Small variant benchmark from a complete assembly of X and Y chromosomes

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Abstract

The sex chromosomes contain complex, important genes impacting medical phenotypes. This benchmark includes 111,725 variants across the Genome in a Bottle HG002 reference material, advancing variant accuracy across the X and Y chromosomes. This work demonstrates how complete assemblies can expand benchmarks to more difficult regions, while highlighting remaining challenges in variant benchmarking in complex genomic regions such as gene conversions, copy number variable gene arrays, and human satellites.

Introduction

The complete human karyotype includes 22 pairs of autosomes and two sex chromosomes (X and Y). The unique biology of the X and Y chromosomes makes their analysis more difficult than the autosomes in some ways. Indeed, the X and Y chromosomes contain many medically relevant genes, as well as very challenging repetitive regions.[1–5] Chromosomes X and Y mostly have distinct sequences, but two pseudoautosomal regions (PARs) experience crossover events similar to autosomes, and the recently X transposed region (XTR) retains relatively high sequence identity between X and Y.[2,6] Benchmark sets from well-characterized samples are important for understanding variant call accuracy. Previous Genome in a Bottle Consortium (GIAB) benchmarks excluded the X and Y chromosomes due to their mostly-hemizygous (i.e., haploid) nature in half the population,[7,8] which requires customized variant calling methods.[9] However, recently, GIAB has developed approaches to form variant benchmarks by aligning long-read assemblies to the reference,[10,11] enabling the generation of benchmarks for more challenging regions and variants. Here, we create benchmarks that include challenging variants and regions by using complete, polished *de novo* assemblies of the X and Y chromosomes in the GIAB Personal Genome Project Ashkenazi Jewish son HG002 from the Telomere-to-Telomere (T2T) Consortium.[1,12] We also pilot a new systematic approach to evaluate benchmarks to exclude problematic regions and ensure the final benchmark reliably identifies errors in a variety of genome contexts.

Results

Our new benchmark includes many but not all challenging regions. To create a small variant benchmark for SNVs and small indels less than 50 bp in size, we aligned the T2T assembly of HG002's chromosomes X and Y to GRCh38 and called variants with dipcall.[13] We then excluded regions where assembly-based small variant calling or benchmarking tools were unreliable. Based on previous GIAB work,[8] we excluded loci with more than one contig aligning (except in the X PAR), structural variants at least 50 bp in size and associated repeats, large repeats

(segmental duplications, tandem repeats, and satellites) that have any breaks in the assembly-assembly alignment, and regions around gaps in GRCh38.[14]

We refined the benchmark using an active evaluation approach (https://github.com/usnistgov/active-evaluation) as we detail in the Supp Note 1. Based on curation of 12 callsets compared against the draft benchmark (see "Visualizing and curating variants to understand errors" of Olson *et al.*[15]), we further excluded homopolymers longer than 30 bp, tandem repeats discordant with a different HG002 assembly, homopolymers discordant with Element avidity-based sequencing variant calls, as well as regions with ambiguous or incorrect assembled sequence or assembly-assembly alignments (Supp Note 2).

The resulting benchmark contains substantially more challenging variants and regions than previous benchmarks. It includes 94% of chromosome X and 63% of chromosome Y, after excluding the 0.7% of chromosome X and 53% of chromosome Y missing from GRCh38. Of 294 medically relevant genes on these chromosomes,[8] 270 are >90% included; of which 251 are >99% included in the small variant benchmark regions. The benchmark includes some challenging regions like 68% of segmental duplications, and 99% of the XTR.

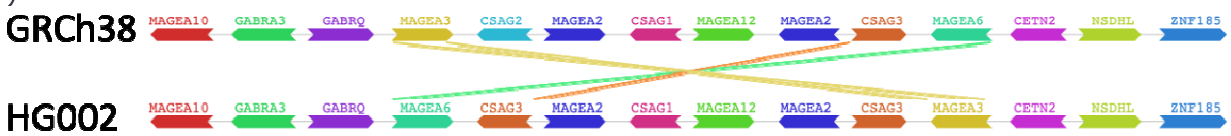
Although the new benchmark excludes the most challenging regions, it correctly identifies many errors in challenging regions, including variants missed by short- and long-read mapping-based approaches. Table 1 describes how older short-read and long-read variant calls from the 2020 precisionFDA Challenge[16,17] perform substantially worse against the assembly-based autosomal Challenging Medically Relevant Gene (CMRG)[8] and XY benchmarks relative to the v4.2.1 benchmark,[7] which used mapping-based approaches except in the MHC[18] (major histocompatibility complex region on chromosome 6). This drop in performance is particularly evident for SNVs in segmental duplications, large indels, and indels in tandem repeats and homopolymers. The benchmark regions also have substantially lower variant density on Y (170/Mbp) compared to X (746/Mbp), as expected from previous work.[19]

Table 1: F1 scores for short-read (Illumina with DeepVariant) and long-read callsets (PacBio HiFi with DeepVariant) from 2020 precisionFDA challenge against three recent GIAB benchmarks: the mapping-based v4.2.1 small variant benchmark for autosomes, the small variant benchmark for 273 challenging medically-relevant autosomal genes (CMRG) in autosomes, and the new XY benchmark in this work. These do not reflect current accuracy, but exemplify how performance metrics can depend substantially on the difficulty of variants and regions included in the benchmark. The lower F1 score for insertions > 15 bp seen against the XY benchmark, especially with long reads, can be attributed to a higher rate of genotype errors.

		Short read (2020)			**Long read (2020)**		
Variant Type	**Region**	**v4.2.1**	**CMRG**	**XY**	**v4.2.1**	**CMRG**	**XY**
SNV	All benchmark	0.997	0.977	*0.899*	1.000	0.981	*0.932*
SNV	Segmental duplications	0.951	0.835	*0.600*	0.991	0.893	*0.785*
INDEL	All benchmark	0.997	0.963	*0.815*	0.996	0.967	*0.738*
INDEL	TRs	0.993	0.915	*0.721*	0.997	0.955	*0.645*
INDEL	Homopolymers longer than 11bp	0.998	0.972	*0.789*	0.990	0.959	*0.677*
Insertions >15bp	All benchmark	0.960	0.821	*0.538*	0.997	0.919	*0.505*

By using alignments of the complete assembly to the GRCh38 reference, we were able to include some gene conversion-like events in the benchmark, where in HG002 the sequence of one GRCh38 region is replaced by the sequence of another similar GRCh38 region. For example, the genes *MAGEA6* and *MAGEA3* are swapped and inverted in HG002 relative to GRCh38, as found previously[20] (Figure 1). The gene *CSAG2* is in the same segmental duplication in GRCh38, and in HG002 the region more closely matches *CSAG3*. Unlike *MAGEA3* and *MAGEA6*, which are swapped, HG002 also still has *CSAG3* in its location on GRCh38, so it is more similar to a stereotypical intrachromosomal gene conversion event. Even further towards the middle of these segmental duplications, *MAGEA2* and *MAGEA2B* are identical in GRCh38, and are also almost identical to each other in HG002, but their sequences in HG002 are diverged from GRCh38. In the middle of this pair of segmental duplications, *CSAG1* and *MAGEA12* have some SNVs but do not appear inverted or converted.

(a)



(b)

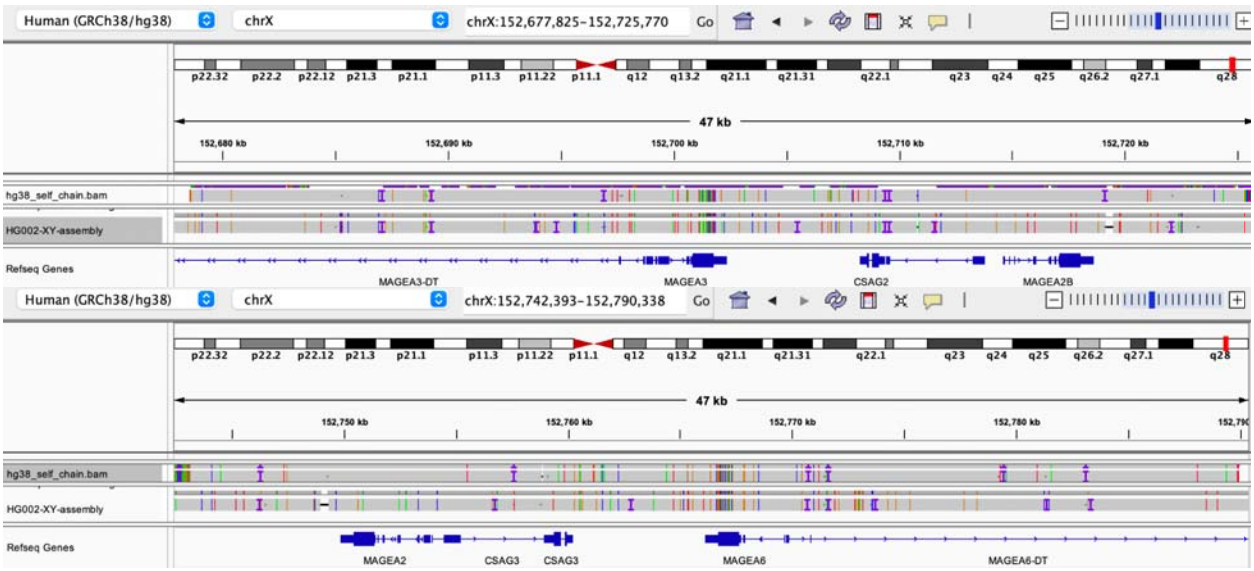


Figure 1: Visualization of gene conversion events included in the benchmark. (a) Visualization of genes in a region containing an inverted segmental duplication pair using the pangene tool (more haplotypes can be seen at http://pangene.liheng.org/view?graph=human98-r183a&gene=MAGEA3&step=3&ori=). (b) IGV visualization showing that many of the variants in *MAGEA3*, *CSAG2*, and *MAGEA6* match the variants in the self-chain alignment of the segmental duplication, indicating a gene conversion-like event.

Beyond gene conversion-like events, the benchmark includes other challenging variants and regions. For example, Supplementary Figure 1 shows an example of complex variants in a tandem repeat in the PAR, where the assembly resolves multiple phased variants on each haplotype within the repeat, which can be challenging for mapping-based approaches. Another challenging region for variant calling in the benchmark is a 856 kbp region on the Y chromosome with a high density of false positives (FPs) due to extra divergent copies of the sequence in HG002 but not GRCh38, shown in Supplementary Figure 2. The assembly resolves the correct sequence in this region, whereas mapping-based methods contain many FPs due to mismapped reads.

We highlight two examples of challenging segmental duplications on chromosome Y that we excluded from the benchmark. The first region is a small known inversion error in the HG002 assembly[1] flanked by a pair of very large segmental duplications (chrY:17,455,804-17,951,260 and chrY:17,954,718-18,450,201). The intervening sequence between these regions is incorrectly inverted and the flanking segmental duplications also contain some small errors. Thus, we excluded the entire region including the segmental duplications (chrY:17,455,804-18,450,201).

The second set of regions excluded involves two different assembly-assembly alignment challenges posed by the TSPY gene family. The TSPY gene family is highly polymorphic in copy number[21] and has been implicated in risk of infertility and cancer.[22,23] The first challenge is that segmental duplications including the *TSPY2* gene were found to be swapped with their homologous sequences about 4 Mbp downstream in HG002 and most individuals relative to GRCh38.[21] We found that standard assembly-based variant calling methods such as dipcall, used in our work, tend to align the assembly contiguously rather than breaking the alignment and aligning *TSPY2* in HG002 to *TSPY2* in GRCh38. This results in variant calls where *TSPY2* is mostly deleted at its location in GRCh38 and inserted in its location in HG002. It is not standardized whether the variants should be called in this way or whether *TSPY2* in HG002 should be aligned to *TSPY2* in GRCh38, resulting in large translocations and smaller variants. Therefore, we chose to exclude these regions from the current benchmark bed file. Since there are multiple segmental duplication pairs annotated in this region of GRCh38 that appear to be swapped in HG002, we specifically excluded the segmental duplication pairs in these regions: chrY:6,234,812-6,532,742 and chrY:9,628,425-9,919,592. In addition to TSPY2 moving about 4 Mbp, the nearby gene *TTTY22* as well as *RBMY2NP, RMBY2GP* and some other TTTY paralogs also swap positions between the segmental duplications chrY:6,234,812-6,532,742 and chrY:9,628,425-9,919,592. Interestingly, all of these genes are annotated differently on T2T-Y by CAT+Liftoff and RefSeq. Where RefSeq seems to match gene sequences, CAT+Liftoff tries to match positions even if the gene sequences differ.

In addition to the *TSPY2* challenge, the TSPY gene array is expanded substantially in HG002 relative to GRCh38, with 46 copies in HG002 versus 9 copies in GRCh38, which has a 40 kb gap (see "Ampliconic genes in composite repeats" in Ref[1]). In this case, no standards exist for whether this should be represented as one or more very large insertions alongside smaller variants, as a copy number variant, as a tandem repeat expansion, or some alternate representation. For these reasons, we excluded the TSPY gene array from the benchmark.

We evaluated the benchmark to test our criteria for Reference Materials to be fit-for-purpose, which in this case is the reliable identification of errors in diverse variant callsets.[15] In this work, we piloted a method for evaluating machine learning systems called Active Evaluation that takes

advantage of stratifying a dataset and estimates a confidence interval for a system's performance. We defined 15 stratifications - as shown in Supplementary Figure 3 - based on homopolymer length, difficult-to-map regions, and pseudoautosomal regions, as well as SNVs vs indels and putative false positives versus false negatives (Supplementary Note 1 and Supplementary Figure 4). We additionally performed long range PCR followed by Sanger sequencing on a subset of variants in challenging genes in chromosomes X and Y as a means of orthogonal validation of the benchmark variants (Supplementary Note 1 and Supplementary Table 2).

Discussion

The chromosome X and Y benchmark generated here, using complete assemblies of HG002 X and Y chromosomes, shows the utility of assembly-based benchmarks to measure accuracy of variant calls, as well as remaining challenges.

We learned several lessons in this work that will be applicable to developing benchmarks in the T2T era. Specifically, better benchmarking tools for tandem repeats, large duplications, and complex SVs are needed. We found that even where assemblies are correct, there is a need for improved assembly-assembly alignment methods and standards for representing variants in regions that do not have 1:1 alignment between an assembly and the reference.[25] The clearest example of this is *TSPY2*, excluded in this current benchmark with plans to revisit this region as part of the HG002 T2T diploid assembly project. Other regions like this include the entire TSPY gene family array due to CNVs and human satellites. Remaining still are a small number of structural errors in these complete X and Y assemblies, such as the inversion on chromosome Y that is also polymorphic in the population as identified by Ref[1]. Additionally, there are still many errors in homopolymers and dinucleotide tandem repeats in current assemblies, which are not completely captured by kmer-based methods typically used to assess assembly error rates. A final consideration is to exclude mosaic variants, which we are in the process of identifying and characterizing.

We acknowledge some limitations of the current HG002 XY v1.0 benchmark, including that it is biased towards HiFi and Element as a result of assembly input and our evaluation process. However, we are working with the HG002 T2T Q100 effort (https://github.com/marbl/HG002) that is polishing diploid assemblies, enabling inclusion of homopolymers in the benchmark that are noisy in HiFi.

Data availability

The benchmark vcf and bed, as well as supporting files, are available at https://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/release/AshkenazimTrio/HG002_NA24385_son/chrXY_v1.0/

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Competing interests

JAL is an employee of PacBio. DF is an employee of Sentieon, Inc., and holds stock options as part of the standard compensation package. PCB sits on the Scientific Advisory Boards of Intersect Diagnostics Inc., Sage Bionetworks and BioSymetrics Inc. LM is an employee and shareholder of Illumina Inc. KS and AC are employees of Google LLC and own Alphabet stock as part of the standard compensation package. FJS has support from ONT, Illumina, Pacbio and Genentech.

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