Longshot: https://github.com/pjedge/longshot
  - NanoCaller: https://github.com/WGLab/NanoCaller
  - Sniffles2: https://github.com/fritzsedlazeck/Sniffles

Heller & Vingron, 2019: 10.1093/bioinformatics/btz041
Edge & Basal, 2019: 10.1038/s41467-019-12493-y
Ahsan et al., 2021: 10.1186/s13059-021-02472-2
Smolka et al., 2022: 10.1101/2022.04.04.487055

Variant Call Format (VCF)

- Chromosome: name of sequence in the reference
- Position: position on the specified sequence
- ID (not relevant in plant biology): variants in humans have IDs
- Reference allele: nucleotide(s) in the reference sequence at the specified position
- Alternative allele: nucleotide(s) in the sample at the same position
- Quality: tool specific value that can be used for filtering
- Filter status: specifies filter name if variant was filtered out
- Info: collection of information
- Format: explains the fields in the following sample columns
- DATA_SET1, DATA_SET2, DATA_SET3, …: one column per sample

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	DATA_SET
R105	10929	.	A	G	58.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-1.242;DP=8;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=44.01;MQRankSum=-2.100;QD=7.33;ReadPosRankSum=0.619;SOR=0.307	GT:AD:DP:GQ:PL	0/1:6,2:8:66:66,0,230
R105	10944	.	A	G	58.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-1.068;DP=8;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=44.01;MQRankSum=-2.100;QD=7.33;ReadPosRankSum=-1.465;SOR=0.307	GT:AD:DP:GQ:PL	0/1:6,2:8:66:66,0,230
R105	10955	.	G	T	61.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-1.068;DP=7;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=46.16;MQRankSum=-1.981;QD=8.81;ReadPosRankSum=-1.465;SOR=0.446	GT:AD:DP:GQ:PL	0/1:5,2:7:69:69,0,204
R105	10962	.	A	G	61.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-1.068;DP=8;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=48.11;MQRankSum=-1.981;QD=8.81;ReadPosRankSum=-1.465;SOR=0.446	GT:AD:DP:GQ:PL	0/1:5,2:7:69:69,0,204
R105	13234	.	T	C	32.64	MQ_filter;QD_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=1.062;DP=35;ExcessHet=3.0103;FS=2.782;MLEAC=1;MLEAF=0.500;MQ=37.70;MQRankSum=-1.106;QD=1.31;ReadPosRankSum=-0.209;SOR=1.911	GT:AD:DP:GQ:PL	0/1:22,3:25:40:40,0,706
R105	13260	.	T	G	72.64	MQ_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=-0.172;DP=36;ExcessHet=3.0103;FS=11.698;MLEAC=1;MLEAF=0.500;MQ=38.68;MQRankSum=-1.349;QD=2.50;ReadPosRankSum=0.243;SOR=3.442	GT:AD:DP:GQ:PL	0/1:23,6:29:80:80,0,695
R105	13395	.	T	A	183.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=1.979;DP=19;ExcessHet=3.0103;FS=8.929;MLEAC=1;MLEAF=0.500;MQ=43.94;MQRankSum=-1.631;QD=9.67;ReadPosRankSum=1.186;SOR=3.126	GT:AD:DP:GQ:PL	0/1:13,6:19:99:191,0,413
R105	13538	.	T	C	61.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=1.359;DP=27;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=46.51;MQRankSum=-1.847;QD=2.47;ReadPosRankSum=1.188;SOR=0.760	GT:AD:DP:GQ:PL	0/1:21,4:25:69:69,0,660
R105	14511	.	C	T	39.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-0.563;DP=13;ExcessHet=3.0103;FS=2.522;MLEAC=1;MLEAF=0.500;MQ=43.42;MQRankSum=-2.771;QD=3.05;ReadPosRankSum=0.217;SOR=1.546	GT:AD:DP:GQ:PL	0/1:9,4:13:47:47,0,352
R105	14542	.	G	A	51.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=-1.825;DP=15;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=42.30;MQRankSum=-2.843;QD=3.44;ReadPosRankSum=-0.919;SOR=1.022	GT:AD:DP:GQ:PL	0/1:10,5:15:59:59,0,390
R105	15681	.	A	G	116.64	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=1.897;DP=43;ExcessHet=3.0103;FS=4.187;MLEAC=1;MLEAF=0.500;MQ=40.84;MQRankSum=-2.008;QD=2.92;ReadPosRankSum=0.303;SOR=1.721	GT:AD:DP:GQ:PL	0/1:34,6:40:99:124,0,1161
R105	15712	.	C	T	70.64	MQ_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=1.307;DP=35;ExcessHet=3.0103;FS=2.026;MLEAC=1;MLEAF=0.500;MQ=39.58;MQRankSum=-2.129;QD=2.08;ReadPosRankSum=-0.994;SOR=1.230	GT:AD:DP:GQ:PL	0/1:30,4:34:78:78,0,1247
R105	15719	.	C	T	64.64	MQ_filter;QD_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=0.166;DP=37;ExcessHet=3.0103;FS=2.037;MLEAC=1;MLEAF=0.500;MQ=38.62;MQRankSum=-2.020;QD=1.80;ReadPosRankSum=-1.360;SOR=1.251	GT:AD:DP:GQ:PL	0/1:32,4:36:72:72,0,1310
R105	16037	.	C	A	50.64	MQ_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=0.000;DP=7;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=34.75;MQRankSum=0.303;QD=7.23;ReadPosRankSum=-0.712;SOR=1.802	GT:AD:DP:GQ:PL	0/1:5,2:7:58:58,0,171
R105	16582	.	CATTGTAAG	C	58.60	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=0.712;DP=8;ExcessHet=3.0103;FS=3.680;MLEAC=1;MLEAF=0.500;MQ=38.22;MQRankSum=0.319;QD=7.33;ReadPosRankSum=0.366;SOR=2.236	GT:AD:DP:GQ:PL	0/1:6,2:8:66:66,0,246
R105	17718	.	A	G	79.64	MQ_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=-0.722;DP=18;ExcessHet=3.0103;FS=7.656;MLEAC=1;MLEAF=0.500;MQ=29.01;MQRankSum=-0.858;QD=4.42;ReadPosRankSum=0.099;SOR=0.041	GT:AD:DP:GQ:PL	0/1:13,5:18:87:87,0,392
R105	37909	.	A	G	51.64	MQ_filter	AC=1;AF=0.500;AN=2;BaseQRankSum=-0.431;DP=9;ExcessHet=3.0103;FS=10.792;MLEAC=1;MLEAF=0.500;MQ=37.28;MQRankSum=0.549;QD=5.74;ReadPosRankSum=-0.549;SOR=2.206	GT:AD:DP:GQ:PL	0/1:7,2:9:59:59,0,254
R105	45724	.	C	CTTATA	61.60	PASS	AC=1;AF=0.500;AN=2;BaseQRankSum=0.210;DP=7;ExcessHet=3.0103;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=43.83;MQRankSum=0.967;QD=8.80;ReadPosRankSum=-0.210;SOR=0.446	GT:AD:DP:GQ:PL	0/1:5,2:7:69:69,0,204

genomic Variant Call Format (gVCF)

- Generally similar to VCF file
- One entry for each position in the reference
- Not just variants, but also non-variants are listed

Variant Annotation - SnpEff

- High throughput annotation of SNVs and larger variants
- Reference sequence (FASTA) and structural annotation (GFF) required
- Freely available software: http://pcingola.github.io/SnpEff/
- Addition of one annotation field to VCF file

```
chrA01  179964  .  T  A  .  PASS  GSL_High,GSL_Low;ANN=A|stop_gained|HIGH|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.863T>A|p.Leu288*|863/1834|863/1834|288/610|   GT:AD:DP:GQ:PL
chrA01  179967  .  T  C  .  PASS  GSL_High,GSL_Low;ANN=C|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.866T>C|p.Phe289Ser|866/1834|866/1834|289/610|       GT:AD:DP:GQ:PL
chrA01  179979  .  C  T  .  PASS  GSL_Low;ANN=T|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.878C>T|p.Thr293Met|878/1834|878/1834|293/610|         GT:AD:DP:GQ:PL
chrA01  179988  .  A  T  .  PASS  GSL_Low;ANN=T|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.887A>T|p.Tyr296Phe|887/1834|887/1834|296/610|         GT:AD:DP:GQ:PL
chrA01  180003  .  G  A  .  PASS  GSL_Low;ANN=A|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.902G>A|p.Gly301Asp|902/1834|902/1834|301/610|         GT:AD:DP:GQ:PL
chrA01  180024  .  A  G  .  PASS  GSL_High,GSL_Low;ANN=G|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.923A>G|p.His308Arg|923/1834|923/1834|308/610|       GT:AD:DP:GQ:PL
chrA01  180039  .  T  C  .  PASS  GSL_High,GSL_Low;ANN=C|missense_variant|MODERATE|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.938T>C|p.Ile313Thr|938/1834|938/1834|313/610|       GT:AD:DP:GQ:PL
chrA01  180048  .  G  A  .  PASS  GSL_High,GSL_Low;ANN=A|stop_retained_variant|LOW|Gene_BnaA01g00340D|Gene_BnaA01g00340D|transcript|BnaA01g00340D|Coding|5/9|c.947G>A|p.Ter316Ter|947/1834|947/1834|316/610|       GT:AD:DP:GQ:PL
```

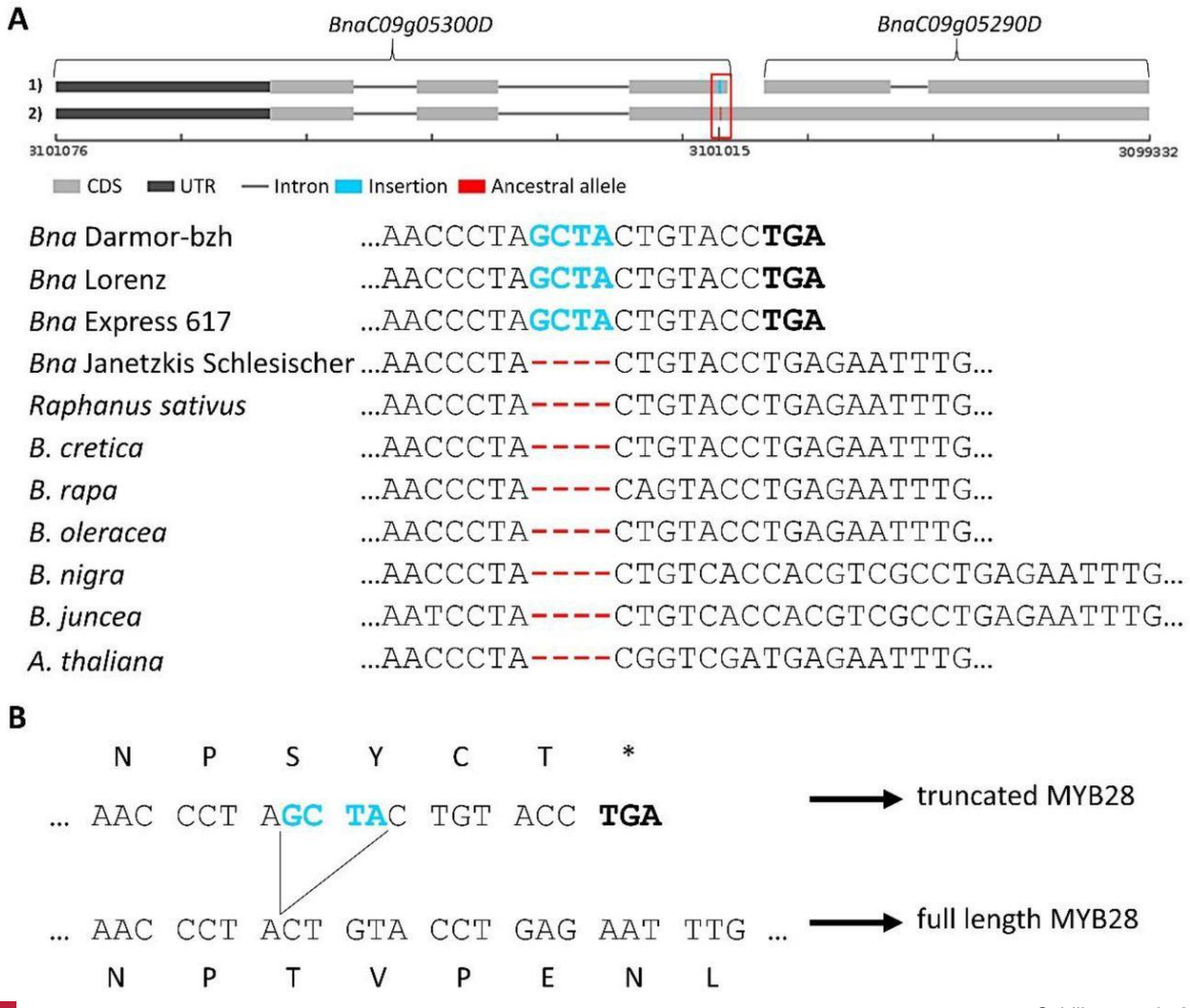
Cingolani et al., 2012: 10.4161/fly.19695

SnpEff predictions

Type	Meaning	Example
SNP	Single Nucleotide Polymorphism	Reference = ‘A’, Sample = ‘C’
Ins	Insertion	Reference = ‘A’, Sample = ‘AGT’
Del	Deletion	Reference = ‘AC’, Sample = ‘C’
MNP	Multiple Nucleotide Polymorphism	Reference = ‘ATA’, Sample = ‘GTC’
MIXED	MNP and InDel	Reference = ‘ATA’, Sample = ‘GTCAGT’

http://pcingola.github.io/SnpEff/se_introduction/

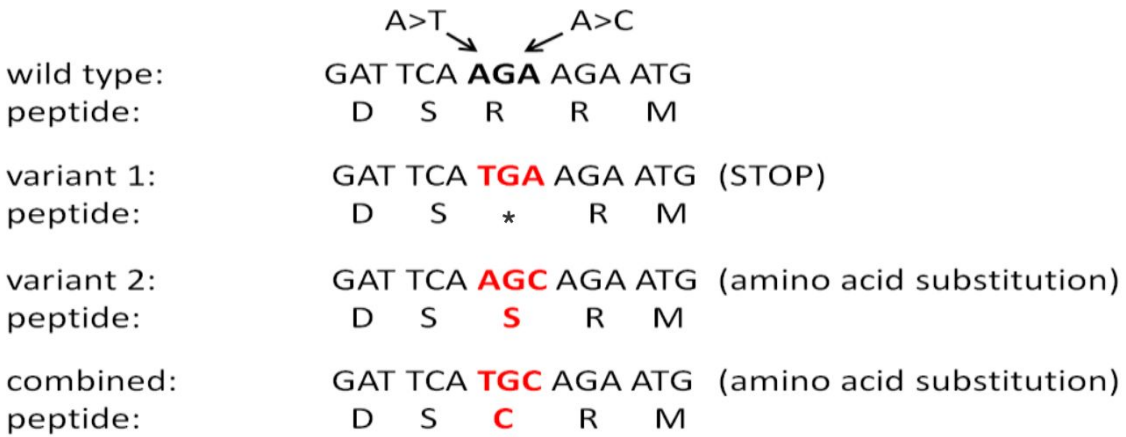
Example: Glucosinolate biosynthesis in rapeseed



Schilbert et al., 2022: 10.1101/2022.06.01.494149

Variant Annotation - NAVIP

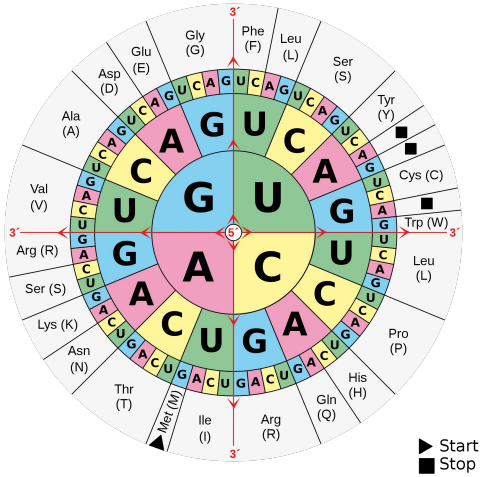
- NAVIP = Neighborhood-Aware Variant Impact Predictor
- Variants can have interacting effects e.g. compensate each other
- Considering multiple variants while predicting effects
- Tool freely available: https://github.com/bpucker/NAVIP

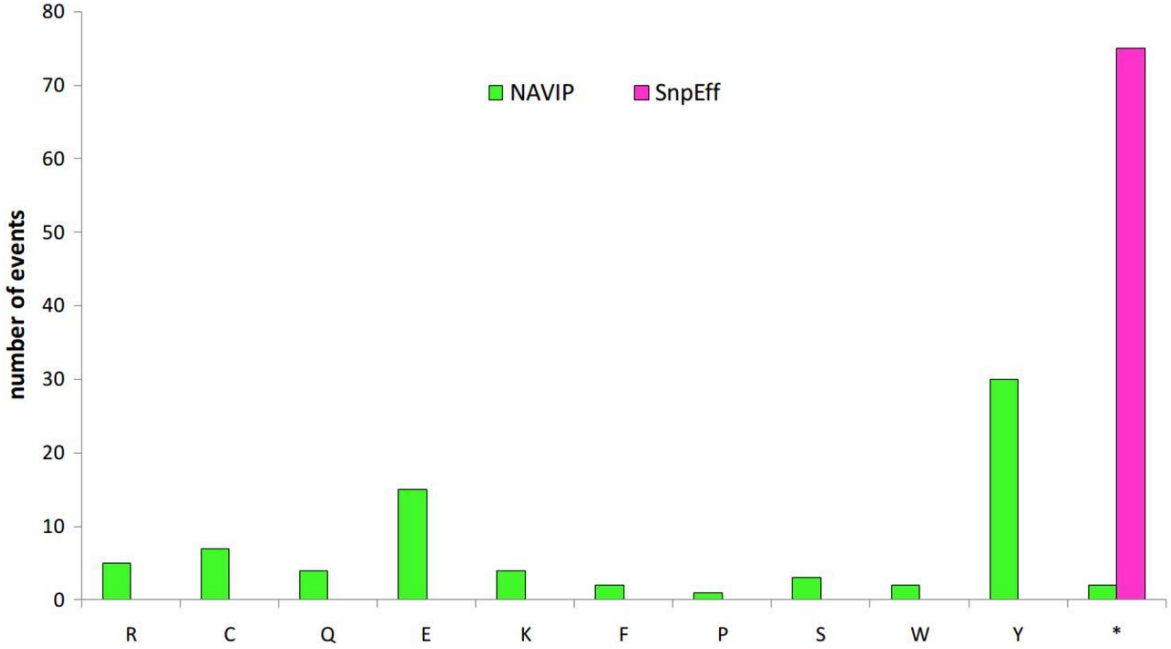


Baasner et al., 2019: 10.1101/596718

Connected SNVs

	A>T ↘ ↙ A>C	
wild type:	GAT TCA **AGA** AGA ATG	
peptide:	D S R R M	
variant 1:	GAT TCA **TGA** AGA ATG	(STOP)
peptide:	D S * R M	
variant 2:	GAT TCA **AGC** AGA ATG	(amino acid substitution)
peptide:	D S **S** R M	
combined:	GAT TCA **TGC** AGA ATG	(amino acid substitution)
peptide:	D S **C** R M	

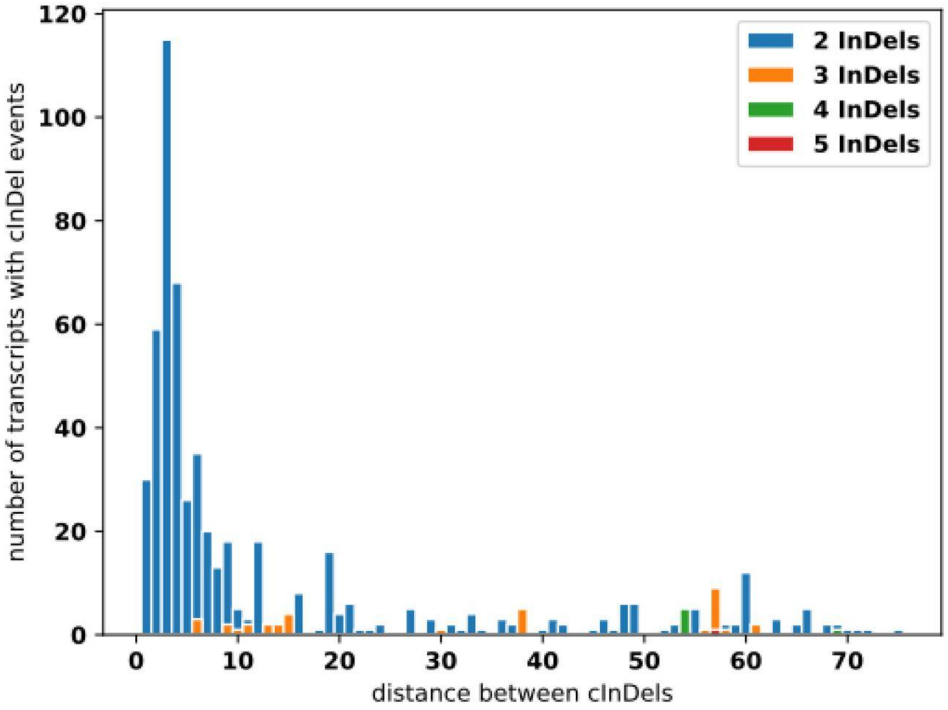




Baasner et al., 2019: 10.1101/596718

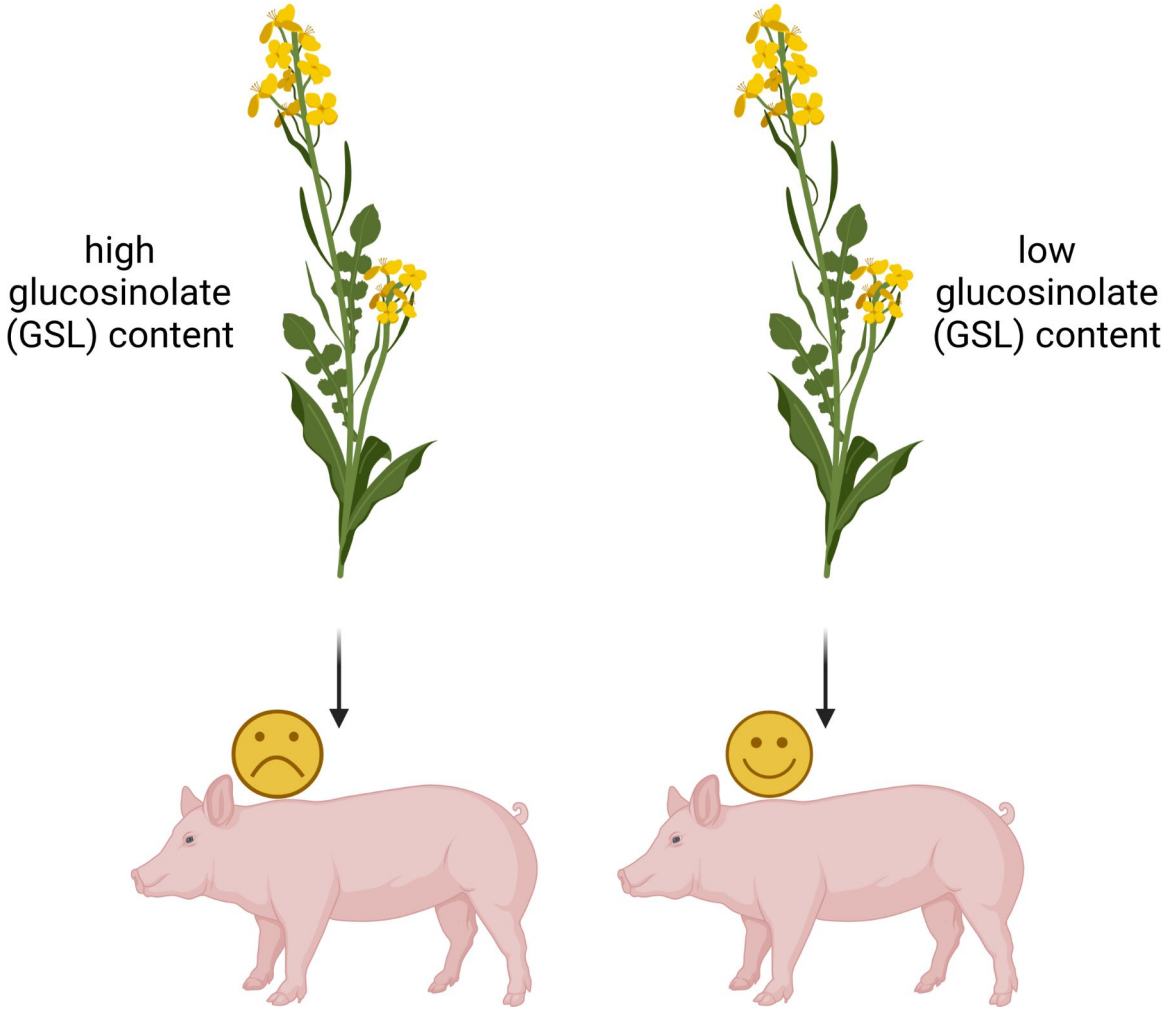
Compensating InDels

```
                  T>TA          C>CGC
wild type:    GTG TAT CTG CGC ATT
peptide:        V   Y   L   R   I

variant 1:    GTG TAT ACT GCG CAT T      (frameshift)
peptide:        V   Y   T   A   H

variant 2:    GTG TAT CTG CGC GCA TT     (frameshift)
peptide:        V   Y   L   R   A

combined:     GTG TAT ACT GCG CGC ATT
peptide:        V   Y   L   A   R   I
```

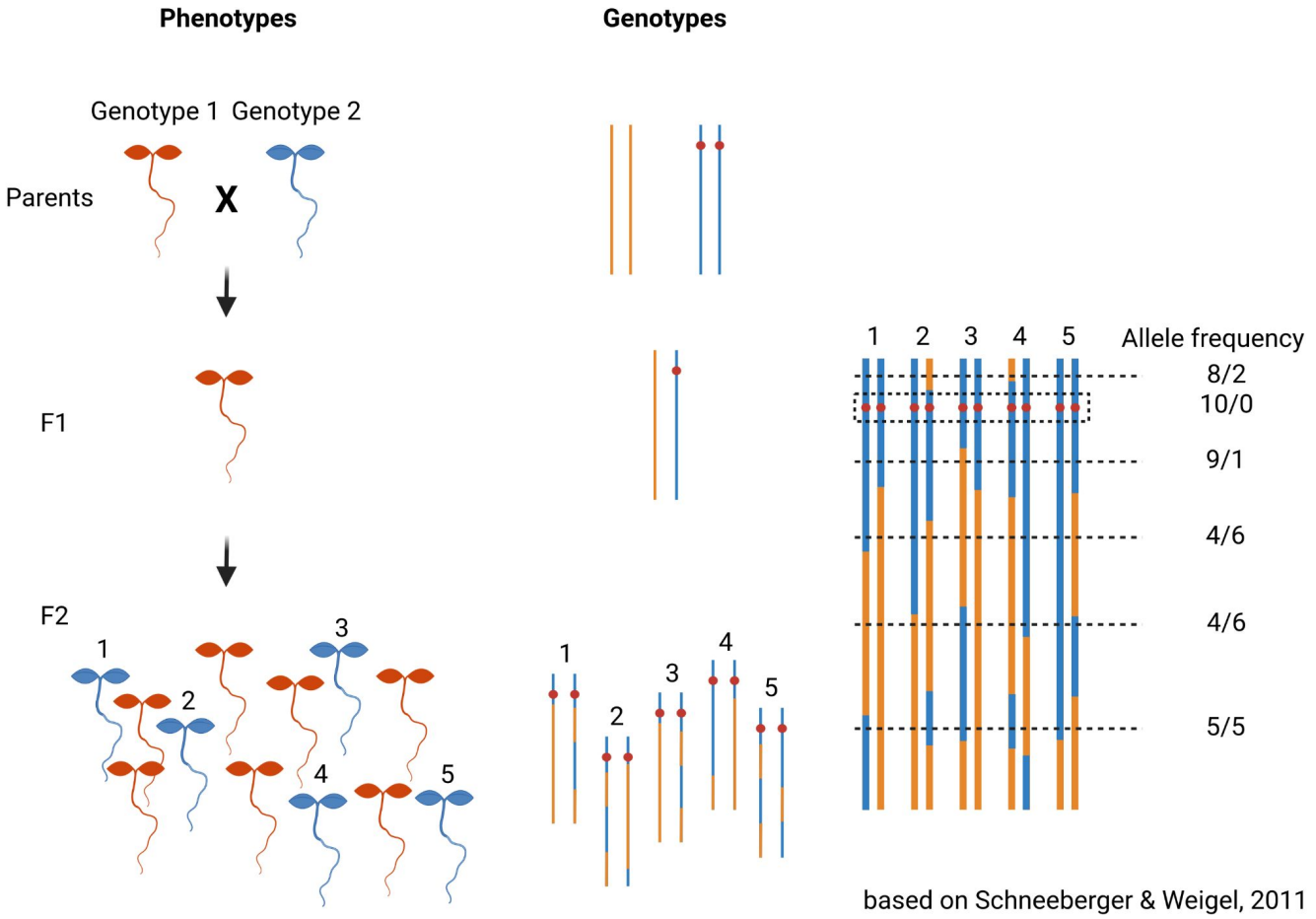


Baasner et al., 2019: 10.1101/596718

Question: What determines differences between plants?

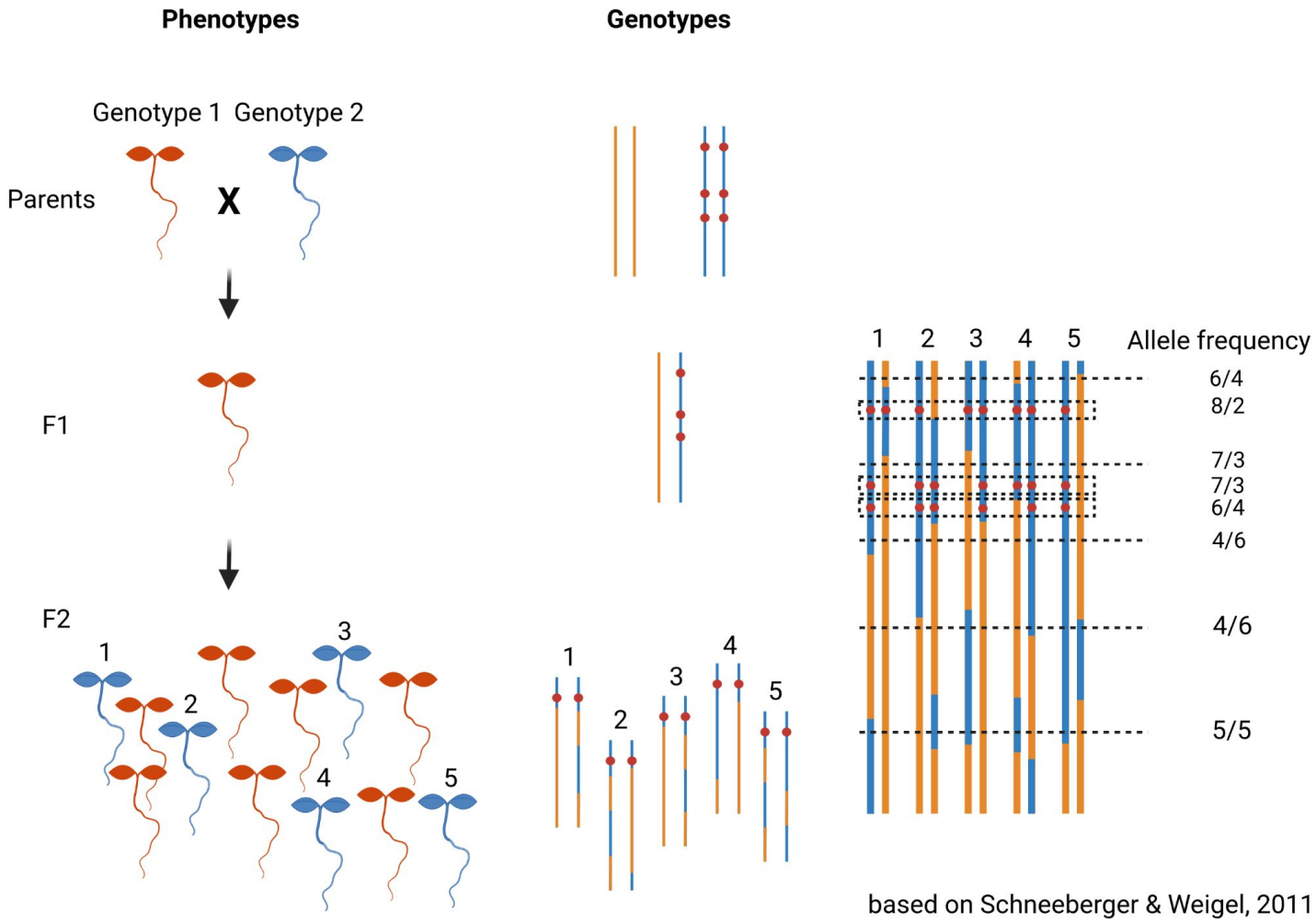


Mapping-by-sequencing (1)



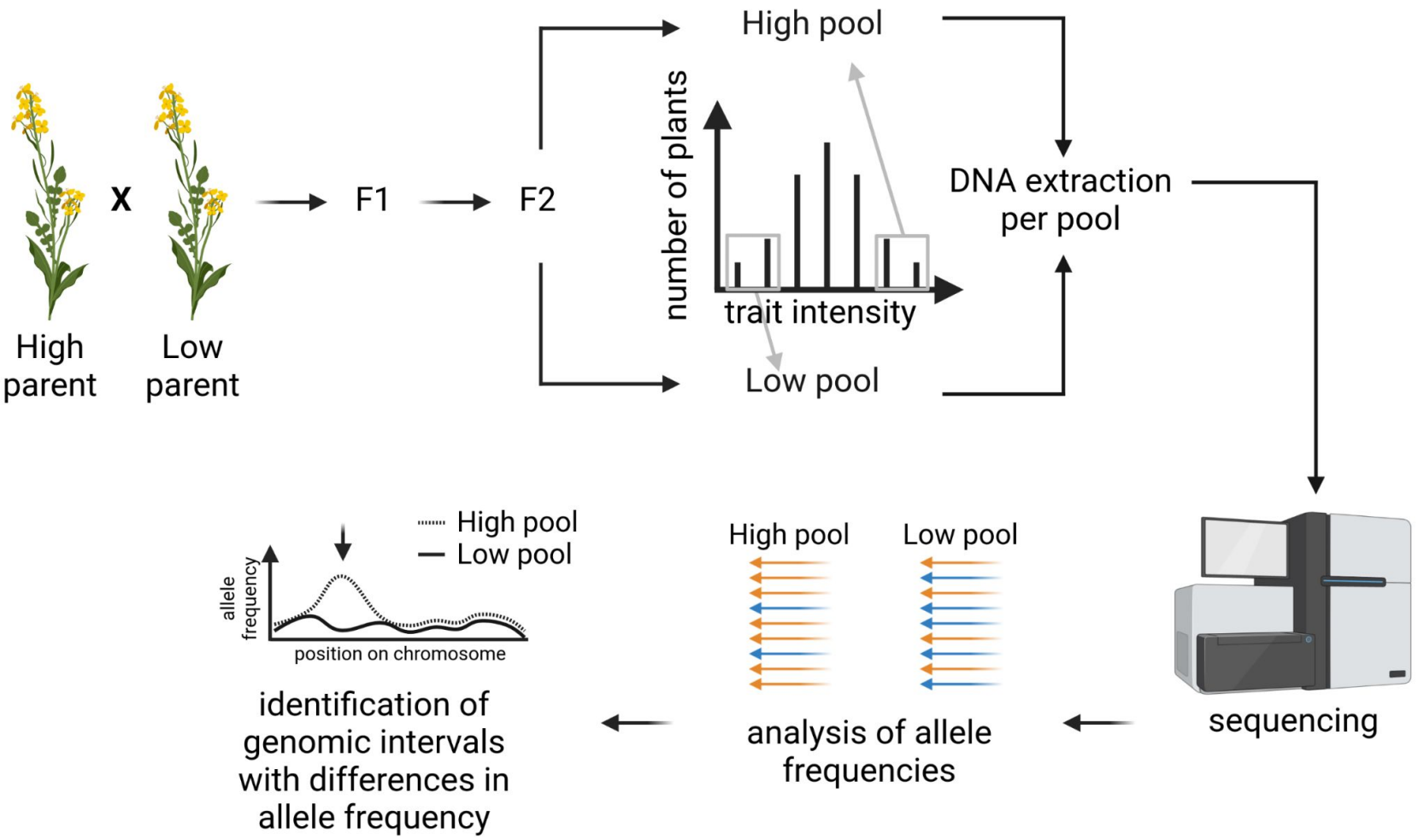
based on Schneeberger & Weigel, 2011

Mapping-by-sequencing (2)

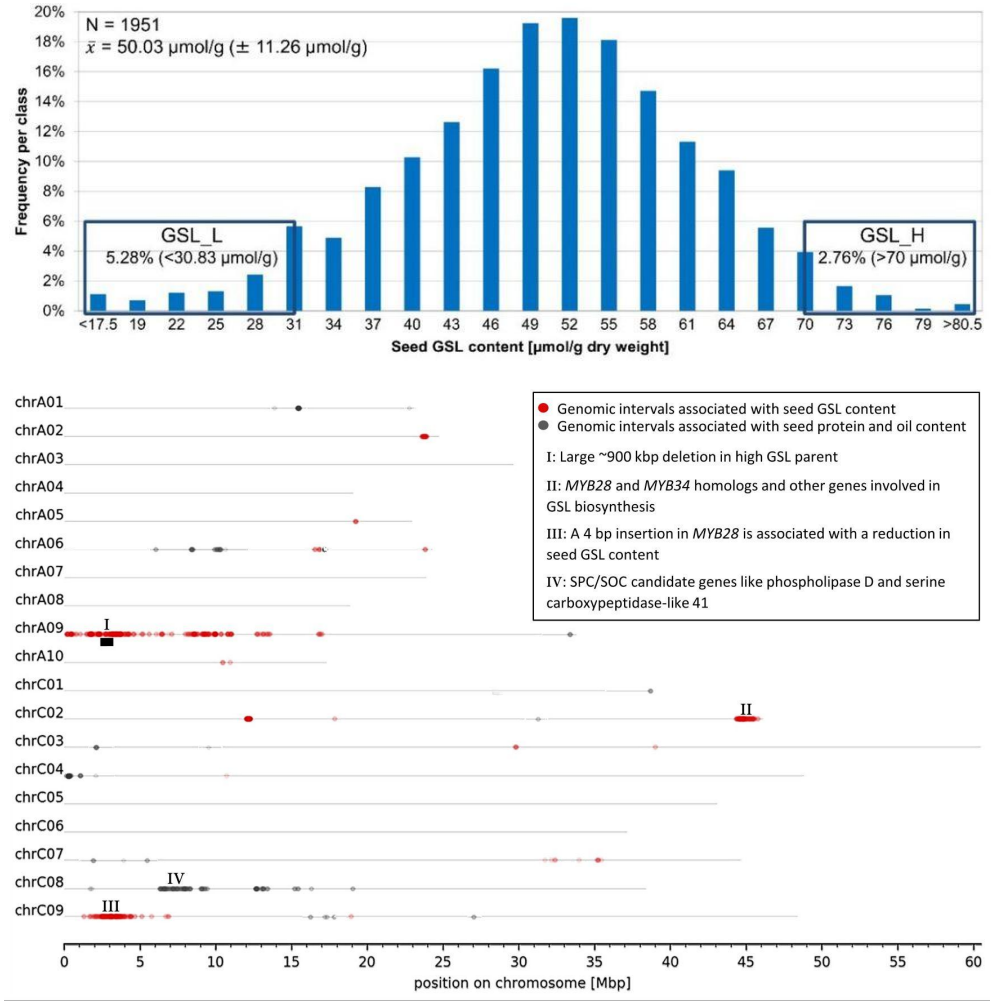


based on Schneeberger & Weigel, 2011

Mapping-by-sequencing (3)



Mapping-by-sequencing (4)



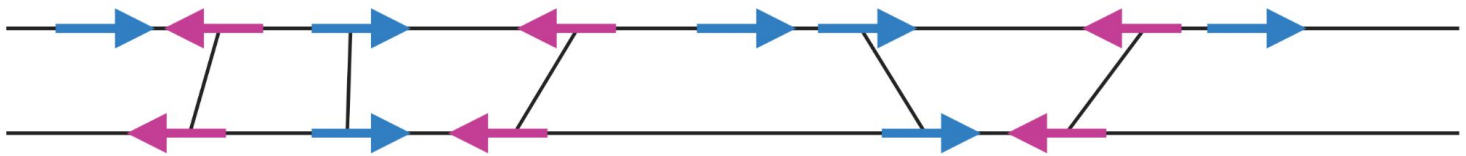
Schilbert et al., 2022: 10.1101/2022.06.01.494149

Variant annotation

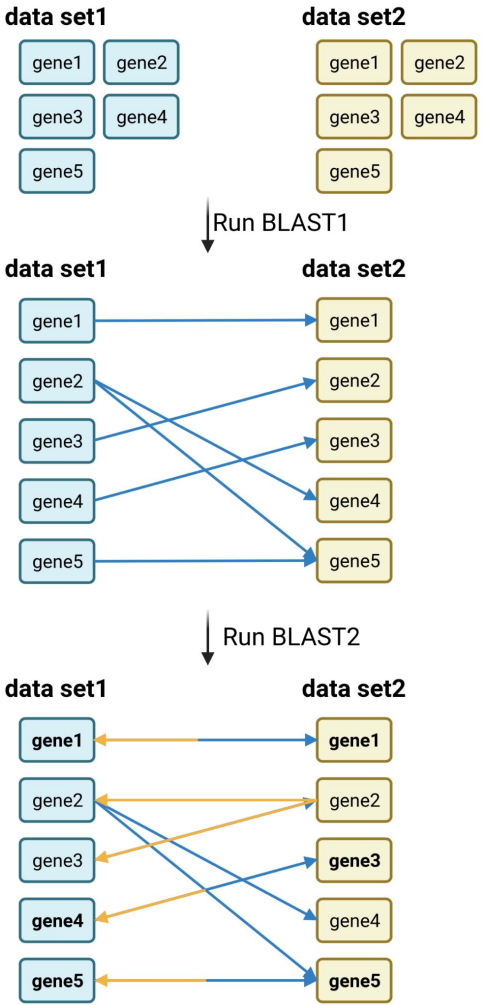
- Variants can have functional consequences
- Variants in coding sequences can change amino acids
- Variants outside CDS can have regulatory consequences

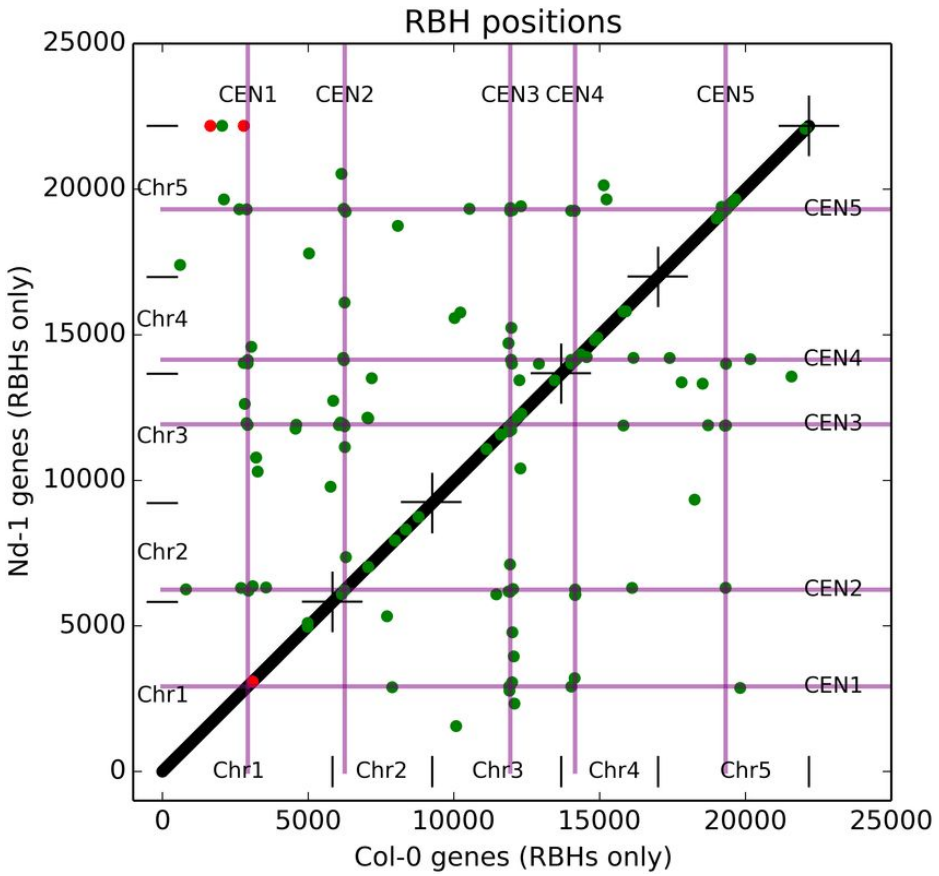
Synteny

Synteny = Same order of genes in different genomes



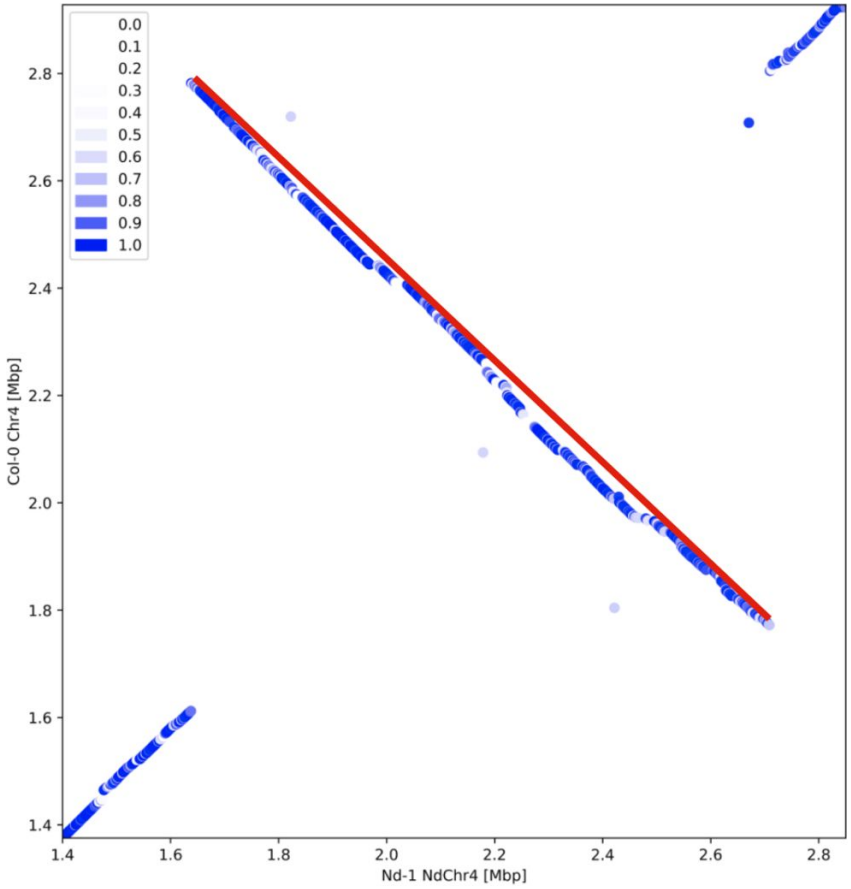
Reciprocal Best BLAST hits (RBHs)

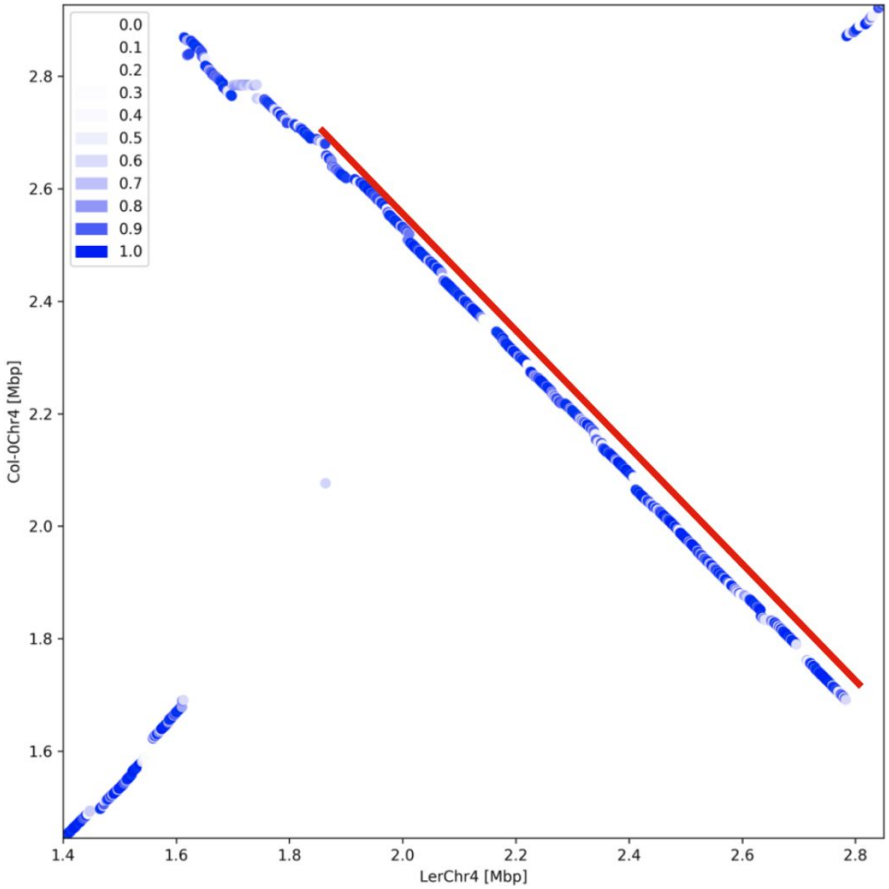




Pucker et al., 2016: 10.1371/journal.pone.0164321

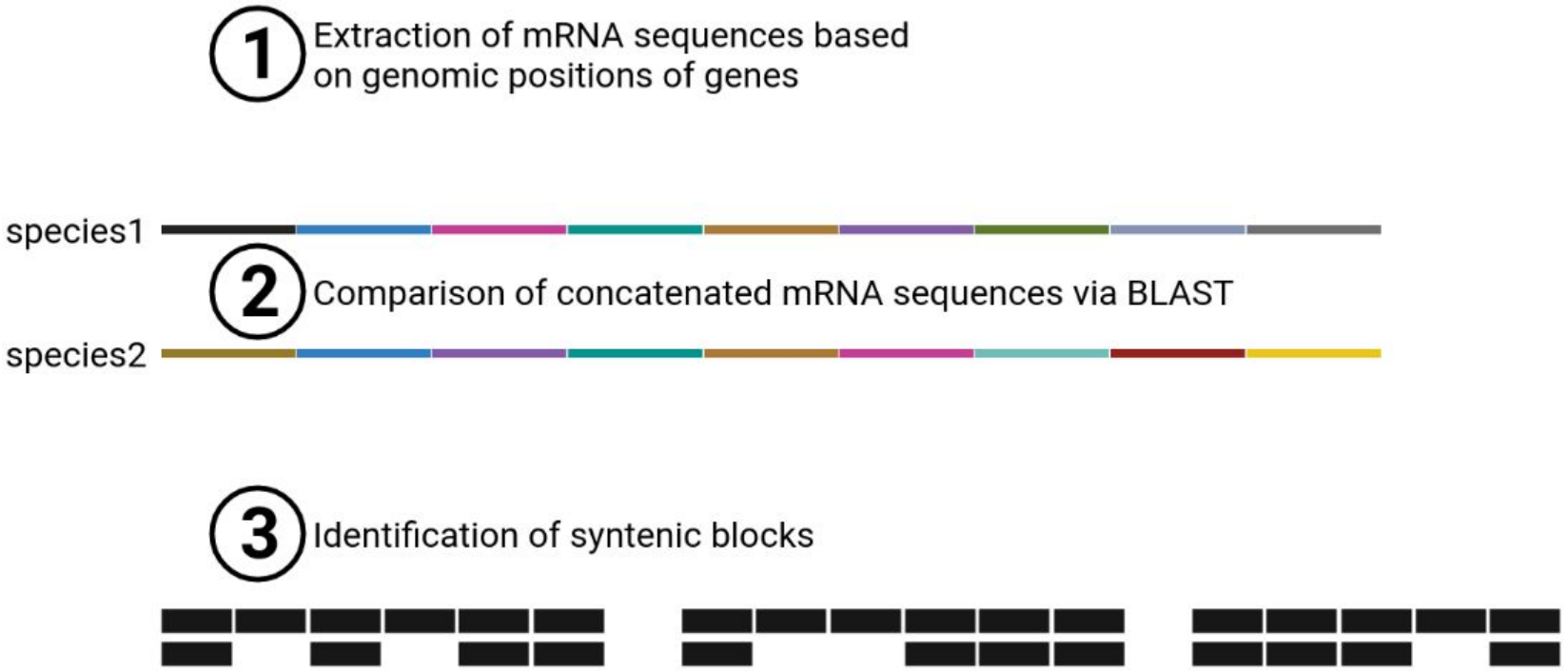
Dot plots





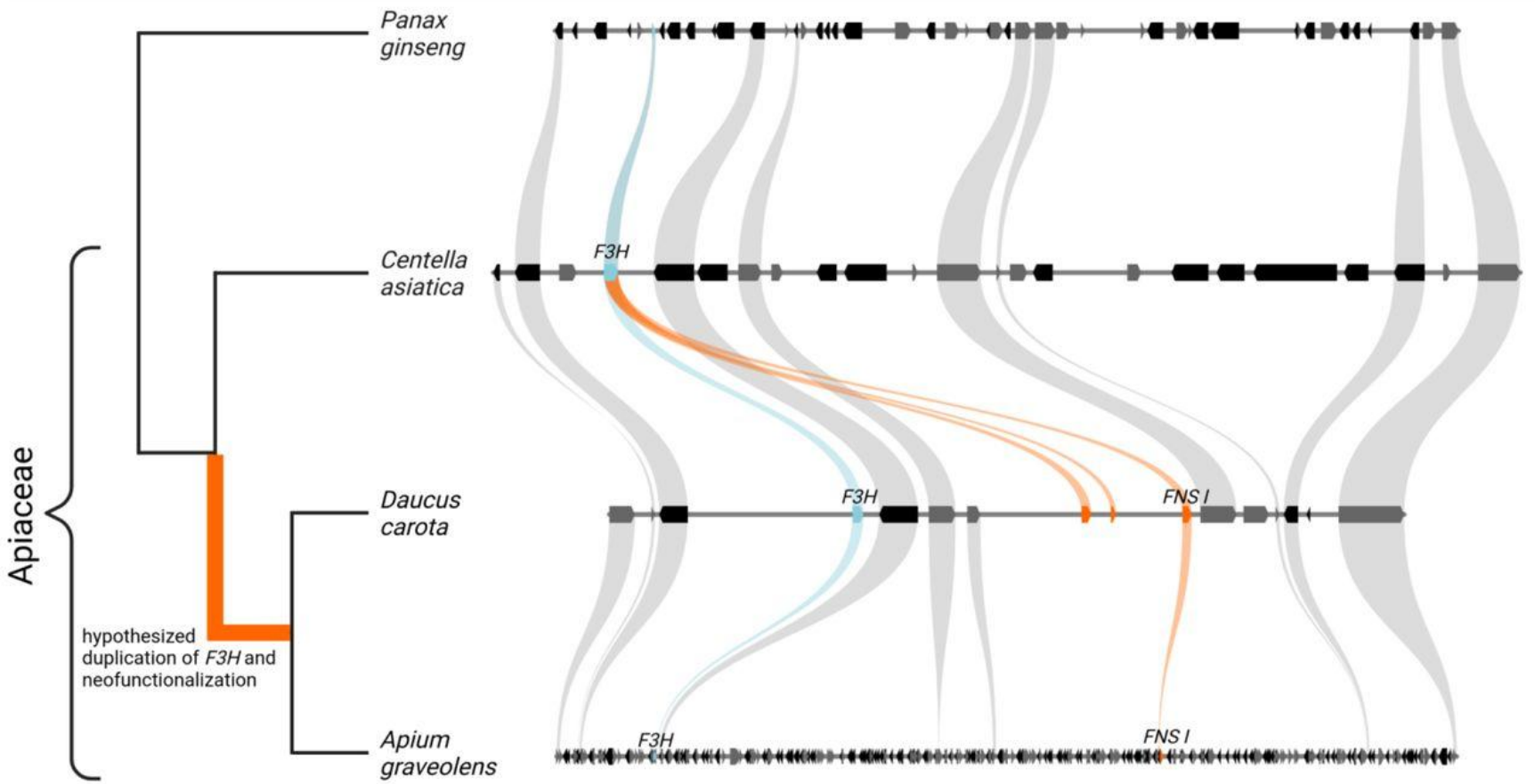
Pucker et al., 2019: 10.1371/journal.pone.0216233

JCVI/MCscan



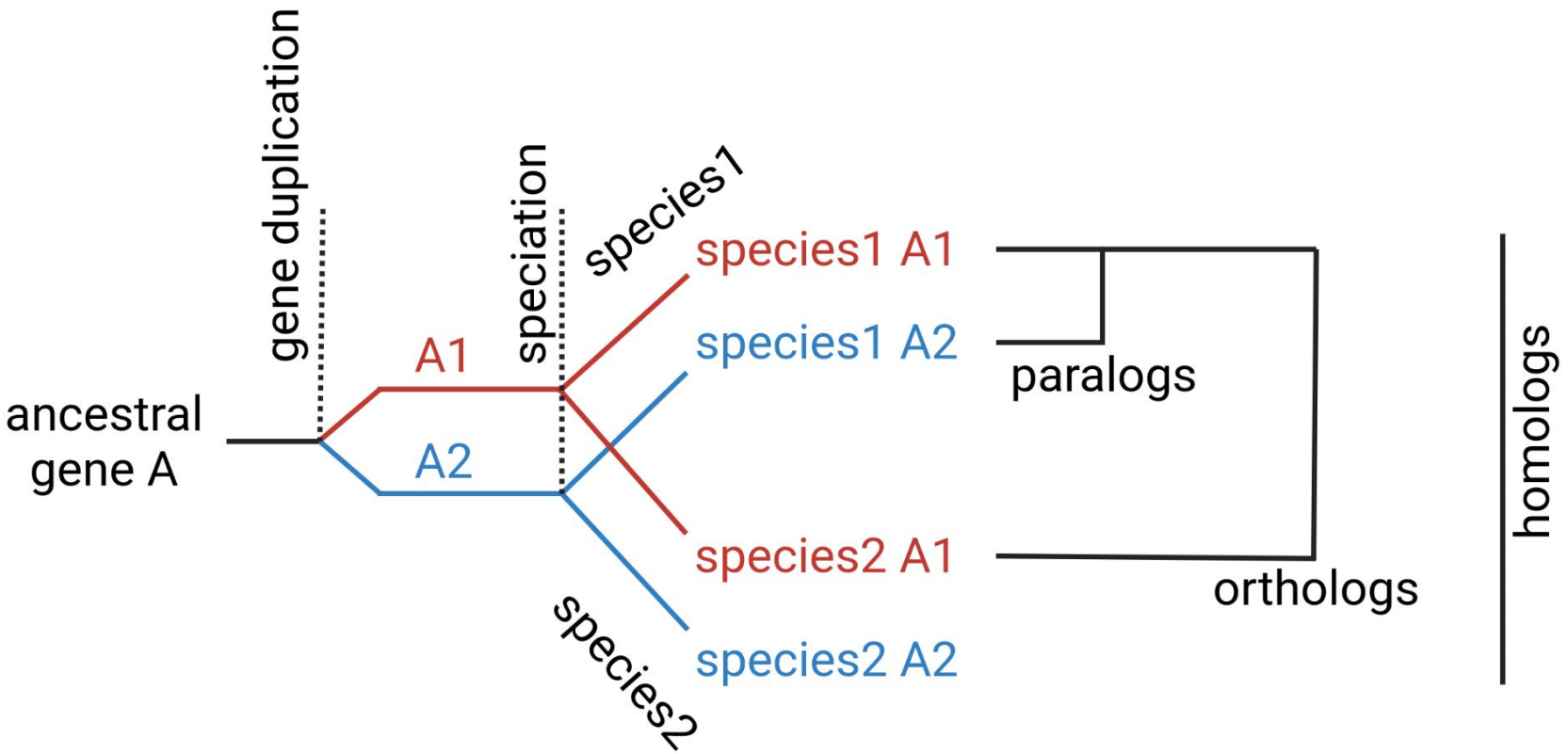
https://github.com/tanghaibao/jcvi/wiki/MCscan-(Python-version)

Evolution of FNS I in the Apiaceae



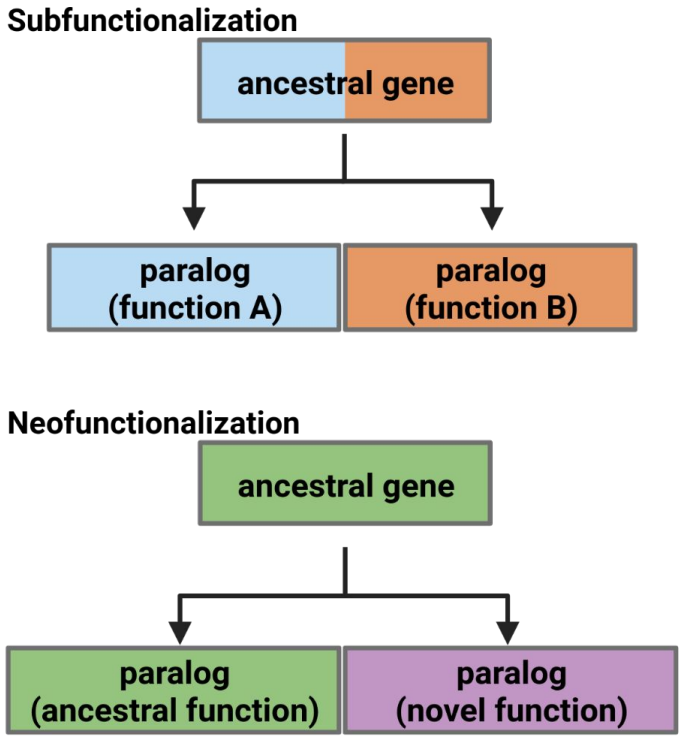
Pucker & Iorizzo, 2022: 10.1101/2022.02.16.480750

Paralogs & orthologs

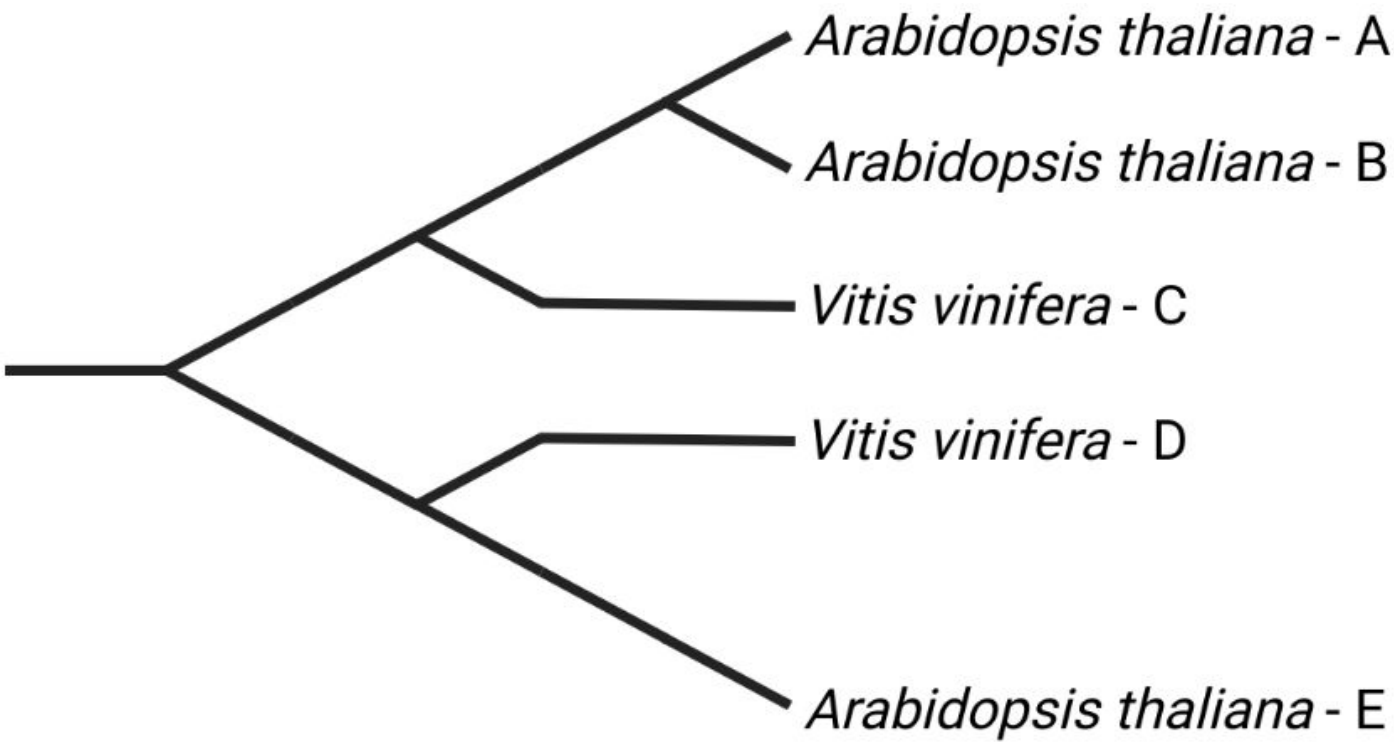


Sub/Neofunctionalization

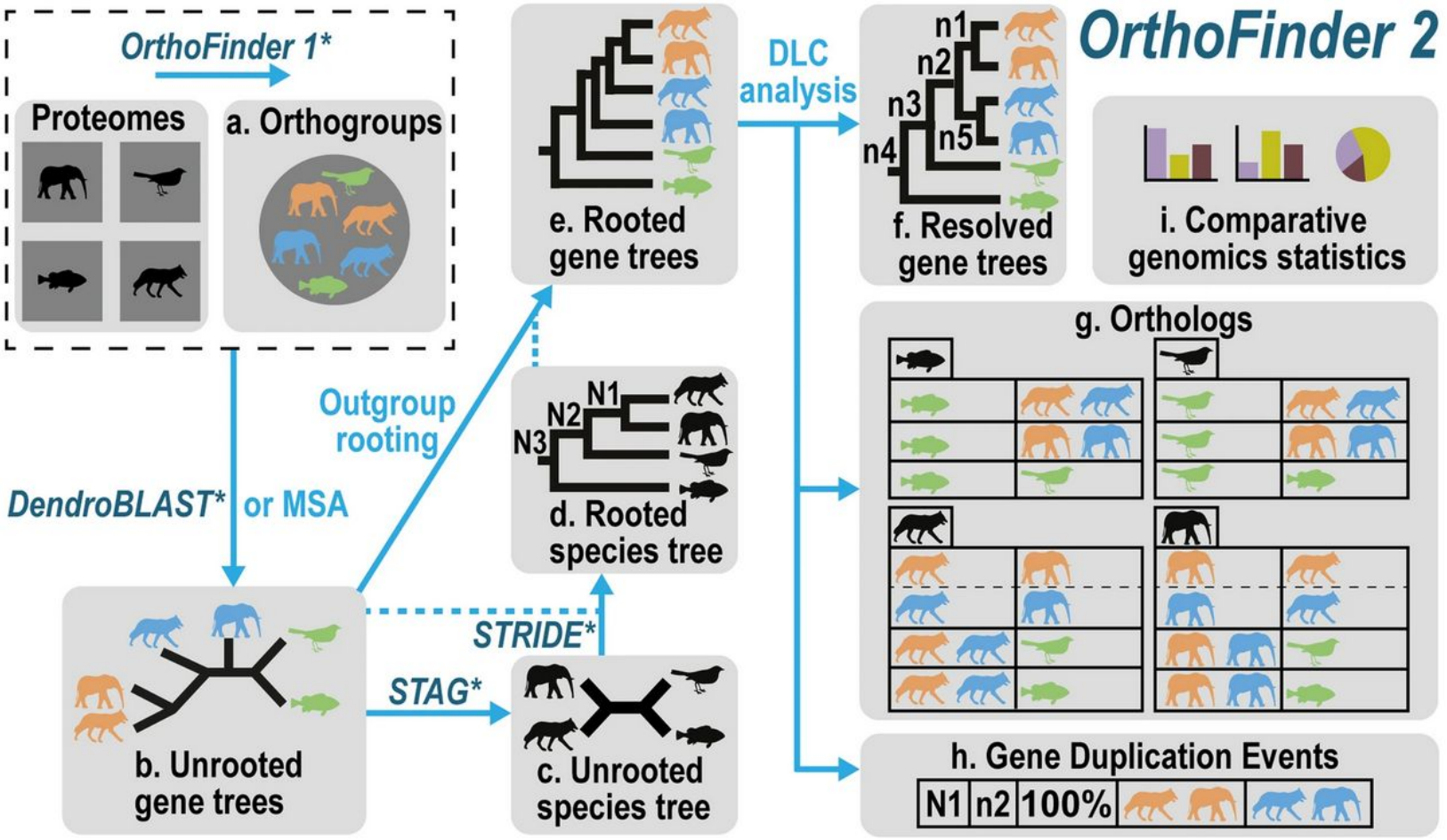
- Sub/neofunctionalization can be achieved through changes in gene expression following the duplication
- Gene copies can be restricted to specific tissues/conditions



Which are orthologs/paralogs?

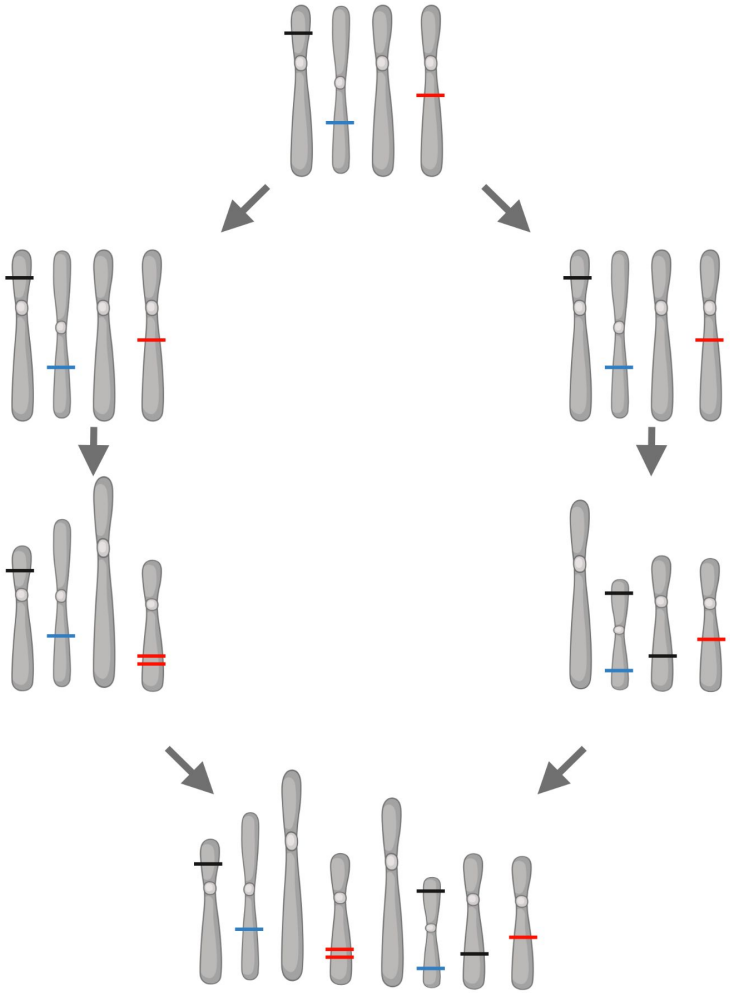


OrthoFinder2



Emms et al., 2019: 10.1186/s13059-019-1832-y

Homeologs



Same color indicates **orthologs**

Same color indicates **homeologs**

Speciation

Gene duplications, translocations, and rearrangements

Hybridization leads to polyploidization

Glover et al., 2016: 10.1016/j.tplants.2016.02.005

Summary

- Structural & functional annotation
- Read mapping, variant calling, variant annotation
- Genome-Wide Association Studies (GWAS) / Mapping-By-Sequencing (MBS)
- Comparative genomics / synteny analysis
- Phylogenetic analyses

Time for questions!

Practical part: outline day3

- Gene prediction (BRAKER3)
- Functional annotation (Mercator) & biosynthesis pathway annotation (KIPEs3)
- Long read mapping (minimap2)
- Variant calling and annotation (SVIM, SnpEff, NAVIP)
- Synteny analysis (MCscan)
- Alignment construction (MAFFT)
- Phylogenetic tree construction (FastTree2, IQ-TREE2)
- Phylogenetic tree vissualization (iTOL)

Exercises: 3-1

- Install HISAT2 and map some RNA-seq reads to the assembly
- Run gene prediction with BRAKER3
- Run BUSCO on structural annotation
- Perform a functional annotation with Mercator and KIPEs3

HISAT2 RNA-seq read mapping

- Split read alignment (RNA-seq vs. genome sequence)
- Availability: http://daehwankimlab.github.io/hisat2/
- Usage:

```
hisat2-build ref_genome.fa ref_index

hisat2 -x ref_index -1 fw_reads.fastq -2 rv_reads.fastq -S output.sam

samtools view -S -b output.sam > output.bam

samtools sort -o sorted_output.bam output.bam

samtools index sorted_output.bam
```

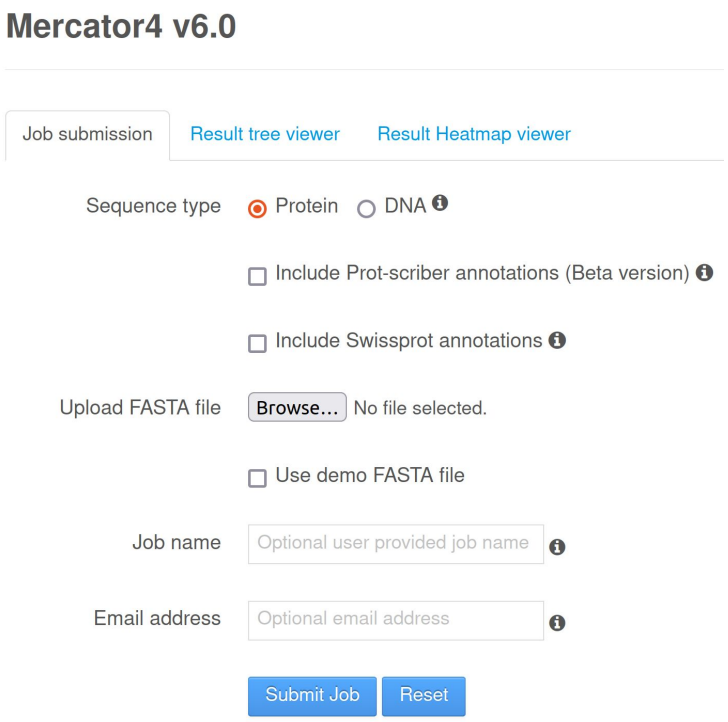
Gene prediction with BRAKER3 (structural annotation)

- Predicting gene models of protein encoding genes
- Availability: https://github.com/Gaius-Augustus/BRAKER
- Usage:

```
braker.pl \
--species=plantX \
--genome=assembly.fasta \
--bam=file1.bam,file2.bam \
--output_dir /vol/data/braker3_output/ \
--threads 10
```

Mercator

- Quick functional annotation of large numbers of polypeptide sequences
- Upload of FASTA file on website: https://www.plabipd.de/mercator_main.html



KIPEs3

- Annotation of genes in a biosynthesis pathway (e.g. flavonoid biosynthesis)
- Availability: https://github.com/bpucker/KIPEs
- Usage:

```
python3 KIPEs3.py \
--baits /vol/data/baits/ \
--positions /vol/data/flavbio_residues/ \
--subject /vol/data/your_peps.fasta \
--out /vol/data/kipes_results/
```

Exercises: 3-2

- Map digi1 and digi4 reads with minimap2 to assembly
- Identify sequence variants with SVIM2 based on the mapping
- Predict variant effects with SnpEff and NAVIP
- Perform a synteny analysis with between a digi1 and digi4 assembly with MCscan
- Generate Circos plots comparing digi1 and digit4 assemblies

Long read mapping with minimap2

- Alignment of long reads to genome sequence
- Availability: https://github.com/lh3/minimap2
- Usage (ONT reads):

```
minimap2 -t 10 -ax map-ont ref.fasta ont_reads.fastq > alignment.sam

samtools view -S -b alignment.sam > alignment.bam

samtools sort -o sorted_alignment.bam alignment.bam

samtools index sorted_alignment.bam
```

Variant calling with SVIM2

- Detect sequence differences between reads and reference sequence (assembly)
- Availability: https://github.com/eldariont/svim
- Usage:

```
svim alignment \
/vol/data/svim_output/ \
--reference reference.fa \
--reads sorted_alignment.bam \
--min_sv_size 50 \
--max_sv_size 100000 \
--min_read_support 5
```

Variant impact prediction with SnpEff

- Predicting the functional consequences of sequence variants
- Availability: https://pcingola.github.io/SnpEff/
- Building a database:

```
nano snpEff.config
my_species.genome : My species
mkdir -p /vol/data/snpeff/data/my_species
cp /path/to/genome.fasta /vol/data/snpeff/data/my_species/sequences.fa
cp /path/to/annotation.gff /vol/data/snpeff/data/my_species/genes.gff
java -jar snpEff.jar build -gff3 -v my_species
java -jar snpEff.jar databases | grep my_species
```

- Running variant annotation:

```
java -jar snpEff.jar -v my_species variants.vcf > variants.annotated.vcf
```

Variant impact prediction with NAVIP

- Predicting the functional consequences of sequence variants
- Availability: https://github.com/bpucker/NAVIP
- Usage:

```
runnavip.sh \
-i input.vcf \
-g ref.gff \
-f ref.fasta \
-o /vol/data/navip_output/
```

Synteny analysis with MCscan

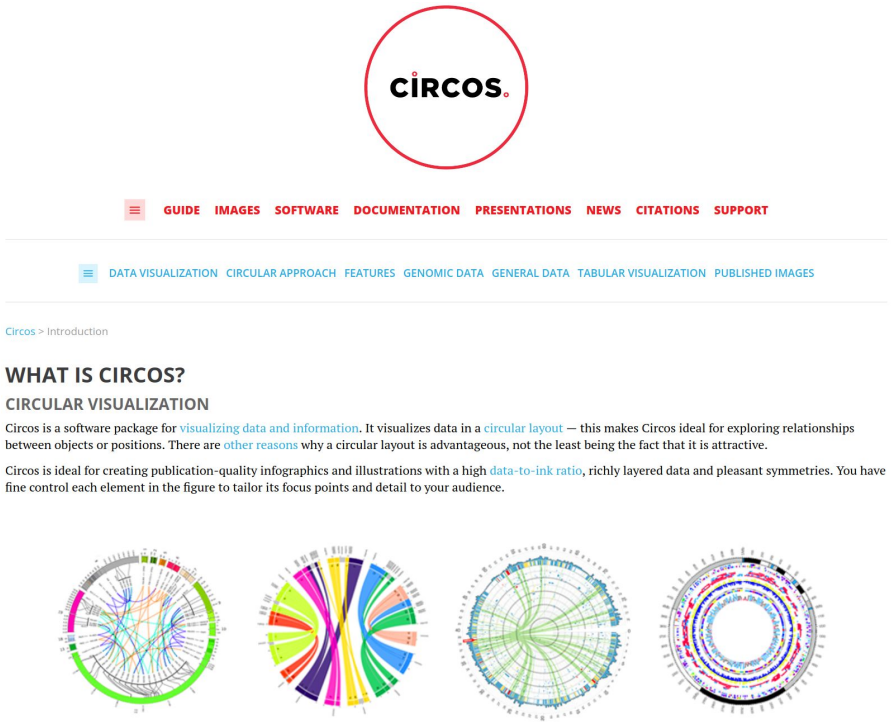
- Compare genomic region across species
- Availability: https://github.com/tanghaibao/jcvi/wiki/MCscan-(Python-version)

- Usage:

```
# Convert GFF to BED format
python -m jcvi.formats.gff bed --type=mRNA /path/to/genome1.gff > genome1.bed
python -m jcvi.formats.gff bed --type=mRNA /path/to/genome2.gff > genome2.bed

# Index the CDS files
samtools faidx /path/to/genome1.cds
samtools faidx /path/to/genome2.cds

# Create BLAST databases
makeblastdb -in /path/to/genome1.cds -dbtype nucl
makeblastdb -in /path/to/genome2.cds -dbtype nucl

# Perform reciprocal BLAST
blastn -query /path/to/genome1.cds -db genome2.cds -out genome1_vs_genome2.blast -evalue 1e-5 -outfmt 6 -num_threads 4
blastn -query /path/to/genome2.cds -db genome1.cds -out genome2_vs_genome1.blast -evalue 1e-5 -outfmt 6 -num_threads 4

# Run JCVI MCScanX
python -m jcvi.compara.catalog ortholog --dbtype nucl --blastp genome1 genome2

# Generate a dot plot
python -m jcvi.graphics.dotplot dotplot genome1.genome2.anchors
```

Circos plot generation

- Comparison of genome sequences
- Inclusion of genome-wide distribution of various elements possible
- Availability: https://circos.ca/

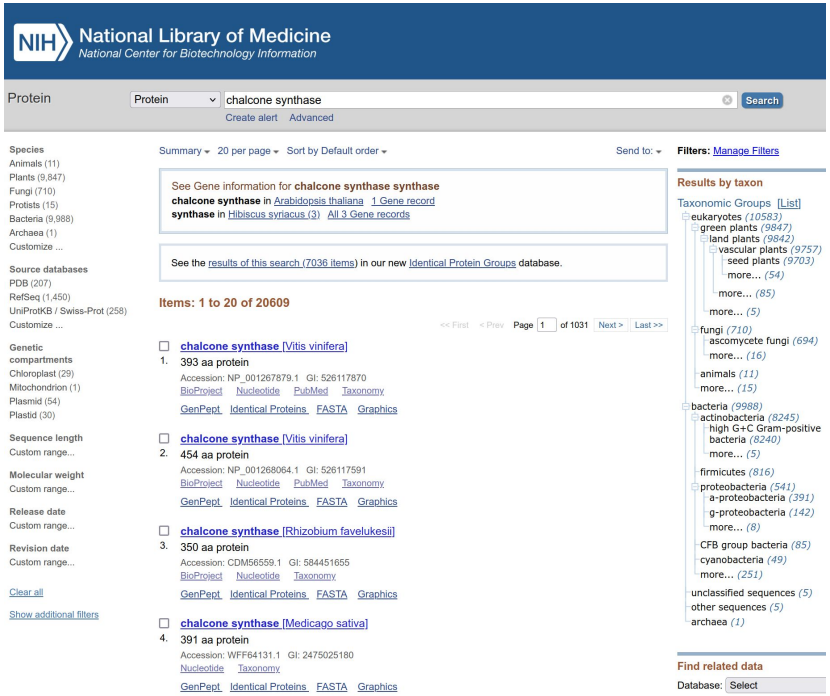


Exercises: 3-3

- Collect sequences (NCBI)
- Construct alignment (MAFFT)
- Phylogenetic tree construction (FastTree2, IQ-TREE2)
- Visualization (iTOL)

NCBI as sequence source

- NCBI offers huge collection of nucleotide and polypeptide sequences
- Links to references (publications) available
- Selection of specific plant taxa possible
- Download in FASTA format possible



chalcone synthase [Vitis vinifera]

NCBI Reference Sequence: NP_001267879.1

GenPept Identical Proteins Graphics

```
>NP_001267879.1 chalcone synthase [Vitis vinifera]
MVSVAEIRKAQRAEGPATVLAIGTATPANCVYQADYPDYYFRITNSEHMTELKEKFKRMCEKSMINKRYM
HLTEEILKENPNVCAYMAPSLDARQDMVVVEVPKLGKEAAAKAIKEWGQPKSKITHLVFCTTSGVDMPGA
DYQLTKLLGLKPSVKRLMMYQQGCFAGGTVLRLAKDLAENNAGSRVLVVCSEITAVTFRGPSDTHLDSLV
GQALFGDGAAAVIIGADPDTKIERPLFELVSAAQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNIE
KSLVEAFTPIGISDWNSLFWIAHPGGPAILDQVELKLGLKEEKLRATRHVLSEYGNMSSACVLFILDEMR
KKSIEEGKGSTGEGLEWGVLFGFGPGLTVETVVLHSVSAPAAH
```

Alignment construction with MAFFT

- Generation of multiple sequence alignment
- Thousands of sequences can be processed efficiently
- Input and output of MAFFT is a FASTA file by default
- Availability: https://mafft.cbrc.jp/alignment/software/
- Usage:

```
mafft sequences.fasta > sequences.fasta.aln
```

Phylogenetic tree construction (FastTree2, IQ-TREE2)

- Construction of a phylogenetic tree from alignment

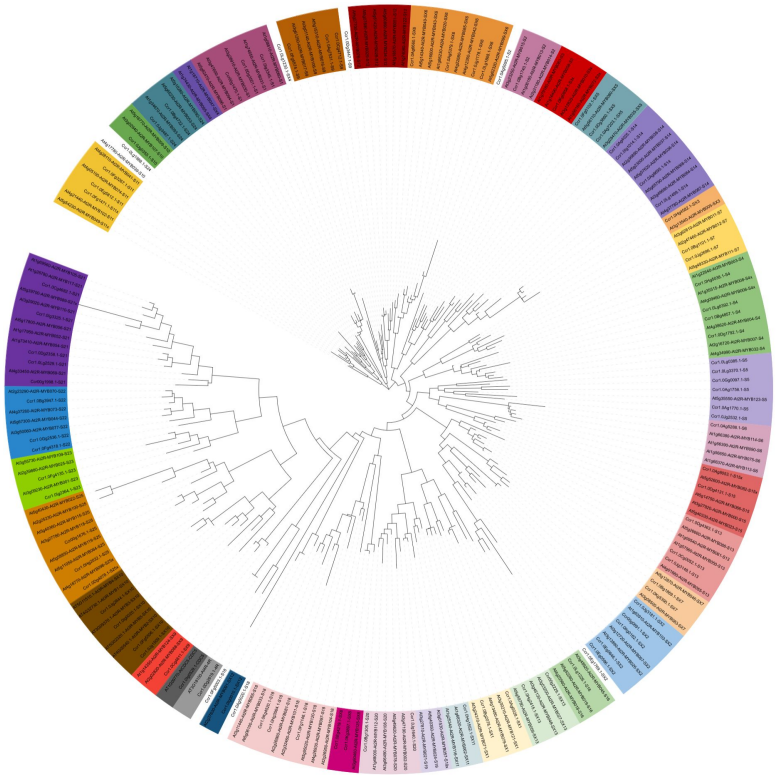
- Availability:
  - FastTree2: http://www.microbesonline.org/fasttree/
  - IQ-TRE2: http://www.iqtree.org/

- Usage:

```
FastTree -wag alignment.fasta > tree.nwk

iqtree2 -s alignment.fasta -m MFP -bb 1000 -alrt 1000
```

Visualize tree (iTOL)

- Visualization of phylogenetic trees
- Modification, highlighting and labeling of tree
- Availability: https://itol.embl.de/



Pucker, 2022: 10.1186/s12864-022-08452-5

Time for questions!