IsoPhase: Phasing Iso-Seq Data for diploid (and possibly tetraploid) genomes

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Elizabeth Tseng, Jan 2018, PAG SMRT Developers Conference

Online Resources:

groups.google.com/forum/#!forum/SMRT_isoseq

github.com/PacificBiosciences/IsoSeq_SA3nUP/
(shortened: **http://tinyurl.com/PBisoseq)**

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PLANT AND ANIMAL ISO-SEQ PUBLICATIONS IN 2017

TYPE	SPECIES	TITLE
Crop	Coffee	Cheng *et al.* Long-read sequencing of the coffee bean transcriptome reveals the diversity of full-length transcripts. *GigaScience* 1–13 (2017).
Medicinal	D. Officinale	He, L. *et al.* Hybrid Sequencing of Full-Length cDNA Transcripts of Stems and Leaves in Dendrobium officinale. *Genes* **8,** 257–13 (2017).
Medicinal	Ginseng	Jo, I.-H. *et al.* Isoform Sequencing Provides a More Comprehensive View of the Panax ginseng Transcriptome. *Genes* **8,** 228–17 (2017).
Medicinal	Huangqi	Li, J. *et al.* Long read reference genome-free reconstruction of a full- length transcriptome from Astragalus membranaceus reveals transcript variants involved in bioactive compound biosynthesis. *Nature Publishing Group* 1–13 (2017).
Crop	Cotton	Wang, M. *et al.* A global survey of alternative splicing in allopolyploid cotton: landscape, complexity and regulation. *New Phytol* **217,** 163–178 (2017).
Animal	Rabbit	Chen, S.-Y., Deng, F., Jia, X., Li, C. & Lai, S.-J. A transcriptome atlas of rabbit revealed by PacBio single-molecule long-read sequencing. *Sci. Rep.* **7,** 1–10 (2017).
Crop	Quinoa	Jarvis, D. E. *et al.* The genome of Chenopodium quinoa. *Nature* **542,** 307–312 (2017).
Crop	Strawberry	Li, Y., Dai, C., Hu, C., Liu, Z. & Kang, C. Global identification of alternative splicing via comparative analysis of SMRT- and Illumina-based RNA-seq in strawberry. *Plant J* **90,** 164–176 (2017).
Crop	Wheat	Clavijo, B. J. *et al.* An improved assembly and annotation of the allohexaploid wheat genome identifies complete families of agronomic genes and provides genomic evidence for chromosomal translocations. *Genome Res.* **27,** 885–896 (2017).

PUBLICLY AVAILABLE SEQUEL ISO-SEQ DATA

Sequel System Data Release: Iso-Seq Results for Hummingbird and Zebra Finch Brain Tissue

Thursday, August 31, 2017

If you're interested in avian vocal learning or want to explore a PacBio Iso-Seq data set generated with the Sequel System, we have good news. We've just released data from Iso-Seq interrogations of brain tissue from two avian models of vocal learning, Anna's hummingbird (*Calypte anna*) and zebra finch (*Taeniopygia guttata*), sequenced in collaboration with the Erich Jarvis and Olivier Fedrigo labs at the Rockefeller University.

Anna's hummingbird photo by Pat Durkin

If you're not familiar with the Iso-Seq method, it's the long-read sequencing answer to short-read RNA-seq studies. By using SMRT Sequencing for a transcriptome project, scientists can generate full-length isoform data, clearly capturing alternative splicing events to see the real diversity of transcripts. Unlike RNA-seq approaches, the Iso-Seq method takes advantage of long-read data to fully span transcript isoforms from the 5' end to their poly-A tails, eliminating the need for error-prone transcript reconstruction and inference processes. With the Sequel System, Isc Seq projects are low cost and time efficient. Currently we recommend only 1-2 SMRT Cells per tissue type for genome annotation.

- 4 Sequel cells
- Barcoded bird brains
- Total: 785k FL reads

	ZEBRAFINCH	HUMMINGBIRD
Runtime	31 hr	28 hr
Unique Genes	7228	7357
Unique Isoforms	17,437	16,898

ISO-SEQ2: COMING TO YOU IN 2018!

- Faster runtime
- Increased transcript recovery
- Reduced false artifacts
- Available in the next SMRTLink release

SMRT CELLS	CCS READS	FL READS	ISOSEQ2 RUNTIME	UNIQUE TRANSCRIPTS	UNIQUE GENES
1	244,804	212,201	15 hr	13,036	7760
3	796,128	676,905	21 hr	30,956	13,044
6	1,908,507	1,562,039	5 days	63,645	18,227

* run using all default options except CCS minimum pass changed to 1

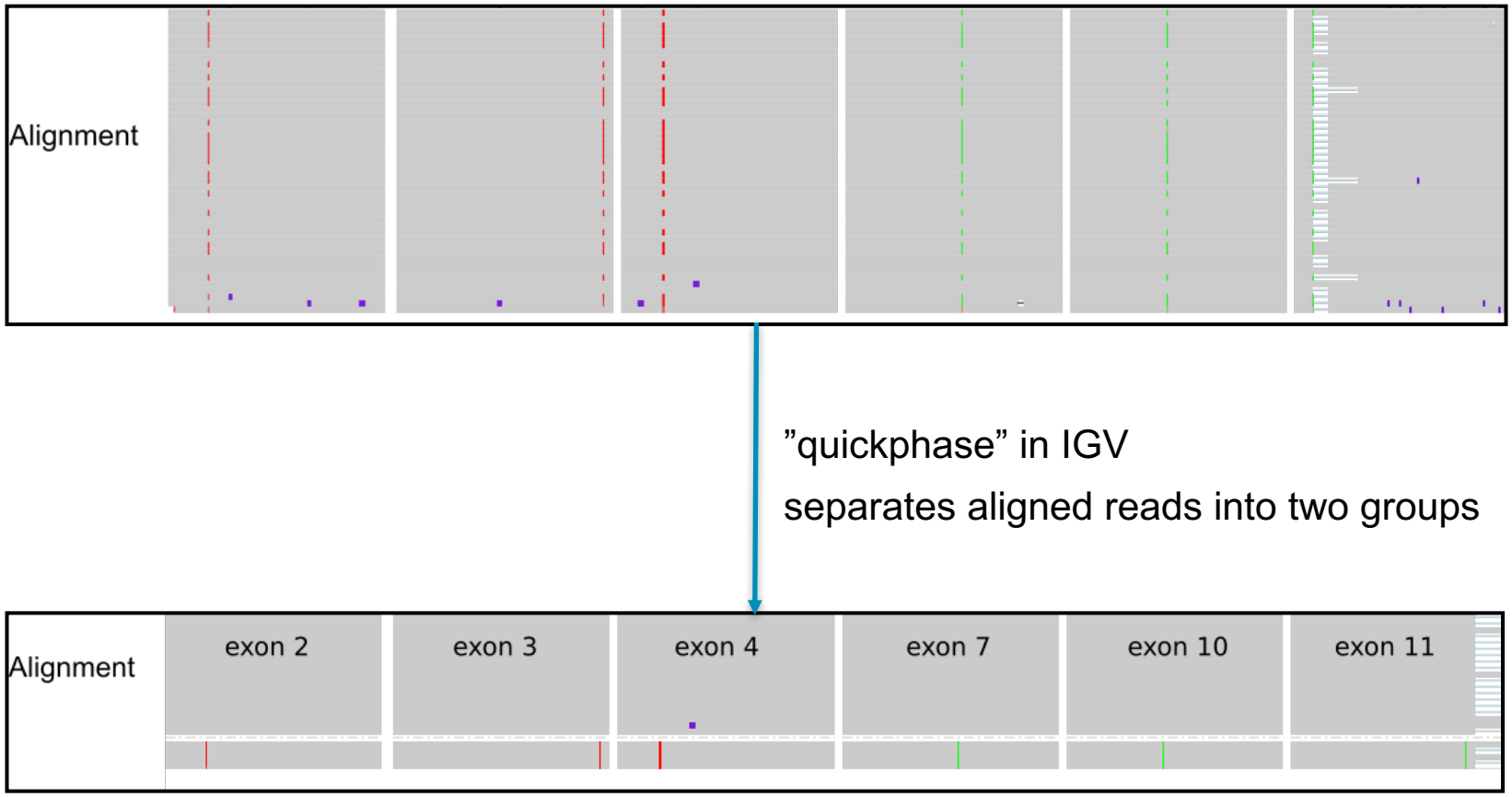
Phasing Iso-Seq Data

MOTIVATION

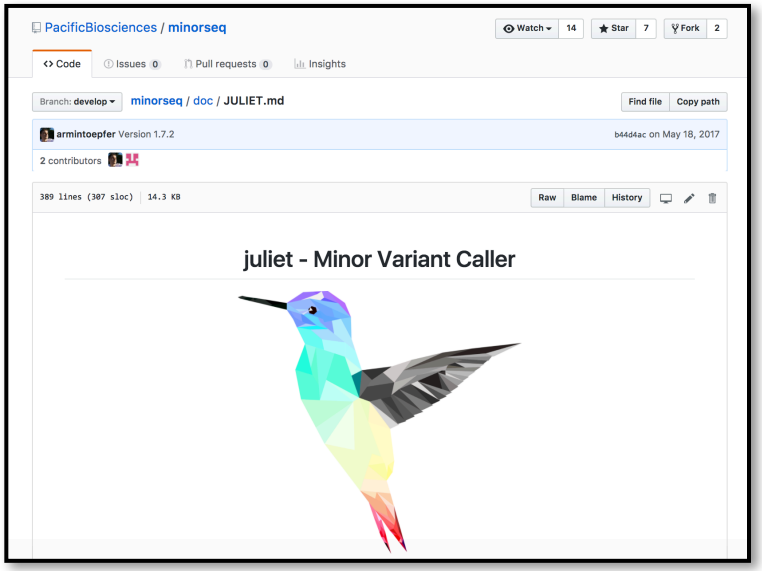
- The Iso-Seq bioinformatics pipeline outputs distinct isoforms (exon skipping, alternative 5’ and 3’ ends), but collapses SNP-level variations.

- SNP information can revealed by aligning full-length (FL) CCS reads back to the unique isoforms after Iso-Seq analysis.

MOTIVATION


Alignment
”quickphase” in IGV
separates aligned reads into two groups
Alignment
exon 2
exon 3
exon 4
exon 7
exon 10
exon 11

MOTIVATION



▼ **Variant Discovery**

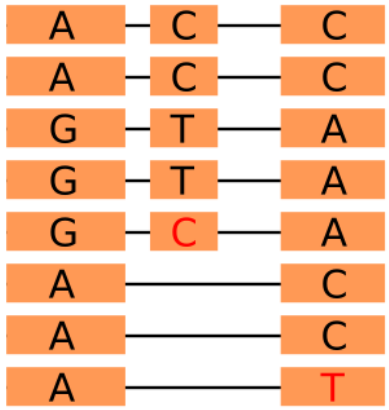
Reverse Transcriptase							
my seq			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
A G C	S	3	S	A G T	99	2905	
G A G	E	40	E	G A A	100	2828	
A T G	M	41	L	T T G	1	2793	fancy drug
G G G	G	45	G	G G A	100	2596	

*DrugDB version x.y.z (last updated YYYY-MM-DD)

- Juliet calls minor variants in viral data
- However,
  - It does not handle splicing
  - Performs best with stringent CCS cutoff (> 99%)
  - Performs best with deep coverage (> 250-fold)

ISOPHASE: ISOFORM PHASING USING ISO-SEQ DATA

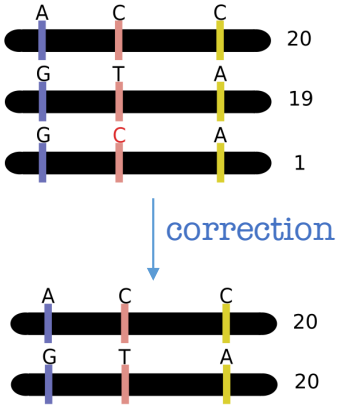
ALIGNMENT



SNP CALLING

Position	SNPs
POS1	A, G
POS2	C, T
POS3	C, A

PHASING



VCF OUTPUT

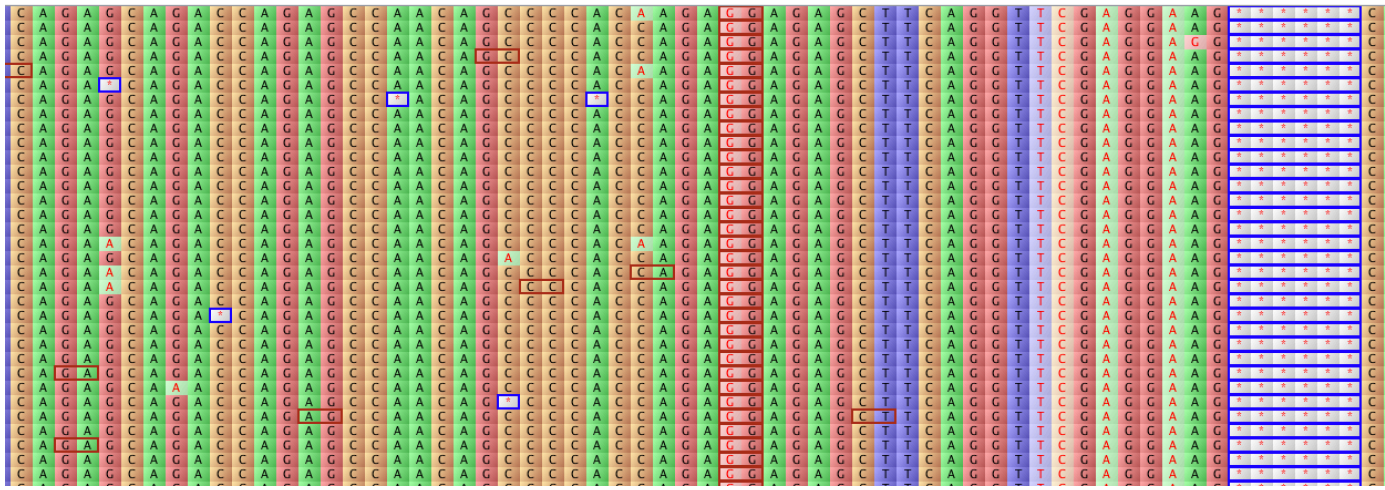
```
##fileformat=VCFv4.2
#CHROM  POS ID  REF ALT QUAL  FILTER  INFO            FORMAT  ISOFORM1   ISOFORM2
chr1    105 .   A   G   .     PASS    DP=40;AF=0.50   GT:HQ   0|1:20,20  0:15
chr1    190 .   C   T   .     PASS    DP=40;AF=0.50   GT:HQ   0|1:20,20  0:15
chr1    336 .   C   A   .     PASS    DP=40;AF=0.50   GT:HQ   0|1:20,20  0:15
```

ISOPHASE METHOD SUMMARY

- Alignment using minimap2, retrieve positions with sufficient coverage
  - If QV is provided, alignment pileup filters out low-quality bases
- SNP calling and phasing using Juliet
- Simple clustering of phased haplotypes to remove errors
- Output VCF denoting SNPs and allele counts for each isoform

SNP CALLING AND PHASING IN JULIET

- ~~Steps across alignment in codon triples.~~ *not applicable for transcriptome*
- Computes a p-value that the observed bases come from purely noise using a Fisher's exact test.
- If the p-value is significant under a Bonferroni correction, call the variant.
- Phase together variant positions by tallying full-length reads that exhibit different combinations of the variant positions and threshold.
- Limits:
  - CCS reads used to minimize impact of noise. Raw read analysis possible.
  - Currently does not estimate indel variants.

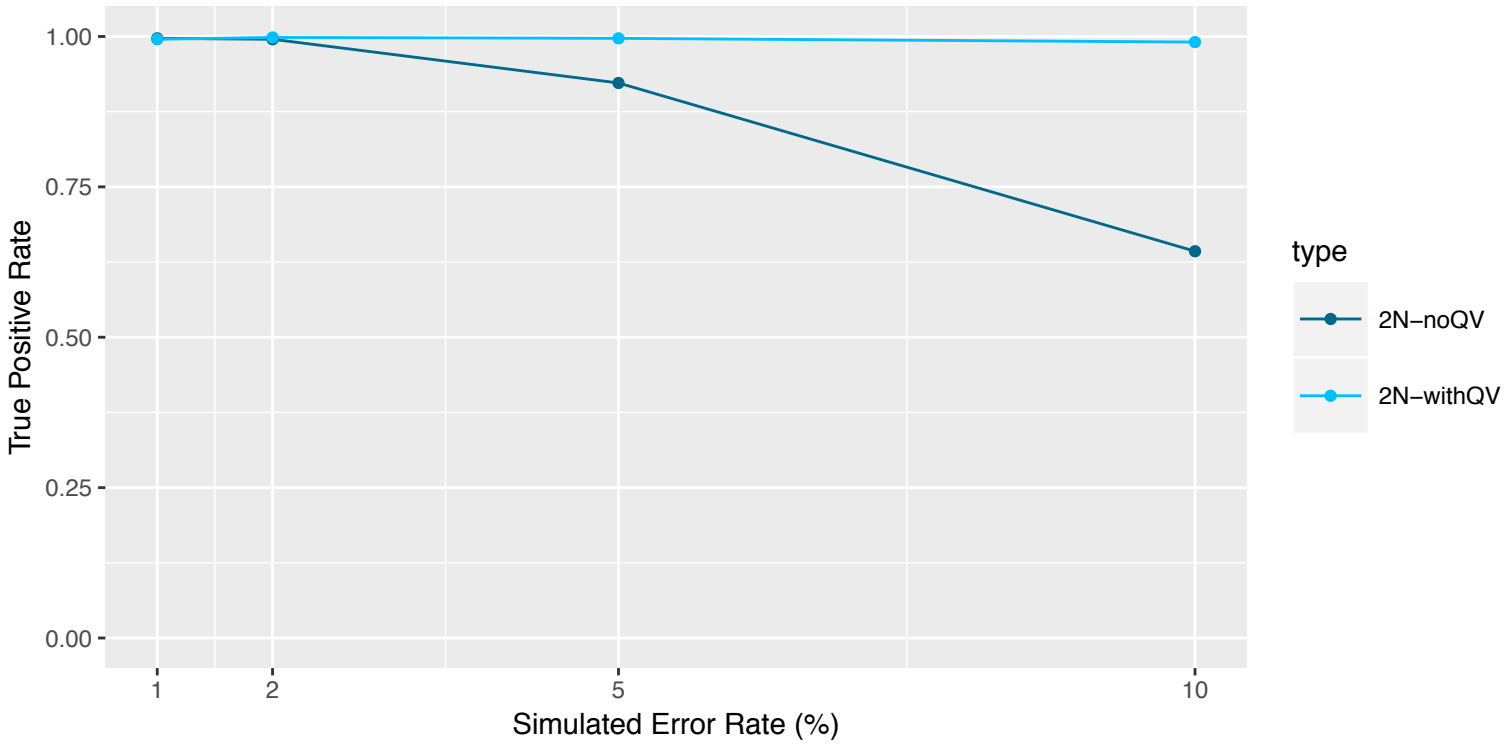


  - Reliably identify 1% true variants from sequencing noise.

Evaluation on Simulated Data

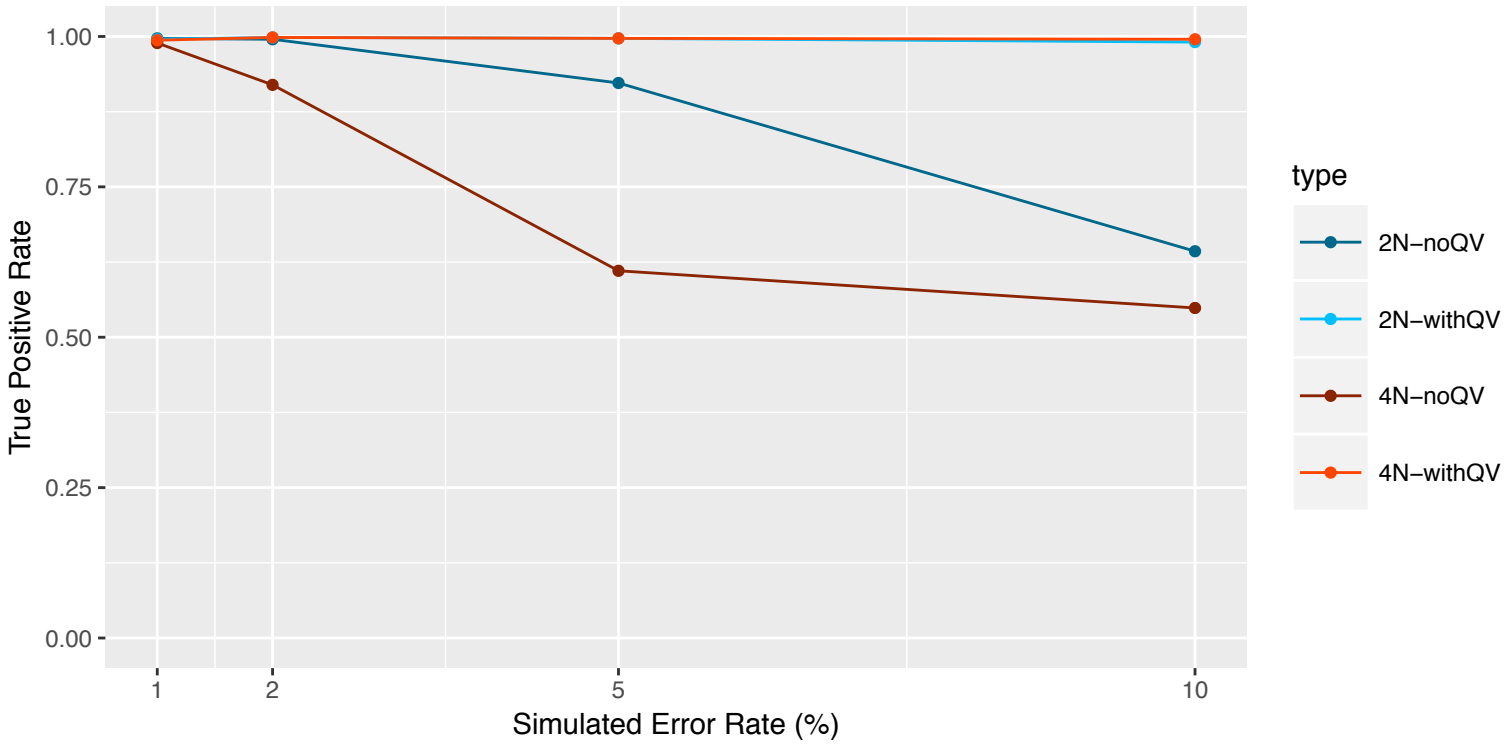
SNP CALLING ON SIMULATED 100 HUMAN GENES

- Random 100 human genes
- Simulated 1 SNP per 300 bp
- Each allele has 20 copies (20-fold coverage)
- Simulated substitution errors at 1%, 2%, 5%, and 10%



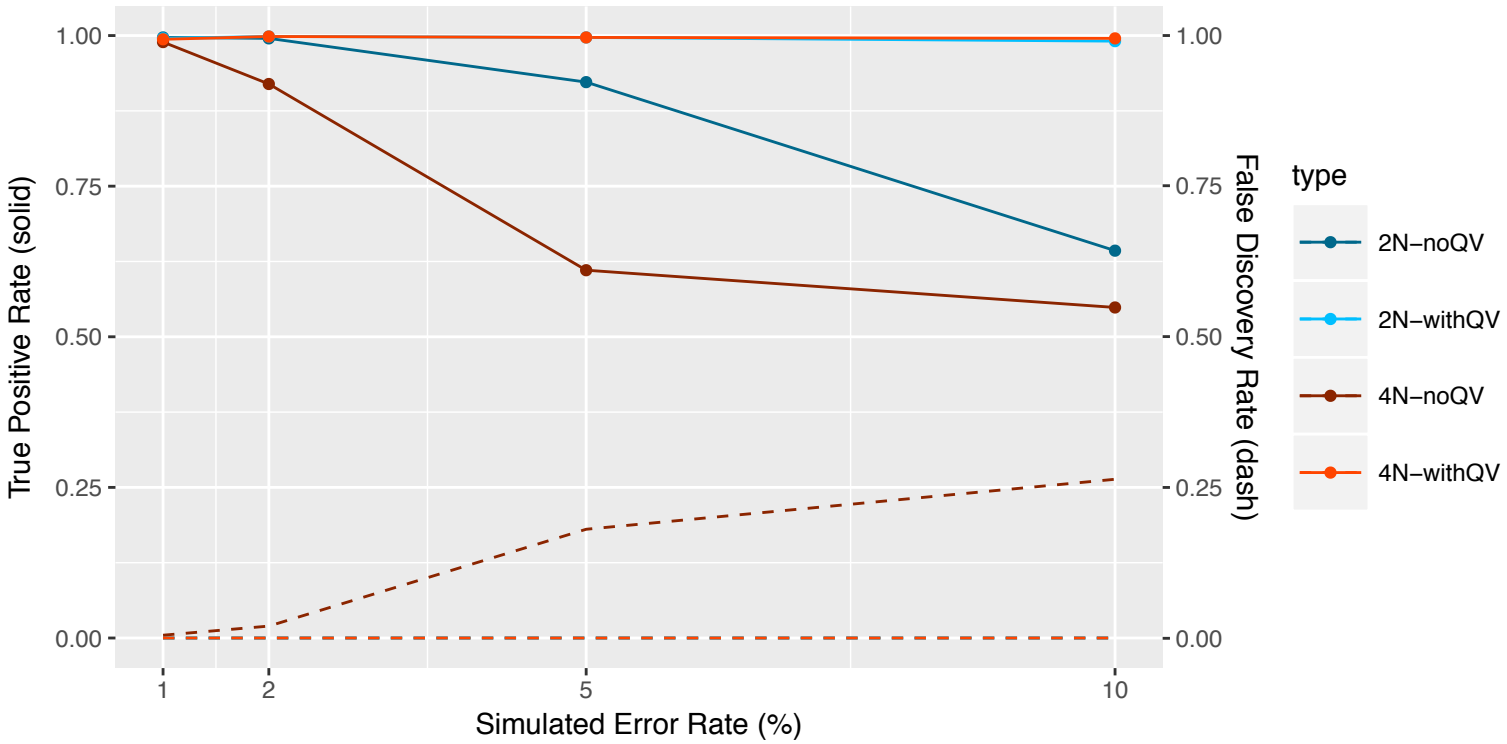
SNP CALLING ON SIMULATED 100 HUMAN GENES

- Using QV improves SNP recovery
- SNP recovery (TPR) remains high for both diploid and tetraploid even at 10% error

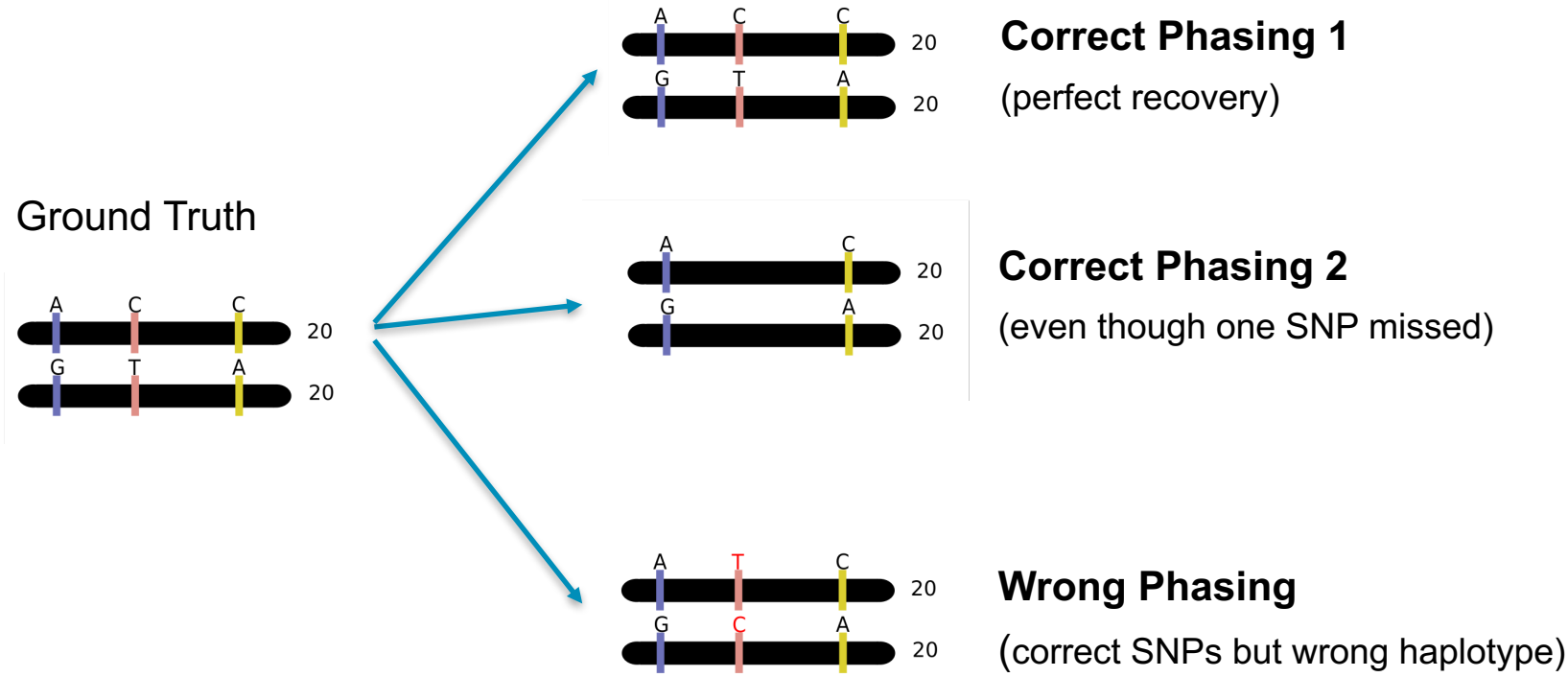


SNP CALLING ON SIMULATED 100 HUMAN GENES

- False discovery rate remains low until 5% error
- False discovery rate increases with erorr rate for tetraploid-noQV



PHASING EVALUATION: CRITERION



For 4N, “correct phasing” means getting all 4 alleles correct. Getting 3 → still wrong.

PHASING EVALUATION ON SIMULATED 100 HUMAN GENES

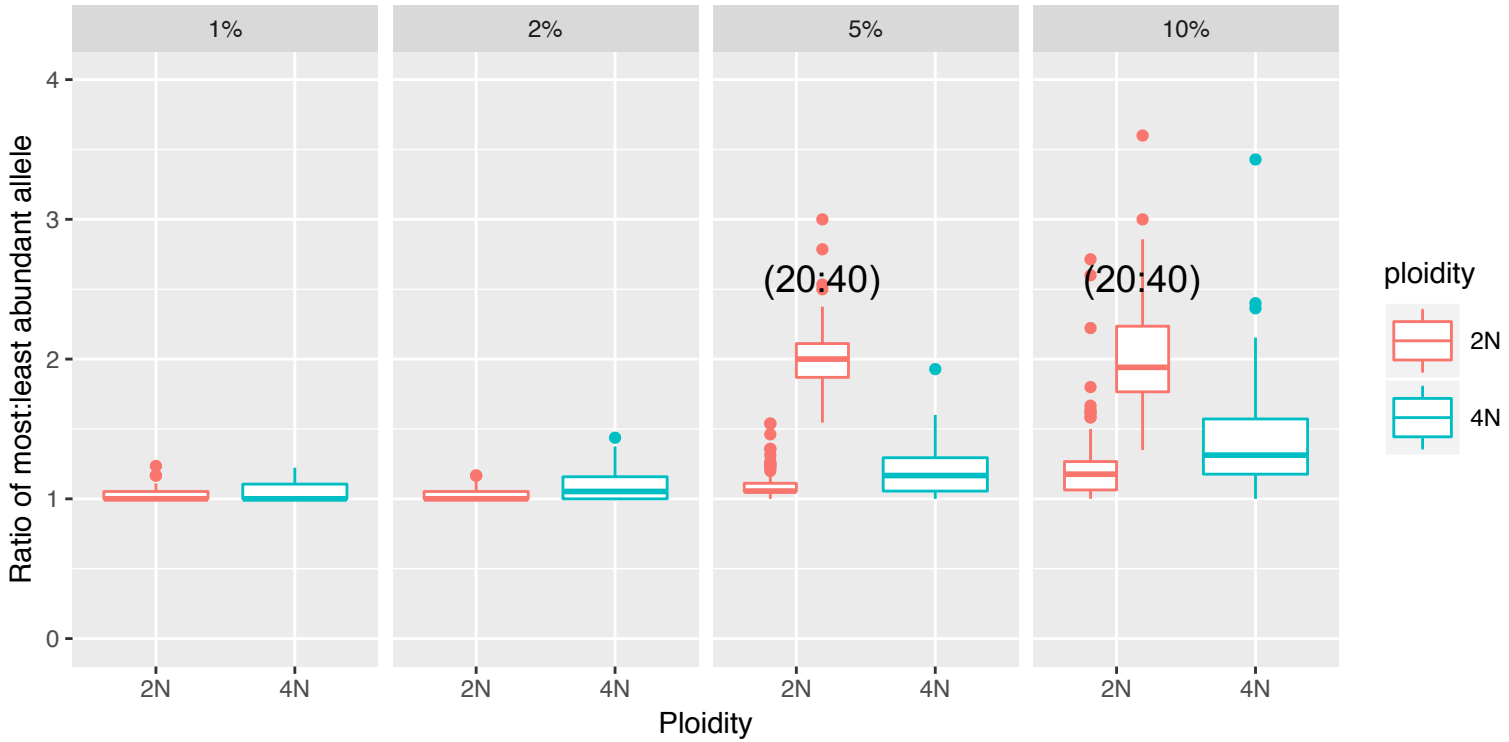
Percentage of 100 genes that were correctly phased.

Error Rate	2N no QV	2N with QV	4N no QV	4N with QV
1%	100%	100%	95%	98%
2%	100%	100%	82%	98%
5%	100%	100%	55%	96%
10%	100%	93%	40%	84%

Worse than no QV due to aggressive dropping of reads
(future work: relax criterion for using reads in haplotyping)

POTENTIAL TO RECOVER ALLELIC SPECIFIC EXPRESSION

Ratio of most abundant : least abundant allele.



Except for (20:40), all 2N simulated with (20:20) read coverage.

All 4N simulated with (20:20:20:20) read coverage.

Evaluation on F1 Cattle Data

ANGUS X BRAHMAN F1 CATTLE

Genome Assembly

- Angus (sire) x Brahman (dam) F1 cattle
- 115-fold coverage on PacBio RS II and Sequel systems
- Assembled using Falcon
- ~90% of genome phased using Unzip

CONTIG	NUMBER	LENGTH	N50	LONGEST
PRIMARY	1427	2.71 Gb	31.4 Mb	65.3 Mb
HAPLOTIGS	5879	2.45 Gb	2.48 Mb	14.0 Mb

Iso-Seq Transcriptome Data

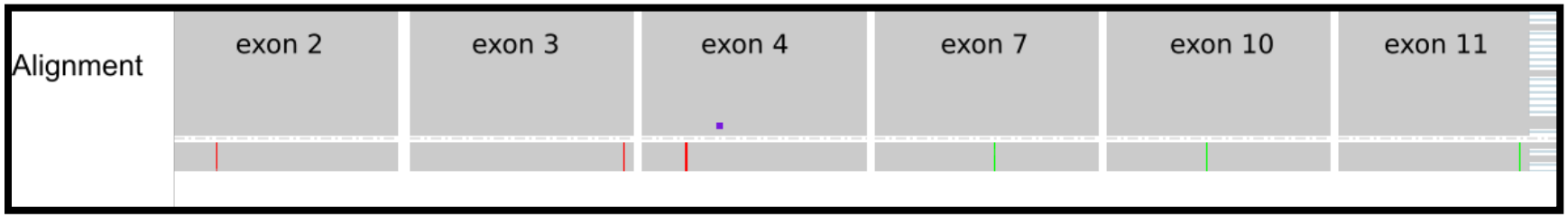
- 8 Sequel cells of tissues from single individual
- Analyzed using IsoSeq2
- Mapped to genome with ≥ 99% coverage, ≥ 95% identity
- 30,137 final isoforms (12,101 genes)
- Selected for phasing: 1758 genes with ≥ 40 full-length CCS read coverage

SNP EVALUATION FOR ANGUS X BRAHMAN

SNP Type	Count
True Positive (called by both)	8334
False Negative (called by genome only)	259
Unphased by Genome (called by transcript only)	1203

Using genome phasing results as truth, IsoPhase SNP calling achieves 97% sensitivity and ~~87% specificity.~~

EXAMPLE OF SNP CALLING VERIFIED BY GENOME

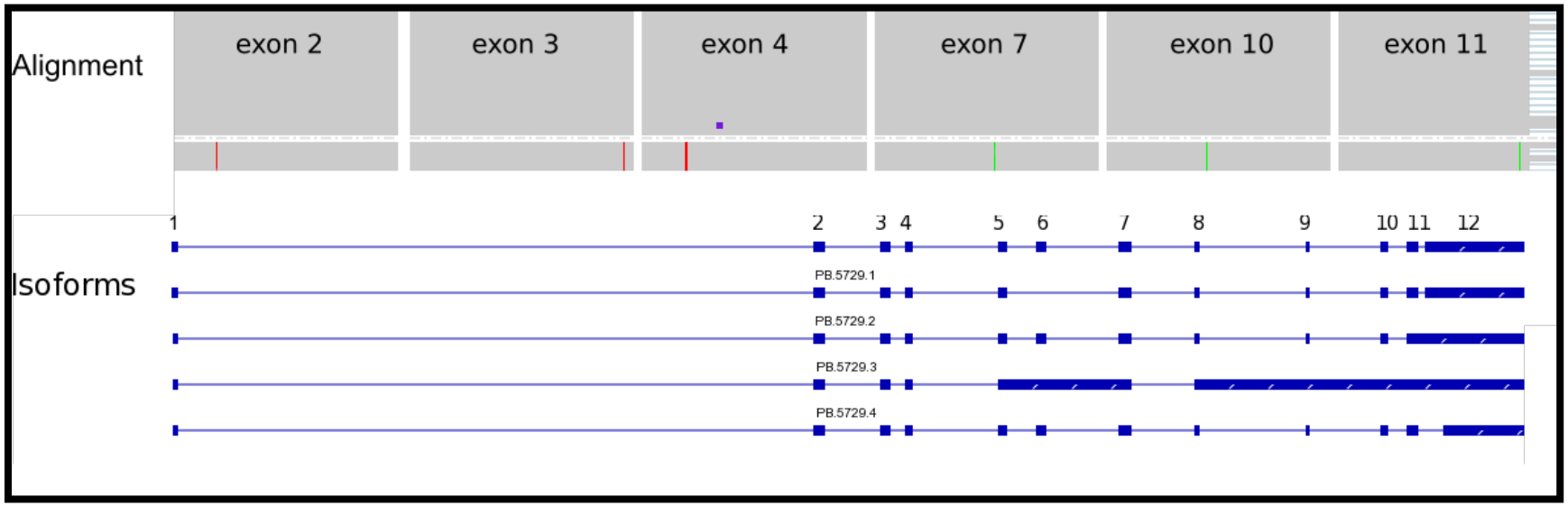


Full-length CCS read alignment, showing only exons with SNPs.

Reads are sorted through “quickphase” in IGV browser showing clear segregation of alleles.

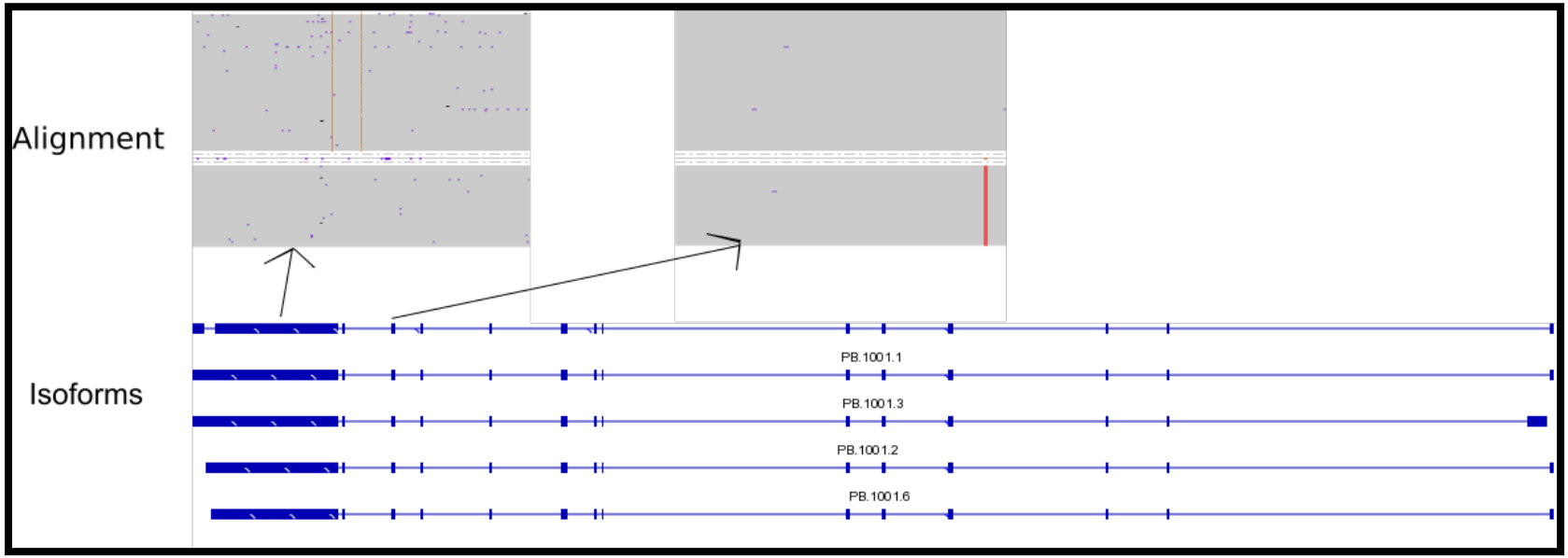
All 6 SNPs validated by genome assembly Unzip results.

EXAMPLE OF SNP CALLING VERIFIED BY GENOME



There are 5 different isoforms for this gene. All isoforms cover all 6 SNP sites.

VPS36 ISOFORMS CALLED SNPS NOT PHASED IN GENOME

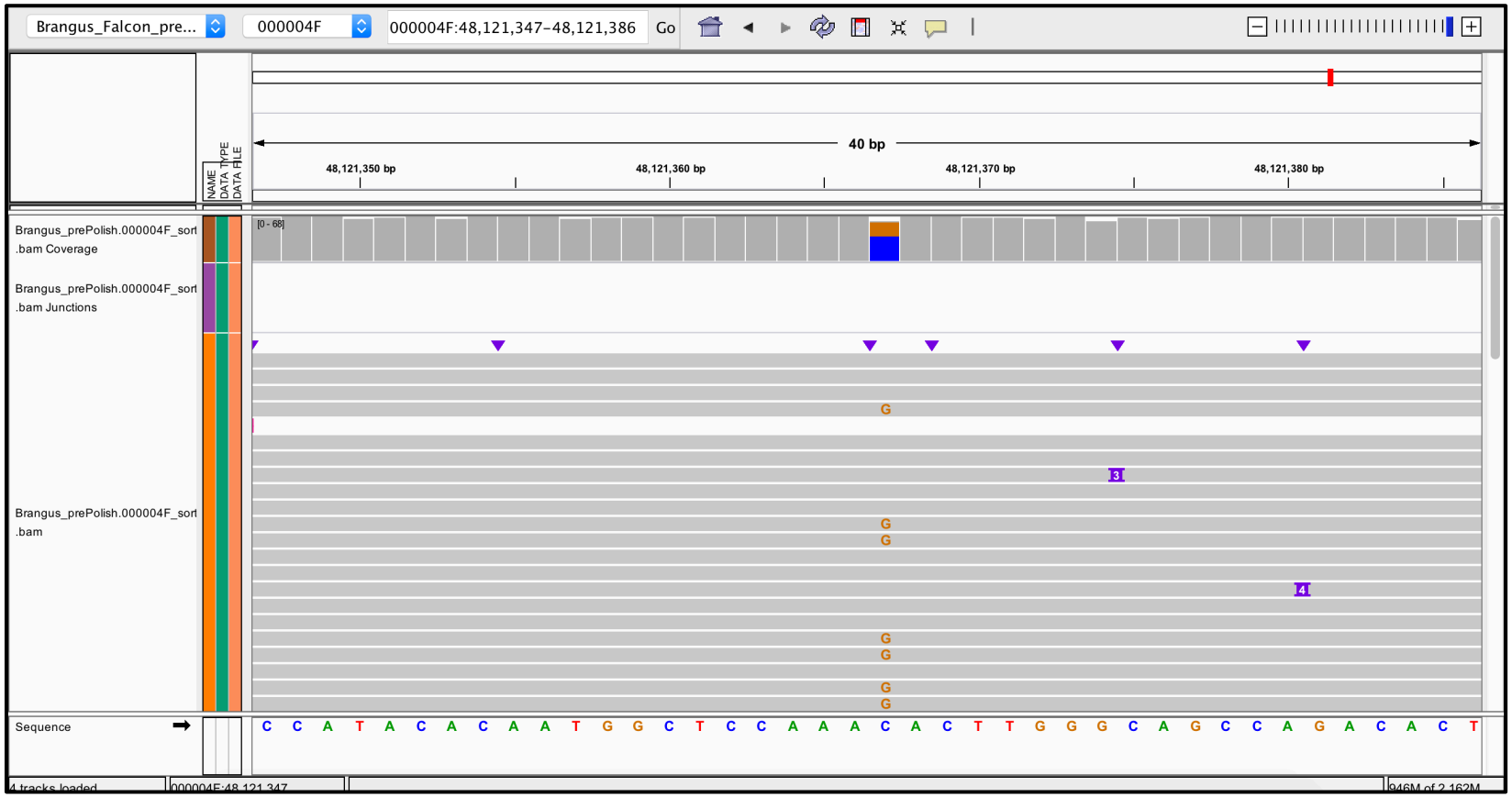


This gene (PB.1001, VPS36) contains 228 FL reads.

- Strong evidence for the 3 SNPs.
- Unzip did not phase this region – so, are the SNPs supported by genome?

VPS36 ISOFORMS CALLED SNPS NOT PHASED IN GENOME

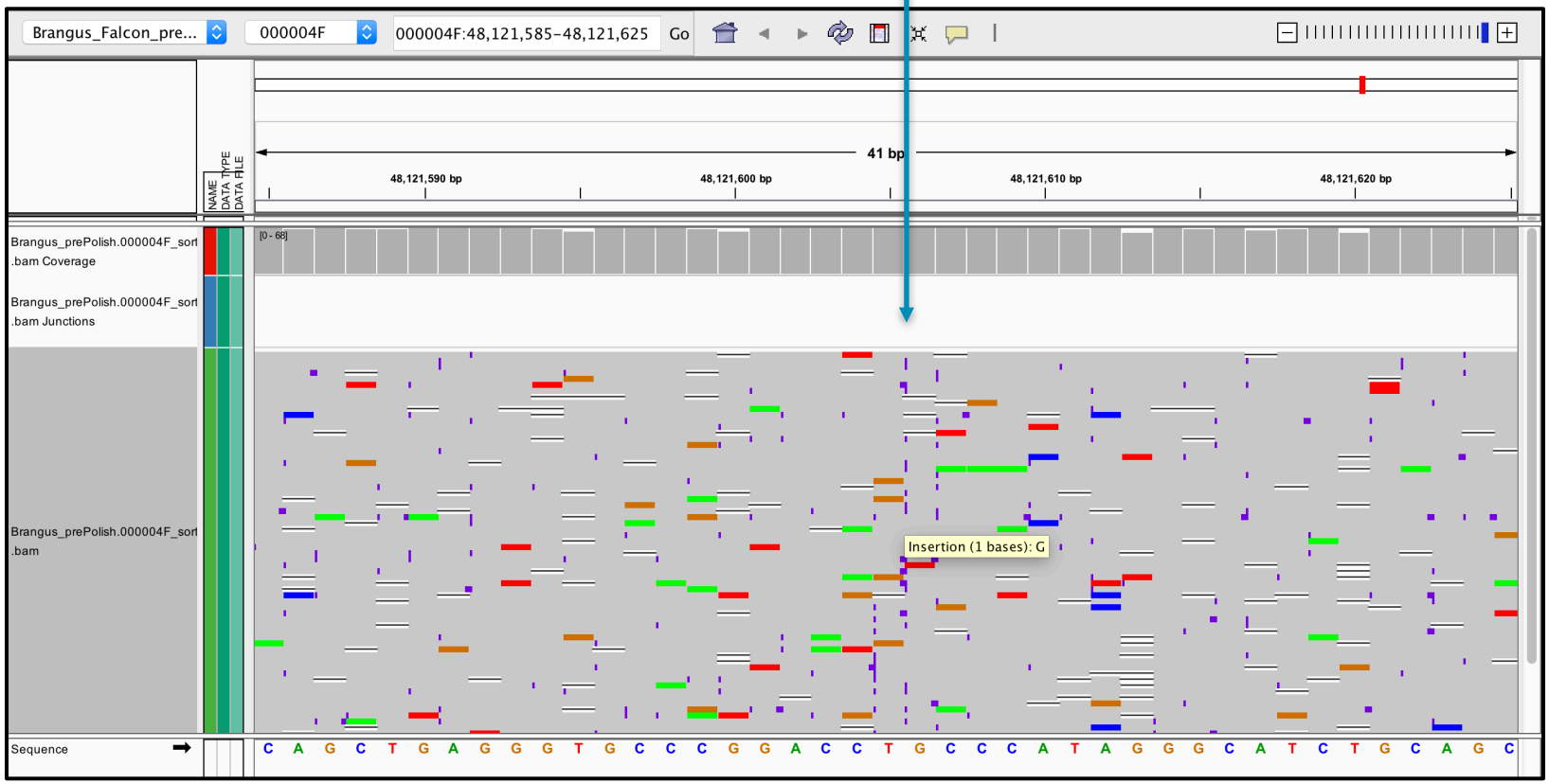
The first SNP 000004F|arrow|arrow:48163477 (C->G) is supported in the pre-polish BAM file.



VPS36 ISOFORMS CALLED SNPS NOT PHASED IN GENOME

The second SNP 000004F|arrow|arrow:48163716 (T->G) is the second T in the sequence context GCCCGGACCT**T**GCCCATAGG which in the pre-polish BAM file shows evidence that the second “T” is either a “T” or a “G”.

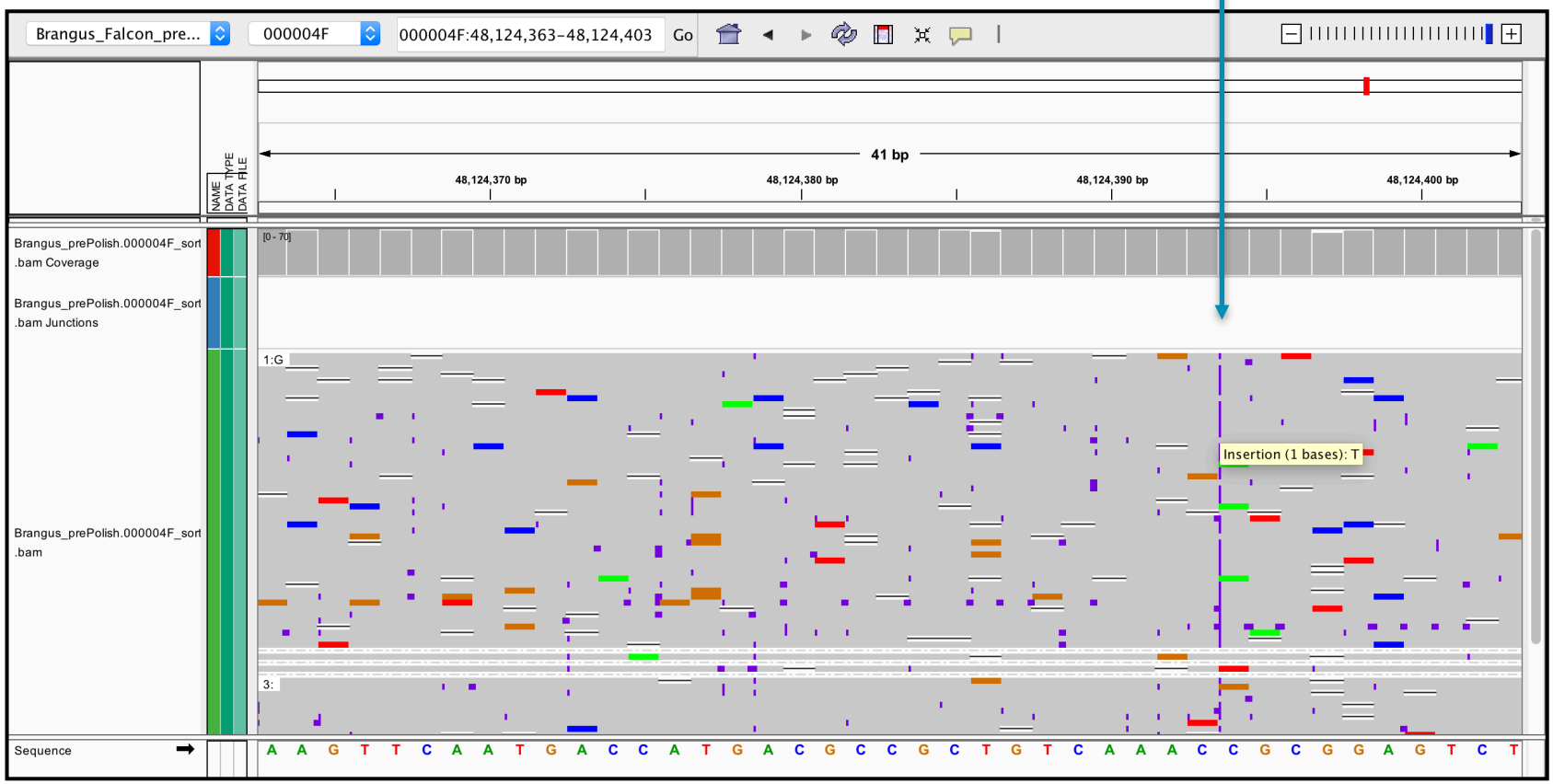
the insertion is either a “T” or a “G” which is the SNP



VPS36 ISOFORMS CALLED SNPS NOT PHASED IN GENOME

The third SNP 000004F|arrow|arrow:48166508 (A->T) is also an insertion against the pre-polish sequence that is supported by the genome subread data.

the insertion is either a “A” or a “T” which is the SNP



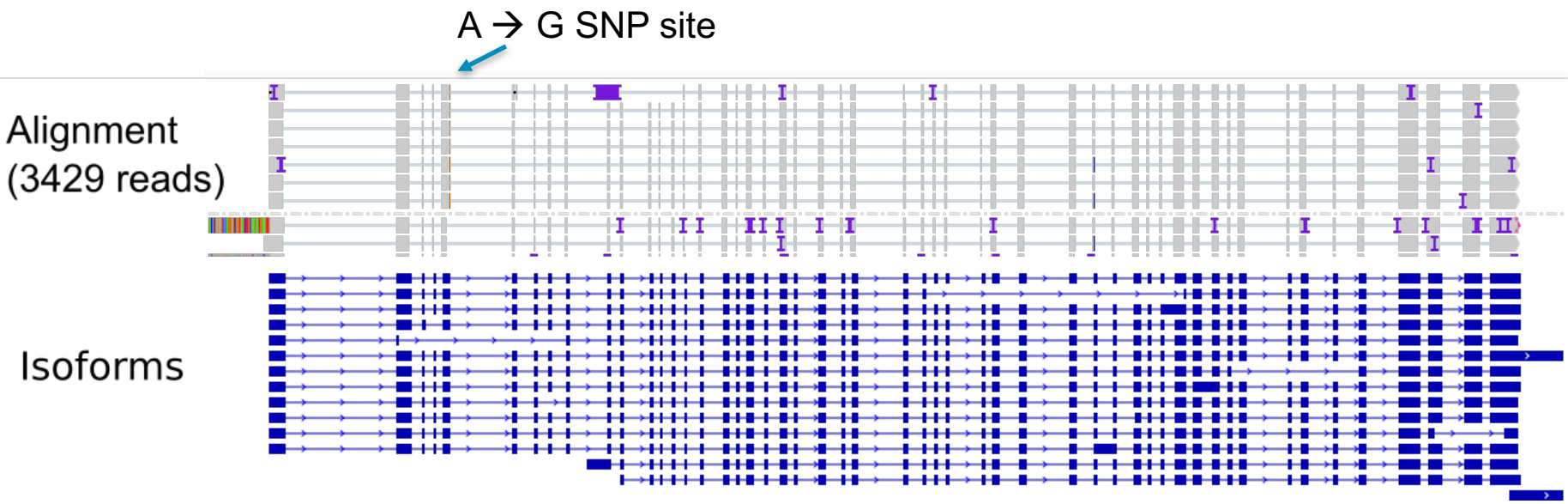
POTENTIAL A → G RNA EDITING IN COL1A1

CHROM	POS	REF	ALT	SNP IN GENOME?
000071F	**7663000**	**A**	**G**	**N**
000071F	7671641	T	C	Y

PB.8679 gene (COL1A1) contains a A → G SNP not supported by genome.

A single alternative contig (000071F_029) covers the whole region.

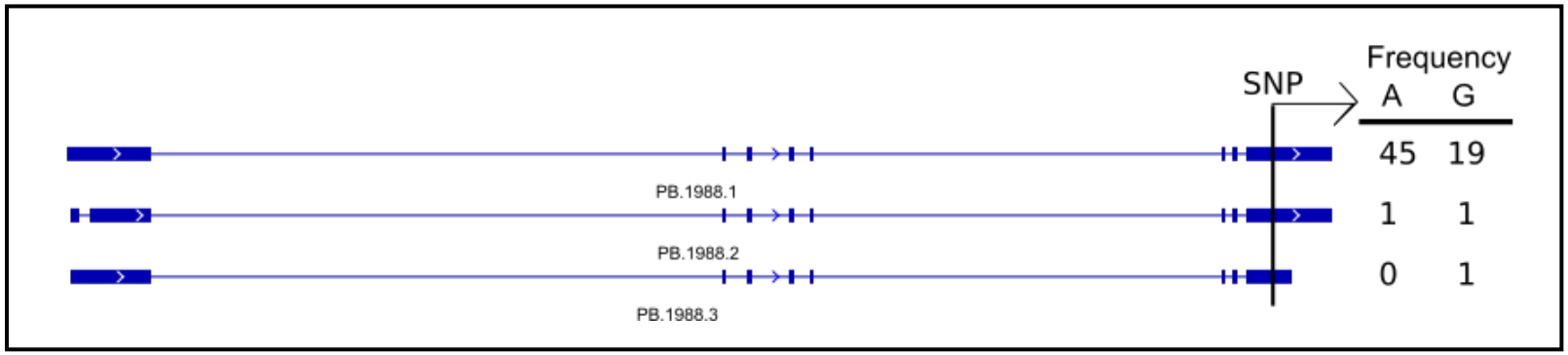
POTENTIAL A → G RNA EDITING IN COL1A1



POTENTIAL A → G RNA EDITING IN COL1A1

Conclusion: COL1A1 contains an non-dominant isoform (PB.8679.1) that uses an alternative donor splice site in exon 5 that includes a potential A → G editing site.

POTENTIAL ALLELE IMBALANCE FOR KIF3C GENE IN BRAIN



- KIF3C is observed in brain only
- The SNP is in the 3' UTR region (A → G) and is verified by genome
- The major isoform expresses the A allele more dominantly

SNP EVALUATION FOR BRAHMAN X ANGUS

SNP Type	Count
True Positive (called by both)	8334
False Negative (called by genome only)	259
Unphased by Genome (called by transcript only)	1203

Using genome phasing results as truth, IsoPhase SNP calling achieves 97% sensitivity and 87% specificity.

However, many of the transcript-only SNPs could be true. They could be not phased by the genome due to low heterozygosity, low coverage, or RNA editing.

ISOPHASE SUMMARY

IsoPhase is a direct extension of the Iso-Seq analysis, utilizing full-length read information to detect SNPs and call haplotypes.

Based on both simulated and real data, it shows **high true discovery rate** and **low false positive rate**.

It has the potential to reveal **allelic specific isoform expressions**.

FUTURE WORK

Detect Indels

- Currently, only substitution SNPs are called
- Calling simple (1-3 bp) indels is conceptually possible, but will require work

Reduce Read Coverage Requirement + Short Read Support

- Currently, requires 40-fold per-gene read coverage
- Reducing read coverage may increase detection but also false calls
- Include short read data for SNP calling; use long reads for phasing

Phasing Without a Reference Genome

- Cogent could be used to reconstruct coding “contigs” to map full-length reads back to. However Cogent will require minor modifications to understand unresolved exonic orderings based solely on Iso-Seq data.

Pipeline for Comparing Genome Phasing Results with IsoPhase

- Automated scripts for showing agreement and disagreement

Please let me know if you have other ideas for making IsoPhase more awesome!

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