

Bioinformatics Workshop Agenda – June 27, 2017

Hosted by the Genomics Resource Center,
University of Maryland School of Medicine



THE LEADER IN LONG-READ SEQUENCING



8:30 – 8:55 a.m. Registration and Continental Breakfast

9:00 – 9:10 a.m. Welcome and Introduction
Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio

9:10 – 9:55 a.m. Introduction to SMRTLink 5.0

Minor Variant Detection with Juliet
Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio
Structural Variant Detection with PBSV
Aaron Wenger, Ph.D., Staff Scientist, PacBio

10:00 – 11:15 a.m. Concurrent Breakout Sessions

SESSION I: So, I Have a Diploid Assembly...Now What?

10:00 – 10:30 a.m. Understanding, Curating, and Analyzing Your Diploid Genome Assembly
Sarah Kingan, Ph.D., Senior Scientist, Bioinformatics, PacBio
10:30 – 10:45 a.m. Chromosome-scale De Novo Assembly of Mammalian Genomes Using Chromatin Interaction Data
Jay Ghurye, Ph.D. Candidate, Department of Computer Science, University of Maryland
10:45 – 11:15 a.m. SESSION I Discussion

SESSION II: Mini-training Session: Best Practices in Multiplexing with PacBio

10:00 – 10:30 a.m. Best Practice for Interpreting Demultiplexed Output
Carmen Guarco, Ph.D., Scientist, Field Applications Support, Bioinformatics, PacBio
10:30 – 10:50 a.m. Downstream Applications: Minor Variant Calling, Microbial Assembly, CCS2, and Iso-Seq
Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio
10:50 – 11:15 a.m. SESSION II Discussion

11:15 – 11:30 a.m. Coffee Break

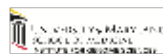
11:30 – 11:45 a.m. Breakout Sessions Wrap-up
11:45 – 12:30 p.m. Keynote: Accurate Detection of Complex Structural Variation
Fritz J. Sedlazeck, Ph.D., Lead Scientific Programmer, Human Genome Sequencing Center, Baylor College of Medicine

12:30 – 12:45 p.m. Open Forum for User Questions, Comments and Feedback on SMRTLink

12:45 – 12:55 p.m. Closing Remarks

1:00 p.m. Lunch

Thanks to our Partners:



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Understanding, Curating, and Analyzing your Diploid Genome Assembly

Sarah B. Kingan, Ph.D.
Senior Scientist, Bioinformatics, PacBio Applications

East Coast UGM, Baltimore, MD
Tuesday June 27th 2017

AGENDA

– Understanding Your Diploid Assembly

- Assembly Workflow
- Heterozygosity and Coverage

– Curating your Assembly

- Filtering Contigs
- Deduplicating Haplotypes

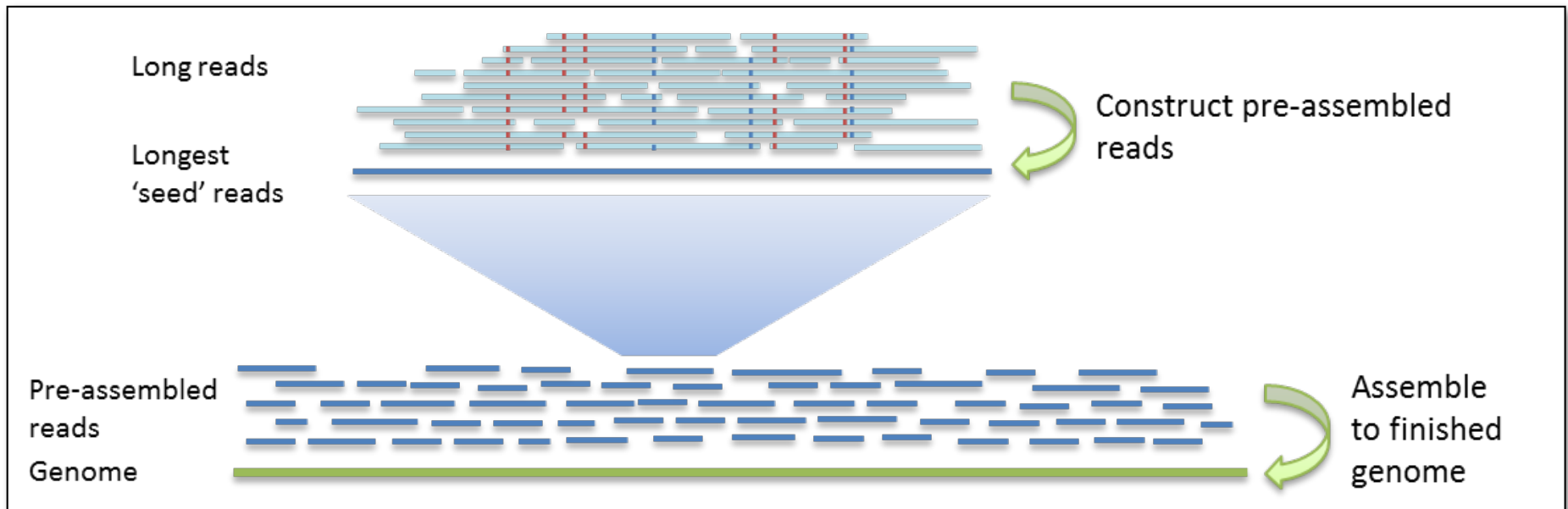
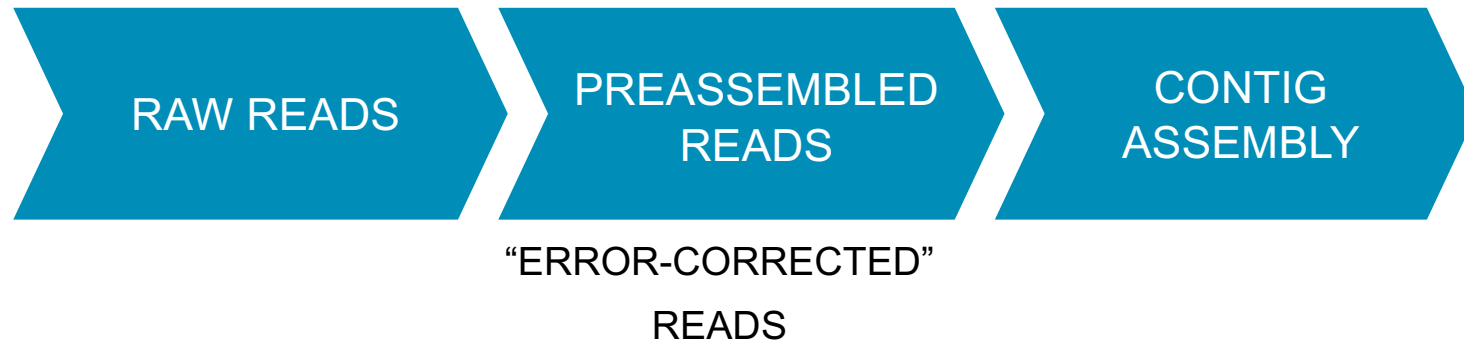
– Submitting Your Assembly to NCBI



Understanding Your Diploid Assembly

Assembly Workflow: From Raw Reads to High Quality
Reference

FALCON / HIERARCHICAL GENOME ASSEMBLY PROCESS (HGAP)



FALCON AND FALCON-UNZIP



Phased diploid genome assembly with single-molecule real-time sequencing.

Chin CS, Peluso P, Sedlazeck FJ, Nattestad M, Concepcion GT, Clum A, Dunn C, O'Malley R, Figueroa-Balderas R, Morales-Cruz A, Cramer GR, Delledonne M, Luo C, Ecker JR, Cantu D, Rank DR, Schatz MC

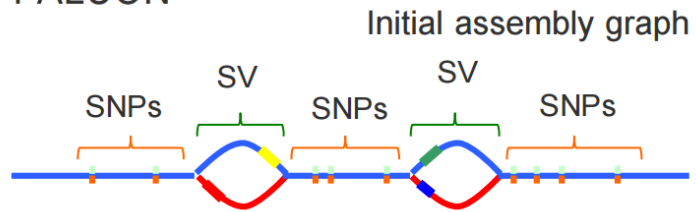
ABSTRACT

While genome assembly projects have been successful in many haploid and inbred species, the assembly of noninbred or rearranged heterozygous genomes remains a major challenge. To address this challenge, we introduce the open-source FALCON and FALCON-Unzip algorithms (<https://github.com/PacificBiosciences/FALCON/>) to assemble long-read sequencing data into highly accurate, contiguous, and correctly phased diploid genomes. We generate new reference sequences for heterozygous samples including an F1 hybrid of *Arabidopsis thaliana*, the widely cultivated *Vitis vinifera* cv. Cabernet Sauvignon, and the coral fungus *Clavicornia pyxidata*, samples that have challenged short-read assembly approaches. The FALCON-based assemblies are substantially more contiguous and complete than alternate short- or long-read approaches. The phased diploid assembly enabled the study of haplotype structure and heterozygosities between homologous chromosomes, including the identification of widespread heterozygous structural variation within coding sequences.

- FALCON is a **diploid-aware assembler**.
- FALCON-Unzip module performs true **phased assembly** for diploid samples.

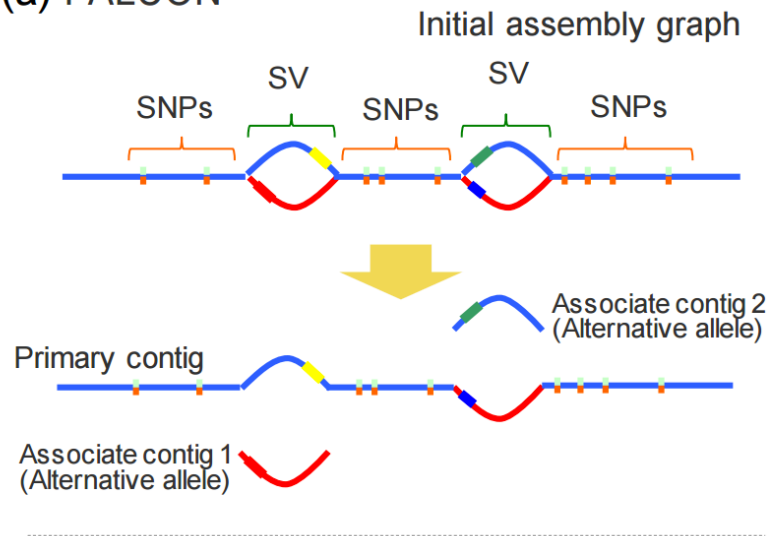
DIPLOID ASSEMBLY WITH FALCON-UNZIP

(a) FALCON



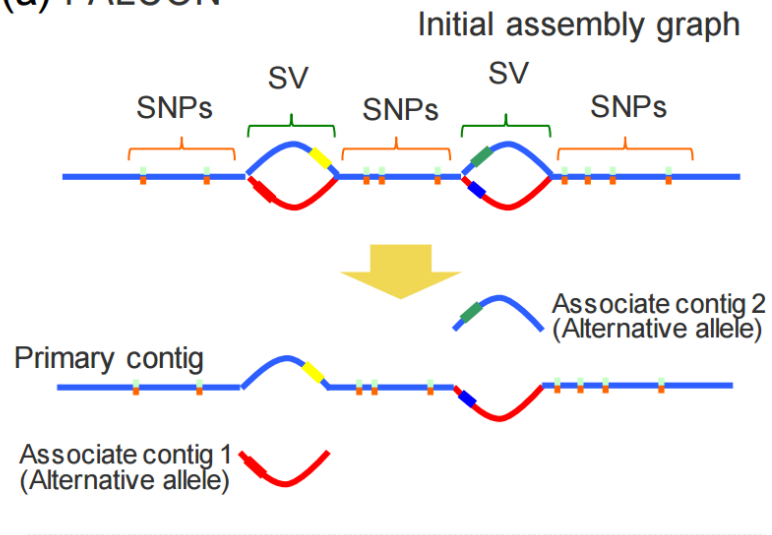
DIPLOID ASSEMBLY WITH FALCON-UNZIP

(a) FALCON

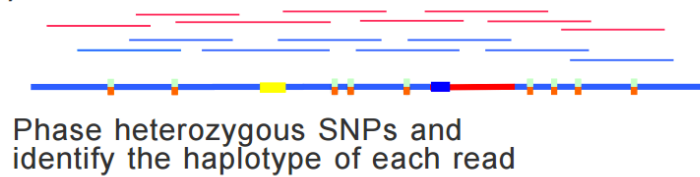


DIPLOID ASSEMBLY WITH FALCON-UNZIP

(a) FALCON

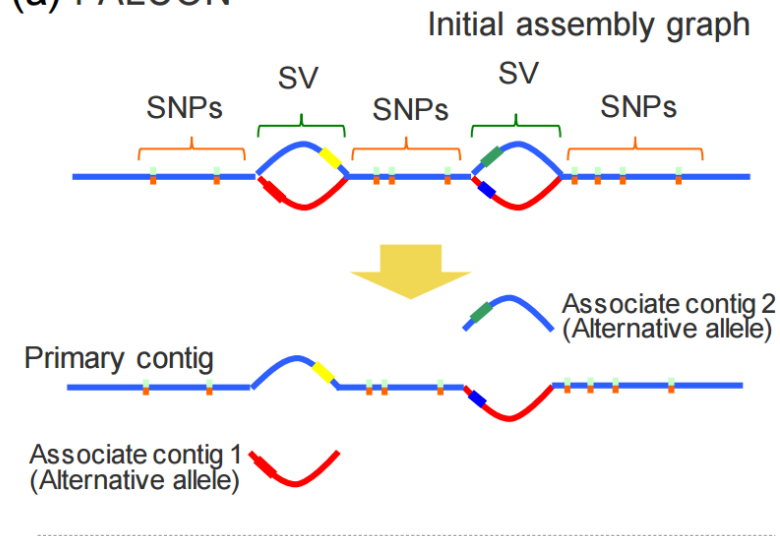


(b)

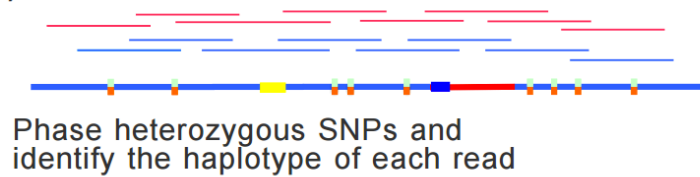


DIPLOID ASSEMBLY WITH FALCON-UNZIP

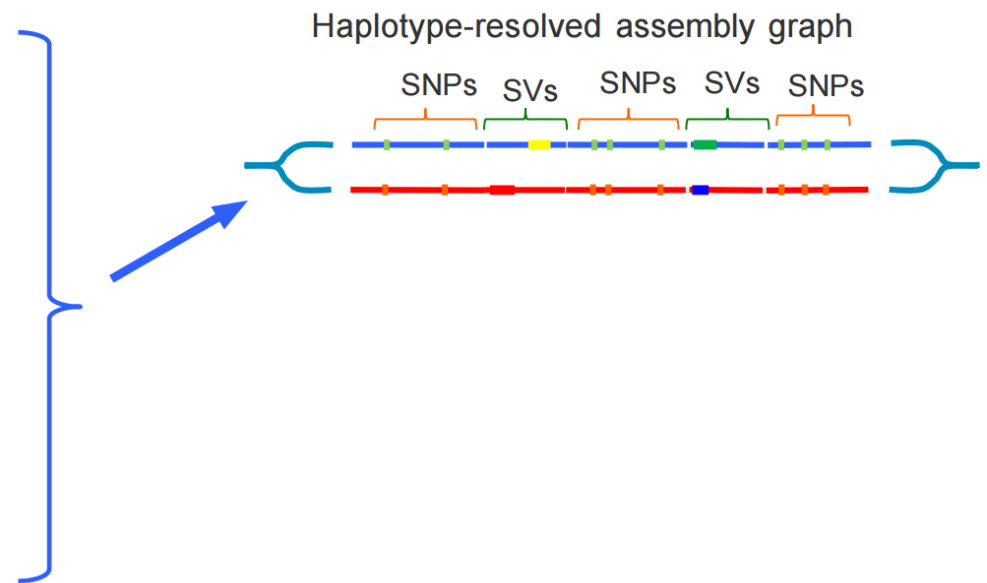
(a) FALCON



(b)

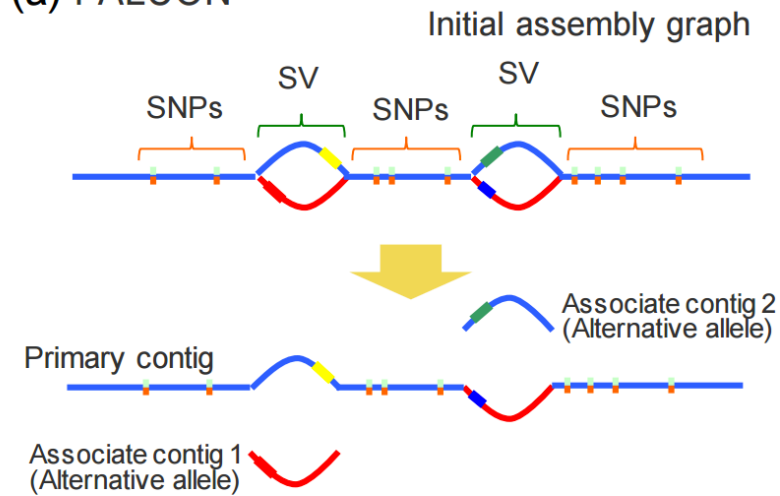


(c) FALCON-Unzip

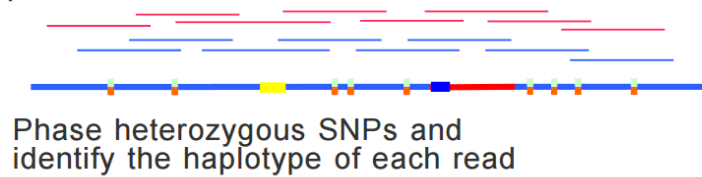


DIPLOID ASSEMBLY WITH FALCON-UNZIP

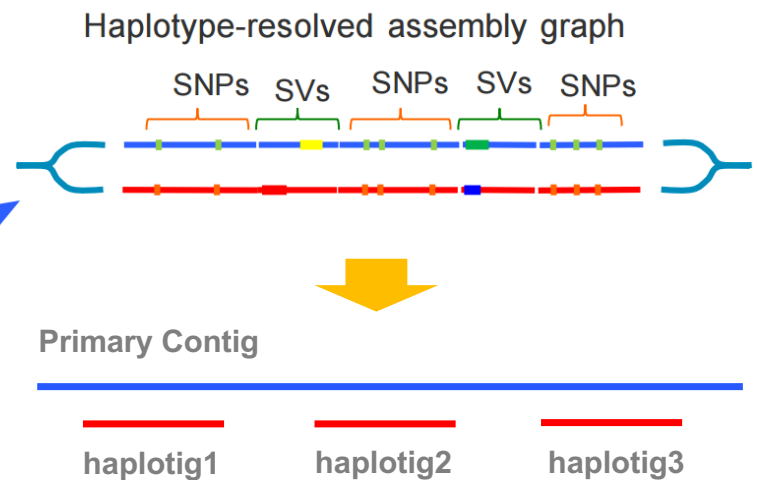
(a) FALCON



(b)

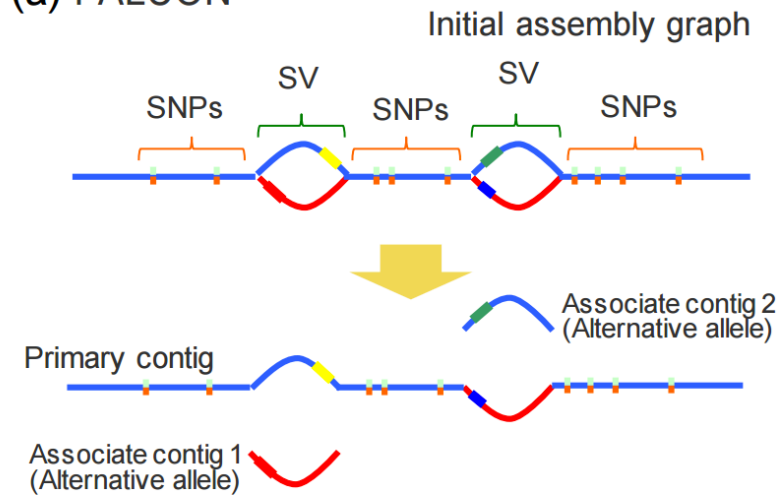


(c) FALCON-Unzip

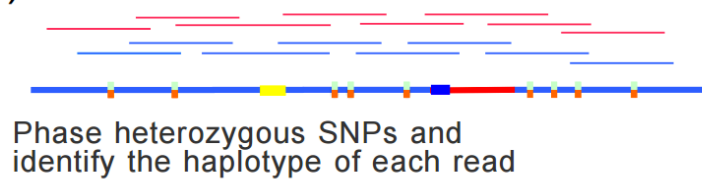


DIPLOID ASSEMBLY WITH FALCON-UNZIP

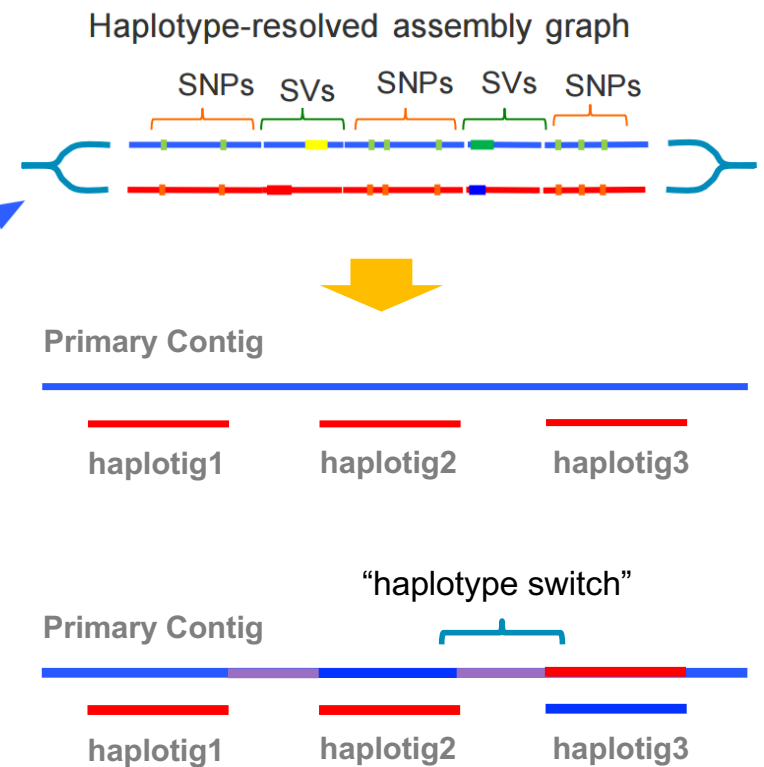
(a) FALCON



(b)

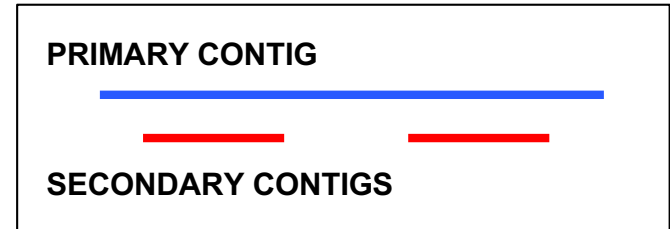


(c) FALCON-Unzip



EXAMPLE ASSEMBLY OF WATER BUFFALO

	FALCON	FALCON-Unzip
Primary Length	2.66 Gb	2.65 Gb
Primary N50	18.7 Mb	18.8 Mb
Secondary Length	0.218 Gb	1.53 Gb
Proportion Phased	8.2 %	58 %



7-fold increase in haplotype phasing with Unzip module



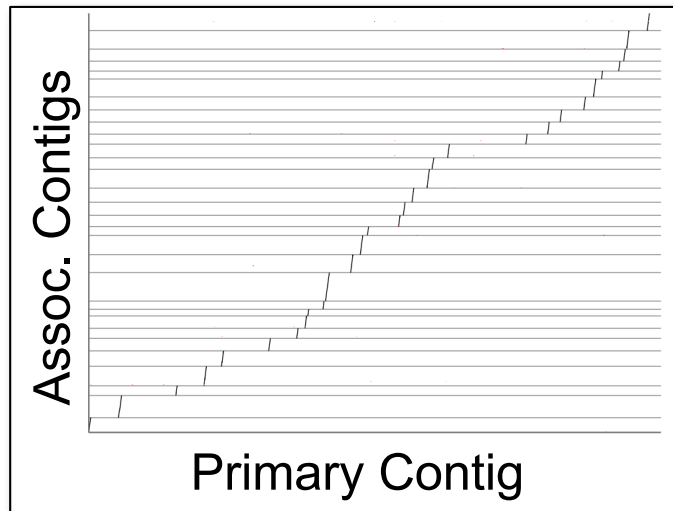
Olimpia

Phot Credit: Caterina Cambuli

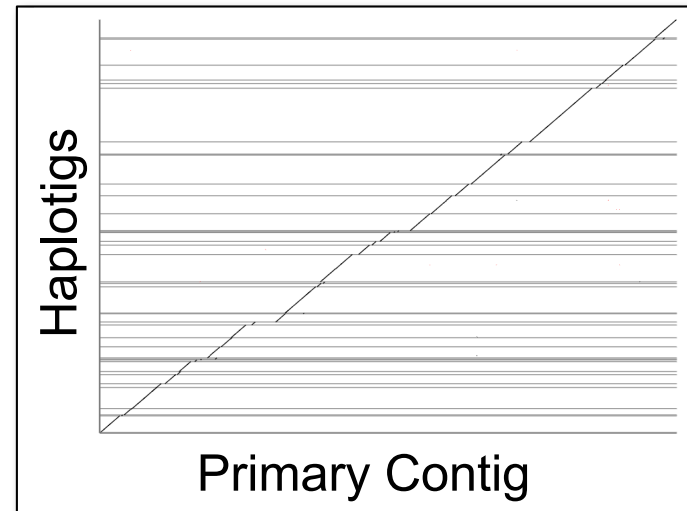
Acknowledgements:
 Tim Smith, USDA-ARS
 John Williams, Lloyd Low, University of Adelaide
 Paolo Ajmone-Marsan, Università Cattolica del S. Cuore
 David Hume, Mick Watson, Roslin Institute

INCREASED HAPLOTIG CONTIGUITY WITH FALCON-UNZIP

FALCON



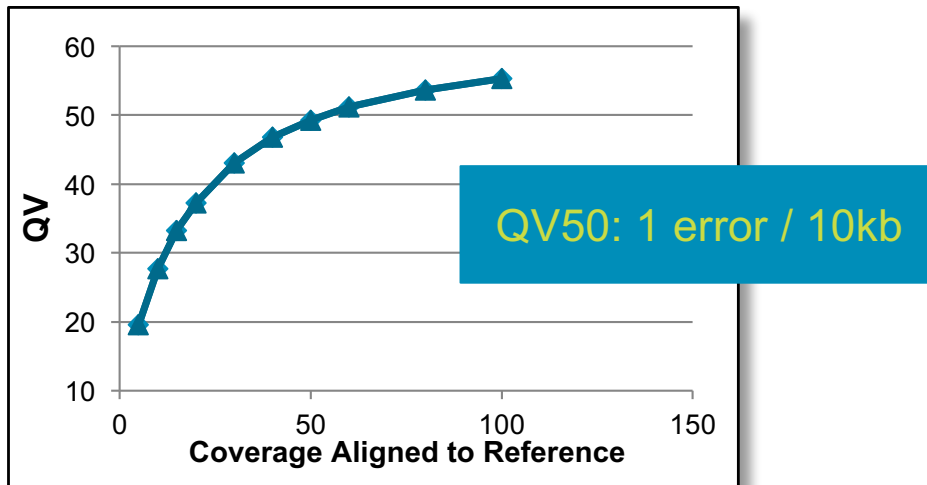
FALCON-UNZIP



CONTIG: 000078F	FALCON	FALCON-Unzip
Primary Contig Length	12.9 Mb	12.9 Mb
Number Secondary Contigs	30	34
Total Secondary Length	1.21 Mb	10.6 Mb
Secondary Contig N50	42.5 kb	470 kb
Proportion Phased	9.3 %	82%

POLISHING WITH ARROW: INCREASED REFERENCE QUALITY

CONSENSUS BASE ACCURACY



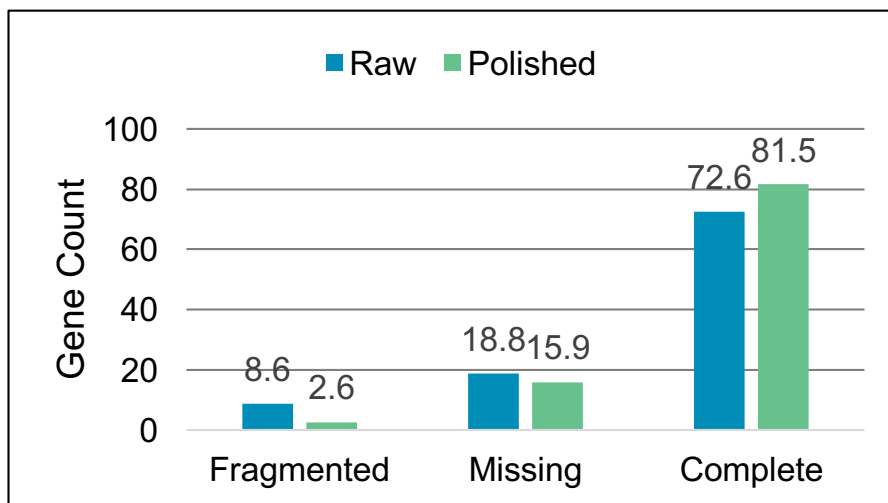
Consensus Base Accuracy

- Sequel 2.0 Chemistry
- Bacterial Genomes

Genome Completeness

- Avian Genome
- 50-fold Raw Coverage
- BUSCO2 analysis with eukaryota geneset

GENOME COMPLETENESS WITH BUSCO



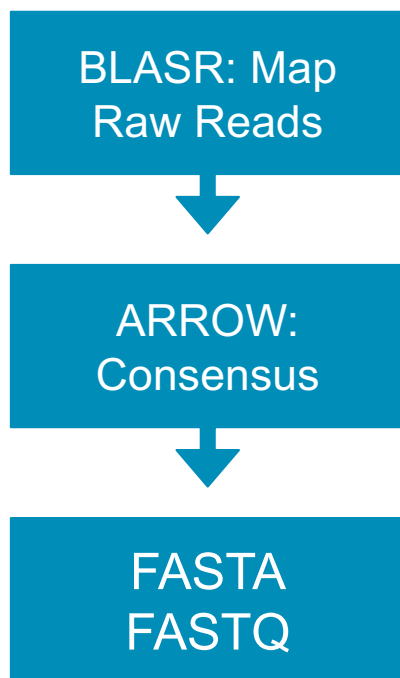
70% reduction in **Fragmented Genes**
 15% reduction in **Missing Genes**
 12% increase in **Complete Genes**

Acknowledgement:

Erich Jarvis, Rockefeller University

POLISHING WITH ARROW: WORKFLOW

METHOD	ASSEMBLY	POLISHING
HGAP4 - SMRT Link	✓	✓
FALCON	✓	resequencing pipeline from pbsmrtpipe/SMRT Link
FALCON-Unzip	✓	✓ (phased) plus optional resequencing



Random Best Mapping

- Random choice of locus with equal BLASR score

Minimum Coverage <5

- <5 reads span 500 bp window
- No consensus call
- Reference base returned as lowercase

Consensus Sequence

```

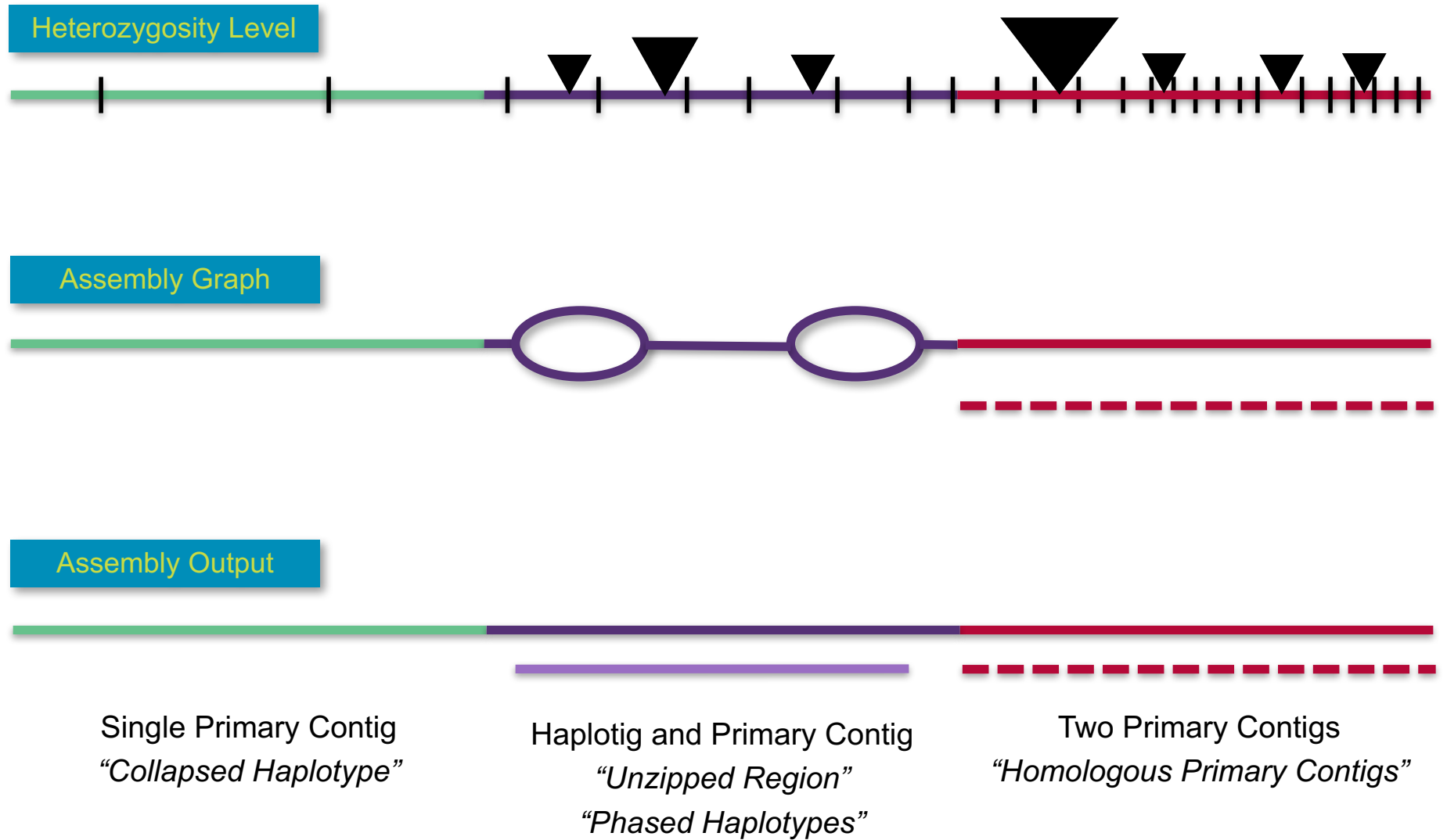
atgcgccgttatatgg
aagctagcTAGCTCTA
GTAGCTAGAGCTAGCT
GCGCGCTAGAATAGGG
CGCCATAGAGCCTTTT
  
```




Understanding Your Diploid Assembly

Heterozygosity, Assembly Structure, and Coverage

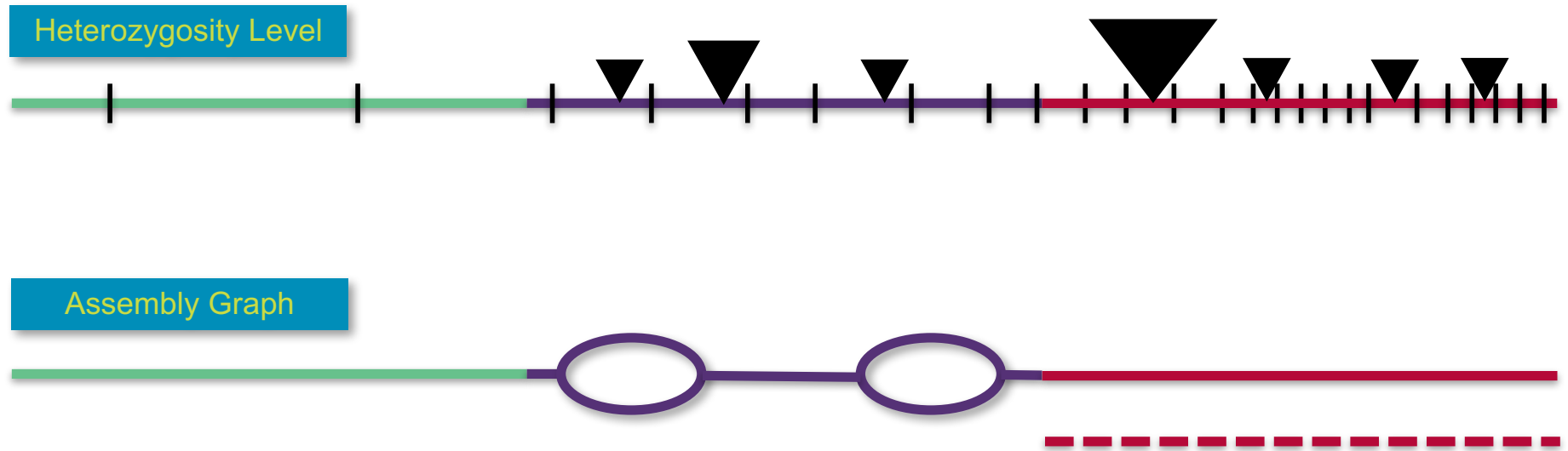
IMPACT OF HETEROZYGOSITY ON ASSEMBLY PROCESS



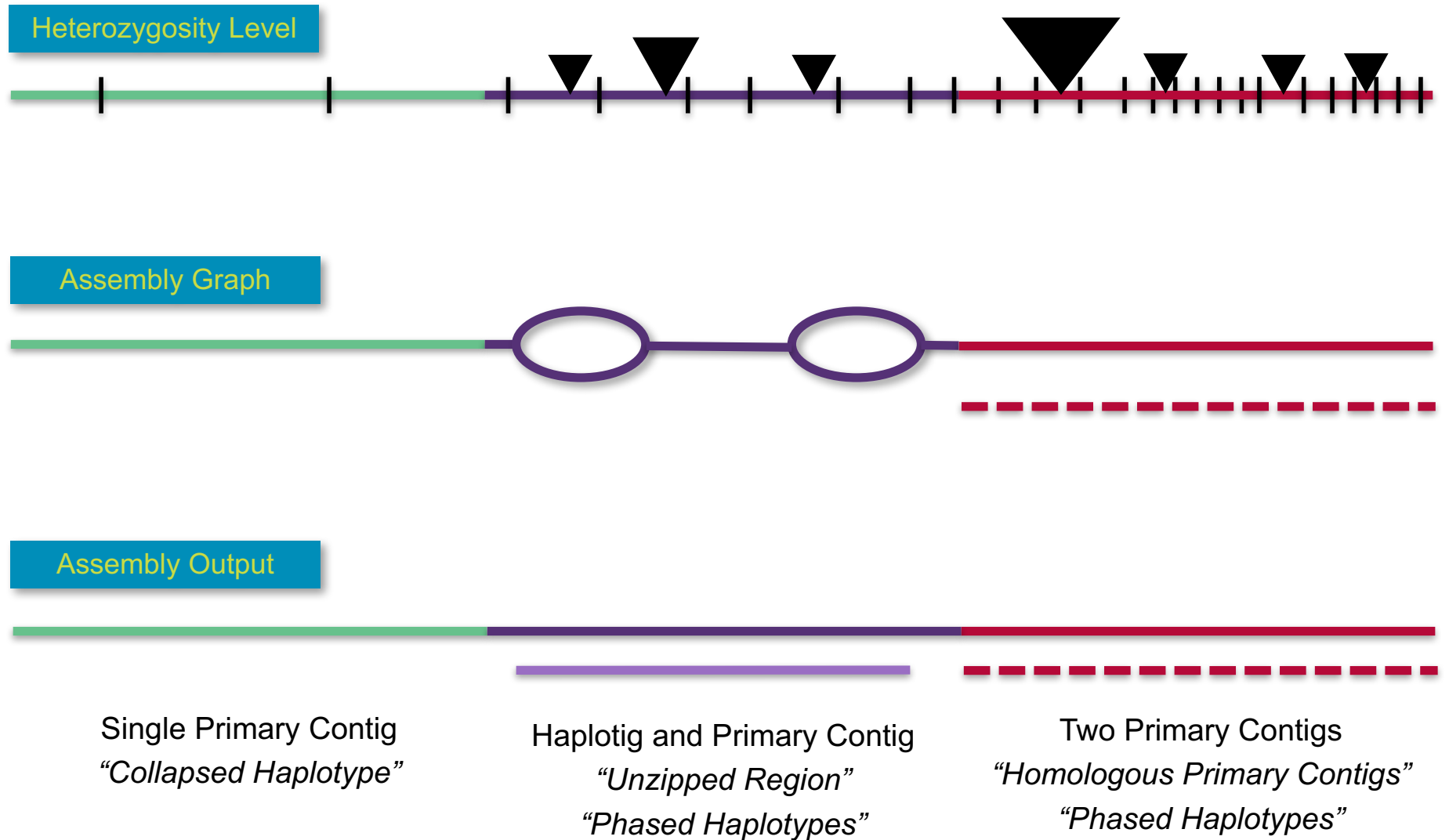
IMPACT OF HETEROZYGOSITY ON ASSEMBLY PROCESS



IMPACT OF HETEROZYGOSITY ON ASSEMBLY PROCESS

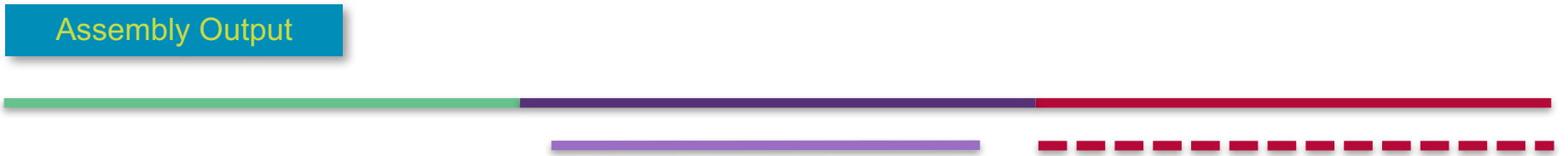


IMPACT OF HETEROZYGOSITY ON ASSEMBLY PROCESS



Modified from Chin *et al.* 2016

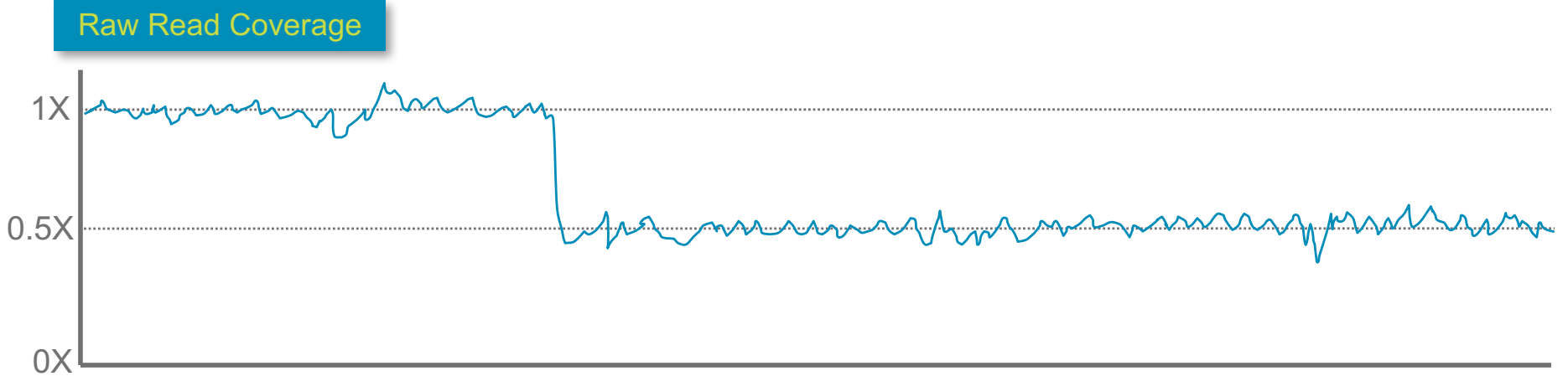
RAW READ COVERAGE AND ASSEMBLY STRUCTURE



Single Primary Contig
"Collapsed Haplotype"

Haplotig and Primary Contig
"Unzipped Region"
"Phased Haplotypes"

Two Primary Contigs
"Homologous Primary Contigs"
"Phased Haplotypes"

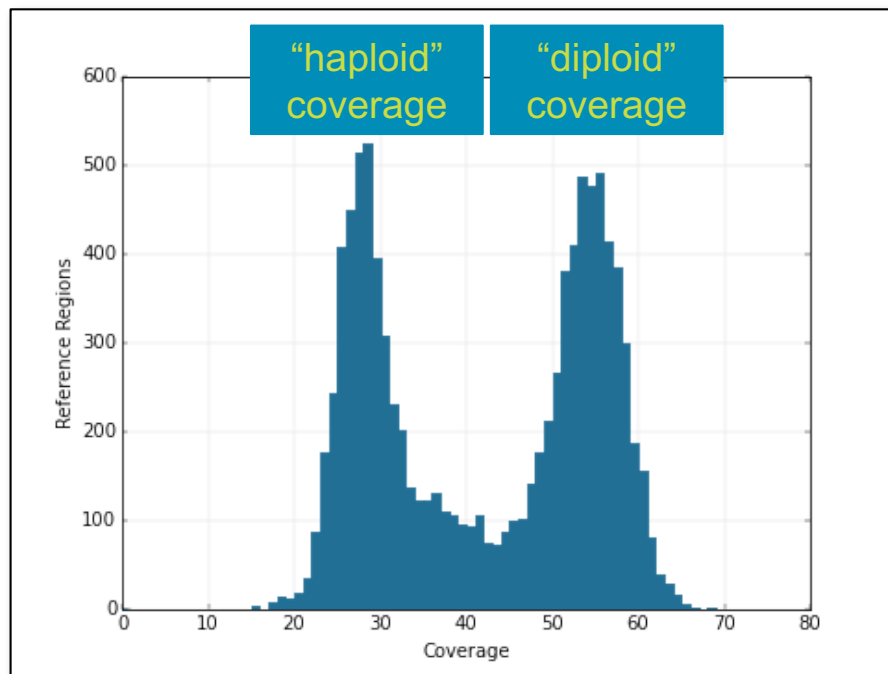


Position Along Chromosome

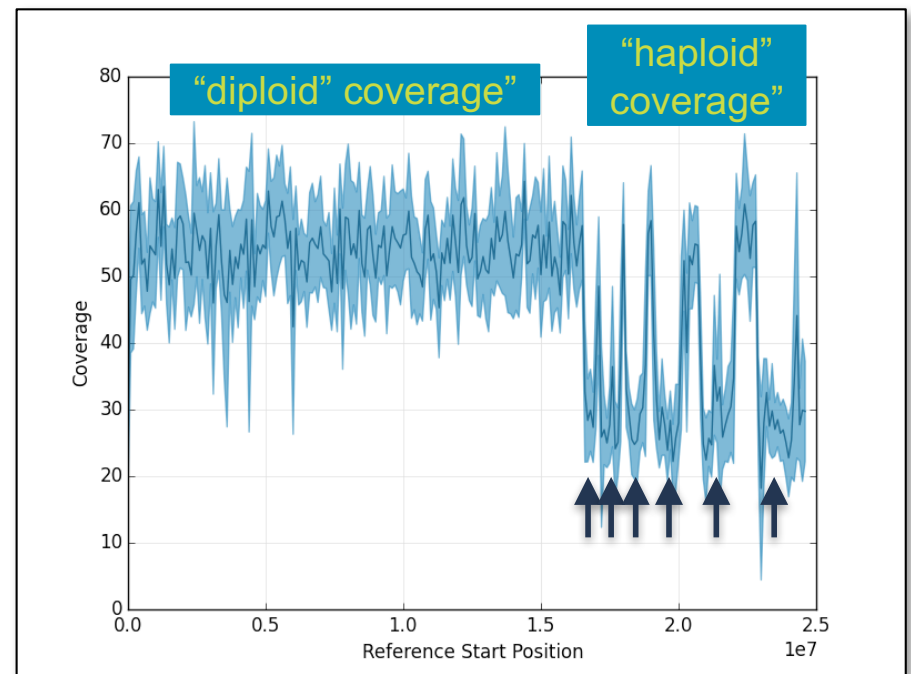
SMRT LINK COVERAGE REPORTS

Graphical Outputs from Resequencing Pipeline / HGAP4

COVERAGE HISTOGRAM: GENOME



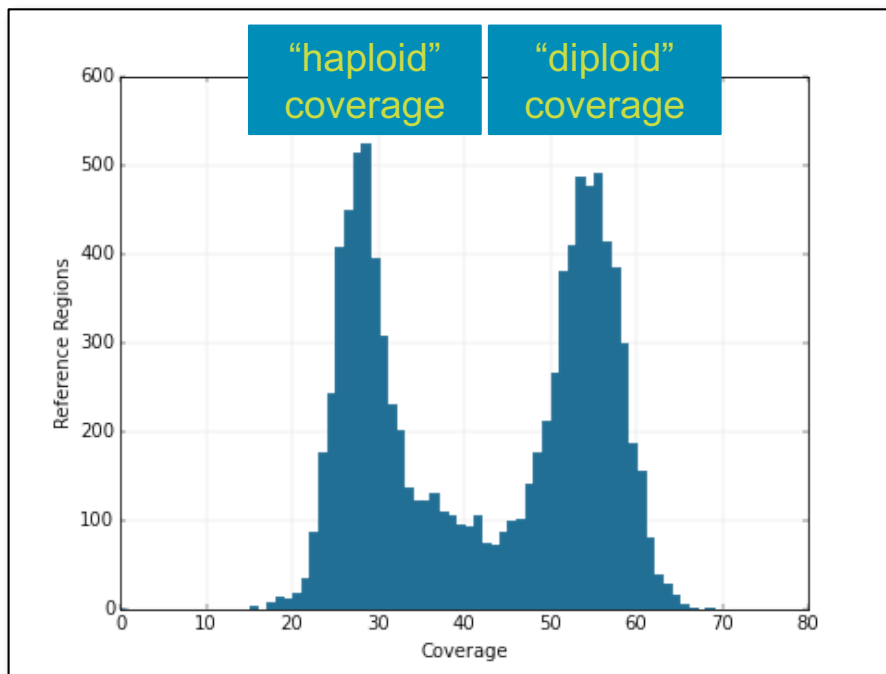
COVERAGE PLOT: CONTIG



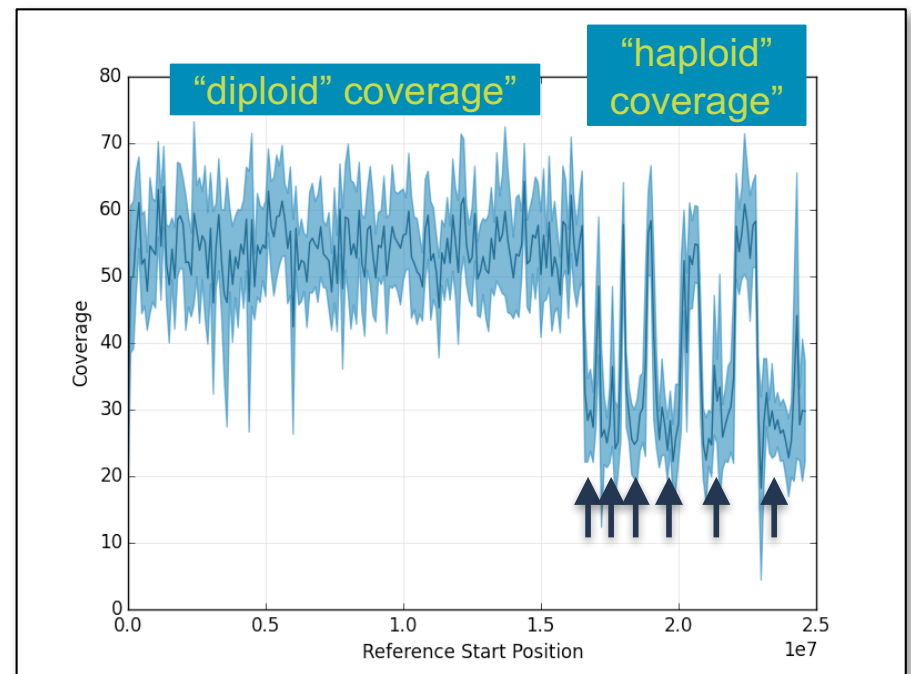
SMRT LINK COVERAGE REPORTS

Graphical Outputs from Resequencing Pipeline / HGAP4

COVERAGE HISTOGRAM: GENOME



COVERAGE PLOT: CONTIG



PRIMARY CONTIG



SECONDARY CONTIGS



SMRT LINK COVERAGE SUMMARY FILES

alignment_summary.gff: coarse coverage across all contigs

—SMRT Link job directory

- myJob/tasks/pbreports.tasks.summarize_coverage-0/alignment_summary.gff
- File Format Specs: <https://github.com/ben-lerch/SAT>

```

skingan — skingan@login14-fas01: /home/skingan — ssh -Y skingan@login14-fas01 — 133x13
000123F|arrow|arrow . region 1 100000 0.00 + . cov=2,52,64;cov2=48.102,10.613;gaps=0,0
000123F|arrow|arrow . region 100001 200000 0.00 + . cov=39,57,76;cov2=56.756,7.613;gaps=0,0
000123F|arrow|arrow . region 200001 300000 0.00 + . cov=35,50,68;cov2=51.148,8.026;gaps=0,0
000123F|arrow|arrow . region 300001 400000 0.00 + . cov=39,55,74;cov2=55.743,7.334;gaps=0,0
000123F|arrow|arrow . region 400001 500000 0.00 + . cov=40,56,76;cov2=56.210,7.317;gaps=0,0
000123F|arrow|arrow . region 500001 600000 0.00 + . cov=37,54,71;cov2=54.493,7.591;gaps=0,0
000123F|arrow|arrow . region 600001 700000 0.00 + . cov=41,59,84;cov2=60.365,8.079;gaps=0,0
000123F|arrow|arrow . region 700001 800000 0.00 + . cov=39,59,84;cov2=59.854,11.002;gaps=0,0
000123F|arrow|arrow . region 800001 900000 0.00 + . cov=36,53,68;cov2=52.339,6.035;gaps=0,0
000123F|arrow|arrow . region 900001 1000000 0.00 + . cov=39,54,73;cov2=53.778,6.807;gaps=0,0
000123F|arrow|arrow . region 1000001 1100000 0.00 + . cov=35,52,73;cov2=52.280,7.533;gaps=0,0
000123F|arrow|arrow . region 1100001 1200000 0.00 + . cov=39,57,71;cov2=56.819,6.163;gaps=0,0
:

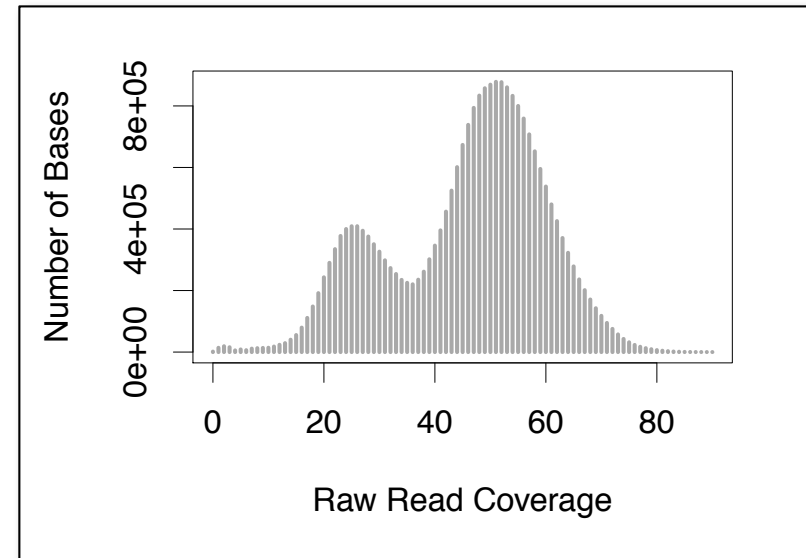
```

cov	cov2	gaps
min	mean	number continuous gaps
median	s.d.	number gap bases
max		

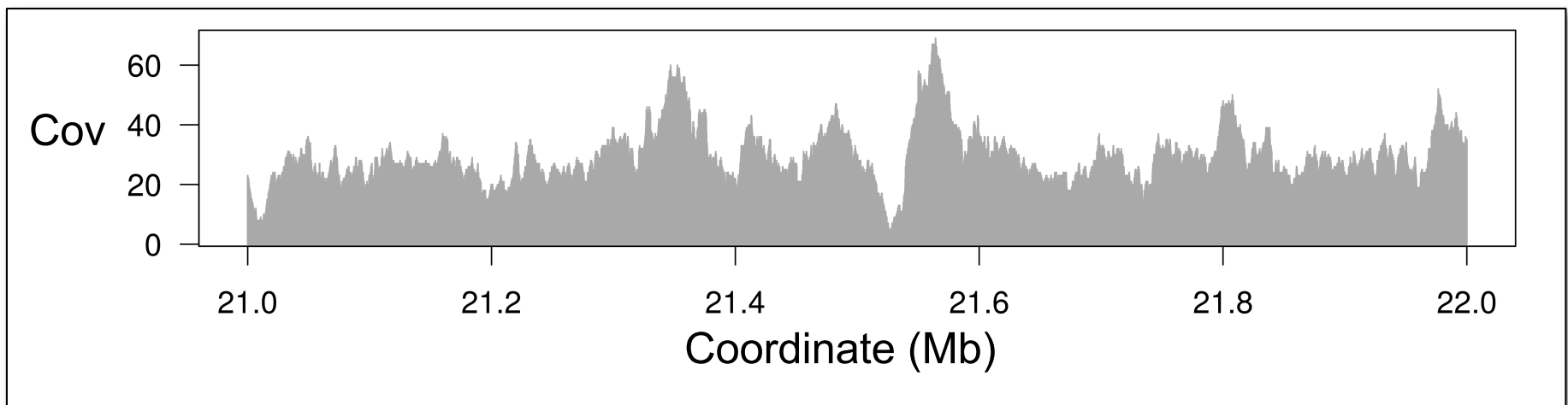
TOOLS FOR CUSTOM COVERAGE ANALYSIS

- Merge BAM files (N=24)
 - pbmerge
 - samtools merge
- Coverage calculation
 - samtools depth
 - bedtools genomecov
- Visualization
 - R - text file/dataframe

CONTIG COVERAGE HISTOGRAM bedtools genomecov, R



CONTIG COVERAGE WINDOW bedtools genomecov/samtools depth, R



ALIGNMENTS AND VISUALIZATION

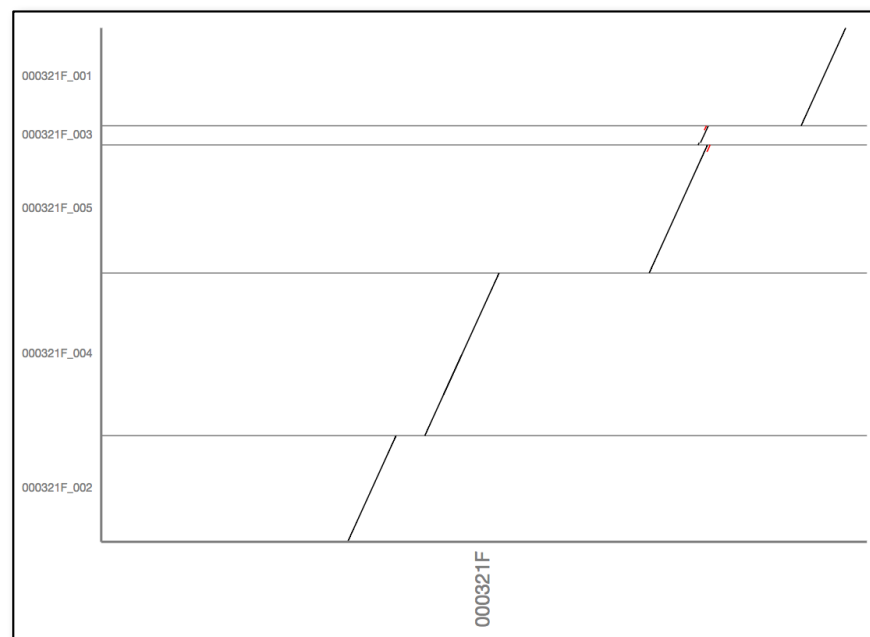
Recommended Tools for Haplotype Alignment and Analysis

- Subset Reference Sequence
 - `samtools faidx`
- Alignment
 - MUMmer (v4, multi-threaded support)
 - NUCmer, delta-filter, show-coords, show-snps, etc
- Visualization
 - `mummerplot`
 - `assemblytics`
 - `gepard` (alignment + vis)
- FALCON Assembly Tools
 - <https://github.com/PacificBiosciences/apps-scripts/>
 - FALCONAssemblyTools repo

HAPLOTIGS TO PRIMARY CONTIG DOTPLOT

`alignHaplotigs2Primary.sh`

Assemblytics





Assembly Finishing

Filtering, Circularizing, Haplotype Deduplication

GUIDELINE FOR CONTIG FILTERING

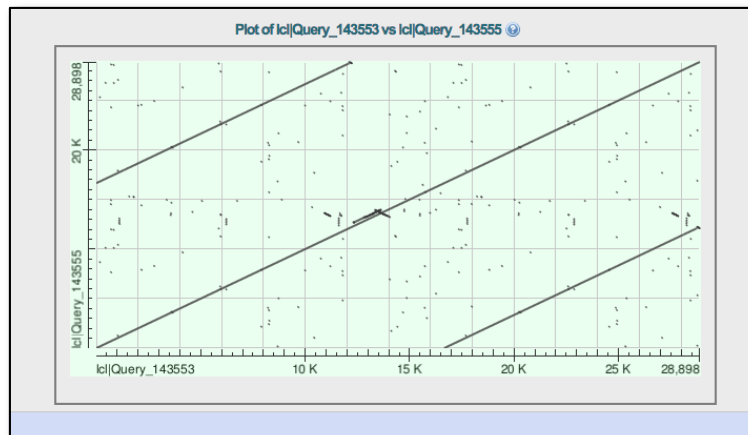
<https://github.com/PacificBiosciences/apps-scripts/tree/master/FALCONAssemblyTools>

Circularize organelle

- Identify
 - high coverage
 - "circular ctg" FALCON annotation
 - blast hit to organelle
- Circularize and polish
 - minimus2, circulator

SELF-ALIGNMENT OF MITO CONTIG

BLAST



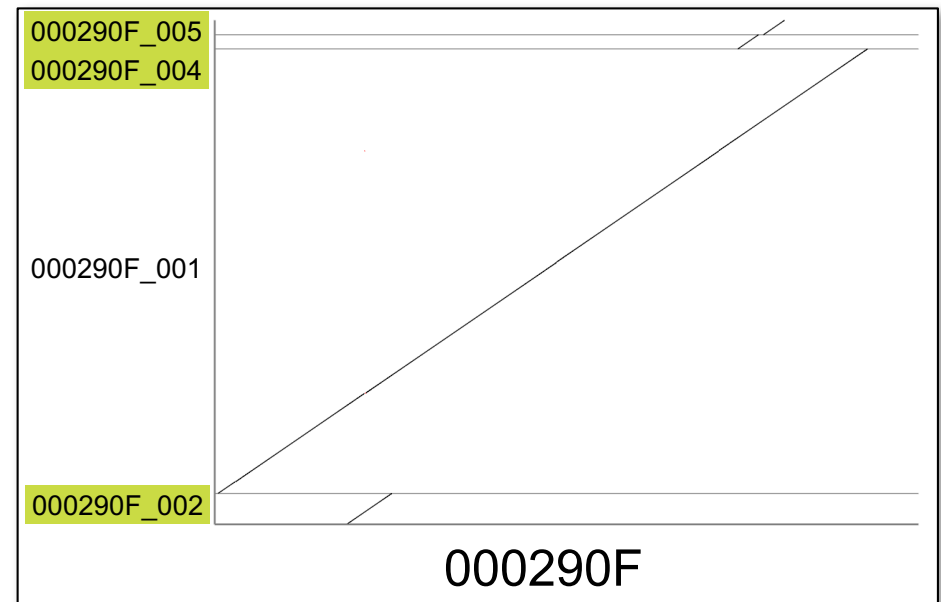
Remove low quality contigs

- Filter out contigs with >50% unpolished bases (lowercase)

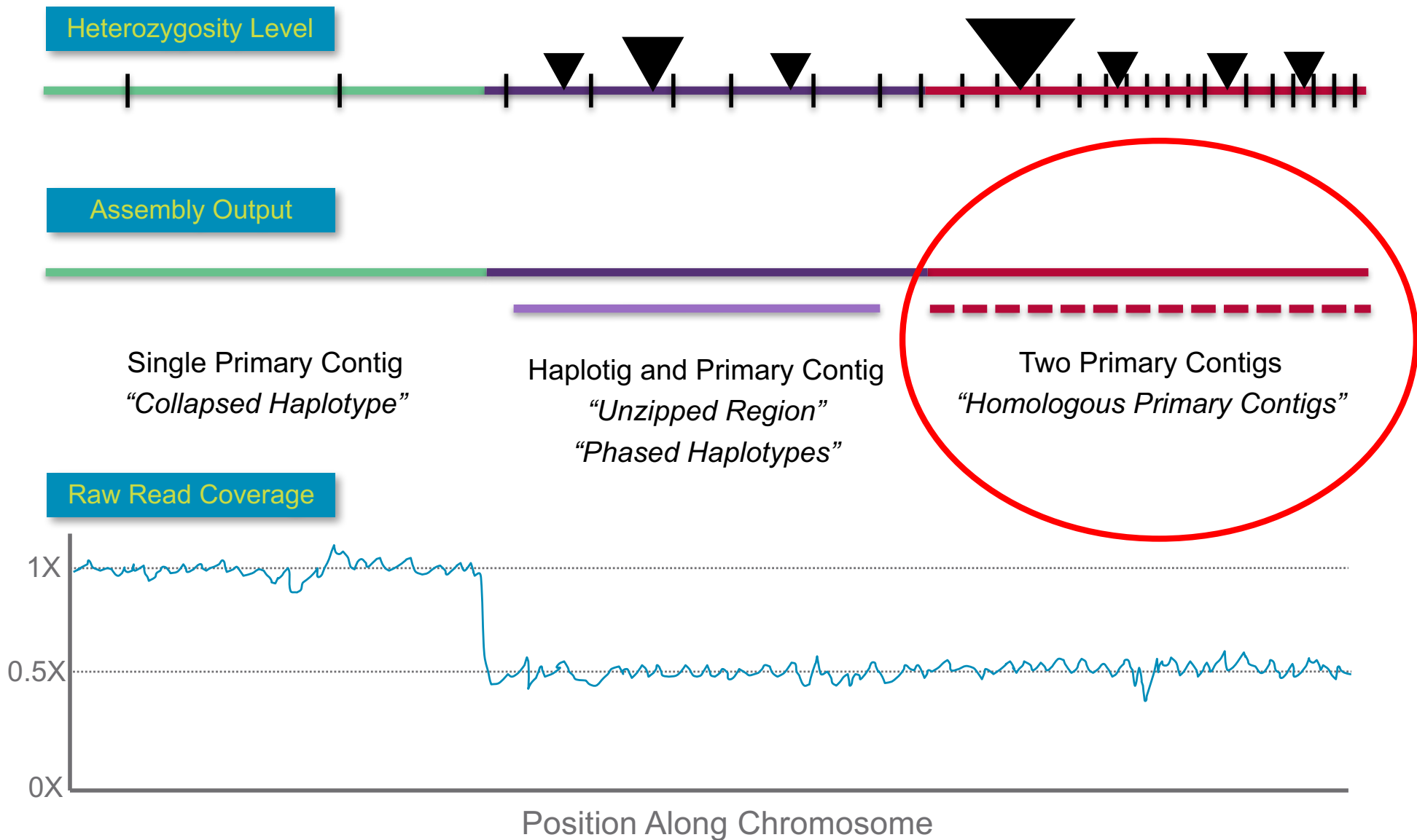
Remove nested haplotigs

- Short haplotigs that align within longer haplotigs

HAPLOTIGS ALIGNED TO PRIMARY



DEDUPLICATING PRIMARY CONTIGS



DOES MY ASSEMBLY HAVE HOMOLOGOUS PRIMARY CONTIGS?

Primary assembly length is longer than haploid genome size

- Inbred individual: diploid assembly: assembly length = $1N$
- F1 hybrid: haploid assembly: assembly length = $2N$

Haploid coverage on primary contigs in regions *without* haplotigs

BUSCO analysis on primary contigs indicates widespread duplicated genes

METHODS TO IDENTIFY HOMOLOGOUS PRIMARY CONTIGS

BUSCO/Gene Annotation

- Pros: simple, works for highly divergent haplotypes
- Cons: unannotated contigs excluded
- Usage Case: high contiguity assembly, highly divergent haplotypes

All-By-All Alignments

- Pros: simple
- Cons: high compute time/manual curation
- Usage Case: small genome (<1 Gb)

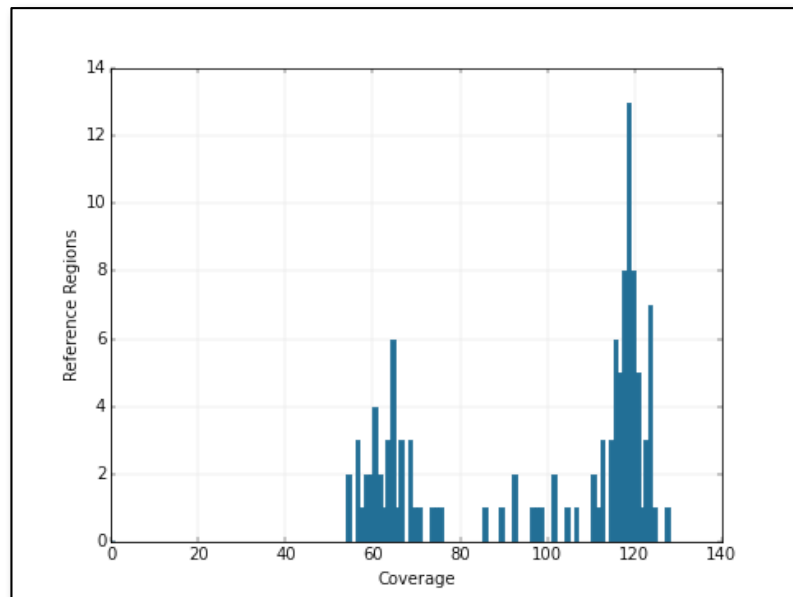
Purge Haplotypes Pipeline (Mike Roach)

- Pros: uses coverage and pairwise identity
- Cons: some manual curation
- Usage Case: many

EXAMPLE: AEDES MOSQUITO FALCON-UNZIP ASSEMBLY

- Expected Genome Size: ~1.3 Gb
- Primary Contig Length: 1.69 Gb

BIMODAL COVERAGE HISTOGRAM



BUSCO ANALYSIS:

ARTHROPOD GENESET (N = 2675)

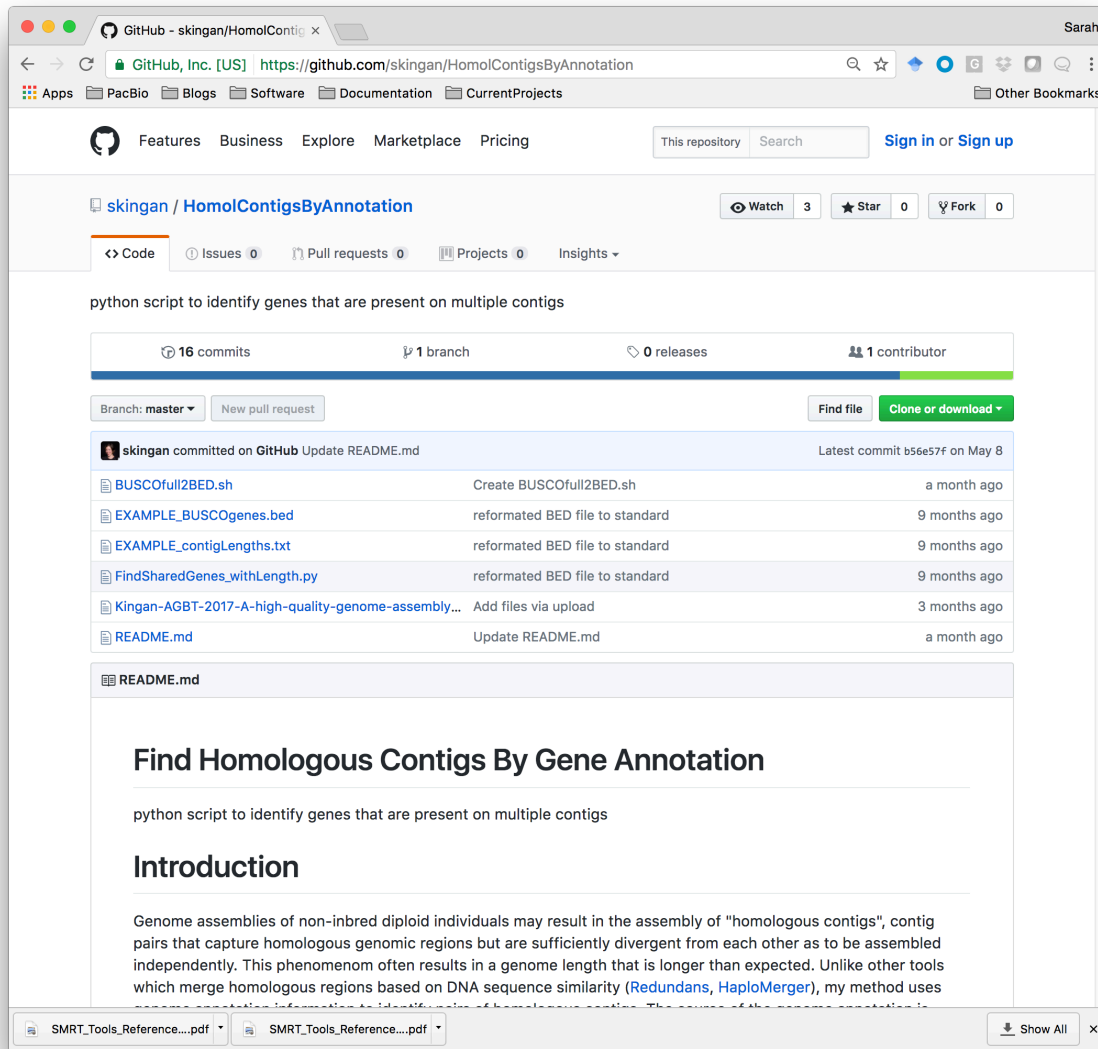
ASSEMBLY	<i>Aedes</i> PACBIO
COMPLETE	98%
MISSING	2%
FRAGMENTED	10%
DUPLICATED	32%

Acknowledgement:

***Aedes* Genome Working Group
 Leslie Vosshall, Ben Matthews,
 Rockefeller University**

BUSCO METHOD

github.com/skingan/HomolContigsByAnnotation



python script to identify genes that are present on multiple contigs

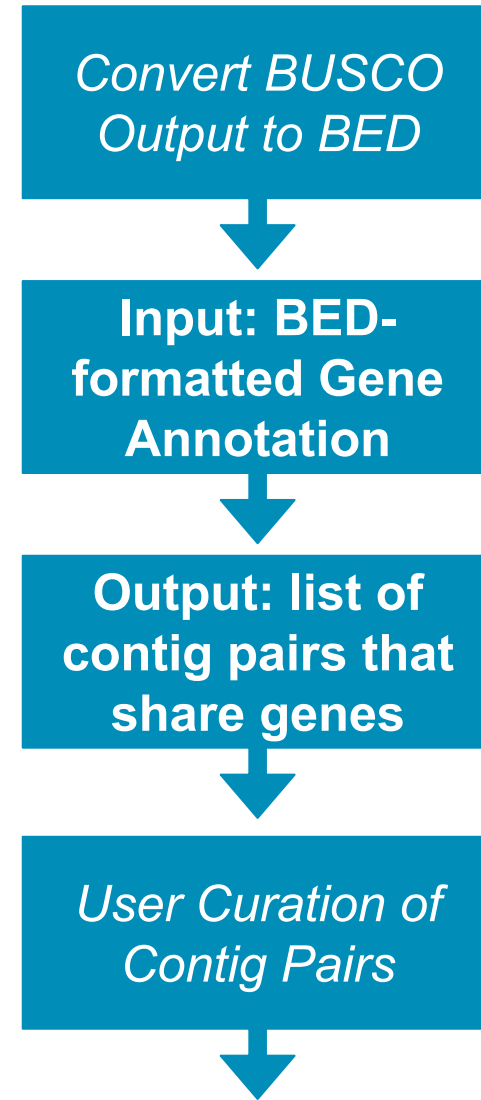
File	Description	Time
BUSCOfull2BED.sh	Create BUSCOfull2BED.sh	a month ago
EXAMPLE_BUSCOgenes.bed	reformated BED file to standard	9 months ago
EXAMPLE_contigLengths.txt	reformated BED file to standard	9 months ago
FindSharedGenes_withLength.py	reformated BED file to standard	9 months ago
Kingan-AGBT-2017-A-high-quality-genome-assembly...	Add files via upload	3 months ago
README.md	Update README.md	a month ago

Find Homologous Contigs By Gene Annotation

python script to identify genes that are present on multiple contigs

Introduction

Genome assemblies of non-inbred diploid individuals may result in the assembly of "homologous contigs", contig pairs that capture homologous genomic regions but are sufficiently divergent from each other as to be assembled independently. This phenomenon often results in a genome length that is longer than expected. Unlike other tools which merge homologous regions based on DNA sequence similarity ([Redundans](#), [HaploMerger](#)), my method uses



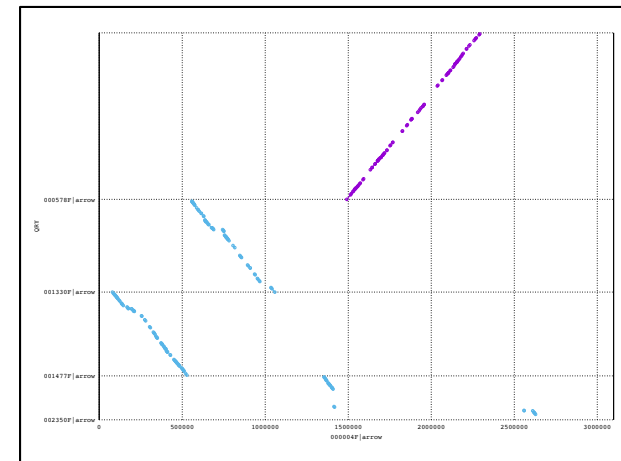
ALL-BY-ALL ALIGNMENTS

https://github.com/PacificBiosciences/apps-scripts/blob/master/FALCONAssemblyTools/get_homologs.py

Identify homologous relationships by alignments

- Each contig aligned to all shorter contigs using NUCmer
 - uses multi-threaded version of MUMmer4
- High quality alignments filtered
- Multi-sequence alignment visualized in mummerplot
- Manual curation of plots

OUTPUT EXAMPLE



PURGE HAPLOTIGS

MIKE ROACH, AUSTRALIAN WINE RESEARCH INSTITUTE

https://bitbucket.org/mroachawri/purge_haplotigs/

Semi-automated pipeline to *remove* haplotigs from primary contigs

- Input: BAM of mapped PacBio reads to primary contigs
- Output: curated **haploid representation** of assembly
 - Record of association between excluded and retained primary contigs

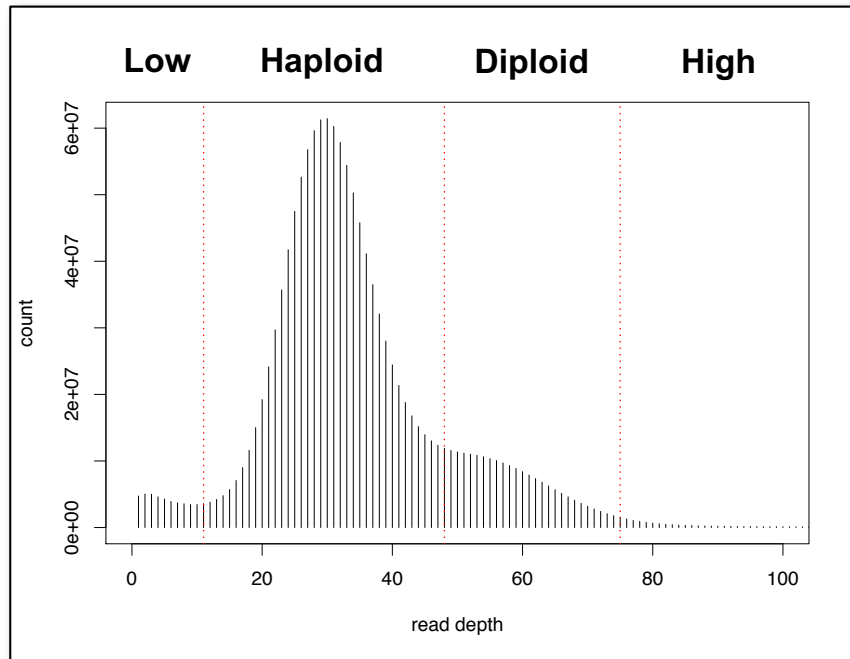
Pipeline

1. coverage histogram and user-defined coverage cut offs
2. contig-specific coverage analysis to identify candidate haplotigs
3. alignments and iterative purging of candidates

PURGE HAPLOTIGS: EXAMPLE FROM BARBERRY BUSH

Acknowledgement:
Iago Hale, UNH

1. PRIMARY CONTIG COVERAGE HISTOGRAM



2. INDIVIDUAL CONTIG COVERAGE ASSESSMENT

- <80% contig length has diploid coverage
- 4470 / 4672 contigs flagged as "suspect"



3. ITERATIVE REASSIGNMENT

- All-by-all BLAST to find two best hits of "suspect" contigs
- NUCmer alignment and summary stats
- Categorization as "repeat" or "haplotig"



OUTPUT: CURATED ASSEMBLY

- Revised haploid genome
- Log file of reassignment

```
000000F, PRIMARY <- 001282F, REPEAT
                  <- 003081F, HAPLOTIG
```

METHODS TO IDENTIFY HOMOLOGOUS PRIMARY CONTIGS

BUSCO/Gene Annotation

— github.com/skingan/HomolContigsByAnnotation

All-By-All Alignments

— https://github.com/PacificBiosciences/apps-scripts/blob/master/FALCONAssemblyTools/get_homologs.py

Purge Haplotypes Pipeline (Mike Roach)

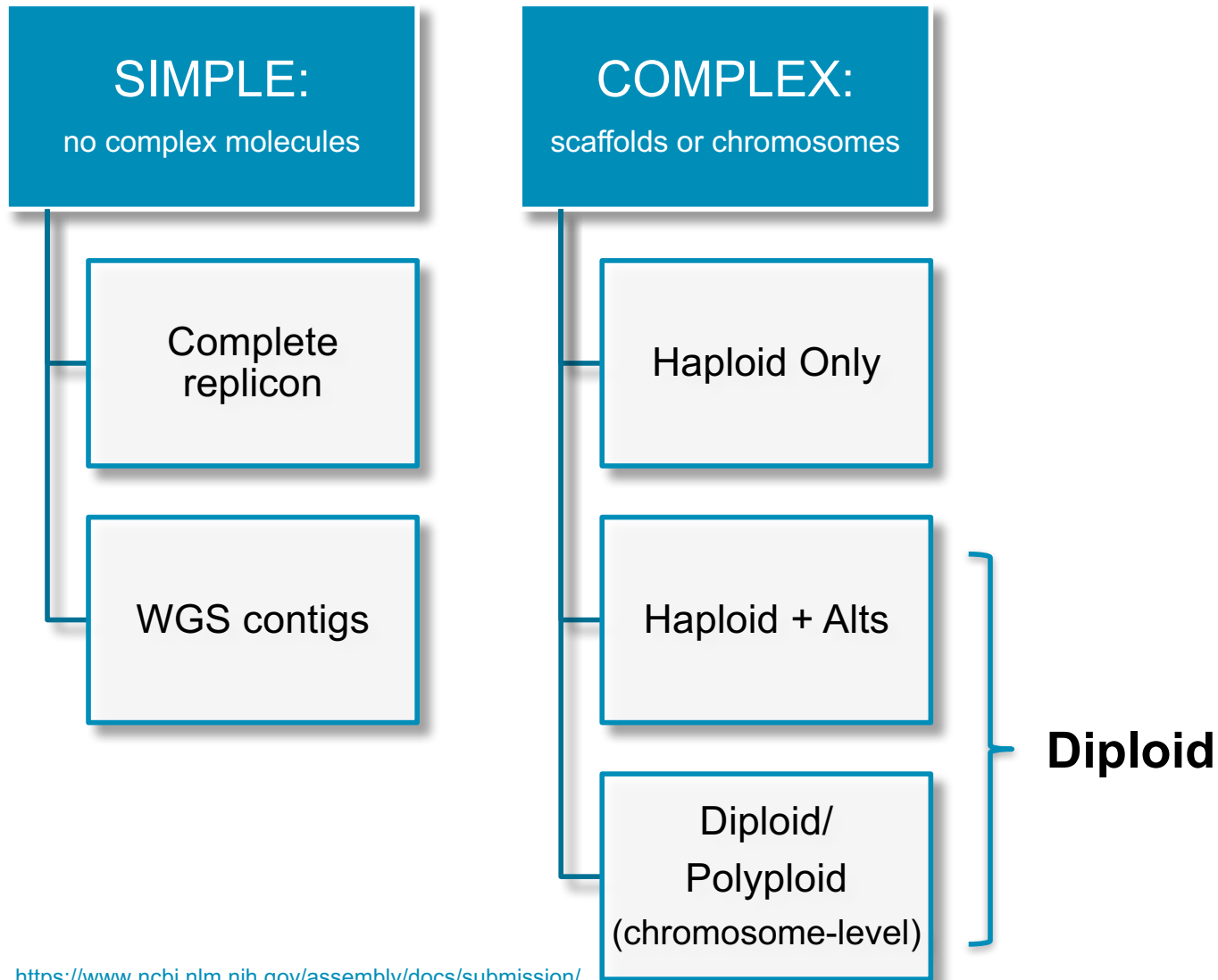
— https://bitbucket.org/mroachawri/purge_haplotigs/



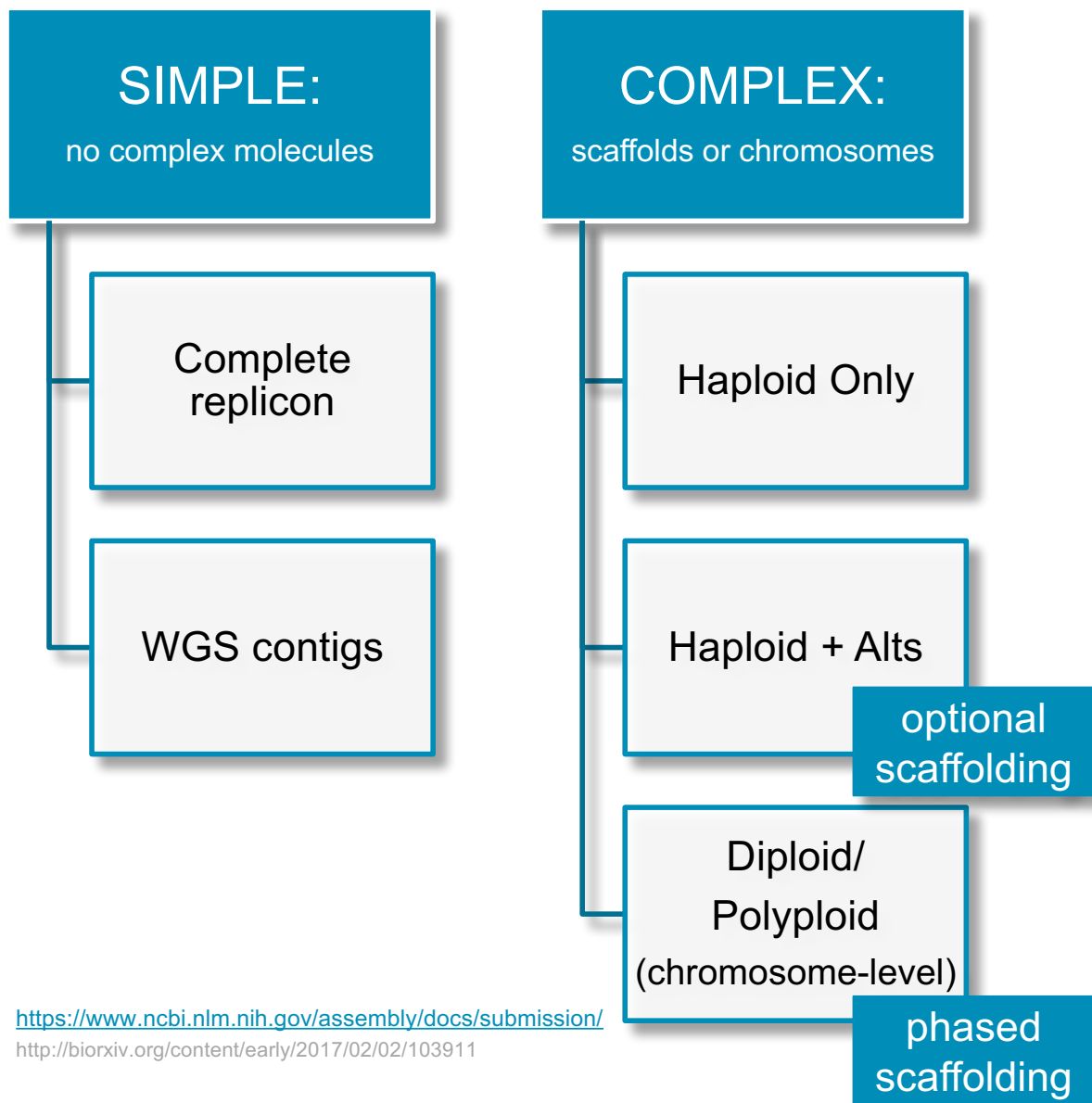
Sharing Your Assembly

Diploid Assembly Submission to NCBI

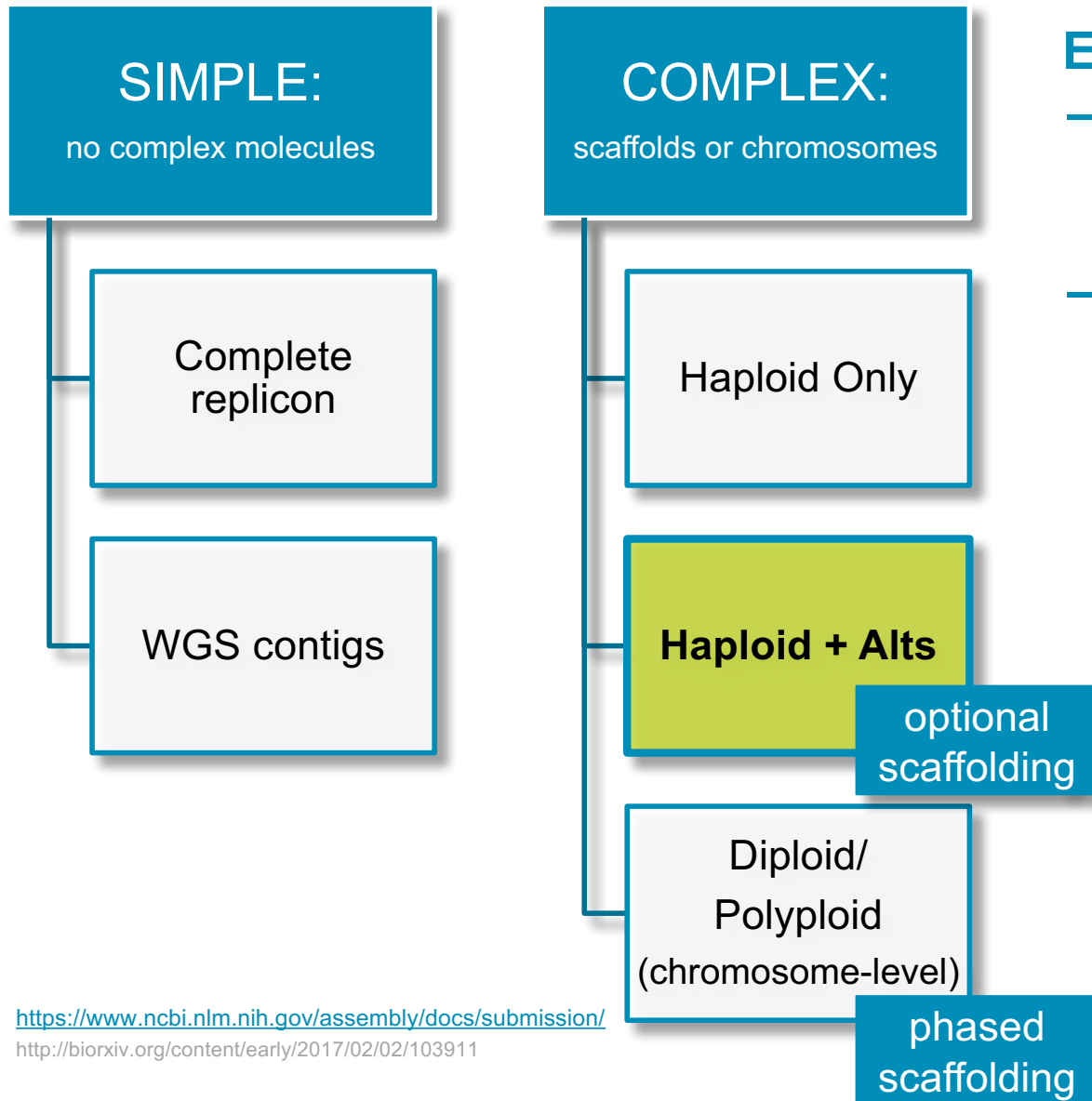
ASSEMBLY SUBMISSION TYPES



ASSEMBLY SUBMISSION TYPES



ASSEMBLY SUBMISSION TYPES

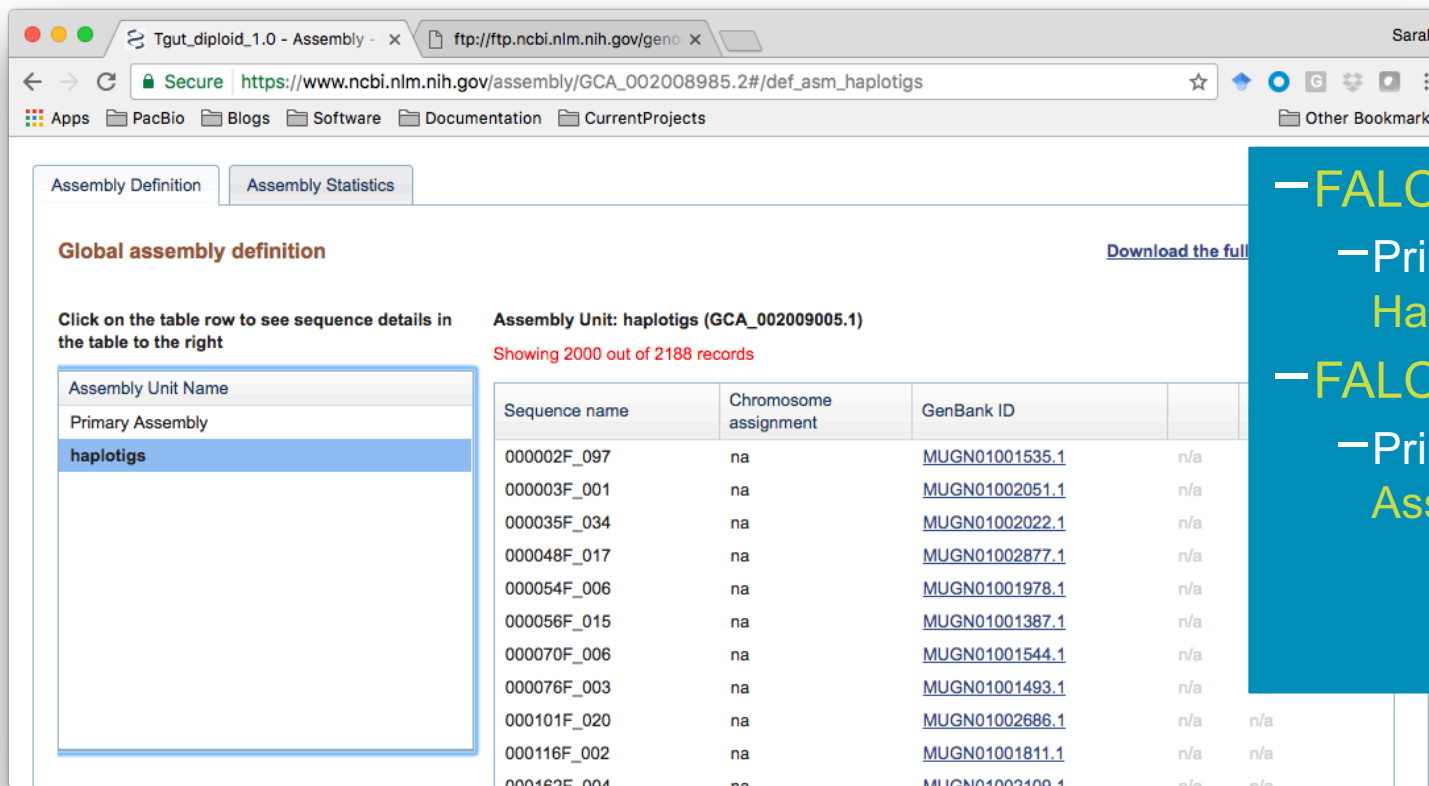


Examples of Haploid + Alts

- GCA_001753755.2
 - *Arabidopsis thaliana* F1 from Chin *et al.* 2016
- GCA_002008985.2
 - zebra finch Korlach *et al.* 2017

ASSEMBLY UNITS: PRIMARY AND ALT

Zebra finch: GCA_002008985.2



Assembly Definition | Assembly Statistics

Global assembly definition [Download the full assembly](#)

Click on the table row to see sequence details in the table to the right

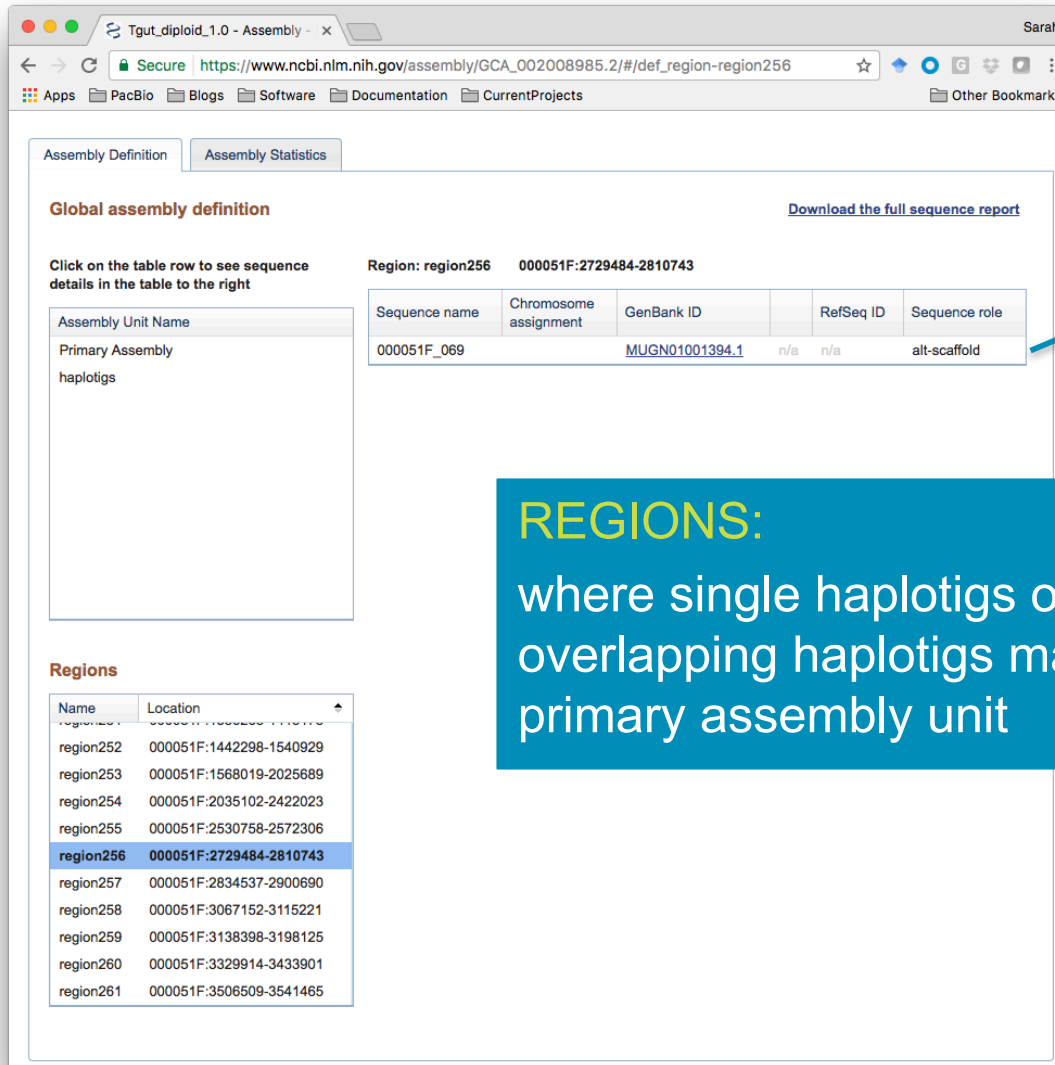
Assembly Unit: **haplotigs (GCA_002009005.1)**
Showing 2000 out of 2188 records

Assembly Unit Name	Sequence name	Chromosome assignment	GenBank ID		
Primary Assembly					
haplotigs	000002F_097	na	MUGN01001535.1	n/a	
	000003F_001	na	MUGN01002051.1	n/a	
	000035F_034	na	MUGN01002022.1	n/a	
	000048F_017	na	MUGN01002877.1	n/a	
	000054F_006	na	MUGN01001978.1	n/a	
	000056F_015	na	MUGN01001387.1	n/a	
	000070F_006	na	MUGN01001544.1	n/a	
	000076F_003	na	MUGN01001493.1	n/a	
	000101F_020	na	MUGN01002686.1	n/a	n/a
	000116F_002	na	MUGN01001811.1	n/a	n/a
	000162F_004	na	MUGN01002109.1	n/a	n/a

- FALCON-Unzip:
 - Primary Contigs and Haplotigs
- FALCON:
 - Primary Contigs and Associated Contigs

REGIONS

Zebra finch: GCA_002008985.2



Assembly Definition | Assembly Statistics

Global assembly definition [Download the full sequence report](#)

Click on the table row to see sequence details in the table to the right

Region: region256 000051F:2729484-2810743

Sequence name	Chromosome assignment	GenBank ID	RefSeq ID	Sequence role
000051F_069		MUGN01001394.1	n/a	alt-scaffold

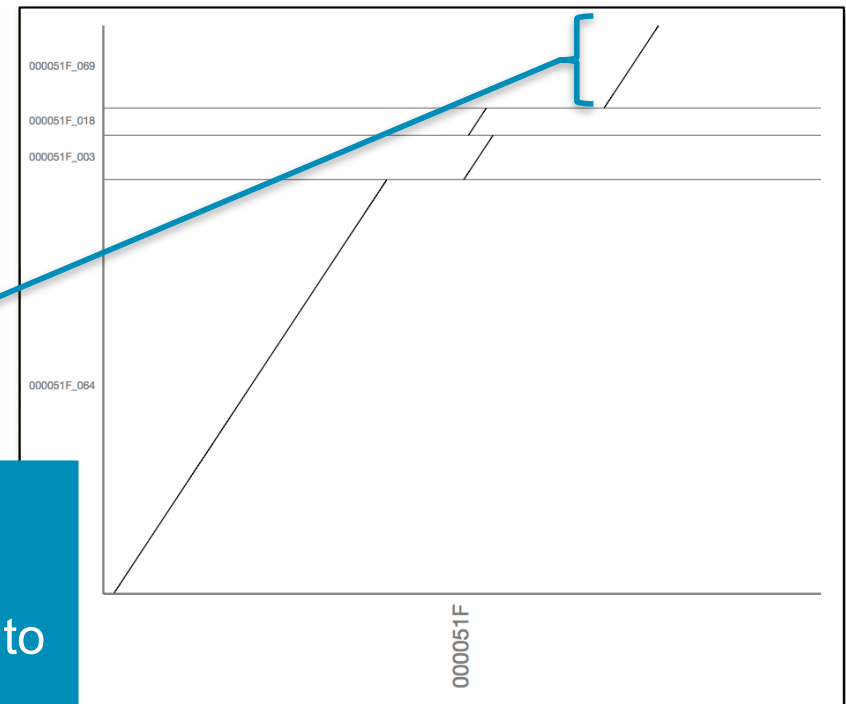
Assembly Unit Name

- Primary Assembly haplotigs

Regions

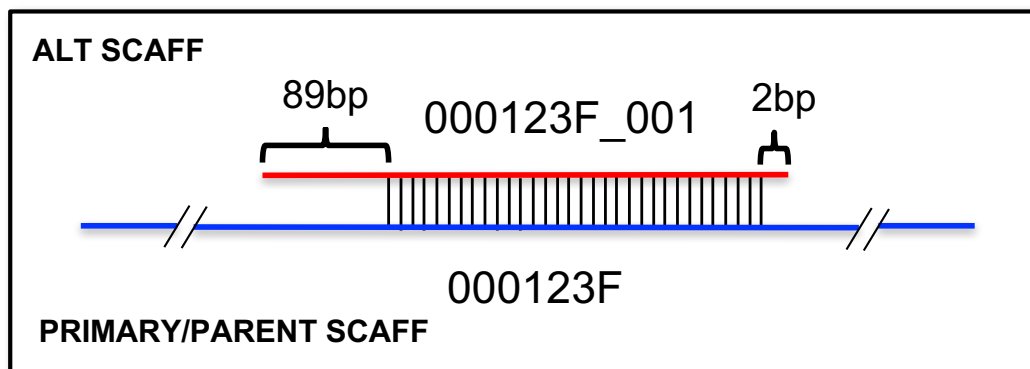
Name	Location
region252	000051F:1442298-1540929
region253	000051F:1568019-2025689
region254	000051F:2035102-2422023
region255	000051F:2530758-2572306
region256	000051F:2729484-2810743
region257	000051F:2834537-2900690
region258	000051F:3067152-3115221
region259	000051F:3138398-3198125
region260	000051F:3329914-3433901
region261	000051F:3506509-3541465

REGIONS:
 where single haplotigs or overlapping haplotigs map to primary assembly unit



ALTERNATE LOCUS PLACEMENT FILE

- Required for haploid + alts submission
- Details placement of alt sequences relative to primary assembly



HEADER	EXAMPLE
alt_asm_name	haplotigs
prim_asm_name	Primary Assembly
alt_scaf_name	000123F_001
parent_type	SCAFFOLD
parent_name	000123F
ori	+
alt_scaf_start	90
alt_scaf_stop	41595
parent_start	8663681
parent_stop	8708427
alt_start_tail	89
alt_stop_tail	2

PLACEMENT FILE TOOLS

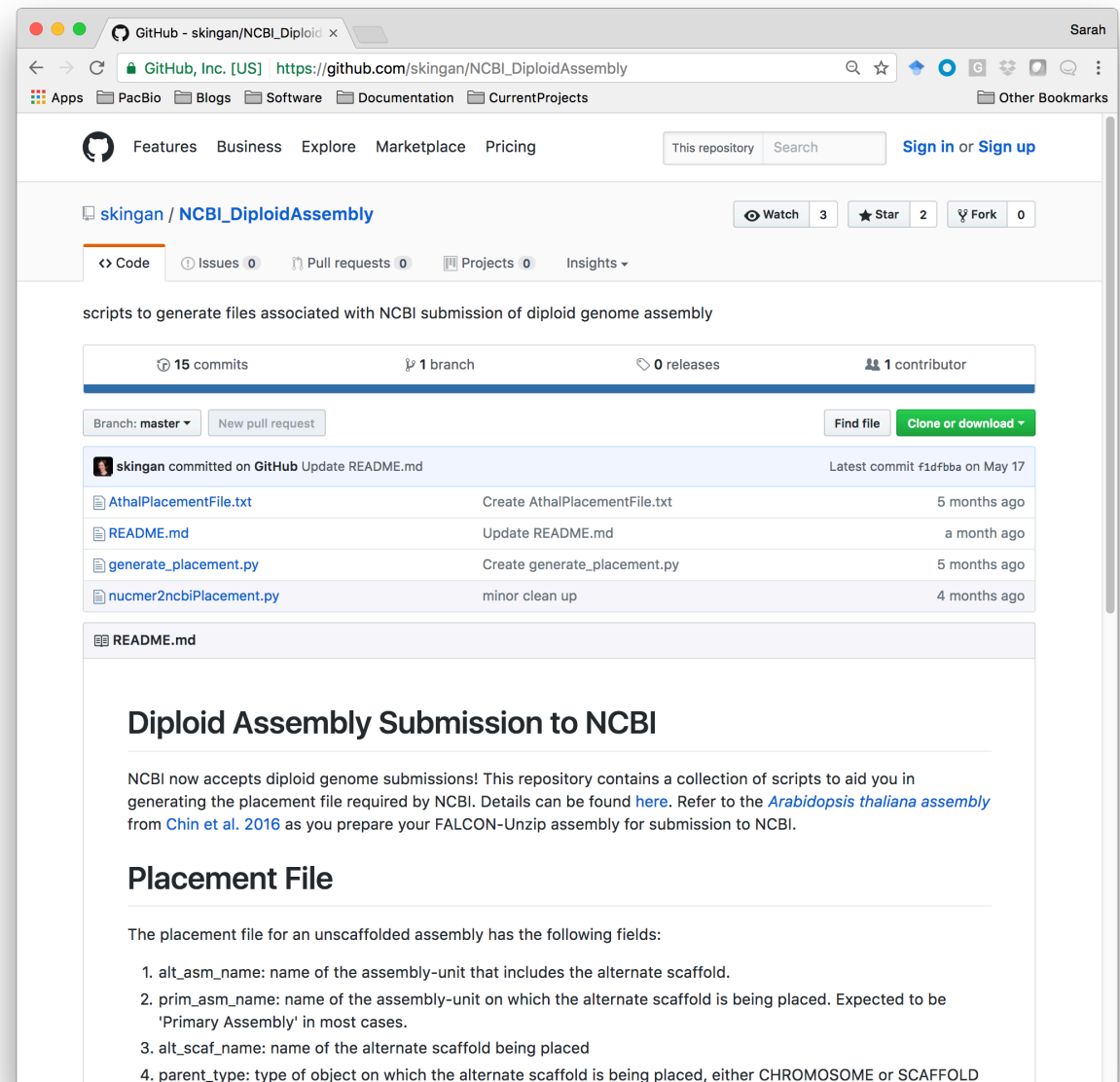
https://github.com/skingan/NCBI_DiploidAssembly

generate_placement.py

- Written by Jason Chin
- Runs NUCmer and generates placement file

nucmer2ncbiPlacement.py

- Written by Sarah Kingan
- generates placement file from directory of filtered NUCmer alignments
- Contains suggested MUMmer commands



The screenshot shows the GitHub repository page for `skingan / NCBI_DiploidAssembly`. The repository has 3 watchers, 2 stars, and 0 forks. It contains 15 commits, 1 branch, 0 releases, and 1 contributor. The commit history shows:

File	Commit Message	Time
AthalPlacementFile.txt	Create AthalPlacementFile.txt	5 months ago
README.md	Update README.md	a month ago
generate_placement.py	Create generate_placement.py	5 months ago
nucmer2ncbiPlacement.py	minor clean up	4 months ago

The README.md file contains the following text:

Diploid Assembly Submission to NCBI

NCBI now accepts diploid genome submissions! This repository contains a collection of scripts to aid you in generating the placement file required by NCBI. Details can be found [here](#). Refer to the *Arabidopsis thaliana assembly* from [Chin et al. 2016](#) as you prepare your FALCON-Unzip assembly for submission to NCBI.

Placement File

The placement file for an unscaffolded assembly has the following fields:

1. `alt_asm_name`: name of the assembly-unit that includes the alternate scaffold.
2. `prim_asm_name`: name of the assembly-unit on which the alternate scaffold is being placed. Expected to be 'Primary Assembly' in most cases.
3. `alt_scaf_name`: name of the alternate scaffold being placed
4. `parent_type`: type of object on which the alternate scaffold is being placed, either CHROMOSOME or SCAFFOLD

RESOURCES

FALCON

- <http://pb-falcon.readthedocs.io/>
- <https://github.com/PacificBiosciences/FALCON-integrate>
- <https://github.com/PacificBiosciences/apps-scripts/tree/master/FALCONAssemblyTools>
- ***Unzip binary to be released with SMRT Analysis 5.0 in Q3***

SMRT Analysis

- <http://www.pacb.com/support/software-downloads/>
- http://programs.pacificbiosciences.com/l/1652/2017-02-01/3rzn6/184345/SMRT_Tools_Reference_Guide__v4.0.0_.pdf



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