3. Assembly and annotation

- Genome assembly
- Metrics and assembly QA/QC
- Re-sequencing and variant calling
- Annotation

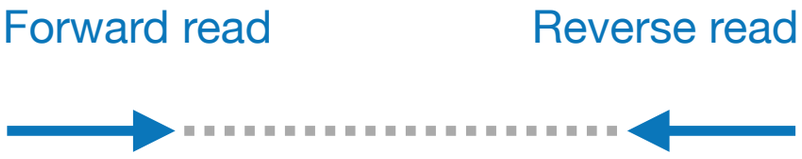
De novo genome assembly

- Reconstructing long, continuous sequence (up to chromosomes) from millions of fragments (reads)

- Computationally difficult due to repetitive (identical or highly similar) DNA
- Levels of assembly: Reads > contigs > scaffolds (> chromosomes)

Short read assembly

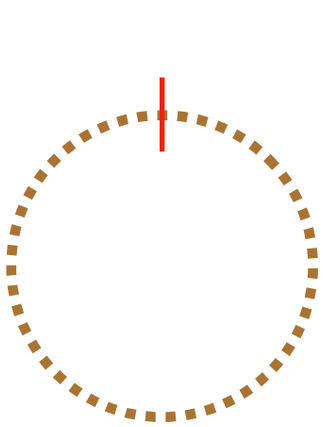
- Paired ends (PE)



Example file names

```
Hpue_raw300_F.fastq
Hpue_raw300_R.fastq
```

- Mate pairs

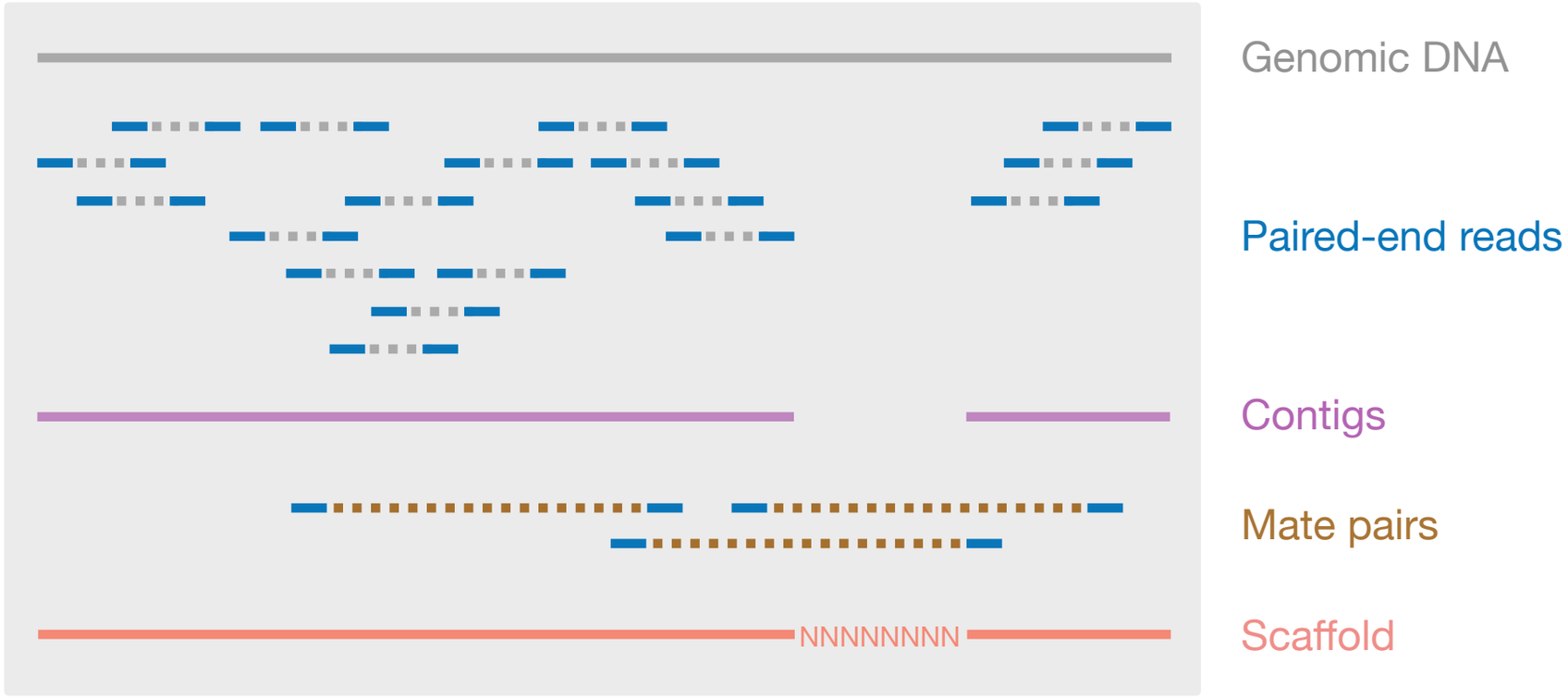


Example sequencing strategy

Library type	Insert sizes
5 × pair	250–580 bp
2 × mate	2.5, 4.3 kb

Short read assembly

- Overlapping reads > contigs
- Ordered, oriented contigs with gaps of known position, length > scaffolds (gaps spanned by mate pairs)

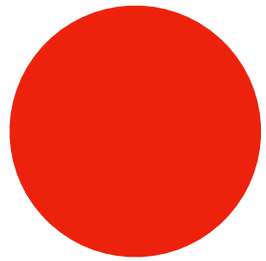


Hybrid strategy

- Combine Illumina with long read sequencing
- Correct long reads with high coverage Illumina data
- Fill gaps in Illumina-based scaffolds with long reads (> up to chromosomes)

Polishing and mapping

- Polishing?
- High-C, optical mapping?
- Merge / purge haplotigs



Assembly example

The FASTA format

```
cd workshop_zmt/project/3_assembly                    # change directory
zcat < Hpue_assembly_01_LG12.fas.gz | head -n 100     # look at compressed file (zcat),
                                                      # display first 100 lines
```

```
>UXGA01000012.1 Hypoplectrus puella genome assembly, contig: LG12, whole genome shotgun
sequence
CCAAAAGTAGTTACAGTCCATGCGGCAAGTCCAGaactctctgcaccagactttTTTGAAGAAATGAGAGAGTTTATGCG
CCGGTCTAAACAAACTGAGGCTGCACTTTTTGAGCAGATTAAACAGCTCAGAAGGGACATGAGACCAAGATCAAGGCAAG
ACTGGACCTCTGAAGAACCTTTTGAACAACCTGTTTCTTCATCTCCTCAGCTGATAGCCAGTGGACCTGTCTCTTCATCT
…
TGGGGATTAAGAGACGAAACACGTTTAAACTCACATGAAACACTAAACTGATACATAGNNNNNNNNNNNNNNNNNNNNN
NNNTCGGTCAGAAAAGTCTGTGCCTGTTTGTCAAAGATGATTTTTGAACTCATGAGCTTGTCTCACTGTCTCTGGTGTAT
GTTAATCAAGTGTACTTCCCGATAATGGAAACCTCTGATAACAGCTCTTTTGTTGCCGCTGCTGATAAGCCCTGCAAAGC
```

Assemblers

- Illumina: ALLPATHS, ABySS, Discovar, Platanus, SOAPdenovo
- PacBio: Falcon, Canu (also Nanopore)
- 10x Genomics: Supernova
- Small genomes: SPAdes, Velvet, HGAP

General advice

- Compare alternate assemblies (consider use case)
- Even finished assemblies should be considered drafts
- Expect lower quality in difficult regions (high repeat content, heterozygosity)

Assessing assembly quality

- Assembly metrics: size distribution of contigs / scaffolds
- Gaps per Gb, unplaced contigs
- Base accuracy (Q score)
- Presence of false duplications
- Gene completeness
- Percentage of assembly assigned to chromosomes

Assembly metrics

- Total size (compare to expected genome size)
- Number of contigs / scaffolds
- Largest scaffold
- N50: contig / scaffold size where 50% of assembly is found on contigs / scaffolds of equal or larger size (measure for sequence continuity)

```
Scaffolds: 530, 760, 1050, 610, 450, 800, 220, and 1200 kb
Reorder: 1200, 1050, 800, 760, 610, 530, 450, 220 kb
Sum / 2: 5620 / 2 = 2810
Add up until reached: 1200 + 1050 + 800 > 2810
N50 = 800 kb
```

Example calculation

Assembly metrics

Using QUAST (command line tool or http://cab.cc.spbu.ru/quast/)

Report Example

E. coli single-cell assemblies

Aligned to "e.coli_reference" | 4 639 675 bp | 50.79% G+C | 884 operons

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Show heatmap

Worst Median Best

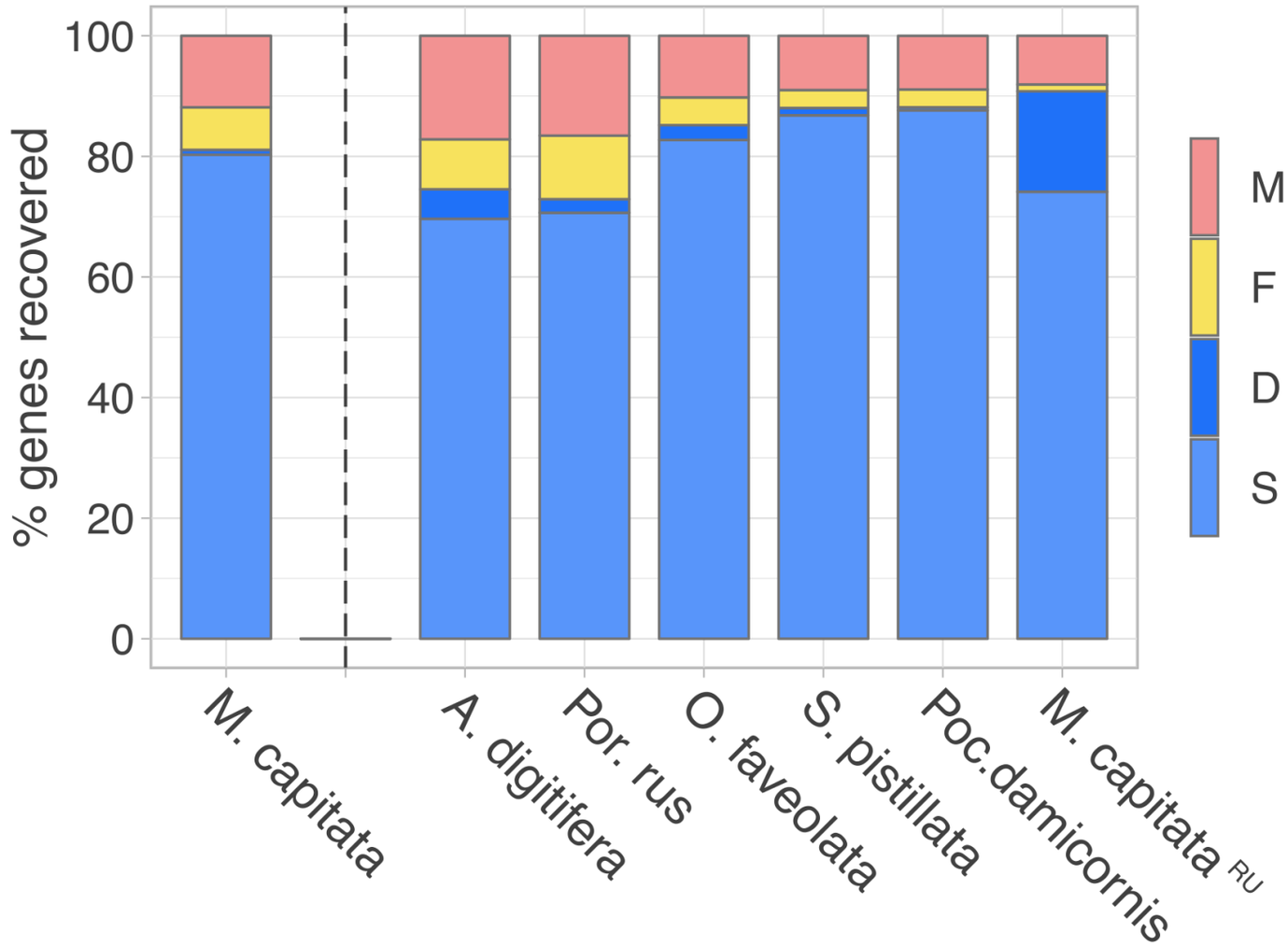
Statistics without reference	E+V-SC	IDBA-UD	SPAdes-2.4	Velvet-SC
# contigs	344	250	277	519
Largest contig	132 865	224 018	269 177	121 367
Total length	4 540 286	4 791 744	4 877 521	4 526 656
N50	33 616	96 947	106 927	20 445
Misassemblies				
# misassemblies	2	9	2	2
Misassembled contigs length	23 485	66 335	26 551	22 359
Mismatches				
# mismatches per 100 kbp	2.26	3.65	5.06	1.77
# indels per 100 kbp	0.7	0.2	0.7	0.92
# N's per 100 kbp	0	0	4.86	0
Genome statistics				
Genome fraction (%)	91.727	94.943	95.759	91.43
Duplication ratio	1.001	1.001	1.004	1.002
# genes	3767 + 160 part	4026 + 80 part	4046 + 102 part	3630 + 288 part
# operons	723 + 87 part	802 + 40 part	809 + 48 part	650 + 158 part
NGA50	32 051	96 947	110 539	19 791
Predicted genes				
# predicted genes (unique)	4258	4394	4417	4331
# predicted genes (>= 0 bp)	4258	4394	4490	4331
# predicted genes (>= 300 bp)	3643	3736	3784	3666
# predicted genes (>= 1500 bp)	524	559	559	515
# predicted genes (>= 3000 bp)	44	49	48	39

Extended report

Gene completeness

Using BUSCO

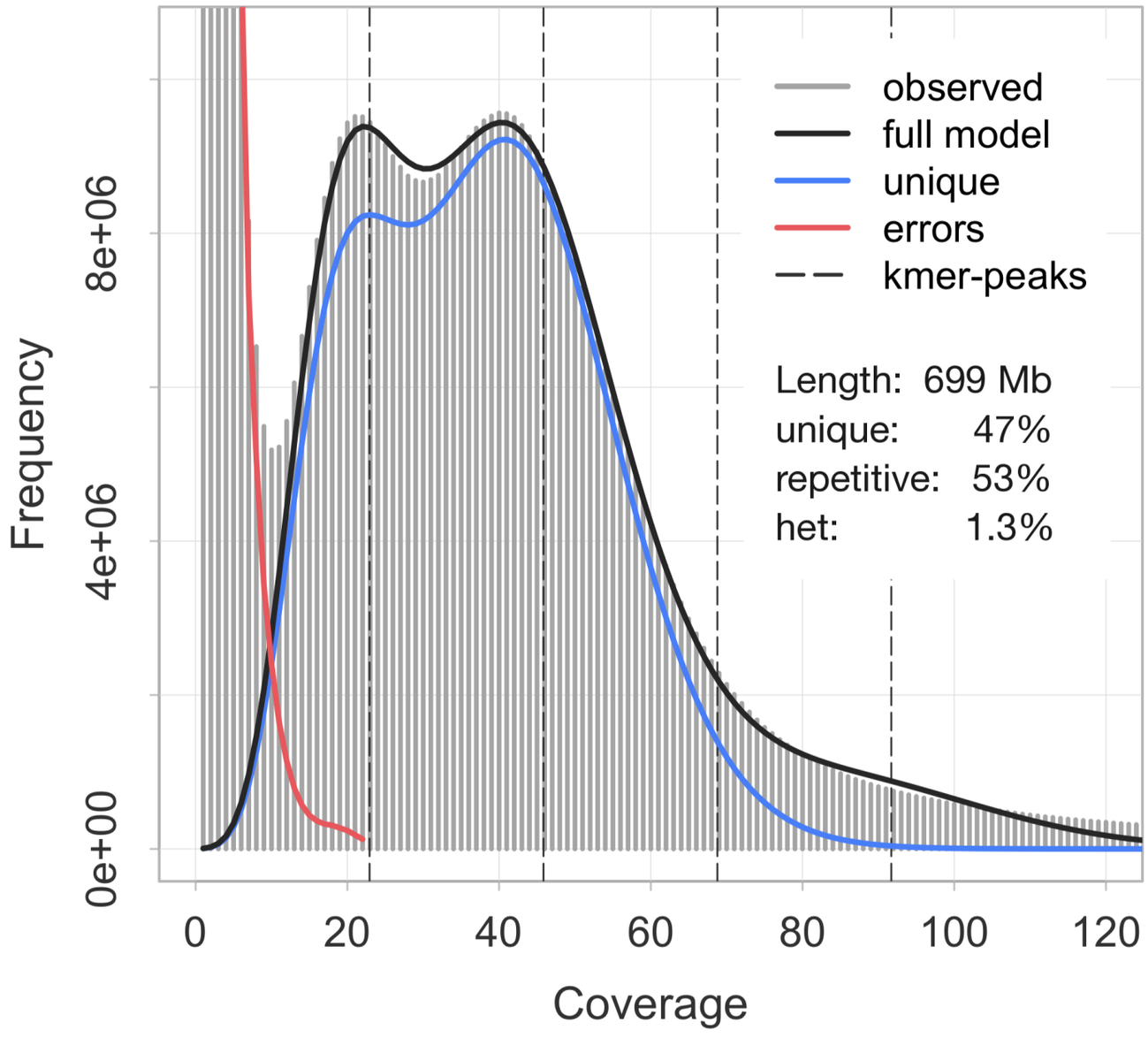
Quantifies assembly completeness based on presence of universal, highly conserved, single-copy genes (e.g. housekeeping genes)



Helmkampf et al. 2019 (Genome Biology and Evolution)

Genome assessment using *k*-mer profiles

Using GenomeScope



Example:

6-mers of 10 bp-sequence

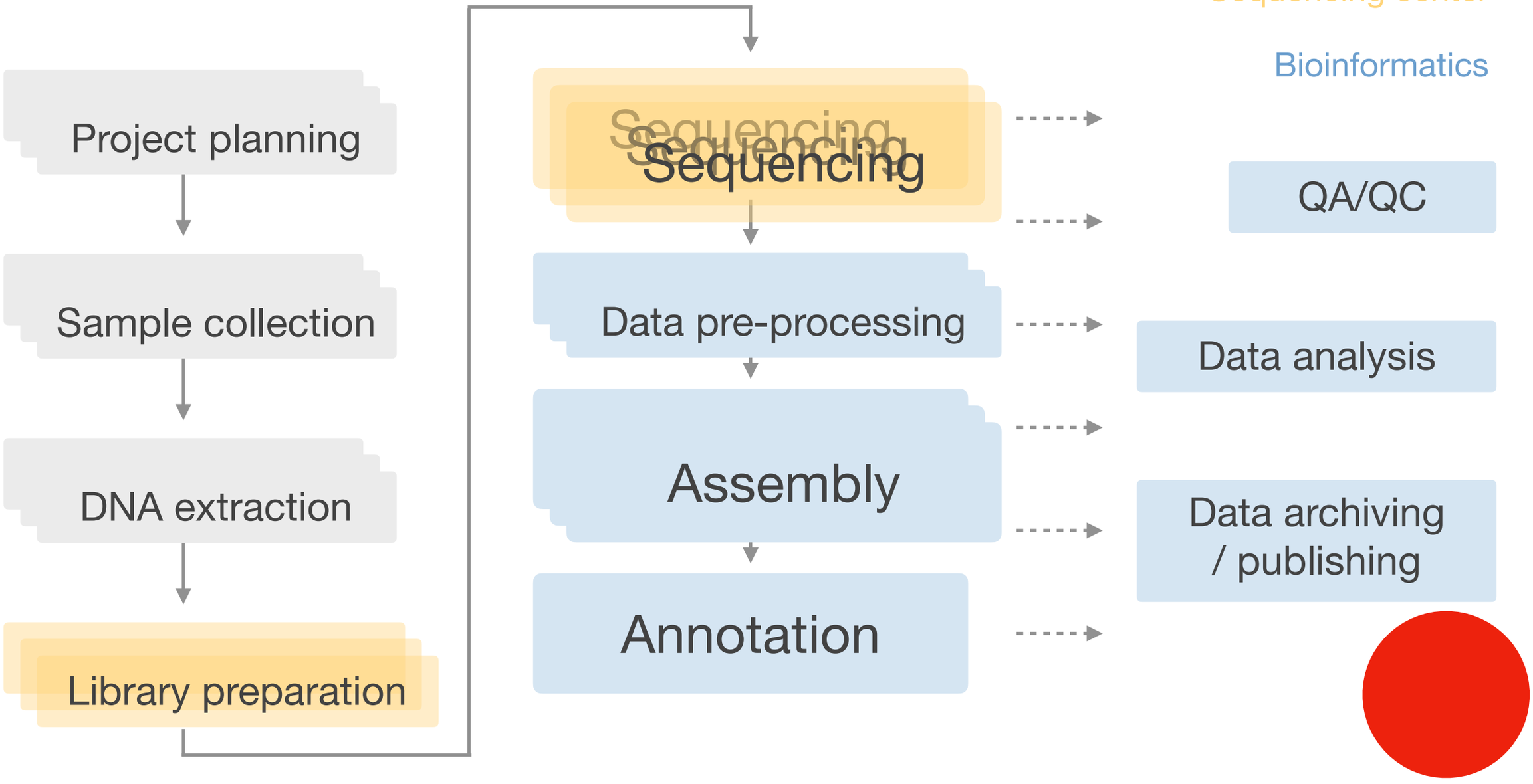
```
AGTCAAGTCT
AGTCAA
 GTCAAG
  TCAAGT
   CAAGTC
    AAGTCT
```

Helmkampf et al. 2019 (Genome Biology and Evolution)

Re-sequencing

- Prerequisite: high-quality reference genome
- Approach
    - low-coverage sequencing of multiple samples
    - aligning reads to reference (mapping)
    - identify differences > SNPs (variant calling / genotyping)

Genome re-sequencing workflow

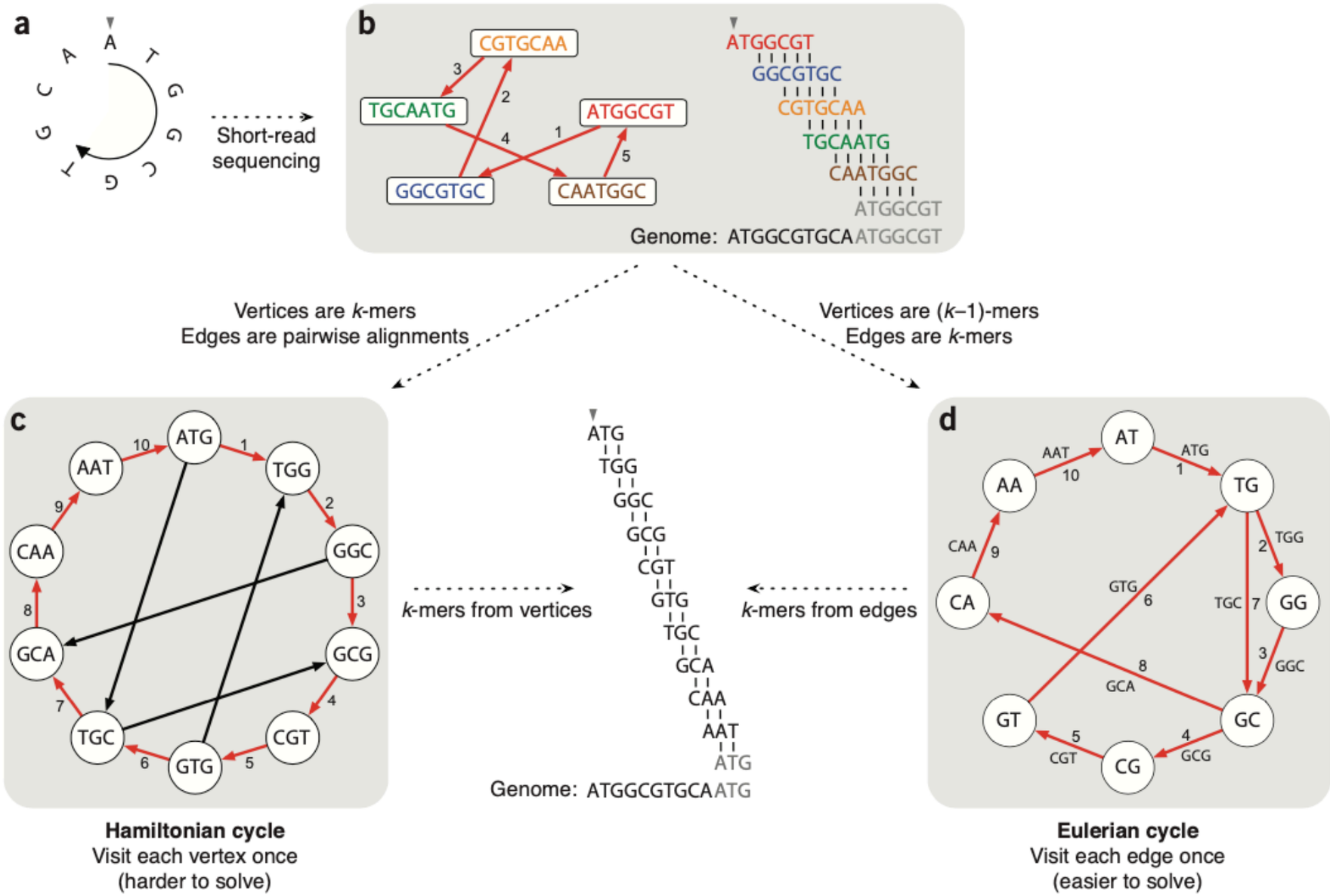


Re-sequencing

The VCF format

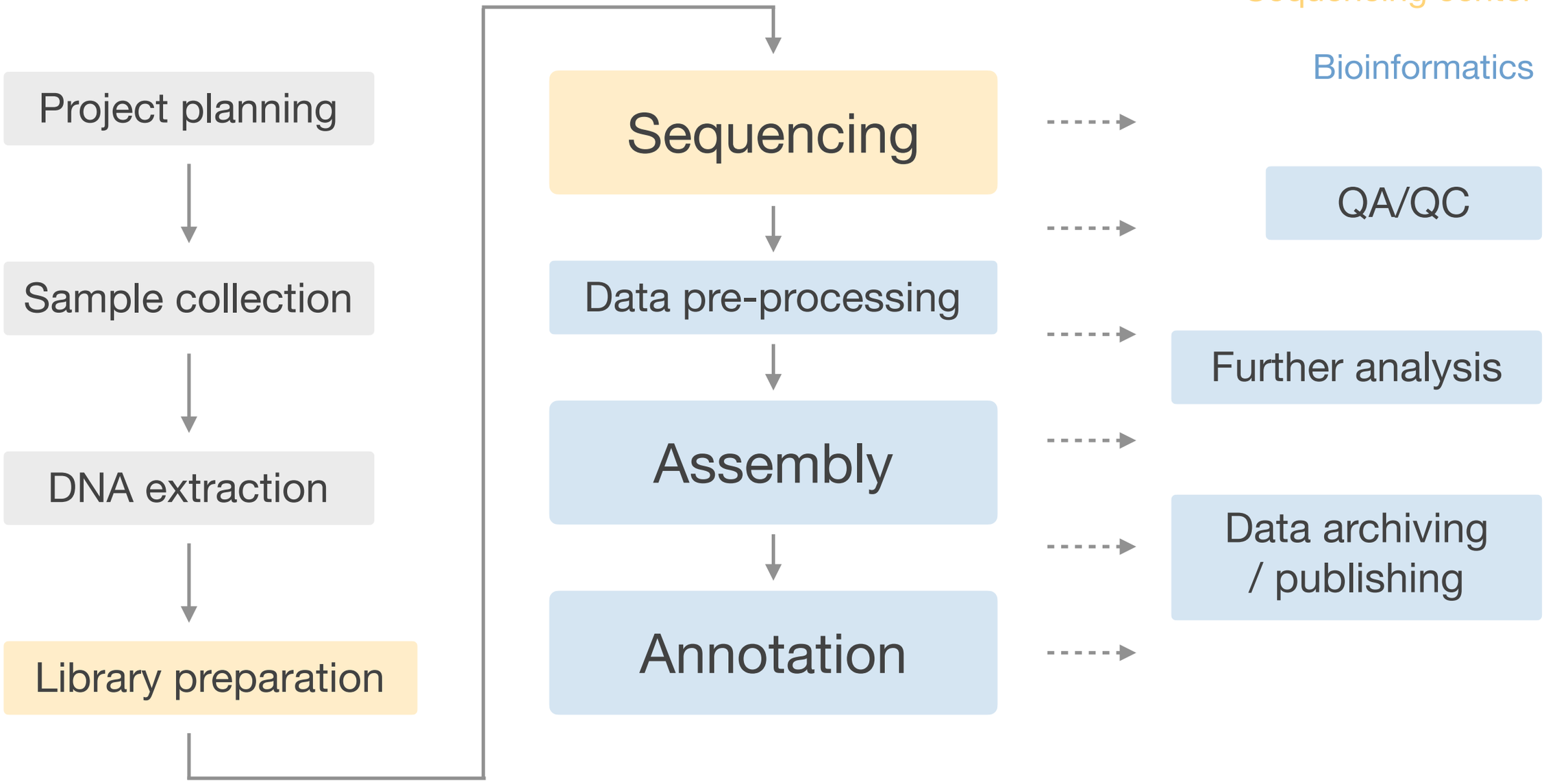
```
##fileformat=VCFv4.1
…
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT sampleA sampleB sampleC sampleD sampleE
LG01 1258 . C T . PASS . GT 0|0 0|0 0|0 0|0 0|0
LG01 1277 . G T . PASS . GT 0|0 0|0 0|0 0|0 0|0
LG01 1292 . G A . PASS . GT 0|0 0|0 0|0 0|0 0|0
LG01 1365 . C A . PASS . GT 0|0 0|0 0|0 0|0 0|0
LG01 1373 . C T . PASS . GT 0|1 0|1 1|1 0|0 1|1
LG01 1398 . G C . PASS . GT 0|1 0|1 1|1 0|0 1|1
LG01 1403 . G C . PASS . GT 0|0 0|0 0|0 0|0 0|0
LG01 1494 . C T . PASS . GT 1|0 0|0 0|0 1|1 0|1
LG01 1495 . G A . PASS . GT 0|1 1|0 1|1 0|0 1|0
…
```

De Bruijn graphs



Compeau and Tessler 2011 (Nature Biotechnology)

De novo genome sequencing workflow



Genome annotation

- Identifying the location, structure, and putative function of genetic elements
  - repetitive and mobile DNA
  - protein-coding genes
  - regulatory elements

Repeat annotation

- Most genomes contain large amounts of repetitive and mobile DNA
- Repeat classes:

Repetitive Elements Identified in the *Montipora capitata* Genome Assembly

	Number	Total Length (Mb)	Fraction of Assembly (%)
Tandem repeats	160,985	16.5	2.7
Interspersed repeats	1,028,006	257.4	41.9
DNA elements	31,667	11.6	1.9
LTR elements	14,753	10.0	1.6
Non-LTR elements	110,728	42.6	6.9
Unclassified	870,858	193.2	31.4

- Repeats may have regulatory or structural functions, but can also bias downstream analyses

Helmkampf et al. 2019 (Genome Biology and Evolution)

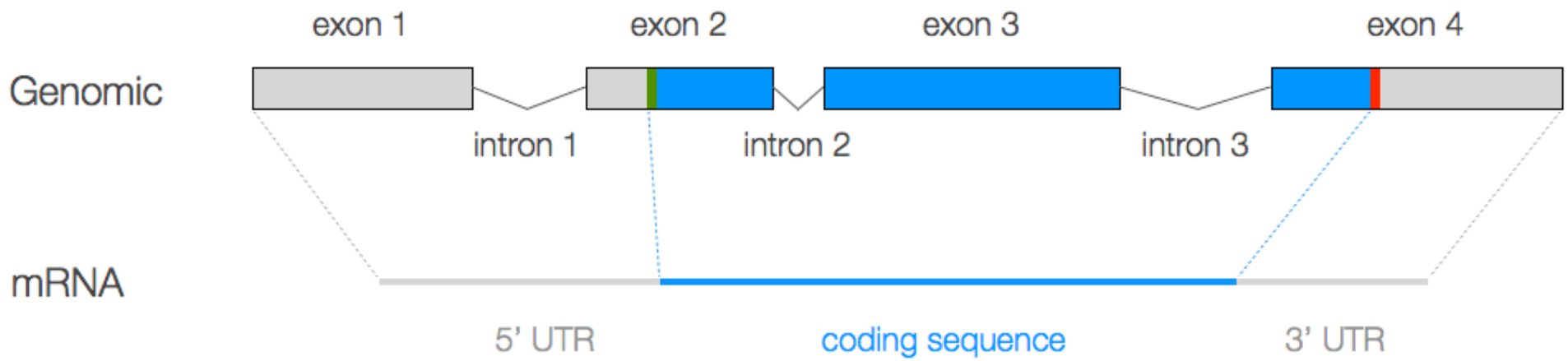
Repeat annotation

- RepeatModeler combines multiple repeat search engines and curated databases (e.g. Repbase) to identify repeats
- RepeatMasker marks repeats in assembly (replacement with Ns or soft-masking)

```
>Sc0000000
TACTGGCAGTCCAATCTGTCACCCTTGATTATAATTTCATTCAAATAATTTCTTTTTTTATCCAATGATCAACCAAATGCATTGCTTGCATTAAAAACTC
TTGTCAGCATTAAGTCCTCAGATATGGATCAACAACACAGTAAAACCATTTAATCACTAAACTTTAAAATAATAATTATTATATTGAAGAATCTAAAtca
tcatttctttcacaggaacacacaagcccaacaaattgacctgttcccaacagattggcttcatagctcagttggttctagcaattgcattcatttggta
tcacagaggtaatgggttcaaatcccattggagctgcctgaatttttgggtgtctTTTAAAGTGACTCAATCAATTCTATCTTCTCCCTGAATGCAGCG
TGTTAAGTATGCTTTTTTGAAACACAAGTTTGAGAATCCTGTACTGCAAACTAAATCCTTTAGTTTGCAGTGTATacacacacacacacacatacTGGAA
AATTACGGGTAAGAAATGTCAATCTCTCCTCATTCCAGTTCTCTCCAAAATAATGTTGAAGTTGAGGTAATGGTGGACCACTGAATAGTGAAATTCCCCT
GTTTTCATCCTTGTTATGTTTTACAATCACATGTCACATTGATTAATGGAAATATAATCCCCCTTTAGAACCAGATTGCAGACAGTCATTCTTTAACTTG
…
```

Gene prediction

In eukaryotes, gene prediction is difficult due to coding sequence (exons) being interrupted by introns



Gene prediction

- Complex pipelines (e.g. AUGUSTUS) combine multiple approaches
    - *ab initio* prediction (pattern-based)
    - extrinsic evidence can include
        - RNA-seq (transcripts)
        - homologous protein sequences
    - Sensitivity and specificity are increased by multiple rounds of training
    - Repeats should be masked
- Computational gene prediction always requires careful quality control

Gene prediction

The GFF format: gene structure, coding sequence

```
# start gene g29
Sc0000000 AUGUSTUS gene 656020 658053 0.9 - . g29
Sc0000000 AUGUSTUS transcript 656020 658053 0.9 - . g29.t1
Sc0000000 AUGUSTUS stop_codon 656020 656022 . - 0 transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS intron 656165 657273 1 - . transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS intron 657509 657956 1 - . transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS CDS 656020 656164 1 - 1 transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS CDS 657274 657508 1 - 2 transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS CDS 657957 658053 0.9 - 0 transcript_id "g29.t1"; gene_id "g29";
Sc0000000 AUGUSTUS start_codon 658051 658053 . - 0 transcript_id "g29.t1"; gene_id "g29";
# coding sequence = [atgacccgcttcgaaagcgacgattctcaattggatgaagatagtgatcatgggaattgttcagcaaaacggttggccc
# ccaaaagaaaaatttcaggtcggttgcgcaggaaatcaaaagaatcatatcgcctgggtcccaaggttgatgtaactccagcgagcagtgacgatgaa
# ctggagcgggaaacgtcttttatgaactctggtacctttaaaccagtcgagggtttttccaaccctatacccgtgagctccggagaggacgactttga
# tgaccttagtgttggagacatgaaaaagcgtcggagctcaaaaacgaaagaattccgacaacgcgaagatacccacgatacacctgatttgacagaca
# atgagggagagctggggtttaacacctttaagcccgcctcttacgtcaacgaaggtgcccacacttcggatgatgccgaatctgaactggaaaaggaa
# aaatga]
# protein sequence = [MTRFESDDSQLDEDSDHGNCSAKRLAPKRKISGRLRRKSKESYRLGPKVDVTPASSDDELERETSFMNSGTFKPVEGF
# SNPIPVSSGEDDFDDLSVGDMKKRRSSKTKEFRQREDTHDTPDLTDNEGELGFNTFKPASYVNEGAHTSDDAESELEKEK]
…
```

Gene prediction

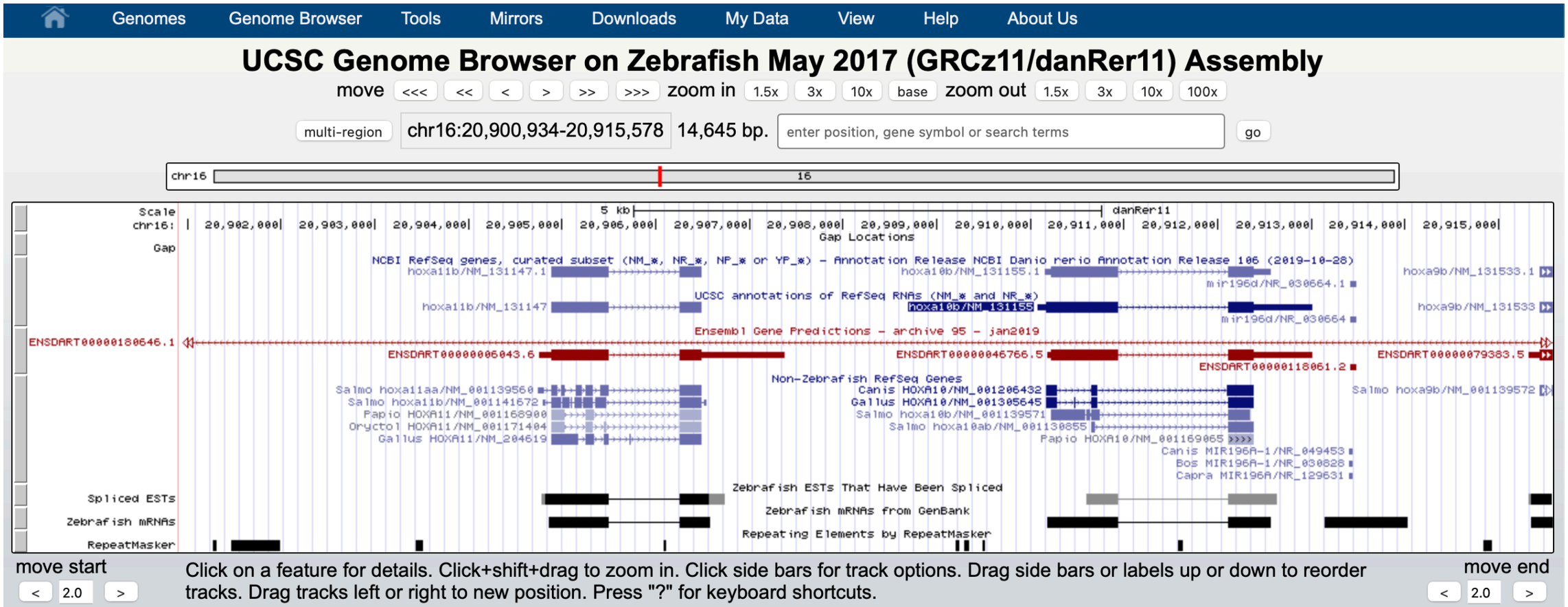
- Gene structure (UTRs, exons, introns)
- Coding sequence
- Transcription status and splice variants
- Putative identity and function

  (homology-based, e.g. by BLAST versus UniProt database)

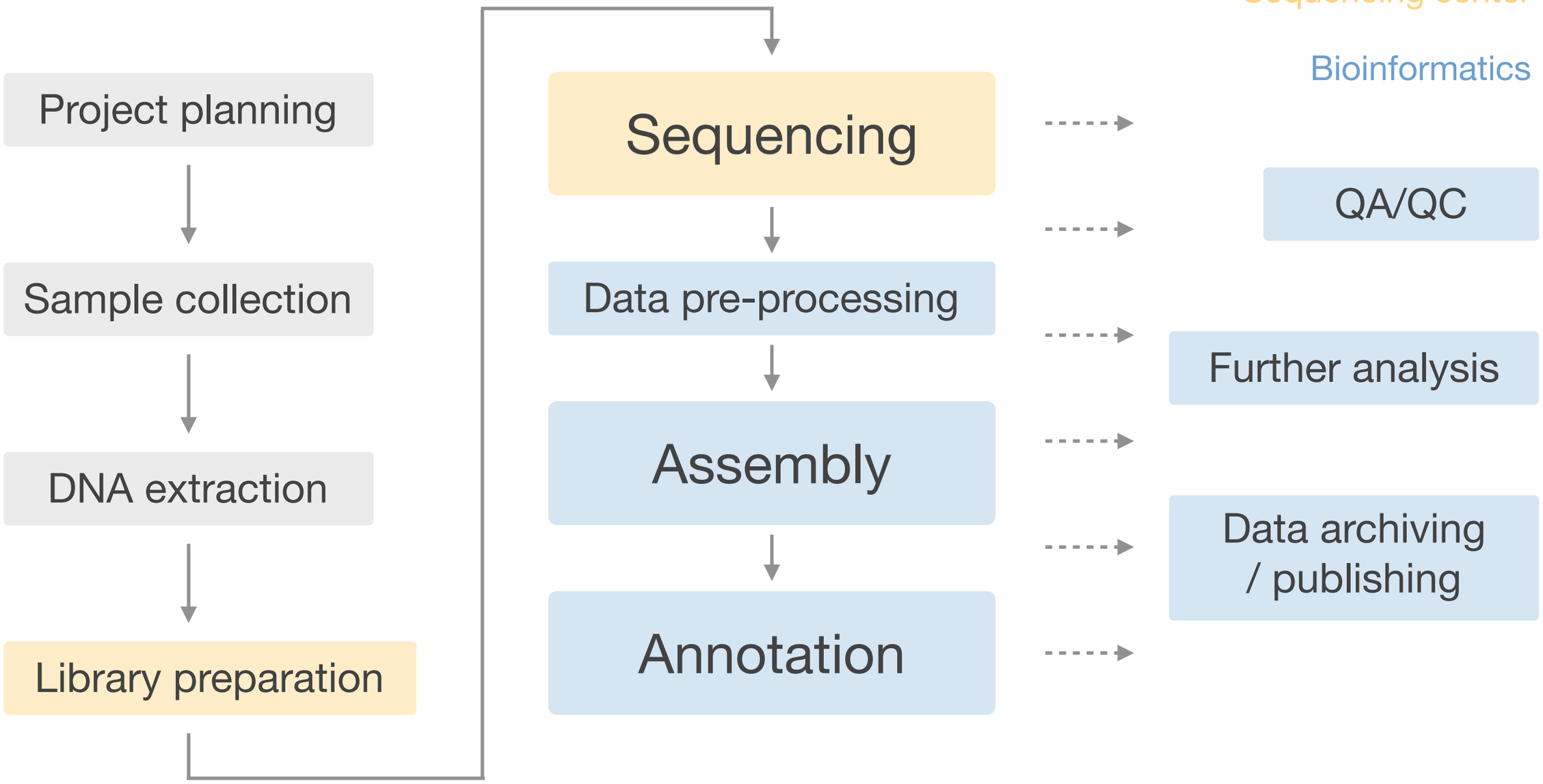
- Computational + manually curated gene models > Official Gene Set

Visualization

GBrowse / JBrowse genome browser



De novo genome sequencing workflow



4. Data management

- Documentation
- Data organization
- Backing up / Version control
- Publishing / Sharing data

Documentation

- Document your workflow in plain text format (Atom / TextEdit / Notepad)
- Include code, software versions, summary results, and comments

Worklog_genome_v4.txt

```
### Assembly v4

## Supernova v2.1.1 (mcap_v4)

supernova run --id=mcap10v4 --fastqs=~/proj/coral/genome/raw_data/raw/ --maxreads='all'

supernova mkoutput --style=pseudohap2 --asmdir=/mcap10v4/outs/assembly --outprefix=ph2

> mcap_v4.fas, mcap_v4h.fas (57431 scaffolds)

supernova upload mhelmkam@hawaii.edu mcap10v4/mcap10v4.mri.tgz

python ~/apps/quast_5.0.0/quast.py -e -s mcap_v4.fas   # no minimum contig length
python ~/apps/quast_5.0.0/quast.py -l -s -k mcap_v4.fas   # imposes -m 3000 -e, adds k-mer
analysis

> quast/mcap_v4

|

### Assembly polishing (v4 and v2 revision)

## Self-Blast to identify duplicates (also performed for v4)

blastn -task megablast -query Mcap_genome_v2.fas -db ../blast_db/Mcap_genome_v2.fas -out v2-
self.blast -evalue 1e-10 -max_target_seqs 2 -outfmt "6 qseqid sseqid pident length qlen slen
qstart qend sstart send evalue" -num_threads 10
blastn -task megablast -query mcap_v4.fas -db ../blast_db/mcap_v4.fas -out v4-self.blast
-evalue 1e-10 -max_target_seqs 2 -outfmt "6 qseqid sseqid pident length qlen slen qstart qend
sstart send evalue" -num_threads 10
```

Data organization

Formats

- Standard file formats (e.g. plain text, pdf, png)
- Standard data formats (e.g. FASTA, FASTQ, VCF, GFF)

Data organization

Naming scheme

- Uniform, persistent
- Comprehensive, includes important information
- No spaces and special characters (use underscores instead)
- Consistent file extensions (e.g. .fastq, .vcf, .gff)
- May include date (YYYYMMDD) or version number

Data organization

Naming scheme – Examples

What is it?

Species acronym

```
Hpue_raw300_F.fastq.gz
Mcap_genemodels_1.1_aa.fas
hyp155_a_0.33_mac4_5kb.raxml.log
```

File / data format

Dataset name

Version number

Important parameters

Data organization

Folder structure

- Be consistent
- Separate ongoing and completed work
- Consider links instead of duplicating files
- Include documentation (readme or worklog)

Example

```
Project
├── 0_metadata/
├── 1_rawdata/
├── 2_processing/
├── 3_assembly/
├── 4_annotation/
└── README.txt   # or WORKLOG.txt
```

Backing up / transferring data

- Keep raw data on dedicated hard drive
- Process and analyze working copies only
- Confirm data integrity using checksums after transfer

```
rsync -arvz <source_file> <destination_file>   # improved copy (-a: archive mode, -r:
                                               # include subdirectories, -z: compress
                                               # during transfer)

md5 <file>                                     # calculate checksum (not working in Git
                                               # Bash)
```

Version control

Typical git usage

```
git init                                    # create git repository based on current folder
git status                                  # show repository status
git add *                                   # track all files (exceptions in .gitignore)
git commit -m “title”                       # record changes

git push                                    # push to remote GitHub repository (if set up)
```

Example .gitignore file

```
1_rawdata/
*.fastq.gz
*.fastq
```

Publishing / sharing genome data

FAIR principles — findable, accessible, interoperable, reusable

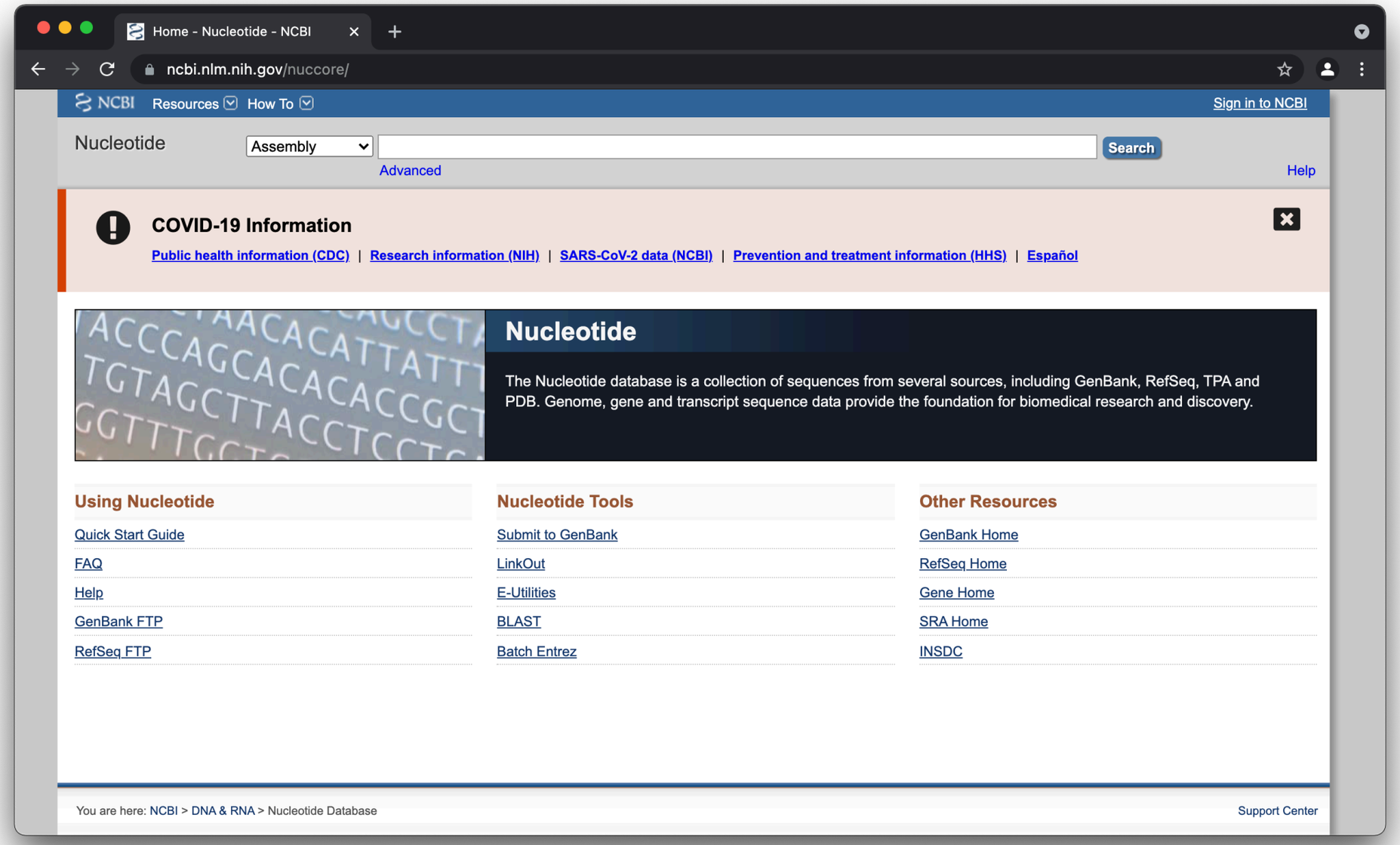
Key attributes:

- unique and persistent identifier
- clear and detailed metadata
- standardized data format and metadata vocabulary
- accessible data usage license (e.g. open access)

Public sequence databases

Public sequence databases

NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/)



Select database:

Assembly

Search term:

Scleractinia

Useful search tags

[ACCN]

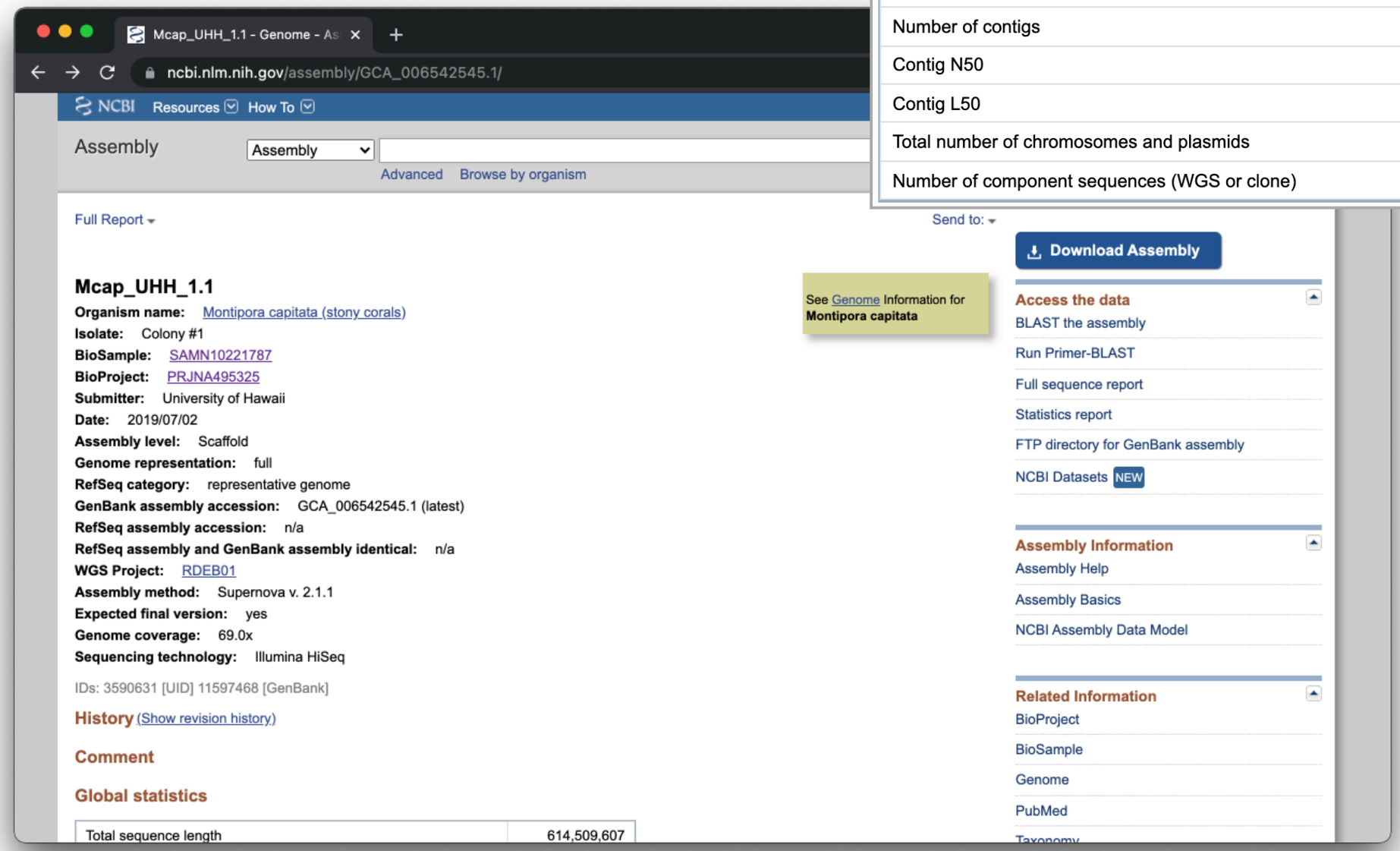
[ORGN]

[AUTH]

[TITL]

Public sequence databases

NCBI Genbank | Assembly report

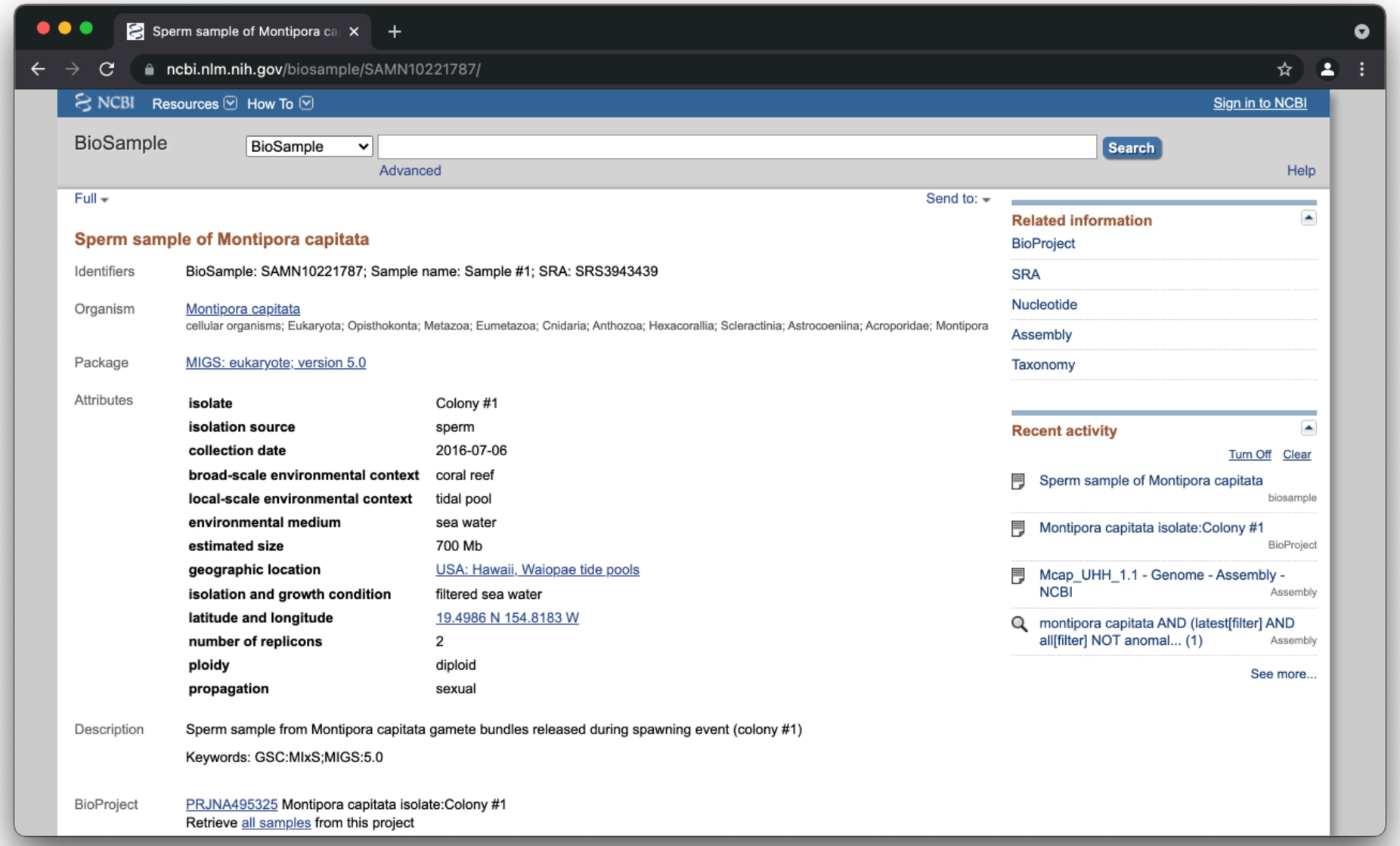


Global statistics

Total sequence length	614,509,607
Total ungapped length	571,886,739
Gaps between scaffolds	0
Number of scaffolds	27,865
Scaffold N50	185,537
Scaffold L50	747
Number of contigs	50,174
Contig N50	24,266
Contig L50	6,689
Total number of chromosomes and plasmids	0
Number of component sequences (WGS or clone)	27,865

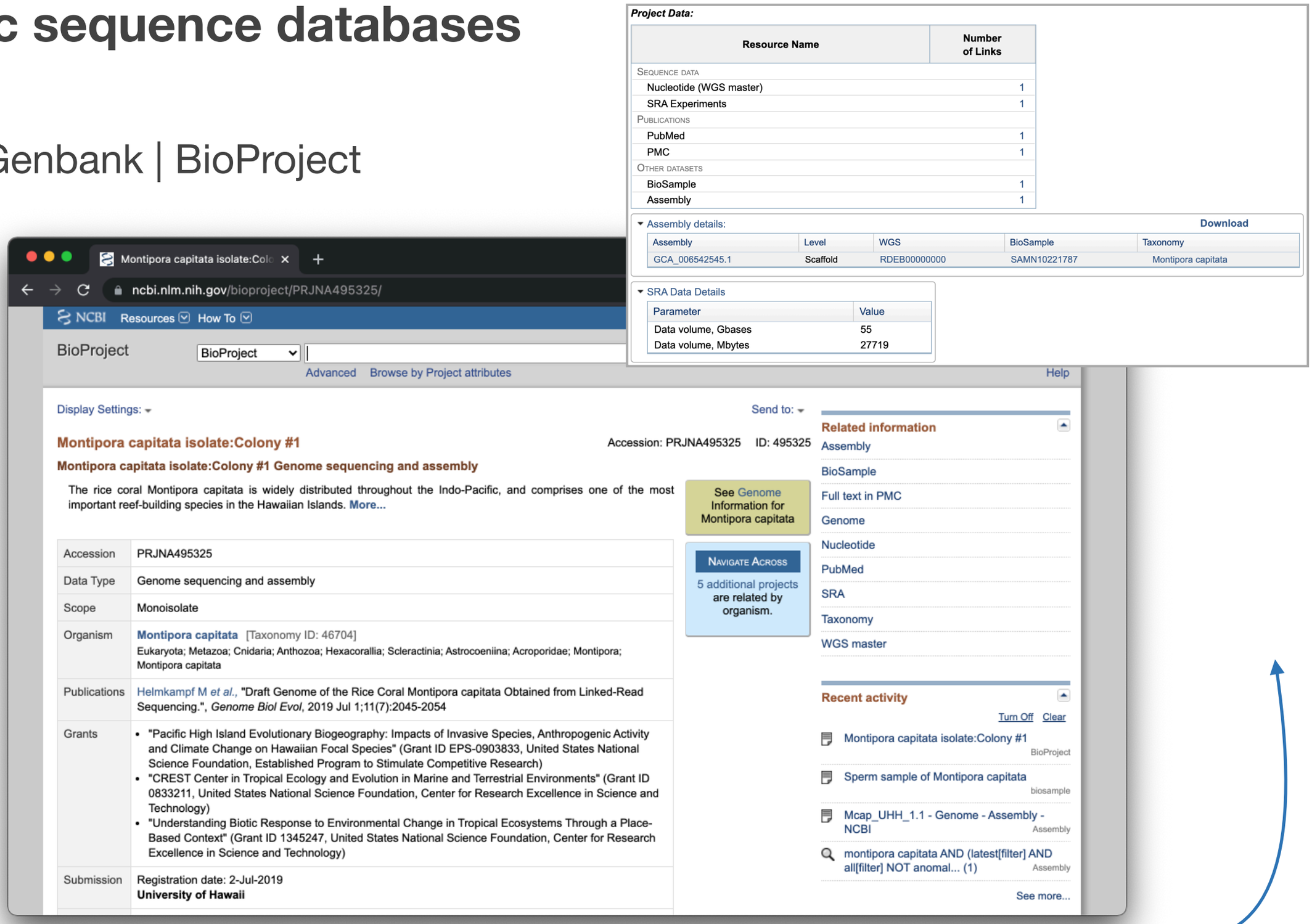
Public sequence databases

NCBI Genbank | BioSample



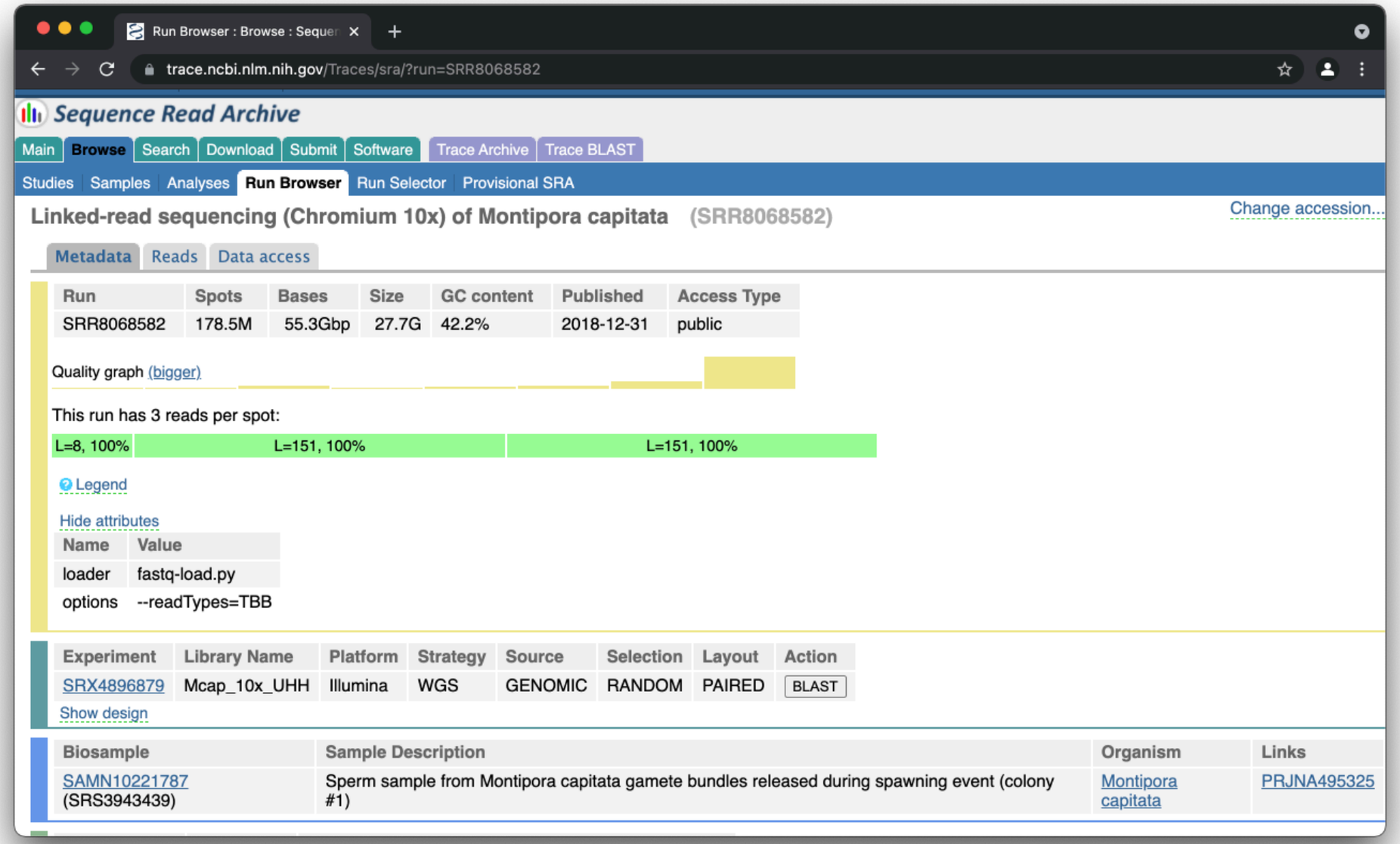
Public sequence databases

NCBI Genbank | BioProject



Public sequence databases

NCBI Genbank | Sequence Read Archive (SRA)



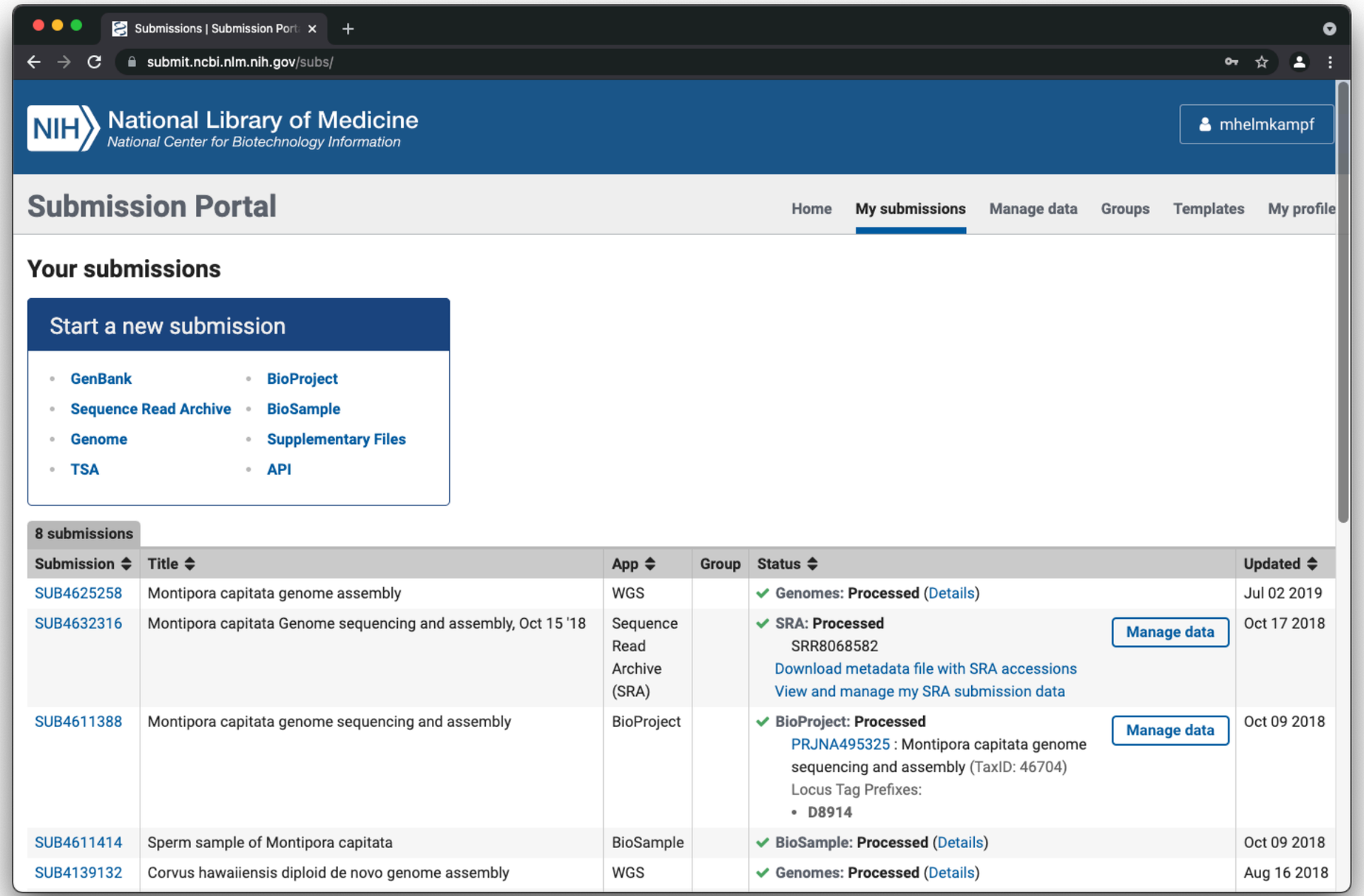
Genome submission

- NCBI genome submission guide:

  https://www.ncbi.nlm.nih.gov/genbank/genomesubmit/

- Submission portal login (https://submit.ncbi.nlm.nih.gov/subs/genome/)
  - Log in with NCBI, Google, or ORCiD etc. account
  - Start new or edit existing submission

Genome submission



New submission / register:

BioProject

BioSample

SRA

Genome (WGS)

Genome submission

- Provide project description, sample metadata, technical details (method, coverage etc.), contact information, publication if applicable
- Upload assembly as single FASTA file (minimum contig length 200 bp)
    - Chromosome-level?
    - What do ’Ns’ represent?
- Upload reads to SRA in FASTQ format
- Batch upload for multiple genomes

Genome submission

- Automated validation:
    - Contamination
    - Adapter sequences
    - Duplicate contigs
- After successful review, accession numbers will be issued
- Public release at requested date or publication date, whichever comes first
- Data may run through database annotation pipelines

Dryad

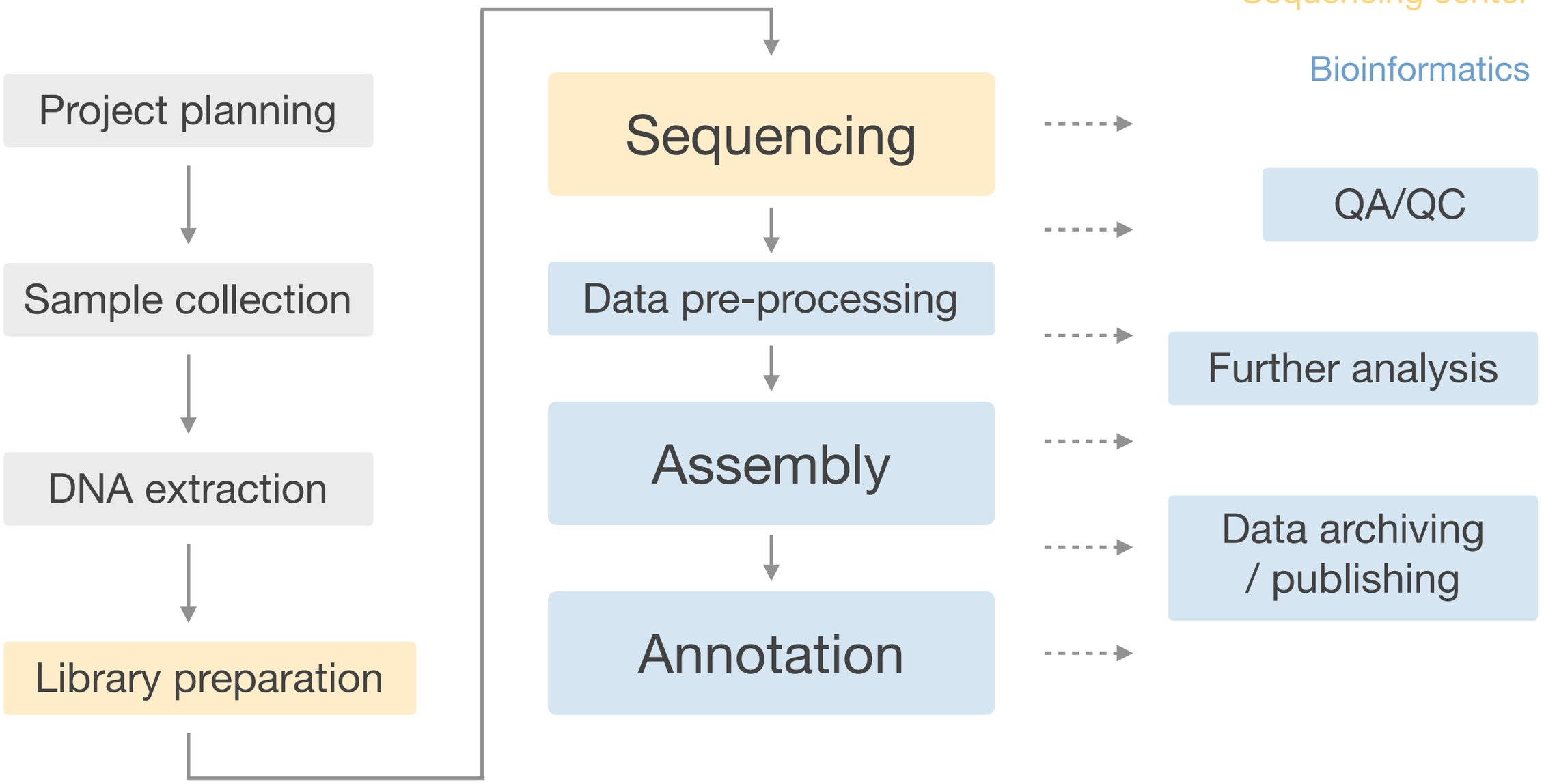
- https://datadryad.org
- Free open-access repository for data in any format

  (e.g. GFF, official gene set FASTA, genotype data in VCF format)
- May or may not be linked to publication
- Data receive permanent DOI

Code and workflow documentation

GitHub – Example: https://k-hench.github.io/hamlet_radiation/

De novo genome sequencing workflow



Thank you!

Please leave feedback for this course here (Google form):

https://tinyurl.com/9hucxfvh

Questions?

Email me at martin.helmkampf@leibniz-zmt.de