GigaScience

Continuous chromosome-scale haplotypes assembled from a single interspecies F1 hybrid of yak and cattle

--Manuscript Draft--

Manuscript Number:	GIGA-D-19-00342R1
Full Title:	Continuous chromosome-scale haplotypes assembled from a single interspecies F1 hybrid of yak and cattle
Article Type:	Data Note
Funding Information:	Agricultural Research Service (3040-32000-034-00D) — Dr. Timothy P. L. Smith Agricultural Research Service (5090-31000-026-00-D) — Dr. Derek M Bickhart Korea Health Industry Development Institute (HI17C2098) — Dr. Arang Rhie
Abstract:	The development of trio binning as an approach for assembling diploid genomes has enabled creation of fully haplotype-resolved reference genomes. This approach is enhanced by heterozygosity of the individual sequenced, rather than hindered by heterozygosity as are other methods of assembling diploid genomes. In order to both maximize heterozygosity and simultaneously assemble reference genomes for two species, we applied trio binning to an interspecies F1 hybrid of yak (Bos grunniens) and cattle (Bos taurus). The genomes of both of these species are comprised of acrocentric autosomes. We produced the most continuous haplotype-resolved assemblies for a diploid animal yet reported. Both the maternal (yak) and paternal (cattle) assemblies have the largest two chromosomes in single haplotigs, and over one third of the autosomes similarly lack gaps. The maximum length haplotig produced was 153 Mb without any scaffolding or gap-filling steps, and represents the longest haplotig reported for any species. The assemblies are also more complete and accurate than those reported for most other vertebrates, with 97% of mammalian universal single-copy orthologs present. The high heterozygosity inherent to interspecies crosses maximizes the effectiveness of the trio binning method. The interspecies trio binning approach we describe is likely to provide the highest-quality assemblies for any pair of species that can interbreed to produce hybrid offspring that develop to sufficient cell numbers for DNA extraction.
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Response to Reviewers:

Reviewer #1: In this manuscript, Rice et al applied the recently developed trio binning technology to an interspecies F1 hybrid of yak (Bos grunniens) and cattle (Bos taurus) and generated high-quality genome assemblies for both parental species simultaneously. Specifically, they sequenced both parents with 30-40X Illumina short reads and their offspring with 125X PacBio long reads. They first used the Illumina short reads from the two parents to identify 21-mers unique to each parent, then they used these unique 21-mers to sort nearly all PacBio long reads into maternal or paternal bins before assembly, so that the assembly process is greatly simplified. Although the trio binning technology is not original in this study, the authors maximized the performance of this technology by applying it to a cross-species hybrid with high heterozygosity. As a result, the authors achieved two haplotype-resolved reference genomes (one for yak and the other for cattle) with impressive continuity.

It is really impressive to see genome assemblies with contig N50 > 70 Mb, and so many chromosome arms are comprised of a single contig. Undoubtedly, the haploid genome assemblies of yak and cattle generated in this study represent the most continuous animal assemblies reported so far. This study also presents a practical example for generating high-quality assemblies for any pair of species that can interbreed to produce viable offspring. In general, the manuscript is well organized and easy to follow. I recommend the publication of this manuscript after some minor comments as listed below are addressed.

We thank the reviewer for the thorough and encouraging review of our manuscript. We appreciate all of the comments and suggested edits, which we find greatly improve the clarity of the manuscript. We address each individual point below.

Page 4, paragraph 3, "15 in maternal and 12 in paternal out of 29": it would be appreciated if the authors could indicate directly which 15 maternal and 12 paternal chromosomes are comprised of a single contig in Fig. 1g and 1h.

We thank the reviewer for this suggestion, which we find makes this figure much easier to interpret as the chromosome ideograms are small. We have noted these chromosomes with asterisks and updated the figure caption.

Table S3: Please explain what "Repeat Consistent", "Repeat Complex" and "No Repeat" represent in this table.

We agree that this table requires a better caption. We have added footnotes to explain these three terms.

Table S4: It is a bit ambiguous what the counts in this table mean. Given that there are 402 gaps identified on the ARS-UCD1.2 reference assembly, there should be 804 gap-flanking regions subject to the intersection of repetitive elements, right? So, do they mean the number of repeat loci (e.g. LINE/L1) found in all the 804 gap-flanking regions, or do they mean the number of gap-flanking regions containing this class of repeat?

We have added the following caption to the table:

Values are the number of repetitive elements identified in gap flanking regions or the

intervening
sequence between flanking sequences.

Page 7, paragraph 2, last sentence: It is a bit hard to understand how the data in Table S5 support the finding of "Inconsistency of flanking elements around gaps in the sire and dam assemblies" in the main text. Table S5 shows the number of ARS-UCD1.2 gaps which are consistently (or not consistently) closed in the yak and cattle assemblies, but it seems to show nothing about flanking elements around gaps.

To help clarify this sentence we have changed it to read as follows:

Inconsistency of the closure status of gaps in the sire and dam assemblies (Supplementary Table S5) suggests that some of these regions may have been sites of non-allelic homologous
recombination that had occurred after the divergence between Bos taurus and Bos grunniens.

Page 8, the last paragraph of results: The authors claim that "The trio assemblies of the cattle and yak haplotype both contain all four subclasses of BOLA in a single contig." This is undoubtedly a good indicator of a high-quality assembly. However, there are no data or figure supporting this result in the manuscript. This is also the case for the coat-color gene KIT.

We thank the reviewer for pointing out the absence of supporting evidence for these claims. We have added a supplementary figure (S6) to the manuscript illustrating the BOLA locus compared to ARS_UCD1.2. However, KIT is also on a single contig in ARS_UCD1.2, so we have decided that pointing out the contiguity of KIT in our assemblies is not useful as it is the same as rather than an improvement over existing work. We have removed discussion of KIT from the manuscript.

According to the Methods section, the authors also generated some RNA-seq data in this study. But what species and tissues were subject to RNA-seq are not clearly indicated. It is also unclear what analyses have been done with these RNA-seq data.

We thank the reviewer for pointing out our oversight in the discussion of these data. We collected RNA-seq data to deposit in SRA to improve NCBI’s gene annotation of the reference genome, and included these methods in our manuscript with the idea that all datasets associated with this manuscript and uploaded to public repositories should be described in this manuscript. However, after further discussion and based on the fact that both reviewers were bothered by the lack of any discussion of RNA-seq in the Results, we have decided that other readers are also likely to find this confusing and these methods are best left out of the manuscript.

Reviewer #2: In this study, Rice et al. applied trio binning to produce the most continuous haplotype-resolved assemblies for an interspecies F1 hybrid of yak (Bos grunniens) and cattle (Bos taurus). This manuscript assemblies an F1 hybrid from two divergent parental genomes by their own trio binning methods, and significantly improves the sequence quality. Although this work shows many advantages of trio binning method and higher-quality sequences, it just focuses on sequence assembly, and has numerous similar results or repetitive findings as compared to the paper by Koren et al., 2018. The biggest issue is that we did not see something new in this work. Furthermore, several points were confusing and needed to be addressed.

We thank the reviewer for the critical but helpful review of our manuscript. While we concede that the manuscript has overlap with Koren et al. in terms of methods, we contend that the application of this method to an interspecies hybrid, and the results of this application, constitute a significant advancement that merits publication for the following reasons: (a) the increased heterozygosity present in an interspecies hybrid allows better distinction between haplotypes, leading to a level of continuity not currently possible with any other method for diploid assembly of large genomes; (b) while intraspecies trio binning produces two reference genomes for a single species, interspecies trio binning produces one reference genome each for two different species from a single individual, of which there are no other examples in the literature to our knowledge; and (c) although a high-quality reference genome of cattle existed prior to our work, the new yak reference we present is several orders of magnitude more complete, contiguous, and continuous than the one published in Qiu et al. 2012. Nonetheless, we appreciate the critique as it has helped us consider how we can better explain the novelty of this work in our manuscript. We have edited our Conclusion accordingly.

Responses to individual points are below:

1. Which tissues are used for RNA-seq and how many libraries and data were sequenced? Why the authors performed RNA-seq? In the results section, we can't find any information about RNA-seq.

As noted in response to Reviewer 1,we collected RNA-seq data to deposit in SRA to improve NCBI’s gene annotation of the reference genome. These methods were included in our manuscript with the idea that all datasets associated with this manuscript and uploaded to public repositories should be described in this manuscript. However, after further discussion and based on the fact that both reviewers were bothered by the lack of any discussion of RNA-seq in the Results, we have decided that other readers are also likely to find this confusing and these methods are best left out of the manuscript.

2. How did the authors get the telomeric repeat location of the genome?

We thank the reviewer for noticing the absence of an explanation of this in our Methods. We have added a Methods subsection describing our technique and added the script we used to github (https://github.com/esrice/misc-tools/blob/master/count_telo_repeats.py).

3. The authors using short reads of twelve yaks and cattle to prove the two assemblies were not likely to be haplotype switch errors. Only SNP rate was selected as the criterion of judgment. More evidences should be provided.

We agree that ideally, a phasing analysis would use additional metrics beyond SNP rate to measure the evolutionary distance between two individuals in a given window of the genome. However, many of the datasets we used for this analysis are too low-coverage (~10x) to confidently call anything besides SNPs, and we are not aware of high-coverage sequencing data for a panel of this size containing both yaks and cattle from verified breeds. Further, we do not believe that analysis of more complex variants is necessary. The SNPs clearly demonstrated regions of introgression and further, SNPs are commonly used to deduce ancestry of segments of genomes in the literature (see, for example, our citation Medugorac et al. 2017).

4. More gaps were filled in the paternal assembly than the maternal assembly using the PBJelly pipeline, but the contig N50s of the maternal assembly improved much more than the paternal assembly. Why?

We greatly appreciate the reviewer’s attention to detail in noticing the seeming discrepancy between the changes in number of gaps versus in contig N50. We were initially also intrigued by this fact after reading this review, but after further consideration, we believe that it is most likely an artefact of the uneven distribution of gaps throughout the assemblies and the discrete nature of N50. For example, filling ten gaps between pairs of 1Mb contigs, while certainly an improvement, will not have any effect on contig N50 if it was already >2Mb before filling these gaps, whereas filling a single gap between two large contigs about the size of the pre-gap-filling contig N50 could double the contig N50.

5. The locus and integrity of BOLA and KIT sequences should be exhibited as Figures (e.g. IGV or UCSC) for better understanding these results.

We thank the reviewer for pointing out the absence of supporting evidence for these claims. We have added a supplementary figure (S6) to the manuscript illustrating the BOLA locus compared to ARS_UCD1.2. However, KIT is also on a single contig in ARS_UCD1.2, so we have decided that pointing out the contiguity of KIT in our assemblies is not useful as it is the same as rather than an improvement over existing

	work. We have removed discussion of KIT from the manuscript. 6. Do you have some data from other full-sibling offspring? Assembling and comparing these samples to estimate recombination and exchange rates in each generation might be reasonable. Esperanza was privately bred, and to our knowledge, no full siblings exist. We agree that sequencing one or more siblings of an individual with a fully phased genome assembly would be an excellent way to study the genomic landscape of meiotic recombination; however, it is not be feasible to breed siblings at this time. Moreover, obtaining a statistically useful estimate of recombination and exchange rates in the manner suggested would require a substantial number of full-sib offspring, which is not practical in a large animal species with a 5-6 year generation interval and low success rate of interspecies breeding. 7. What about assessing molecular mechanisms behind heterosis using trio binning method? Since you can distinguish paternal or maternal origin for all haplotypes of offspring, it might be useful to predict loci leading to heterosis in animals or plants. We agree that the ability to create phased genome assemblies of interspecies hybrid diploid organisms presents an excellent opportunity to explore the molecular mechanisms of various aspects of sexual reproduction such as meiotic recombination as well as to aid in the understanding of allele-specific expression, imprinting, and epistasis. However, work to better understand the mechanisms of heterosis will require the collection of a significant amount of data (genotype and phenotype) from a variety of related admixed and un-admixed individuals. This work would be beyond the scope of the present manuscript, which is focused on describing the results of applying the trio binning method to an interspecies cross for the first time, to generate extremely high-quality assemblies of both species. We thank the reviewer for the thoughtful suggestion just the same.
Additional Information:	
Question	**Response**
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	Yes
Resources A description of all resources used, including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely	Yes

identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	
Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the “Availability of Data and Materials” section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

Continuous chromosome-scale haplotypes assembled from a single interspecies F1 hybrid of yak and cattle

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Abstract

The development of trio binning as an approach for assembling diploid genomes has enabled creation of fully haplotype-resolved reference genomes. Unlike other methods of assembly for diploid genomes, this approach is enhanced, rather than hindered, by the heterozygosity of the individual sequenced. In order to maximize heterozygosity and simultaneously assemble reference genomes for two species, we applied trio binning to an interspecies F1 hybrid of yak (*Bos grunniens*) and cattle (*Bos taurus*), two species that diverged nearly 5Mya. The genomes of both of these species are comprised of acrocentric autosomes. We produced the most continuous haplotype-resolved assemblies for a diploid animal yet reported. Both the maternal (yak) and paternal (cattle) assemblies have the largest two chromosomes in single haplotigs, and over one third of the autosomes similarly lack gaps. The maximum length haplotig produced was 153 Mb without any scaffolding or gap-filling steps, and represents the longest haplotig reported for any species. The assemblies are also more complete and accurate than those reported for most other vertebrates, with 97% of mammalian universal single-copy orthologs present. The high heterozygosity inherent to interspecies crosses maximizes the effectiveness of the trio binning method. The interspecies trio binning approach we describe is likely to provide the highest-quality assemblies for any pair of species that can interbreed to produce hybrid offspring that develop to sufficient cell numbers for DNA extraction.

Keywords: genome assembly, phasing, *Bos taurus*, *Bos grunniens*, Highland cattle

Background

New technologies and algorithms for chromosome-scale genome assembly have improved the contiguity of reference genomes in the past several years [1]. These new methods are more efficient than previous methods, allowing high-quality assemblies of the genomes of a wider variety of organisms, rather than for model organisms only. In addition to increasing assembly efficiency, these technologies have focused on addressing two of the foremost challenges of genome assembly: long repetitive regions and heterozygosity of diploid genomes. Repetitive regions are difficult to assemble due to their low sequence complexity, resulting in gaps in reference genomes [2,3]. Mitigating this issue, advances in long-read sequencing technologies [4,5] have facilitated the generation of reads longer than many of these repetitive regions, spanning what otherwise would be assembly gaps [6,7].

Advances in sequencing technology have thus far not been as successful at resolving heterozygous regions of diploid genomes as they have been at resolving repetitive regions. Heterozygous loci, especially those containing complex structural differences between the haplotypes, add intractable complexity to the assembly graphs used to assemble genomes. Most current long-read genome assemblers, such as canu [8], flye [9], and miniasm [10], choose a random haplotype in each heterozygous region and save the unused haplotype as an alternate, resulting in a single pseudo-haploid assembly containing sequence from both parental haplotypes. Another long-read assembler, FALCON-unzip, uses long reads spanning multiple heterozygous regions to phase the assembly graph as much as possible, but the assemblies it generates still contain numerous haplotype switch errors [11]. The long-range information present in proximity ligation and linked read libraries has also been used to phase diploid assembly graphs with mixed results [12,13].

Trio binning is a new assembly technique that avoids the need for such complex strategies by deconvoluting the problem of diploid genome assembly into a pair of simpler haploid assemblies [14]. Trio binning uses variation present in short reads from two parents to sort long reads from their offspring into bins representing either maternal or paternal haplotypes. The long reads in these bins are then assembled independently of one another, resulting in two haploid assemblies of higher quality and contiguity than would be possible with a diploid assembly. This method's ability to correctly infer haplotype of origin for long reads from the offspring is dependent on how divergent the two parental genomes are, as greater divergence results in more places in their offspring's genome where the two haplotypes are differentiable. Thus, trio binning produced better results for assembly of an intraspecies hybrid of two breeds of cattle (heterozygosity ~0.9%) than for a human trio (heterozygosity ~0.1%) [14].

Here, we apply trio binning to an interspecies F1 hybrid of yak (*Bos grunniens*) and cattle (*Bos taurus*), two species that diverged ~4.9Mya[15] but are capable of producing fertile offspring[16]. The interspecies application of trio binning maximizes the use of heterozygosity to make it easier to bin reads resulting in high-quality reference genomes for both parental species. The resulting fully phased haploid assemblies of both the cattle and yak genomes contain chromosome-arm length haplotigs, representing the most contiguous assemblies to date of large diploid genomes.

Results

We applied trio binning to a trio consisting of a yak cow (*Bos grunniens*) Molly, a Highland bull (*Bos taurus*) Duke, and their F1 hybrid offspring Esperanza **(Figure 1)**. After verifying Esperanza's parentage (**Supplementary Table S1**), we sequenced both parents with Illumina short reads and their offspring with PacBio long reads. We estimated Esperanza's heterozygosity to be ~1.2%, compared to ~0.9% for the cross-breed cattle hybrid assembled by Koren et al. [14], which is consistent with the longer divergence time between yaks and cattle than between indicine and taurine cattle (**Supplementary Figure S1**).

Using the short reads from the two parents, we found ~350 million 21-mers unique to each parental line. More than 99% of the total length of the long reads from Esperanza contained one or more 21-mers unique to one of the parental genomes, allowing them to be sorted into maternal or paternal bins (**Figure 1d,e**), each of which were then independently assembled.

The initial contig assemblies of these two haplotypes are ultra-continuous (**Figure 1f-h)**, with contig N50s of 70.9 Mb for the yak haplotype and 71.7 Mb for the cattle haplotype. In addition, over one third of the 29 autosomal chromosomes in both assemblies are comprised of a single contig: 15 in maternal and 12 in paternal. BUSCO [17] analyses of both genomes show most single-copy orthologs present in the initial contig assemblies. 97.1% of single-copy orthologs are present in the maternal assembly, 95.5% of which are both complete and single-copy. 96.8% of single-copy orthologs are present in the paternal assembly, 95.6% of which are both complete and single-copy.

Trio binning assembly is advantageous not only because removing heterozygous diploidy as a complicating factor leads to more contiguous assemblies, but because it results in two fully phased assemblies. To confirm that the maternal (yak) assembly and the paternal (cattle) assembly were correctly phased, with no switch errors, we again took advantage of the large divergence between the two haplotypes resulting from the interspecies cross by testing the similarity of both assemblies to several cattle and yak individuals (**Figure 2 and Supplementary Figures S2 & S3**).

We aligned short reads from three Highland cattle, three Tarentaise cattle, two wild Asian yaks, and four domestic Asian yaks to the paternal and maternal assemblies, and calculated the number of SNPs for each individual compared to both references in 50kb windows across the genome. In a vast majority of windows, the mean SNP rate of the six cattle is higher than that of the six yaks when compared to the maternal yak reference (98.4%), and the mean SNP rate of the six yaks is higher than that of the six cattle when compared to the paternal cattle reference (99.7%). Notable exceptions to this occur in places like the beginning of maternal chr11, where all six yaks have higher SNP rates compared to the maternal reference than all six cattle, indicating that the maternal reference is more cattle-like at these locations. However, the paternal reference is not more similar to the six yaks at the same locations, indicating that these are not likely to be haplotype switch errors. Rather, we hypothesize that these are regions of cattle introgression into the maternal genome, as introgression among various *Bos* species including cattle and yak is known to be pervasive worldwide [18,19].

Some chromosomes in both genomes are comprised of multiple contigs, so scaffolding the assemblies was still necessary. To this end, we sequenced 250 million reads from a Hi-C library created from a tissue sample of Esperanza. The short read length of a Hi-C short read library presents fewer chances in each read for finding kmers unique to one parent, so we instead aligned all read pairs to both the maternal and paternal haplotype assembly and used alignment scores to bin read pairs. We were able to assign 152M Hi-C pairs to one or the other haplotype using this method, and used the remaining 98M pairs to scaffold both assemblies. The resulting scaffolds had an N50 of 86.2Mb for the paternal and 94.7Mb for the maternal assembly.

Both scaffolded assemblies are highly concordant with the current cattle reference genome (**Supplementary Figures S4 & S5**). Whole genome alignment of the two assemblies to ARS_UCD1.2 revealed a small number of large (>1Mb) structural differences between ARS_UCD1.2 and the yak and cattle haplotypes: four in the yak and five in the cattle haplotype. Further investigation of these discordant segments using a recombination map of cattle [20], an optical map [21], Hi-C heatmaps, the location of telomeric repeats, short read coverage around the breakpoints, and the previous cattle reference UMD3.1 [22], provided sufficient evidence to justify inverting three contigs in the maternal assembly and three contigs in the paternal assembly.

After assigning scaffolds to chromosomes using the recombination map for autosomes and alignment to ARS_UCD1.2 for the X chromosome, we filled gaps created between contigs during scaffolding and chromosome assignment by aligning binned long-reads back to their assemblies using the PBJelly pipeline. This process was able to fill 74 of these gaps in the maternal and 78 in the paternal haplotype assembly, increasing the contig N50s to 79.8Mb for the maternal and 72.8Mb for the paternal assembly. We then finalized both assemblies with a polishing step.

Out of 402 identified gaps on the ARS-UCD1.2 reference assembly, our maternal and paternal assemblies conclusively closed 213 and 219 gaps, respectively (Supplementary Table S3). Gap closure was confirmed by the alignment of 500bp of sequence flanking ARS-UCD1.2

gaps to each assembly and ensuring that the alignments were on the same scaffold, within 100 kb of each other. Gap flanking sequence could not be placed on the same scaffold (trans-scaffold) in 185 and 179 cases for the maternal and paternal assemblies, respectively, suggesting that the cause of ARS-UCD1.2 gaps could be due to scaffolding errors. Of these trans-scaffold closures, 77 and 110 events in the maternal and paternal assemblies were not consistently closed, suggesting structural differences between the assemblies that may indicate true differences between species or individuals.

Intersection of repetitive element annotations with gap flanking sequence revealed that most ARS-UCD1.2 gap regions may have been caused by discrepancies in scaffolding of contigs that were terminated by L1 LINE elements (Supplementary Table S4). These events were followed closely by BovB repetitive elements, which may have also terminated a large proportion of contig ends. While the association of repetitive elements in gap flanking sequence points towards a potential cause for the gap region in ARS-UCD1.2, we cannot rule out the possibility that transposition of L1 LINEs, BovB and other active retroelements may have been spuriously detected in this analysis. Inconsistency of the closure status of gaps in the sire and dam assemblies (Supplementary Table S5) suggests that some of these regions may have been sites of non-allelic homologous recombination that had occurred after the divergence between Bos taurus and Bos grunniens.

The final assemblies of both the cattle and yak genome contain the largest contigs and the fewest gaps of any current assembly of a large diploid genome (**Figure 3**). Both cover the largest two chromosome arms, the q-arms of chr1 (158Mb) and chr2 (136Mb), with a single contig. The maternal yak assembly has 19 gaps on autosomes and 13 gaps on the X chromosome; the paternal highland cattle assembly has 18 gaps on autosomes and 22 gaps on the X chromosome. For comparison, the current cattle reference ARS_UCD1.2 has 260 gaps on autosomes and 55 on the X chromosome; both assemblies reduce this number of gaps by nearly a factor of ten. Furthermore, our trio assemblies of yak and cattle are comparable or superior to other vertebrate reference genomes in terms of contig N50, number of gaps, and size of largest contig compared to size of largest chromosome arm.

Trio binning also resolves heterogeneous loci into haploid sequences. BOLA, the bovine major histocompatibility complex, is a set of highly diverse loci on chr23 containing variants associated with infectious disease susceptibility [23,24]. The trio assembly of the cattle contains all four subclasses of BOLA in a single contig (**Supplementary Figure S6**).

Discussion

The application of trio binning to a yak/cattle hybrid trio demonstrates that this method is capable of producing highly accurate reference assemblies more continuous than those currently available for species with large diploid genomes. The initial contig N50s of the maternal yak and paternal cattle assemblies, at 70.9Mb and 71.7 Mb, respectively, are larger than the contig N50s of the current references for yak (20.4kb) [15] and cattle (ARS_UCD1.2, 25.9Mb) [25]. Our assemblies are also more continuous than the previous trio binning assemblies of bovines, at 23.3Mb and 26.6Mb for the maternal and paternal haplotypes of an Angus × Brahman cross[14]. Thus, our initial haplotig assemblies, even before scaffolding and gap-filling, represent large improvements over existing assemblies of the cattle and yak genomes.

These assemblies not only represent large improvements compared to the current cattle reference genome, but are more contiguous by some measures than even the highest-quality reference genomes of organisms such as human (hg38), chicken (galgal5), and goat [26]. For example, the largest contig in hg38 is a 132Mb contig containing most of the 140Mb q-arm of chr4, whereas over one third of the q-arms of the all-acrocentric autosomes in our assemblies are comprised of a single contig.

Moreover, these assemblies used as input only long reads from a single individual and short reads from its parents, including the mitochondrial genomes, which were assembled from parental short reads. We also used a Hi-C library to scaffold the assemblies and various orthogonal data types to correct errors in the scaffolding and assign scaffolds to chromosomes, but many chromosomes in both haplotypes were assembled into single contigs in the initial long read assembly and thus did not require these additional data types. By comparison, recent chromosome-scale assemblies of other non-model mammals such as horse [27] and goat [26] required many additional data types, such as Sanger sequence, BAC clones, Chicago libraries, optical maps, and linked reads, to achieve their levels of contiguity and composition. We used a pre-existing genetic map for validation of our assemblies, but the high contiguity and accuracy of our scaffolded but otherwise unedited contig assemblies demonstrates that long reads plus a Hi-C library are sufficient for producing high-quality assemblies using trio binning.

Furthermore, our results demonstrate that it is now technologically feasible to assemble full chromosome arms gap-free with only long reads. The remaining gaps in our assembly are likely the result of repetitive regions such as rDNA, centromeres, and large segmental duplications too large to be spanned by the long reads we have, but the ever-increasing maximum read lengths achievable with SMRT [4] and nanopore [5] sequencing continue to surpass the sizes of new repetitive regions. We predict that these improvements to existing technologies, along with algorithmic advances such as those that enabled assembly of the human Y centromere[28], will therefore make gap-free assemblies of vertebrate genomes possible in the near future.

These assemblies are not only highly contiguous, but have the additional advantage of being fully haploid rather than pseudo-haploid as in most current reference assemblies of large diploid genomes. This is especially valuable in highly heterogeneous regions of the genome where the two haplotypes in an individual are most likely to be divergent. We show that the haploid assemblies produced by trio binning can fully resolve difficult to assemble heterogeneous loci such as MHC without the need for additional phasing data. This technique is likely to represent a large benefit in the assembly of out-bred or wild vertebrate species that are known to produce viable hybrids.

Trio binning using a cross-species hybrid, in addition to allowing for easier binning of long reads through increased heterozygosity, also has the advantage of producing reference genomes for two species with long reads from only a single individual. Thus, this approach will be especially useful for comparative genomics studies in which contiguous haploid reference genomes for two related species can be used to identify evolutionary breakpoints with high accuracy.

Conclusions

Our assembly of chromosome-length haplotigs for the yak and cattle genomes using trio binning suggests that trio binning is the best approach currently available for assembling the genomes of diploid organisms that either can be cross-bred with a closely related species or at least have enough population structure within the species to allow breeding two unrelated parents with divergent genomes. The application of the trio binning method to an interspecies cross represents a significant advancement over existing methodology because the high heterozygosity present in an interspecies cross results in phased diploid assemblies of higher continuity than currently possible with any other method, and because it allows the creation of reference genomes for two species from a single individual. While many organisms of biological interest are polyploid or unable to be bred in a controlled setting, many model organisms and other highly studied species would be good candidates for trio binning. We expect that this method will therefore soon be used to assemble new reference genomes for a variety of species.

Methods

Sample collection and preparation

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln, an AAALAC International Accredited institution (IACUC Project ID 1648). Whole blood (EDTA) was collected via jugular venipuncture from the Highland bull and yak cow. Tissue sampling of the yaklander heifer was conducted after euthanization using pentobarbital administered intravenously (1ml/10lb). Lung tissue was flash frozen and stored at -80°C until DNA isolation and sequencing.

Long-read library preparation and sequencing

Genomic DNA was extracted from Esperanza lung tissue using high salt extraction method as described previously [26]. The DNA was converted into sequencing libraries using the SMRTbell Express Template Prep Kit (Pacific Biosciences, Menlo Park CA) as directed, except without any shearing step. Three libraries were prepared, one with a 25 kb cutoff setting on the BluePippin instrument (Sage Science, Beverly MA) and two with a 30 kb cutoff setting. The libraries were sequenced with 44 cells on a Sequel instrument using Sequel Sequencing kit v2.1 chemistry (Pacific Biosciences, Menlo Park CA).

Short-read library preparation and sequencing

Genomic DNA from Esperanza lung (used also for long read sequencing, above) was converted into sequencing libraries using the TruSeq DNA PCR-Free LT Library Preparation Kit (Illumina Inc., San Diego CA) as directed. The shearing was conducted on a Covaris S220 instrument (Covaris Inc., Woburn MA) with setting to 350 base pair fragment size. The same procedure was used to create libraries for parental and unrelated yak samples, except the DNA was prepared from blood using a standard phenol:chloroform extraction as described previously[29]. Sequencing was performed by 2x150 base paired end sequencing on a NextSeq500 instrument (Illumina Inc., San Diego CA) using High Output Kit v2 (300 cycles) kits.

Hi-C library preparation and sequencing

33.36 mg frozen lung from Esperanza was removed from cold storage and homogenized by chopping with a sterile scalpel. The resulting lung paste was transferred to a microcentrifuge tube along with one milliliter PBS. Paraformaldehyde (EMS Cat. No. 15714) was added to a final concentration of three percent and the sample was vortexed briefly before rotation for twenty minutes at room temperature. Collagenase from a Dovetail Hi-C Library Preparation Kit (Catalog No. 21004) was added to the crosslinked tissue and incubated for 1 hour at 37°C in an agitating thermal mixer. The liquid phase was taken from this reaction and brought to a final concentration of one percent SDS.

Crosslinked chromatin was bound to SPRI beads and washed thoroughly before digesting with DpnII (20 U, NEB Catalog No. R0543S) for 1 hour at 37°C in an agitating thermal mixer. Biotin-11-dCTP (ChemCyte Catalog No. CC-6002-1) was incorporated by DNA Polymerase I, Klenow Fragment (10 U, NEB Catalog No. M0210L) for thirty minutes at 25°C. Following another wash, intra-aggregate ligation with T4 DNA Ligase (4000 U, NEB Catalog No. 0202T) was carried out overnight at 16°C. Crosslinks were reversed in an eight percent SDS solution with Proteinase K (30 g, Qiagen Catalog No. 19133) for fifteen minutes at 55°C followed by forty-five minutes at 68°C. After SPRI bead purification, DNA was split into two replicates and sonicated to an average length of 350 bp using a Diagenode Bioruptor NGS platform.

Sheared DNA samples were run through the NEBNext Ultra II DNA Library Prep Kit for Illumina (Catalog No. E7645S) End Preparation, Adaptor Ligation with custom Y-adaptors, and SPRI bead purification steps before Biotin enrichment via Dynabeads MyOne Streptavidin C1 beads (ThermoFisher Catalog No. 65002). Indexing PCR was performed on streptavidin beads using KAPA HiFi HotStart ReadyMix (Catalog No. KK2602) and subsequently size selected with SPRI beads.

We sequenced 250M reads of this library on a 2x151bp run of an Illumina NextSeq500 using High Output Kit v2 (300 cycle) kits (Illumina Inc., San Diego CA).

Heterozygosity estimation

Heterozygosity of Esperanza was estimated using Genomescope [30].

Parentage confirmation

Using the Illumina whole genome shotgun sequence data generated for this project as well as other data already published to the public domain, we calculated the number of sites relative to the bovine reference genome that did not follow the expected pattern of inheritance. For example if the sire was homozygous for allele A, the dam homozygous for allele B, and the progeny homozygous for B, in the absence of a genotyping error, this pattern suggests that the reported sire is not in fact the sire. We expect some genotyping errors[31,32], but whatever exclusions are identified when analyzing the verifiable trio should be dwarfed in number when one of the actual parents is swapped in the analysis with an unrelated animal. For this comparison we did trio analysis of the yak × cattle offspring versus the reported Highland sire and yak dam as well as the reported dam versus four unrelated Highland bulls, and the reported sire versus an unrelated yak dam.

The UnifiedGenotyper [33] was used in gt_mode=DISCOVERY to analyze the mapped datasets (bam files) for cattle × yak progeny in turn vs. a prospective sire/dam pair to identify sites polymorphic in the trio, then genotype those positions producing a vcf file. A custom java program was written to search the dataset for exclusions. Given the nature of this cross it was expected that the majority of the sites identified would be those specific to the interspecies mating. Specifically, we ignored all polymorphic sites with the interspecies cross signature of the bovine sire homozygous for an allele A (consistent with the bovine reference allele), the yak dam homozygous for allele B (likely consistent with the allele fixed in yak) and the progeny heterozygous A/B. Since this pattern would be common to any cattle × yak mating, it would not be suitably specific for a parentage test. The sequence data from the animals not part of the trio were generated for another study with a much lower fold coverage requirement. The coverage for these other animals is on average ~14×. It has been demonstrated previously (cattle and sheep genotyping papers) that a genotyping accuracy of ~98% can be attained at this level of coverage. The ~2% error rate in that work was attributable to an undersampling of the second allele for heterozygous genotypes or allele dropout in the assay based genotyping platform. This will have the effect of increasing the rate of exclusions in those animals not reported to be the parents, but it should be at a rate of approximately 1% of the total genotypes analyzed. This error amounts to a small contribution to the observed exclusion count for the negative controls. The results are shown in **Supplementary Table S1**. The reported yak dam produced ~12 fold fewer exclusions than the negative control dam (4.99% vs 0.42%) and the reported Highland sire produced ~32 fold fewer exclusions when compared with the negative control sires (6.24%,5.70%,5.76%, and 7.06% vs 0.19%). These results indicate a correct parental assignment.

Contig assembly

The trioBinning scripts from (https://github.com/skoren/triobinningScripts) were used to classify the reads. Briefly, meryl from canu 1.7.1 was used to count all parental k-mers. The k-mers specific to both the maternal and paternal haplotype were identified via the meryl difference command. Finally, any paternal k-mer occurring at least 6 times and any maternal k-mer occurring at least 4 times were retained for classification:

```
meryl -B -C -m 31 -s maternalIlluma.fa -o mom -threads 28 -memory 60000

meryl -B -C -m 31 -s paternallIlluma.fa -o dad -threads 28 -memory 60000

meryl -M difference -s dad -s mom -o dad.only
meryl -M difference -s mom -s dad -o mom.only

meryl -Dt -n 6 -s dad.only |awk '{if (match($1,">")) { COUNT=substr($1, 2, length($1)); } else {print $1" "COUNT}}' |awk '{if ($NF < 100) print $0}' > dad.counts

meryl -Dt -n 4 -s mom.only |awk '{if (match($1,">")) { COUNT=substr($1, 2, length($1)); } else {print $1" "COUNT}}' |awk '{if ($NF < 100) print $0}' > mom.counts
```

Reads with no parental marker were not used in downstream analysis. Classified reads were assembled with Canu 1.7.1 with the patch for truncated consensus (git commit e42d54d4f1b1133b8e944b09733806bfe63bc600) command 'genomesize=2.8g' 'correctedErrorRate=0.105' 'cnsErrorRate=0.15' 'corMhapSensitivity=normal' 'ovlMerThreshold=500'.

Scaffolding

We preprocessed the Hi-C reads by trimming to the DpnII junction sequence GATCGATC. To separate the junction-split Hi-C read pairs into maternal and paternal bins, we aligned all reads to both maternal and paternal contig assemblies using bwa mem v0.7 [34] with default parameters. We then ran the classify_by_alignment program (https://github.com/esrice/trio_binning v0.2.1) to determine based on the 'AS' tag of the resulting bam files whether each read pair aligned better to the maternal contigs, the paternal contigs, or both equally. If the read pair aligned better to one haplotype than the other, we used it to scaffold only this haplotype, but if it aligned equally well to both, we used it to scaffold both haplotypes. We then ran SALSA2 v2.2 [35] to scaffold both assemblies using the parameters '-e GATC -m yes'.

Quality control

To find possible mis-assemblies in the scaffolds, we aligned them to ARS_UCD1.2 [25] using mashmap v2.0 [36] with parameter '--perc_identity 95'. We also aligned probes from a recombination map of cattle [20] to the scaffolds using bwa mem v0.7 [34]. We examined resulting alignments for each chromosome for evidence of disagreements between our assembly and ARS_UCD1.2 or the recombination map. Where such disagreements existed, we used the combination of evidence from Hi-C heatmaps, ARS_UCD1.2, the recombination map [20], an optical map [21], telomeric repeat location, the previous cattle reference UMD3.1 [22], and short read coverage around the breakpoint to determine whether there was sufficient evidence to edit our assembly to better match the reference. In total, we inverted the orientation of three haplotigs in the paternal assembly and three haplotigs in the maternal assembly.

Chromosome assignment

We used the alignments of recombination map probe sequences as described above to order and orient scaffolds onto chromosomes. As the recombination map does not include the X chromosome, we used the mashmap alignments between our assemblies and ARS_UCD1.2 to order and orient scaffolds onto the X chromosome.

Gap filling

We filled remaining gaps in each assembly using the PBJelly pipeline [37], which we modified for compatibility with current versions of the software upon which it depends: blasr [38] v5.3.2 and networkx[39] v2.2. This modified pipeline is available at https://github.com/esrice/PBJelly.

Gap analysis

Gap flanking sequence consisting of 500 bp of sequence from the 5’ and 3’ ends of each gap region was extracted from the ARS-UCD1.2 reference genome. These flanking sequences were aligned to the sire and dam haplotig assemblies using bwa mem v0.7 [34] and checked for consistency. If both gap flanking sequences were on the same scaffold, were within 100 kb [40] distance of each other, and had no intersecting gaps from the same assembly, the gap was considered closed. Repetitive elements were identified using RepeatMasker (http://repeatmasker.org), with the settings “-q”, “-species cow” and “-no_is.” Repeat annotations were converted to bed coordinates and were intersected with gap flanking regions using Bedtools [40].

Polishing

Arrow from SMRTanalysis v5.1.0.26412 (pbcommand v0.6.7, arrow 2.2.2 ConsensusCore v1.0.2, ConsensusCore2 v3.0.0, pbalign version: 0.3.1) was used via the ArrowGrid pipeline (https://github.com/skoren/ArrowGrid). Only classified reads were used to polish each haplotype. Initial contigs were polished with two rounds of Arrow. Final gap-filled assemblies were again polished with two rounds of Arrow, using SMRTanalysis v6.0.0.47841 (pbcommand v1.1.1, arrow 2.2.2, ConsensusCore v1.0.7, ConsensusCore2 v3.0.0, pbalign version: 0.3.1).

Telomere location

We tested for the presence of telomeric repeats at the ends of each chromosome sequence by counting the number of exact occurrences of the telomeric repeat sequence (TTAGGG) in the final kilobase of each chromosome. We consider a chromosome sequence to end with a telomere if the telomeric repeat sequence occurs >= 5 times in the last kilobase, which is roughly equivalent to $p < 10^{-7}$. The script we wrote for this purpose can be found at https://github.com/esrice/misc-tools/blob/master/count_telo_repeats.py.

Phasing confirmation

We confirmed that our assemblies are phased correctly by comparing both references to several yak and cattle genomes. We downloaded short reads from SRA for three Highland cattle, three Tarentaise cattle, four domestic yaks, and two wild yaks. Supplementary Table S2 lists the IDs and SRA accessions of these individuals. We aligned short reads to both maternal and paternal haplotype assemblies using bwa mem v0.7 [34] with default parameters and sorted alignments and removed PCR duplicates using samtools sort and rmdup [41] with default parameters. Finally, we called SNPs and calculated window SNP rates, which we define as (# homozygous SNPs + 0.5 * # heterozygous SNPs) / (# bases genotyped in window), using samtools mpileup output piped to a custom script available at https://github.com/esrice/misc-tools/blob/master/pileup2windows.py. We used the mpileup parameters "-Q 20 -q 20" to exclude low-quality base calls or alignments from the pileup, and we did not call SNPs for positions where the sequencing depth was below the 2.5th percentile or above the 97.5th percentile position-depth for that sample.

List of abbreviations

Mb: megabases; kb: kilobases; MYA: millions of years ago; MHC: major histocompatibility complex; SMRT: single molecule real time

Declarations

Ethics approval and consent to participate All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska - Lincoln (UNL, Protocol 1648). UNL is an AAALAC International accredited institution.

Availability of data and material The datasets generated and/or analysed during the current study are available in the NCBI BioProject repository under accessions PRJNA551500 and PRJNA552915.

Competing interests The authors declare that they have no competing interests.

Funding This work was supported by ARS Project Number 3040-32000-034-00D. ESR and sample collection costs were supported by funding from an Enhanced Research Collaboration grant from the University of Nebraska-Lincoln, Institute of Agriculture and Natural Resources, Agricultural Research Division and the USDA Meat Animal Research Center. DMB was supported by USDA CRIS project number: 5090-31000-026-00-D. SK, AR, and AMP were supported by the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health. AR was also supported by the Korean Visiting Scientist Training Award (KVSTA) through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HI17C2098).

Authors' contributions This study was conceived and designed by TPLS with input from JLP and AMP. TH and PH identified the animals and collected ante-mortem samples; BVL, MPH, and JLP were responsible for handling the euthanasia and tissue collection postmortem. TPLS was responsible for long and short read genomic and RNA sequencing, except NWM and ESR prepared Hi-C sequencing libraries. ESR, AR and SK generated the assemblies. ESR, SK, DMB, and BDR conducted quality control and assessment of the assemblies. TSK performed additional computational analyses. MPH, BVL, and REG provided additional input. ESR, SK, AMP, JLP, and TPLS wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements We thank Beth Nelson and Nedda F. Saremi (UC Santa Cruz), Kristen Kuhn, Kelsey McClure, Jacky Carnahan, and Bob Lee (USMARC) for technical assistance, and Sarah Rada-Scott for providing animals, tissue samples, and photography. Tissue sampling was conducted with help from the following: David Steffen, John Kammermann, Shauna Tietze, Kristin Beede, Erin Duffy, Rachel Burrack, Brent Johnson, Matthew Quinn, Shawna Clement, Lianna Walker, Kylee Sutton, Hiruni Wijesena, Lizzy Dorwart, Katie Howser, Robert Posont, Kerri Bochantin, Taylor Barnes, Lauren Kett, Rebecca Swanson, Joslyn Beard, Aaron Cowell, Emma Winters, Devon Lockman, Jaden Carlson, Madeline Pelster, and Adam Bassett (University of Nebraska-Lincoln); and Leah Treffer (Nebraska Wesleyan University). This work used the computational resources of the NIH HPC Biowulf cluster (https://hpc.nih.gov), the USDA CERES cluster (a part of the SCINet initiative: https://www.ars.usda.gov/scinet/), and the University of Nebraska - Lincoln Holland Computing Center (https://hcc.unl.edu/). Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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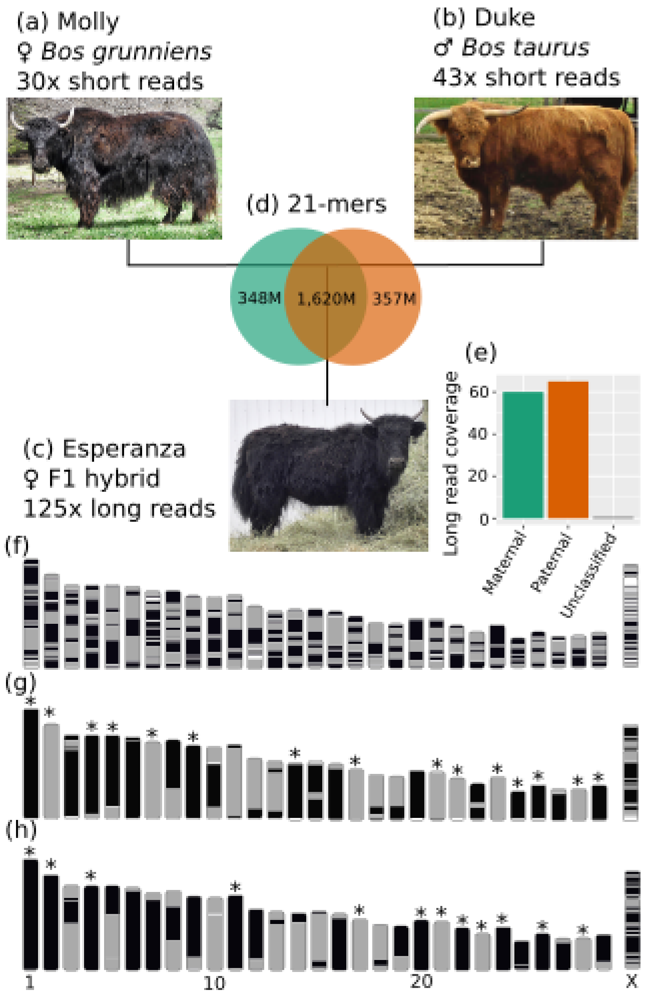


Figure 1. Trio binning of a yak/cattle hybrid. (a-c) We collected short reads from a female yak and a male cattle, and long reads from their F1 hybrid offspring. (d) Counts of 21-mers shared by Molly and Duke and those unique to a single parent. (e) Long-read coverage of the maternal and paternal haplotypes after binning reads from Esperanza using 21-mers from (d). (f-g) Ideograms of contigs on chromosomes for (f) ARS_UCD1.2, (g) Esperanza's maternal (yak) haplotype assembly, and (h) Esperanza's paternal (cattle) haplotype assembly, with contigs represented as solid blocks of a single color and full chromosome arms in single contigs noted with an asterisk.

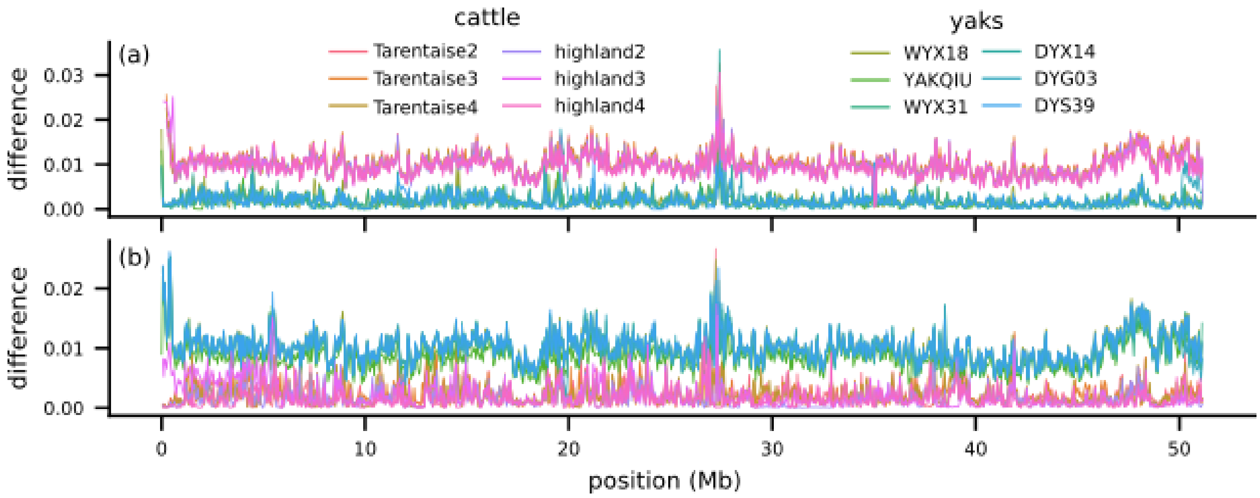


Figure 2. Alignment of six cattle and six yaks to chr29 of our (a) maternal and (b) paternal assemblies shows that the maternal haplotype assembly is more similar to yak genomes than cattle and the paternal haplotype assembly is more similar to cattle genomes, demonstrating that they are phased correctly.

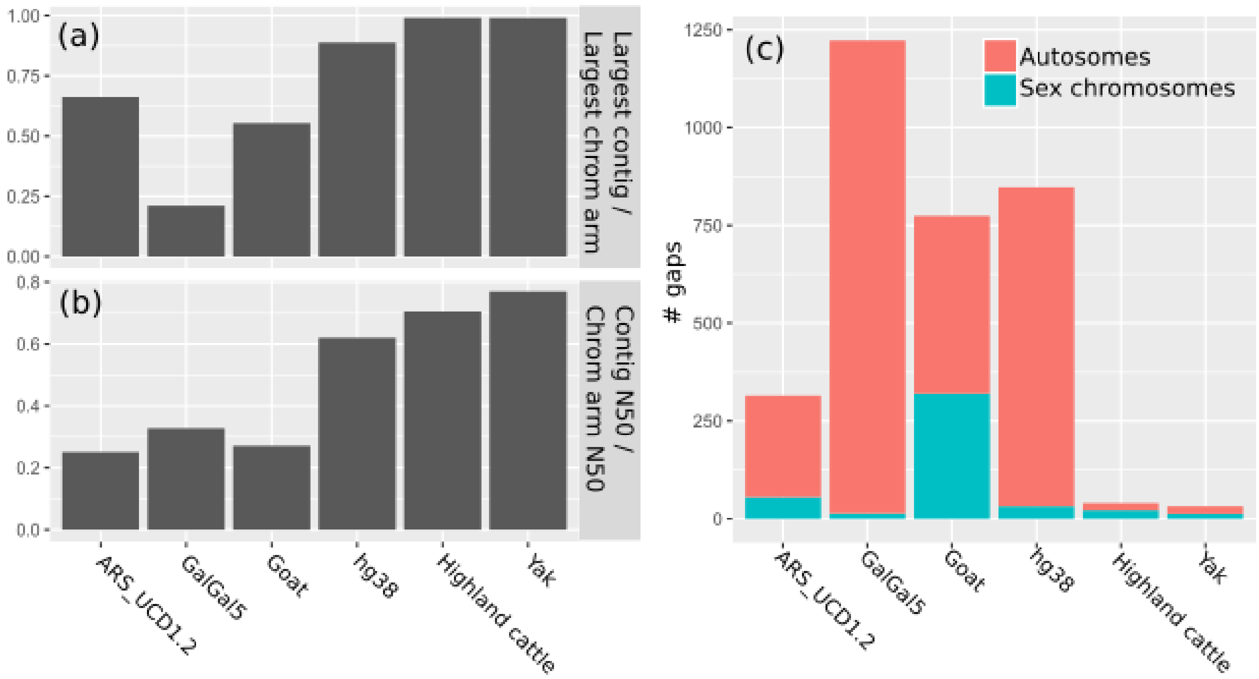
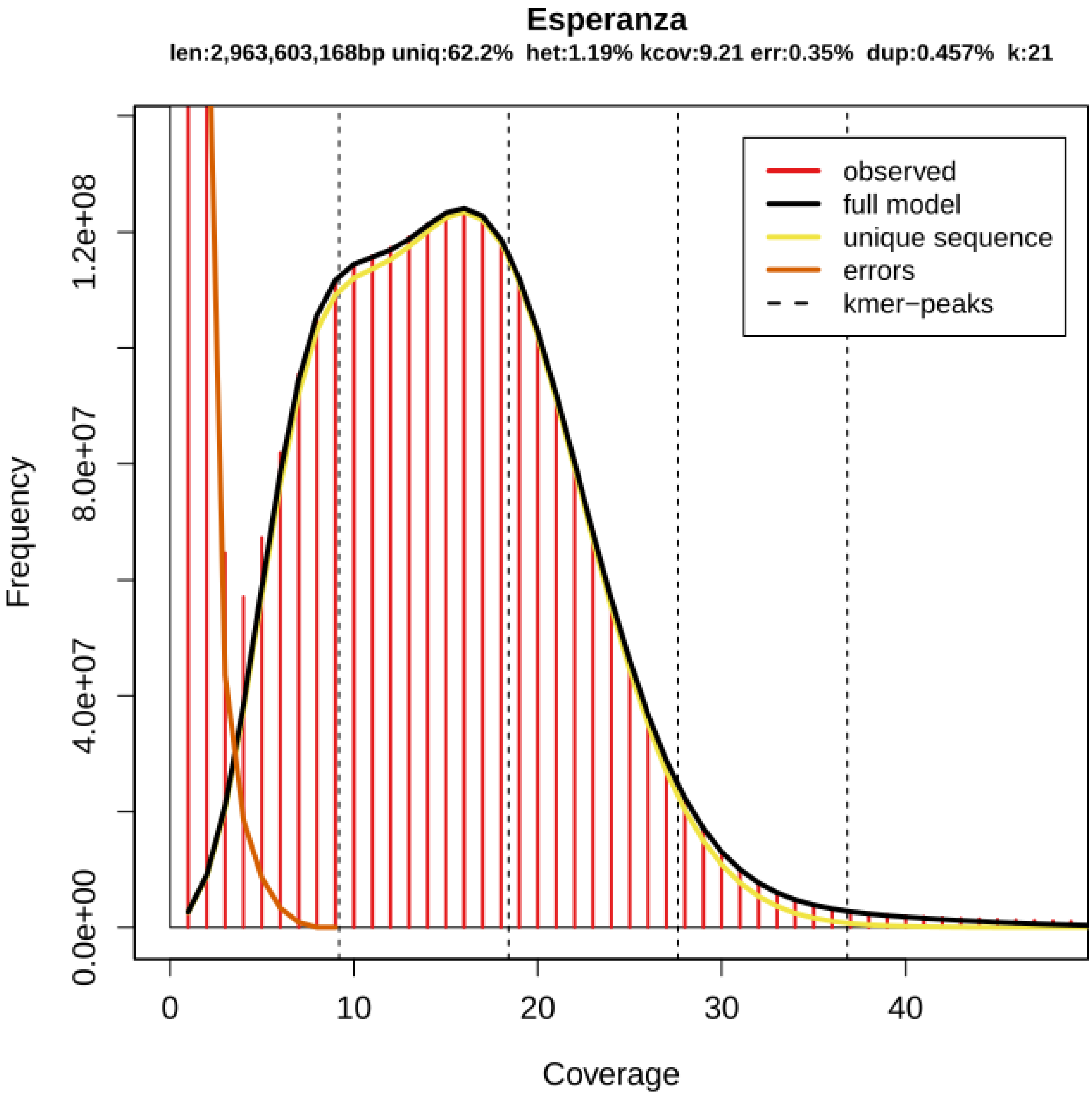


Figure 3. Comparison of trio highland and yak assemblies to current cattle, chicken, goat, and human reference assemblies, based on ratio of largest contig size to largest chromosome arm size (a), ratio of contig N50 to chromosome arm N50 (b), and number of gaps in autosomes and the major sex chromosome, i.e., X in cattle, yak, goat, and human and Z in chicken (c). We note that the number of gaps in hg38 is somewhat inflated due to its gapped assembly of centromeres.

Supplementary Figures



Supplementary Figure S1. Histogram of 21-mer coverage in short reads from Esperanza gives a genome heterozygosity estimate of ~1.2%.

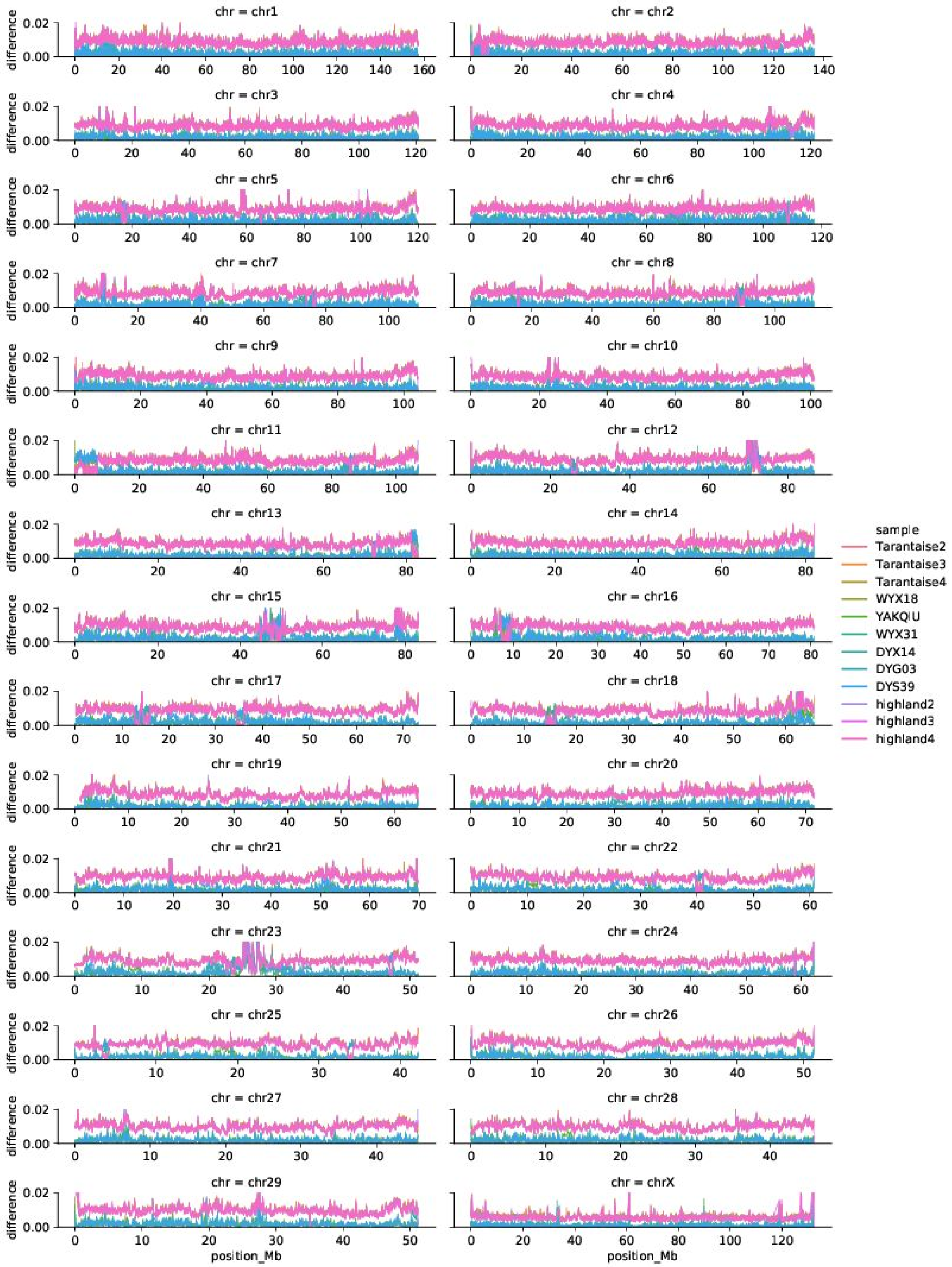


Figure S2. Comparison of twelve yak and cattle genomes to maternal haplotype assembly.

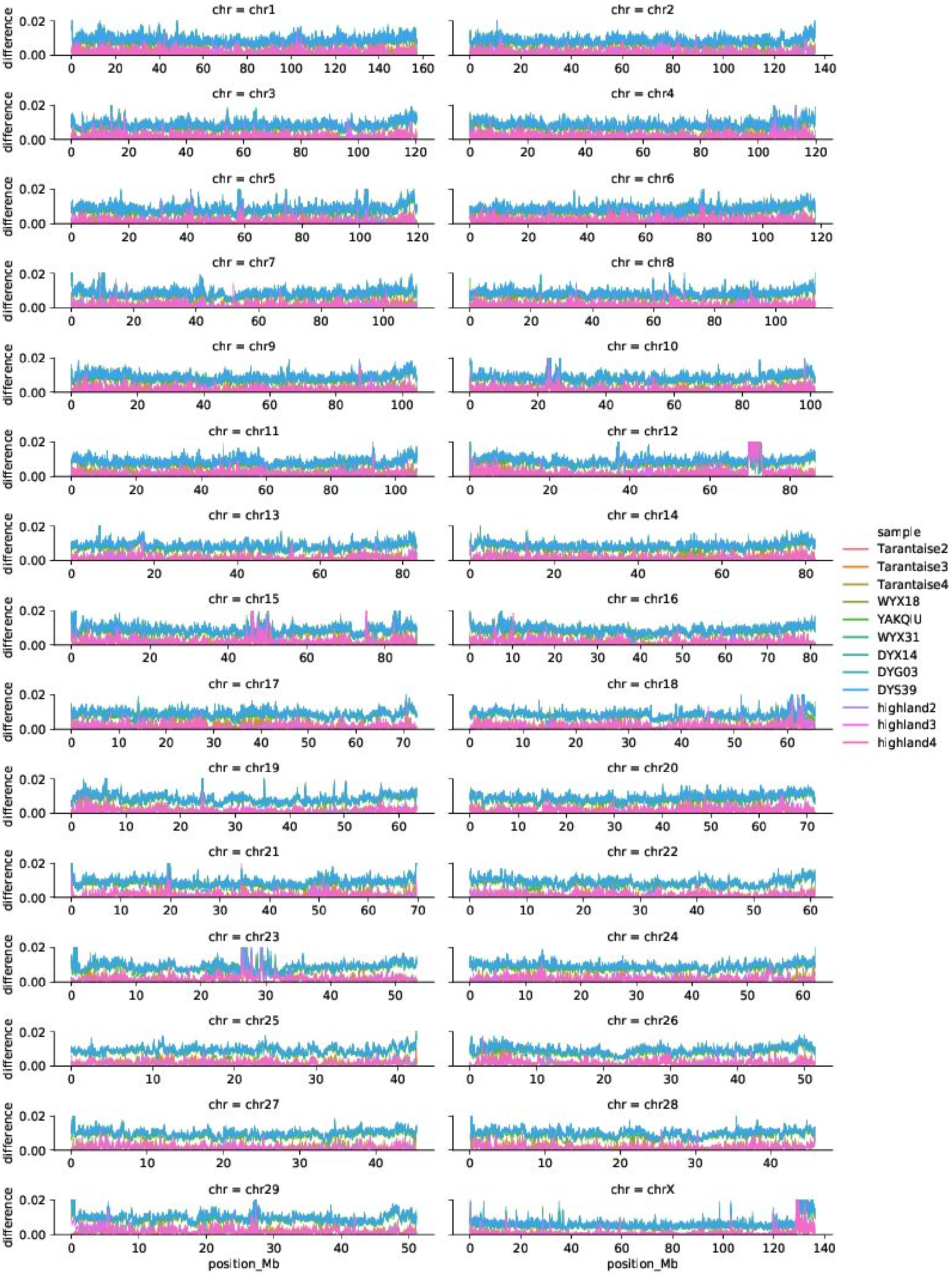
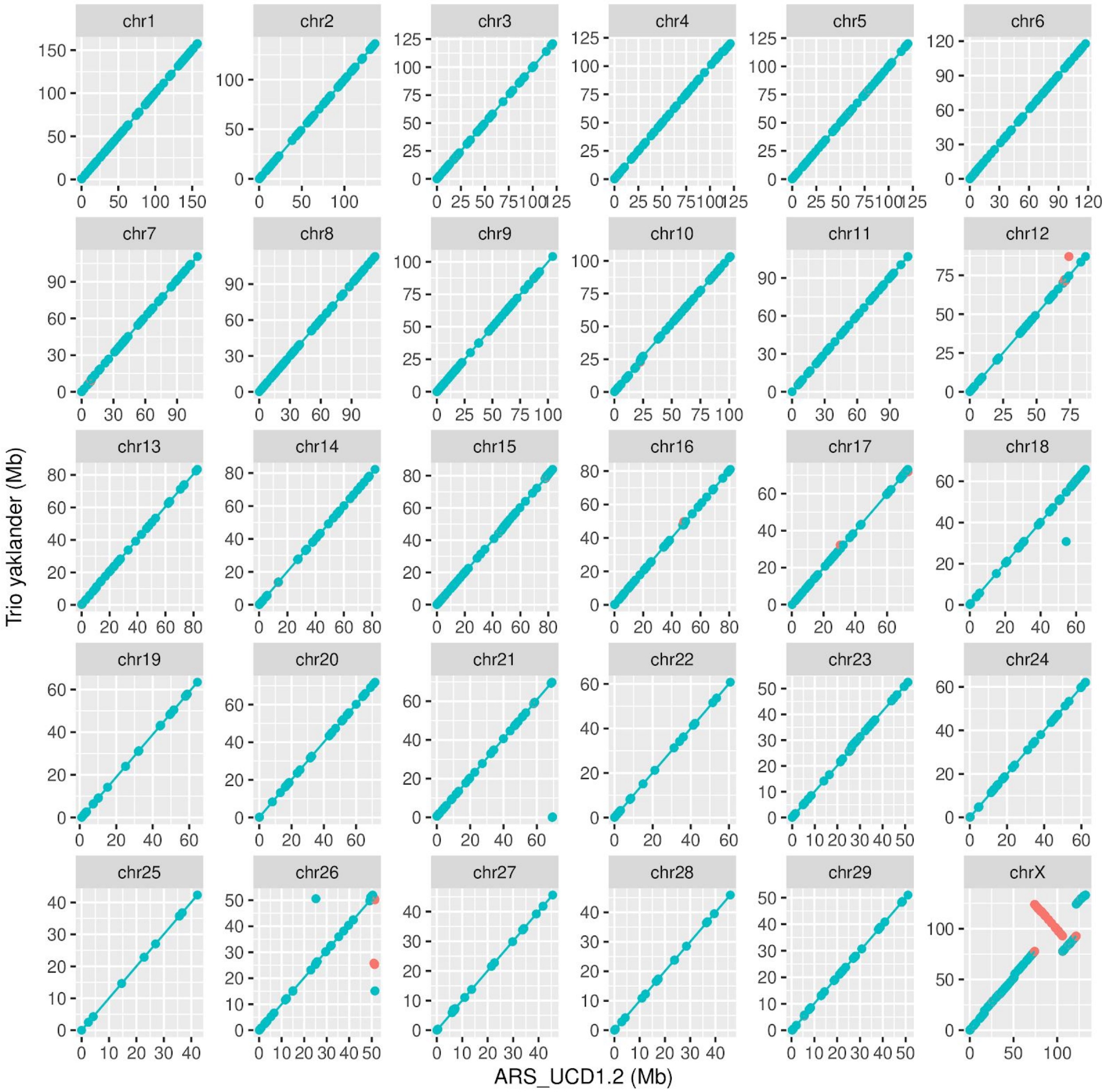
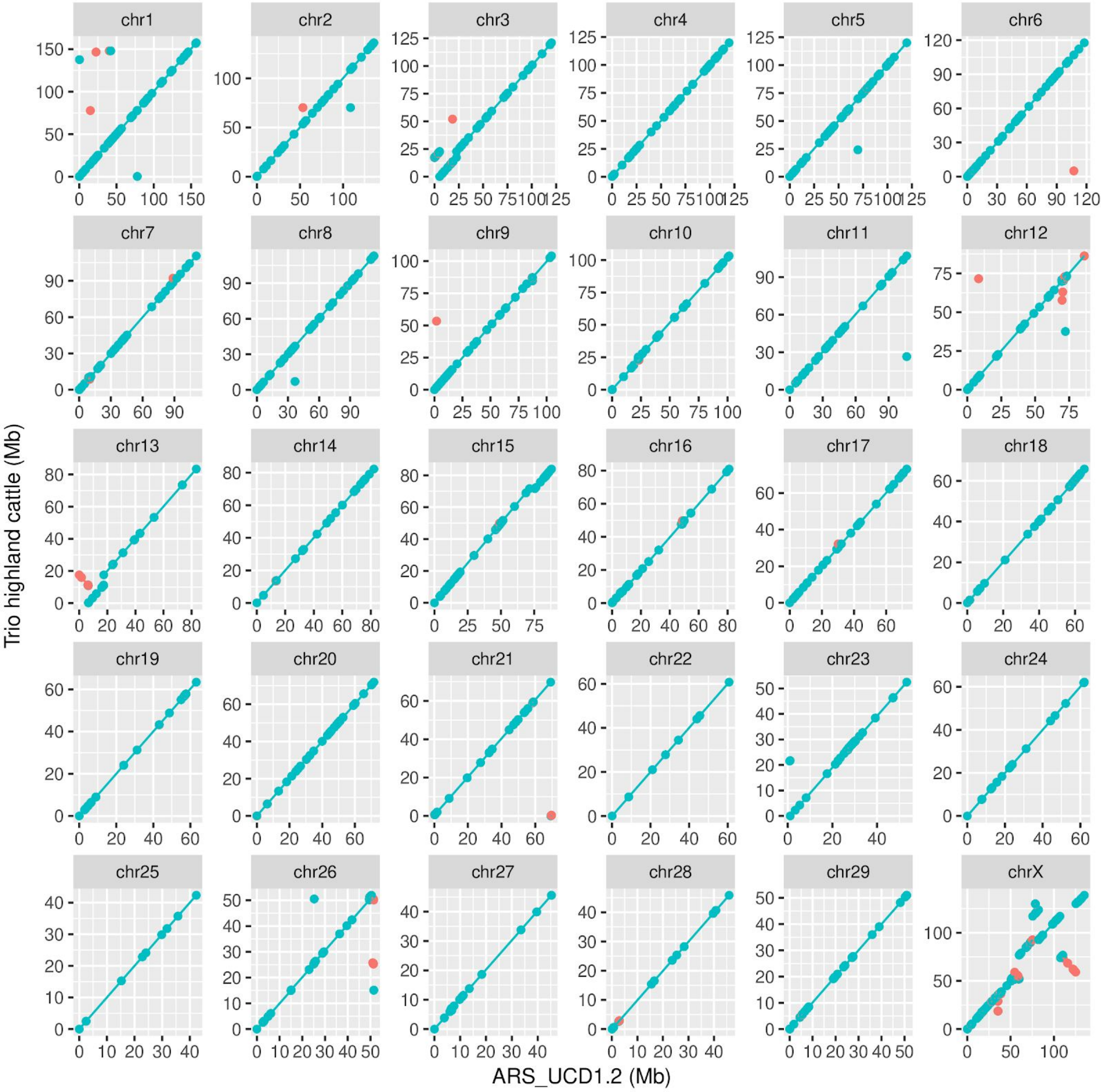


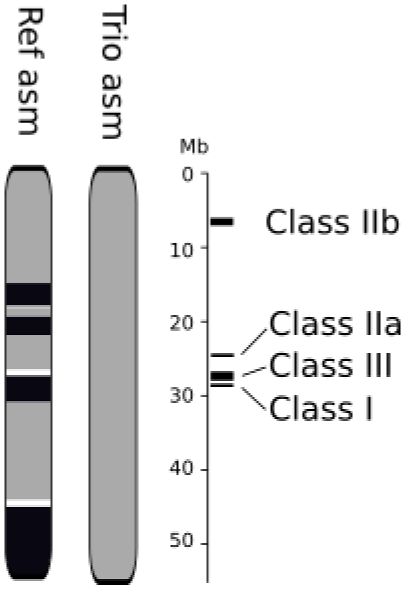
Figure S3. Comparison of twelve yak and cattle genomes to paternal haplotype assembly.



Supplementary Figure S4. Dot plots of alignment of maternal/yak assembly vs. ARS_UCD1.2, the current cattle reference genome, by chromosome. Blue and red colors denote forward and reverse matches, respectively.



Supplementary Figure S5. Dot plots of alignment of paternal/highland cattle assembly vs. ARS_UCD1.2, the current cattle reference genome, by chromosome. Blue and red colors denote forward and reverse matches, respectively.



Supplementary Figure S6. Contiguous haplotypes of chromosome 23 in ARS_UCD1.2 vs. the trio assembly of highland cattle, alongside the locations of the four subclasses of BOLA, the bovine MHC. The four subclasses are all on the same haplotig of the highland cattle assemble, while they are on different contigs in ARS_UCD1.2.

Supplementary Tables

Supplementary Table S1 (attached): Results of verification of Esperanza's parentage.

Supplementary Table S2 (attached): SRA accessions of yak and cattle libraries used for phasing confirmation.

Supplementary Table S3: Intersection of repetitive elements with gaps

	Sire		**Dam**	
Repeat Status	**Closed**	**Trans-Scaffold**	**Closed**	**Trans-Scaffold**
Repeat Consistent[1]	35	25	29	25
Repeat Complex[2]	175	142	172	152
No Repeat[3]	9	12	12	8

[1] The same repetitive element was found on both flank sequences adjacent to the gap. In the case of "Closed" gaps, the intervening sequence also had to have the same repetitive element.

[2] Repeats were either inconsistent on flanking regions, not identified in the intervening sequence, or were present only on one of the flanking sequences and not the other.

[3] No repetitive elements were identified in gap flanking sequences.

Supplementary Table S4: Frequency of repetitive elements flanking gap regions. Values are the number of repetitive elements identified in gap flanking regions or the intervening sequence between flanking sequences.

Repeat Class	**Count**
Line/L1	391
Line/RTE-BovB	288
Sine/tRNA-Core-RTE	167
Sine(other)	223
Simple Repeat	118
LTR-ERV1	72
Satellite/Centromere	65

Supplementary Table S5: Consistency of gap closures between sire and dam assemblies

	Consistent Gap status	Not Consistent
Closed	110	109
Trans-Scaffold	102	77[1]

[1] These discrepancies indicate a structural deviation between the assemblies that may be sub-species specific.