BIO392 file formats and tools

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Talk typesetting

- Commands/options are in `typewriter font`
- URLs are highlighted in blue

Unix: recap

- Lecture from SIB
  https://edu.sib.swiss/pluginfile.php/2878/mod_resource/content/4/couselab-html/content.html

Exercises

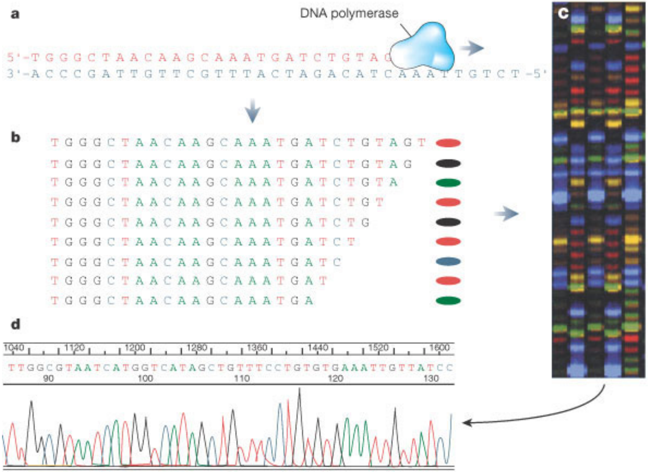
- Run the exercises till number 4

Commonly used formats

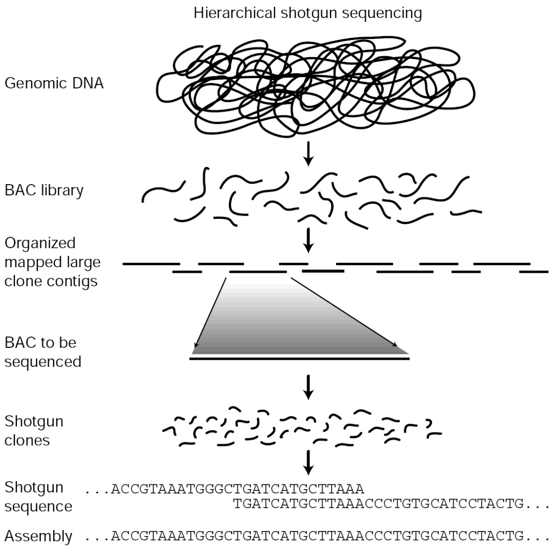
- Reference genomes
- Fasta and FastQ (Unaligned sequences)
- SAM/BAM (Alignments)
- BED (Genomic ranges)
- GFF/GTF (Gene annotation)
- Wiggle files, BEDgraphs (Genomic scores).
- Indexed file formats
- VCFs (variants)

Reference genomes

- Reference genomes describe the 'consensus' DNA sequence
- (Who is the human consensus for DNA sequencing?)
- Aside of human variation, multiple assemblies have been released


a
DNA polymerase
5'-TGGGCTAACAAGCAAATGATCTGTAG
3'-ACCCGATTGTTCGTTTACTAGACATCAAATTGTCT-5'
b
TGGGCTAACAAGCAAATGATCTGTAGT
TGGGCTAACAAGCAAATGATCTGTAG
TGGGCTAACAAGCAAATGATCTGTA
TGGGCTAACAAGCAAATGATCTGT
TGGGCTAACAAGCAAATGATCTG
TGGGCTAACAAGCAAATGATCT
TGGGCTAACAAGCAAATGATC
TGGGCTAACAAGCAAATGAT
TGGGCTAACAAGCAAATGA
c
d
1040
1120
1200
1280
1360
1440
1520
1600
TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCC
90
100
110
120
130

Hierarchical shotgun Nature 409, 863 (2001)



Reference genomes

GRCh stands for ‘Genome Reference Consortium‘

- Human GRCh37 (hg19)
- Human GRCh38
- Mouse mm10
- Mouse GRCm38
- Zebrafish, chicken and others: https://www.ncbi.nlm.nih.gov/grcThe Genome Reference consortium

Reference genomes: FASTA

- A reference genome is a collection of contigs/scaffolds
- A contig is a stretch of DNA sequence encoded as A,G,C,T,N.
- Typically comes in FASTA format.
- ">" line contains the scaffold name
- Following lines contain the sequence (single line, 80 nt-column sized...)

Reference genomes: FASTA

```
>NC_009902.1 Babesia bovis T2Bo mitochondrion, complete genome
TTTAAAAAAGTGTTAAAAACTTTATACATTAAAAAATTTAAACAAGTGATCATGTATAAAGTACACTTGT
TACTGTGTAAATATCAAAAACAATTTAATTTCAAAATTTTTGAAATATGTTTTTTGTGTTGTGTTATAAA
GTTTTTTTTCAAAATTATATATGTTTGCATTTGCTGGATATAGTTCGGTCTCTGCAAACCATAAAGTCAT
CGGTATATCCTACATATGGCTTTCATATTGGTTTGGAGTTATTGGATTTTATATGAGTATTTTGATAAGA
ACAGAATTGAGTATGAGTGGTTTAAAGATTATGACAATGGATACTCTTGAGATATACAATATGATGTTTT
```

Patches, alternate loci and primary assembly

- Primary assembly: the best known assembly of a haploid genome
  - Chromosome assembly
  - Unlocalized sequence (associated to a chromosome but whose order/orientation is unknown)
  - Unplaced sequence (not linked to any chromosome)
- Alternate loci: An alternate representation of a locus (usually highly polimorphic regions, such as the MHC region)
- Patches: A contig sequence that is released outside of the full assembly release
  - Fix: error correction
  - Novel: new sequences that will be included into the next full assemblty release

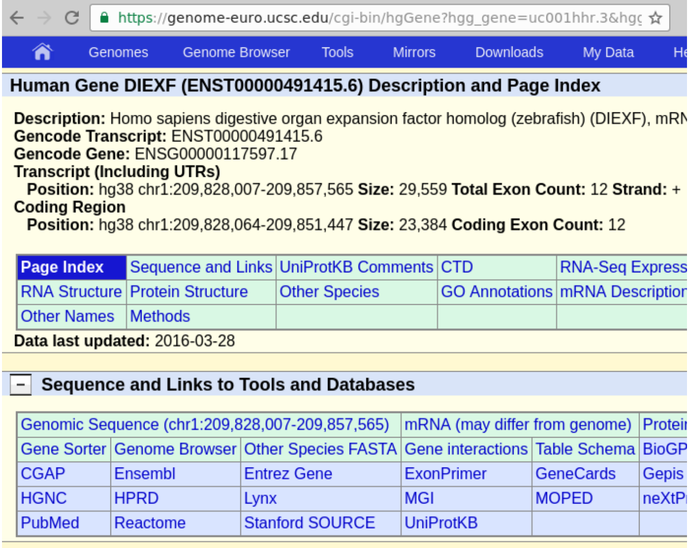
Browsing genomic patches

- Activity: browse genomic patches, i.e. ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/001/405/GCA_000001405.27_GRCh38.p12/README_patch_release.txt

Retrieving fasta sequences manually (UCSC)

- Try to retrieve the `DIEXF` gene promoter
- (What is a promoter in terms of sequence?)
- Go to an assembly `https://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg38`
- Query gene symbol (i.e. `DIEXF`)
- Click into the gene (gencode track)
- Click into the `sequence and links` item
- Specify your promoter definition

Manually downloading the DIEXF promoter



Manually downloading the DIEXF promoter

Genomes | Genome Browser | Tools | Mirrors | Downloads | My Data

Genomic Sequence Near Gene

Get Genomic Sequence Near Gene

Note: if you would prefer to get DNA for more than one feature of this track at a time, try the Table Browser using the output format sequence.

Sequence Retrieval Region Options:

- [x] Promoter/Upstream by 1000 bases
- [ ] 5' UTR Exons
- [ ] CDS Exons
- [ ] 3' UTR Exons
- [ ] Introns
- [ ] Downstream by 1000 bases

(•) One FASTA record per gene.
( ) One FASTA record per region (exon, intron, etc.) with 0 extra bases upstream (5') and 0 extra downstream (3')

- [ ] Split UTR and CDS parts of an exon into separate FASTA records

Note: if a feature is close to the beginning or end of a chromosome and upstream/downstrea bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.

Manually downloading the DIEXF promoter

```
>hg38_knownGene_uc001hhr.3 range=chr1:209827007-2098
gtttctgctgttgttaaatggggaatgctggaacagatttgtttgcgggg
actcttccaatactttcagaaaatgcgagaatagggtgagggtgggaatc
tcagacttgtgggccccatgattgatataacacacacaggcggcagaccc
taatgggtaaaagcatgtggttgcatcagttaaggtttttctctcttctc
ttgctagcgtgttatcttttcttttcttttcttcttttttttttttcg
agatggagtctagcttttgtcgcccaggctggagtaggctggagtgcagt
ggagtgatctcggctcattgcaacctccacctcccgggttccagcgattc
tcctgcctcacctcctgagtagctgggattacaggcgcccgctaccacgc
ccggctgatttttgtactttttagtagagacggggtttcaccatgtttggc
catgctggtctcgaactcctgacctcaggtgatccgcccatctcggcctc
ccaaagtgttgagattacaggcgtgagccaccgcgcccggccgctagcgt
gttatcttttctaagcatcagtttccttatctgcaacaccaggcttatta
acaagacctatctgtacactgttgtggtgatgaagtgagatgttcaggca
cccttaaatgttggttgatatttttattgcagtatactgtaaagtcactg
cattcgactatctccgctactacacatttacgcagactgatttccataac
caaaacacaagcacaaagctcatgcccccgactcacgcaacccgggaagc
ccagctgcccacgttctagggctctgagaacactagtgaacgaactcccg
tgctttcaaagagctgcggtagggggcagaaccgggaaccggatgttcta
agcctgtcgtacgagcgcgacgtaaagcggatctgctttatggcaccttg
ctttcgccgtaaagcgcagtcagcgagcccacgtgcttgtgttgactgga
```

How do we do this in a reproducible manner?

- Scripting. We store an up-to-date reference genome in our computer (once)...
- ... and then use specific file standards to specify the genome annotation (i.e. GTF, BED files).
- Activity: read the documentation of UCSC on how to download sequences http://genome.ucsc.edu/FAQ/FAQdownloads.html (section Extracting sequence in batch from an assembly)

How to specify the DIEXF promoter in a reproducible manner?

Same concept that when we did manually:

1. Download the human genome sequence
2. Download a file with all the genes (transcripts) locations (not sequences, but their coordinates)
3. Then select the gene we are looking for (DIEXF)
4. Decide what a promoter is (i.e. 2 kb upstream of the gene) and update the coordinates accordingly
5. Then use a specific tool to slice the full genome to only report the DIEXF promoter

How to specify the DIEXF promoter? standards

Same concept that when we did manually + some standards

1. Download the human genome sequence (fasta)
2. Download a file with all the genes (transcripts) locations (GTF)
3. Then select the gene we are looking for (DIEXF) (grep/awk)
4. Decide what a promoter is (i.e. 2 kb upstream of the gene) and update the coordinates accordingly (BEDfile, bedtools/awk)
5. Then use a specific tool to slice the full genome to only report the DIEXF promoter (bedtools)

Standards

- This we can do because the genome consortia and the science community release free data, software/toolsets
- We benefit from the Unix-like operating systems
- But the access to the same data and tools is not enough: the need for data standards

Commonly used formats

- **Fasta and FastQ**
- SAM/BAM (Alignments)
- BED (Genomic ranges)
- GFF/GTF (Gene annotation)
- BEDgraphs (Genomic ranges)
- Wiggle files, BEDgraphs and BigWigs (Genomic scores).
- Indexed BEDgraphs/Wiggles
- VCFs (variants)

Short reads sequencing

- Sequencing very short reads (50 to 150 nucleotides) is common practice
- We get hundreds of millions of short reads for each experiment
- Instead of assembling them, we map them into a reference genome
- Activity: read `https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2836519/`
- Sequencers provide sequence and error rates assessment: `fasta` format is not suitable, but `fastq` is

FASTQ: Short read sequencing

- Next step to FASTA: including quality data
- Standard de facto for short read, high-throughput sequencing instruments such (i.e. Illumina)

```
@SRR001666.1 071112_SLXA-EAS1_s_7:5:1:817:345 length=36
GGGTGATGGCCGCTGCCGATGGCGTCAAATCCCACC
+
IIIIIIIIIIIIIIIIIIIIIIIIIIIIII9IG9IC
```

phred scores

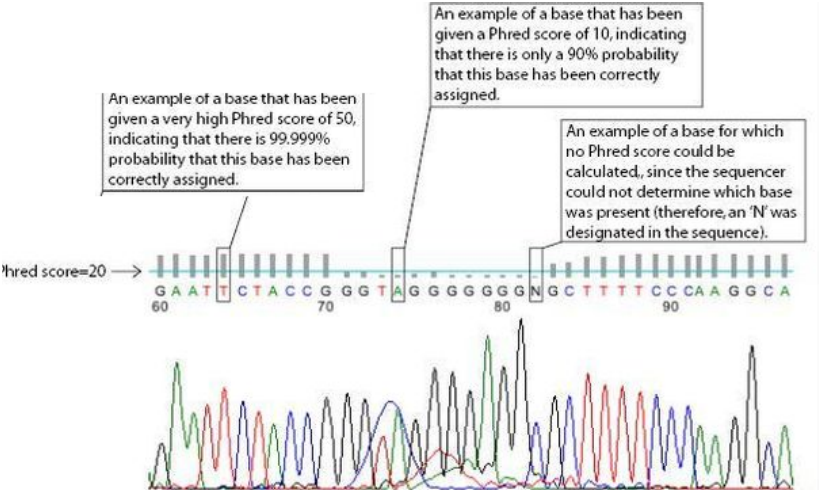
- Sequence quality is represented using Phred scores
- The sequencing quality score of a given base Q is defined by as
- $Q = -10 \ \log_{10} P$

phred scores

Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

phred scores (old school Sanger electrophoretogram)



Phred scores encoding

- There are several Phred score encodings:
- Activity: read about the Quality scores offsets at https://en.wikipedia.org/wiki/FASTQ_format and https://wiki.bits.vib.be/index.php/Identify_the_Phred_scale_of_quality_scores_used_in_fastQ

Phred scores encoding (Wikipedia)

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX......................
 ...............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII......................
 .................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
 |                         |    |        |                              |                     |
33                        59   64       73                            104                   126
 0........................26...31.......40
                          -5....0........9.............................40
                                0........9.............................40
                                   3.....9..............................41
 0.2......................26...31........41

S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 41)
    with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
    (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

Unaligned sequences (from sequencers): FASTQ

- FASTQs stands for FASTA with Qualities
- Plain text files with chunks of four lines:
  - @ identifier line
  - Sequence
  - "+" (sometimes the sequence name, again)
  - Quality scores (different encodings exist)

Example FASTQ entry

```
@SRR001666.1 071112_SLXA-EAS1_s_7:5:1:817:345 length=36
GGGTGATGGCCGCTGCCGATGGCGTCAAATCCCACC
+
IIIIIIIIIIIIIIIIIIIIIIIIIIIIII9IG9IC
```

In class exercises for fastq

- Activity: FASTQ/A exercises (exercises 5 to 14)

Working with fastq files

```
## retrieving an example fastq file
curl  https://molb7621.github.io/workshop/_downloads/SP1.fq \
 > file.fastq

## counting number of reads
awk 'END{print NR/4}' file.fastq

## transforming into fasta
awk 'NR % 4 == 1 {print ">"$1};
     NR % 4 == 2 {print}' file.fasta
```

Further manuals on awk

- https://en.wikipedia.org/wiki/AWK
- http://www.grymoire.com/Unix/Awk.html

awk: Counting the number of items in a fastq

So in fastq each data chunk is stored in four different lines. We'll need to be able to extract the first, second, third of fourth line for each block of four lines. Using awk,

```
awk 'END{print NR/4}' file.fastq
```

- NR gives the number of records (line numbers)
- FASTQ are chunks of 4 lines for each sequence
- NR/4 at the END of the file indicates the number of sequences

Working with fastq files

```
## retrieving an example fasta file
curl  https://molb7621.github.io/workshop/_downloads/SP1.fq \
 > file.fastq

## counting number of reads
awk 'END{print NR/4}' file.fastq

## transforming into fasta
awk 'NR%4==1{a=substr($0,2);} NR%4==2 {print ">"a"\n"$0}' \
  file.fastq
```

awk: fastq to fasta

```
awk 'NR%4==1{a=substr($0,2);} NR%4==2 {print ">"a"\n"$0}' \
  file.fastq
```

- % is a modulo operator
- NR%4==1 will retrieve the first line of a fastq chunk (header)
- NR%4==2 will retrieve the second line (the sequence)
- the id line will be preprended with the > and reduced to a substring (chopped)
- This will be applied to all lines!

- Discussion: how to get rid of the sequences and to have a smaller data representation?
- Trying to transform sequence to reference genome coordinates (= aligning to the genome/mapping)
- i.e. transforming `ACGCACGCACGCACGCCCC` to human genome hg19 ‘chr10:10010-10030‘

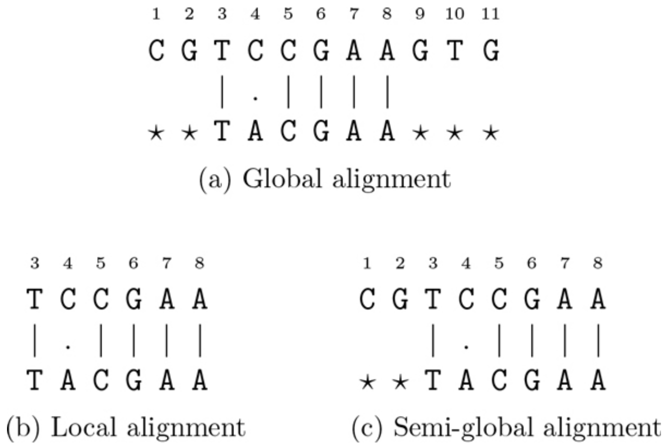
Alignment file format

- SAM - Sequence Alignment Map.
- The standard stores where the reads (i.e. the ones we had as FASTQs) map in the reference genome
- Recognised by majority of software and browsers: standard

What is an alignment?

- Sequence alignment: arrange a set of sequences to identify regions of similarity/identity
- Mapping short reads against a reference genome: aligning large amounts short reads to a reference genome

Local alignments vs global alignment



Alachiotis et al, 2013

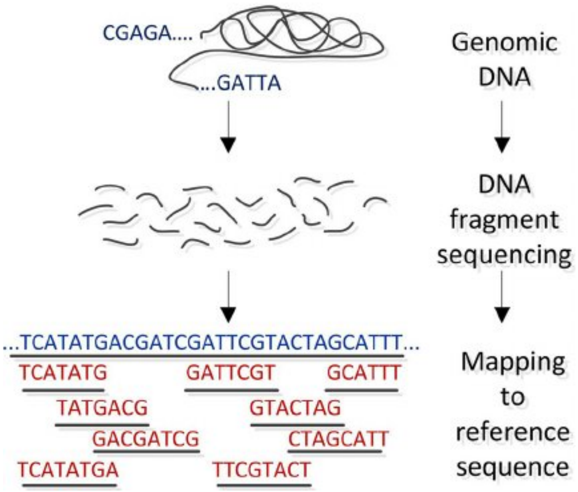
SAM format

- Chromosome
- Locus (coordinate)
- CIGAR string, i.e.
- 30M1D2M - 30 bases continuously match, 1 deletion from reference, 2 base match
- Some flags (https://broadinstitute.github.io/picard/explain-flags.html)

CIGAR strings

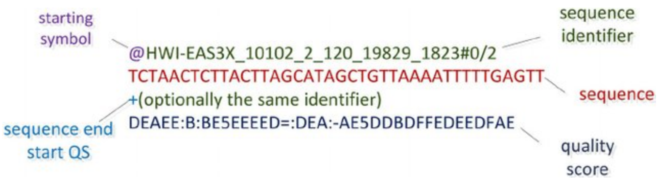
- Activity: read a post on CIGAR encoding http://bioinformatics.cvr.ac.uk/blog/tag/cigar-string/ (please skip the Java code)

Next generation sequencing to SAM



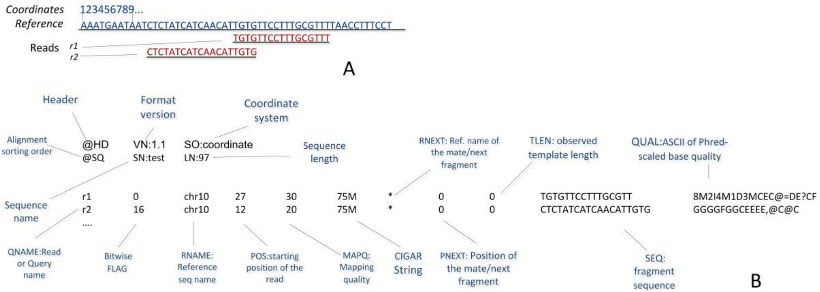
Pavlopoulos et al 2013

Next generation sequencing to SAM



Pavlopoulos et al 2013

Next generation sequencing to SAM



Pavlopoulos et al 2013

SAM format

- Activity: read the SAM format specification
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2723002/

Exercises on SAM files

- Exercise number 15

From SAM to BED: counts

- BED files are simpler data representations, usually the next step after getting the SAM files
- Why? they are smaller and easier to handle
- For instance, after mapping a new genome-wide sequencing BED files with the genomic coverages are generated
- Discussion: how to handle expression data, i.e. transcripts without introns etc? how do we count them?
- Activity: read https://bedtools.readthedocs.io/en/latest/content/tools/genomecov.html

Keep it simple: count and transform into BED files

- BED (Browser Extensible Data) files come in different flavours
- BED3: 3 tab separated columns, chromosome (scaffold), start, end
- BED6: BED3 plus name, score, strand

BED3

```
chr22 1000 5000
chr22 2000 6000
```

BED6

```
chr22 1000 5000 cloneA 960 +
chr22 2000 6000 cloneB 900 -
```

BED12

```
chr22 1000 5000 cloneA 960 + 1000 5000 0 2 567,488, 0,3512
chr22 2000 6000 cloneB 900 - 2000 6000 0 2 433,399, 0,3601
```

How do we count? 0s and 1s

- Even though BED files are standard how to count nucleotides is not
- 0-start vs. 1-start : Does counting start at 0 or 1?
- For a counted range, is the specified interval fully-open, fully-closed, or a hybrid-interval (e.g., half-open)?
- Activity: read http://genome.ucsc.edu/blog/the-ucsc-genome-browser-coordinate-counting-systems/

Activities

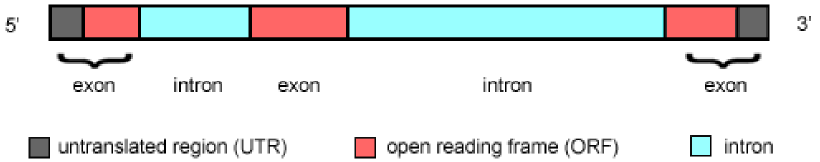
- Exercises 16 to 24

The need for further data formats

- So to sum up until now generally we have a reference genome, reads that were retrieved as FASTQ files, mapped and transformed to SAM files
- (Which step was the last one with actual sequences on them?)
- So, at last, we can answer questions
- Which fraction of the human genome is covered by exons?
- Genomic locations of SNPs associated with prostate cancer?
- Are gene bodies more variable (in terms of SNPs) than intergenic regions?
- For this we need further data analysis tools (i.e. BEDtools) on BED files and other data formats (annotations and variation)

What does annotation mean?

- Genomic annotations are layers to genomic coordinates specifying their nature



Henrik Lantz, BILS/SciLifeLab

How to store genomic annotations? GFF3

Seqid	source	type	start	end	score	strand	phase	attributes
Chr1	Snap	gene	234	3657	.	+	.	ID=gene1; Name=Snap1;
Chr1	Snap	mRNA	234	3657	.	+	.	ID=gene1.m1; Parent=gene1;
Chr1	Snap	exon	234	1543	.	+	.	ID=gene1.m1.exon1; Parent=gene1.m1;
Chr1	Snap	CDS	577	1543	.	+	0	ID=gene1.m1.CDS; Parent=gene1.m1;
Chr1	Snap	exon	1822	2674	.	+	.	ID=gene1.m1.exon2; Parent=gene1.m1;
Chr1	Snap	CDS	1822	2674	.	+	2	ID=gene1.m1.CDS; Parent=gene1.m1;
		start_ codon						Alias, note, ontology_term ...
		stop_c odon						

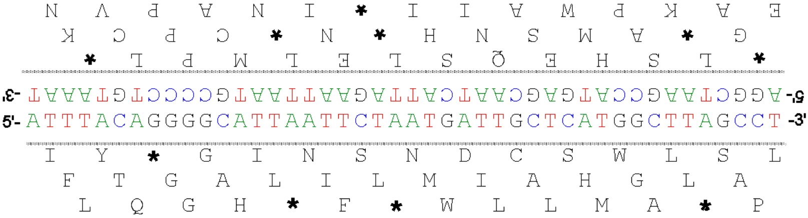
Henrik Lantz, BILS/SciLifeLab

How to store genomic annotations? GTF

Seqid	source	type	start	end	score	strand	phase	attributes
Chr1	Snap	exon	234	1543	.	+	.	gene_id “gene1”; transcript_id “transcript1”;
Chr1	Snap	CDS	577	1543	.	+	0	gene_id “gene1”; transcript_id “transcript1”;
Chr1	Snap	exon	1822	2674	.	+	.	gene_id “gene1”; transcript_id “transcript1”;
Chr1	Snap	CDS	1822	2674	.	+	2	gene_id “gene1”; transcript_id “transcript1”;
		start_ codon						
		stop_c odon						

Henrik Lantz, BILS/SciLifeLab

Why so complex? Open reading frames



Steven M. Carr

Why so complex? CDS, exons, introns, stop codons

- How many transcript does a gene have?
- Are they tissue dependent?
- How can we annotate the different between transcription and translation?

GTF and GFF3 fileformats

- Reading: https://www.ensembl.org/info/website/upload/gff.html

Activity

- Run exercises 25 to 27

BEDgraph

```
chromA   chromStartA   chromEndA   dataValueA
chromB   chromStartB   chromEndB   dataValueB

chr19 49303800 49304100 0.50
chr19 49304100 49304400 0.75
chr19 49304400 49304700 1.00
```

- To display continuous-valued data in track format.
- Uuseful for probability scores

Which are the differences between BEDgraphs and BED?

- BED, BED, BED12?
- Advantages: the coordinates are specified, so sparsity is allowed
- Next step in file formats: trying to cover all the genome (that is, no sparsity anymore)
- Example: does it make sense to generate a BED file with GC content? (GC content at Wikipedia)
- How can we store features with definite start and ends but for which the value is the primary purpose, but not their starts and ends?

Wig files

- To display continuos-value data
- GC percent, probability scores, and transcriptome data.
- Data is not sparse! Wiggle data elements must be equally sized (step)

Wig

```
variableStep chrom=chr2
300701 12.5
300702 12.5
300703 12.5
300704 12.5
300705 12.5
```

Wig with added span

```
variableStep chrom=chr2 span=5
300701 12.5
```

Wig with fixedStep and span

```
fixedStep chrom=chr3 start=400601 step=100 span=5
11
22
33
```

Activity

- Read more on Wig files at https://genome.ucsc.edu/goldenpath/help/wiggle.html

On coordinates, 0s vs 1s and open and closed intervals

- Remember the unusual 0 and 1 counting when convertig BED, Wiggle and/or when using genomic positions at the UCSC genome browser (very last part of the article http://genome.ucsc.edu/blog/the-ucsc-genome-browser-coordinate-counting-systems/)

VCF

- Standard file format for storing variation data
- Unambiguous, scalable and flexible
- 8 columns:
    1. CHROM
    2. POS
    3. ID
    4. REF
    5. ALT
    6. QUAL
    7. FILTER
    8. INFO

VCF

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	NA19909
11	5248232	rs334	T	A	100	PASS	AA=T|||;AC=1;AF=0.0273562;AFR_AF=0.0998;AMR_AF=0.0072;AN=2;DP=22876;EAS_AF=0;EUR_AF=0;EX_TARGET;NS=2504;SAS_AF=0;VT=SNP	GT	0|1

EMBL/EBI training

Quality values: which one?

- Phred-scaled quality score for the assertion made in ALT. i.e. $Q = -10 \ \log_{10} P$ being P(call in ALT is wrong)
- Read quality
- Mapping quality
- Variant calling quality

Variant calling

- Lecture by Michael Lawrence (VariantExplore package)
- https://www.bioconductor.org/help/course-materials/2014/CSAMA2014/3_Wednesday/lectures/VariantCallingLecture.pdf

The VCF format

- Activity: read http://www.internationalgenome.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-40

Further activities

- Run exercises from 28 on