A comparison of software for analysis of rare and common short tandem repeat (STR) variation using human genome sequences from clinical and population-based samples.

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Abstract

Short tandem repeat (STR) variation is an often overlooked source of variation between genomes. STRs comprise about 3% of the human genome sequence, are highly polymorphic, and some are known to cause not only Mendelian disease, but extensively affect gene expression. Nevertheless, their contribution to common disease is not well-understood, but recent software tools designed to genotype STRs using short read sequencing data are beginning to address this. Here, we compare software that genotypes common STRs and rarer STR expansions genome-wide, with the initial aim of applying them to population-scale genomes. By using the Genome-In-A-Bottle (GIAB) consortium and 1000 Genomes Project sequencing data, we compare performance in terms of sequence length, depth, computing resources needed, genotyping accuracy and number of STRs genotyped. To ensure broad applicability of our findings, we also measure genotyping performance against a set of genomes from clinical samples with known STR expansions, and a set of STRs commonly used for forensic identification. By using analysis of Mendelian inheritance patterns and comparison with capillary electrophoresis genotypes, we find that HipSTR and GangSTR perform well in genotyping common STRs, with GangSTR outperforming HipSTR for genotyping call rate and memory usage. Analysis for expanded STRs showed ExpansionHunter denovo (EHdn) and GangSTR outperformed STRetch, but EHdn used considerably less processor time compared to GangSTR. Analysis on shared genomic sequence data provided by the GIAB consortium allows future performance comparisons of new software approaches on a common set of data, facilitating comparisons and allowing researchers to choose the best software that fulfils their needs.

Introduction

Over the past two decades, most research on the contribution of genetic variation in the human genome to disease has focused on single nucleotide variation. Short tandem repeat (STR) variation has been generally overlooked, as it is not readily assayed by chip-based hybridisation approaches. STRs are short DNA sequence motifs, typically 2-6bp in size, that are repeated multiple times in tandem. They are distributed throughout the human genome, comprising about 3% of the genome sequence, and have a mutation rate generally much higher than single nucleotide variants (around $2x10^{-3}$ per locus per generation for STRs compared to 10^{-8} for single nucleotide variants) (Brinkmann et al., 1998; Burgarella and Navascués, 2011; Willems et al., 2014; Gymrek, 2017). They are frequently polymorphic with multiple alleles in a population, as expected by neutral population genetics theory for loci with high mutation rates.

STRs cause a variety of severe rare monogenic diseases that are inherited in a Mendelian manner (Hannan, 2018). These include triplet-repeat expansion diseases, such as Huntington's disease and myotonic dystrophy, caused by expansion of 3 bp repeat STRs in the coding region of *HTT*, and *DMPK* genes respectively. Some disorders also occur with other repeat motifs in both coding and in non-coding regions (Hannan, 2018; Paulson, 2018; Lieberman et al., 2019; van Kuilenburg et al., 2019). Emerging evidence shows that STRs modulate disease risk in several complex diseases such as autism and neurodegenerative disorders (Hannan, 2020; Trost et al., 2020).

STRs can affect the function of genes in three main ways, either by directly altering the coding region of genes, by disrupting introns, or by affecting expression levels as part of enhancer elements. For example, in addition to STRs encoding coding CAG-repeats leading to polyglutamine tracts in genes such as *HTT*. Polymorphic STRs with GCA, GCC, GCG or GCT motifs also encode polyalanine tracts of varying length in a variety of genes (Lavoie et al., 2003). Other STRs can disrupt introns, such as the expansion of an intronic ATTCT-repeat in spinocerebellar ataxia type 10 (Matsuura et al, 2000). It is well-established that STR variation is associated with expression levels of genes both at a single gene level and at a genome-wide level (Contente et al., 2002; Gymrek et al., 2016; Fotsing et al., 2019). It is unclear the extent to which STRs are responsible for genemowide association signals detected using SNPs. Linkage disequilibrium between STRs and SNPs varies, but is generally lower than between two SNPs at the same recombination distance because the high mutation rate of STRs rapidly breaks down linkage disequilibrium over time. This is shown in attempts to impute STR length genotypes from SNP genotypes, as biallelic STRs can generally be reliably imputed but

multiallelic STRs are much less reliably imputed (Saini et al., 2018).

Given the potential importance of STRs in human disease, genome-wide studies examining STR variation are clearly warranted. The first generation of STR-calling software, including LobSTR (Gymrek et al., 2012), RepeatSeq (Highnam et al., 2013) and HipSTR (Willems et al., 2017), aimed to genotype STRs using reads that completely cover the STR. This led to the limitation that STRs longer than the sequencing read length could not be genotyped. The second generation of STR-calling software uses information from the distance between the paired sequencing reads, in addition to direct sequence information across the repeat, to genotype the locus. This attempts to capture information on STRs that are longer than the sequencing read length and is particularly effective at identifying large expansions at an STR (Bahlo et al., 2018).

We selected software tools that had at least one of the following characteristics: not limited by read length, able to estimate STR allele length, or had not been compared previously. Two recent pieces of software are focused on calling large expansions: Expansion Hunter de Novo (EHdn) (Dolzhenko et al., 2020) and STRetch (Dashnow et al., 2018). Both use sequence reads that span STRs and mate pair distance information to identify large expansions, but the key difference between the two is that EHdn does not require a predefined catalogue of STRs to genotype, unlike STRetch. We also compare two that focus on calling both short and expanded repeats; ExpansionHunter (EH) and GangSTR. Both these software tools use sequence mate pair distance information together with STR-spanning sequence reads. For further assessment, we included HipSTR. Although limited by read length, HipSTR is more robust and was shown to outperform its counterparts (Willems et al., 2017). HipSTR was compared to both GangSTR and EH to genotyping common STR genome wide. Both GangSTR and EH can also identify large expansions and were compared against EHdn and STRetch.

The software tools also differ in the repeat unit sizes they can detect, HipSTR and STRetch are restricted to 1bp-6bp repeat units while GangSTR, EH and EHdn can genotype both STRs (1-6bp repeats) and VNTRs (>6bp repeats). Two additional tools excluded in this study that aim at calling STRs expansions are exSTRa and Tredparse. exSTRa doesn’t estimate the allele length and Tredparse cannot estimate repeat lengths longer than sequencing mate-pair length (Bahlo et al., 2018, Mousavi et al., 2019).

Our motivation for this study was to identify software to genotype common STRs and identify rarer STR expansions on a large dataset suitable for biobank-scale data analysis. We assess five software tools in their ability to genotype known common STRs and identify rarer STR expansions, and their application to studies involving large datasets, with a view to identifying an approach to investigate the role of STRs in human diseases. We use different sequence coverages, read lengths and genomes from different populations to estimate practical parameters with broad applicability, such as run time, memory usage and proportion of STRs

called. We use both Mendelian inheritance in parent-offspring trios and comparison with capillary electrophoresis-based genotypes to measure the genotyping error rate of different approaches. Our genome data includes those from the Genome in a Bottle consortium and 1000 Genomes project, which are collections of publicly available samples analysed by different methods ensuring data can be cross validated as new technical approaches become available (Zook et al., 2016).

Methods

Samples and datasets analysed

An overview of the approaches used in this paper is shown in Figure 1.

The five tools were benchmarked by analysing three different datasets. Firstly, ten gold standard human genome sequences from the Genome in a Bottle consortium (https://github.com/genome-in-a-bottle/giab_data_indexes), secondly, four child-parent trios from the 1000 Genomes project (https://www.internationalgenome.org/data) and thirdly, 116 samples with known trinucleotide repeat mutations obtained from the Coriell Institute (https://ega-archive.org/datasets/EGAD00001003562). The accession numbers for samples obtained from the Genome in a Bottle (GIAB) and the 1000 Genomes Project are listed in **Table 1**. The collated dataset was subsequently analysed as described below.

The 116 whole genomes including previously validated disease-causing STR expansions obtained from Coriell Institute (Dolzhenko et al., 2017) have been validated with one the following repeat expansions: Fragile X Syndrome (FMR1), Huntington disease (HTT), Friedreich's ataxia (FXN), Myotonic Dystrophy (DM1), Spinocerebellar Ataxia 1/3 (ATXN1/3), Spinal and Bulbar Muscular Atrophy (SBMA). The samples were sequenced at 2x150 bp reads on Illumina HiSeqX and repeat expansions detected using ExpansionHunter and standard PCR techniques (Dolzhenko et., al 2017).

Bam file preparation

The seven GIAB bam files were downsampled from their original coverage of either 300x or 100x to a final coverage of ~30x. In summary, a total of 5/7 GIAB samples had an initial genome coverage of ~300x and 2/7 had a genome coverage of ~100x. The 300x genomes were downsampled to 100x and further all samples downsampled to ~30x genome coverage. The bam files were refined to remove sequence duplicates and the quality of the bam files assessed using Qualimap v2.2.1 (Okonechnikov et., al 2016). This data set was collated with 12 30x genomes obtained from the 1000G Project (1 KGP). Overall, we generated 3 datasets: 300x (n = 5), 100x (n = 7) and 30x (n =19) genomes, including 6 child-parent trios, with three individuals sequenced using both 150bp and 250bp paired-end reads (Table 1). These sequences had been aligned to the GRCh38 human genome assembly. All the bam files were sorted and indexed using samtools v1.9 (Li et., 2009) before calling for STR genotypes. The final 30x coverage was chosen to assess the applicability of STR genotyping tools on large cohorts of genomes sequenced at 30x genome coverage. STR genotyping was

performed at the three levels of genome coverage and calls compared across coverage and between the tools.

Table 1 - Samples used in this study

Sample ID	Population	Trio	Collection	Average fold sequence coverage	Paired end Read length (bp)	Accession number
HG002	Askanazi	Son	GIAB	30, 100, 300	150, 250	NA24385
HG003	Askanazi	Father	GIAB	30, 100, 300	150, 250	NA24149
HG004	Askanazi	Mother	GIAB	30, 100, 300	150, 250	NA24143
HG005	Chinese	Son	GIAB	30, 100, 300	250	NA24631
HG006	Chinese	Father	GIAB	30, 100	150	NA24694
HG007	Chinese	Mother	GIAB	30, 100	150	NA24691
NA12878	CEU	-	GIAB	30, 100, 300	150	NA12878
HG00403	CHS	Father	1000G	30	150	HG00403
HG00404	CHS	Mother	1000G	30	150	HG00404
HG00405	CHS	Son	1000G	30	150	HG00405
NA18485	YRI	Son	1000G	30	150	NA18485
NA18487	YRI	Father	1000G	30	150	NA18487
NA18489	YRI	Mother	1000G	30	150	NA18489
HG01500	IBS	Father	1000G	30	150	HG01500
HG01501	IBS	Mother	1000G	30	150	HG01501

HG01502	IBS	Son	1000G	30	150	HG01502
NA06984	CEU	Father	1000G	30	150	NA06984
NA06989	CEU	Mother	1000G	30	150	NA06989
NA12329	CEU	Daughter	1000G	30	150	NA12329

GIAB=Genome in a Bottle

1000G=1000 Genomes Project

STR calling

GangSTR v.2.5.4 was run using default parameters. The raw STR calls were filtered using the DumpSTR tool as recommended by the authors (Mousavi et al 2019). HipSTR v.0.6.2 was run using some non-default parameters (see Data availability). We used reference panels recommended by the software providers (https://github.com/HipSTR-Tool/HipSTR and https://github.com/gymreklab/GangSTR), which are of very different sizes, with GangSTR listing 832,379 STR loci and HipSTR listing 1,638,944 loci. These catalogues have been previously tested and provided by each tool. ExpansionHunter v.5.0.0 was run at default parameters using a catalog provided by the tool (https://github.com/Illumina/RepeatCatalogs). This catalogue contains 174,293 STRs in size of 2-6bps. A STRetch custom reference panel was created using the STRetch pipeline (https://github.com/Oshlack/STRetch/wiki/Reference-Data). This panel contained 353,943 STRs in sizes of 2-6bp. STRetch was run using a whole genome sequencing pipeline (STRetch_wgs_bam_pipeline.groovy) with the default settings. In this study, samples were compared against each other at each loci at the different levels of genome coverage (i.e. 30x,100x and 300x). The putative expanded loci (p value <0.05) were tabulated for each sample. STR calls were filtered to remove 1 bp repeat unit that are prone to sequencing errors. For some analyses, GangSTR and HipSTR were compared using the same reference panel, but this could not be applied to ExpansionHunter as it is sensitive to the presence of Ns in the reference genome.

ExpansionHunter denovo (EHdn) does not require a predefined catalogue of STR genomic coordinates, instead it scans for expanded repeats longer than the read length or fragment length (mate-pair length) by searching for two types of STR reads: (a) Anchored In Repeat Reads (IRR) whose mates confidently align to genomic regions adjacent to the repeat region and (b) IRR where both mates bind within an STR (Dolzhenko et. al 2020). The read counts are compared and Z- scores are calculated for a case sample with counts that exceed the mean distribution. Putative expanded loci with z-score greater than 1.0 are reported. To explore STR expansion profiles in each sample, the sample to be examined was treated as a case sample and the rest of the samples as controls and performed outlier analysis. Putative expanded loci were recorded for each sample.

To assess the sensitivity of GangSTR, EHdn and STRetch at detecting STR expansions, the three tools were run on samples with known clinical STR expansions. These samples were obtained from the Coriell institute as aligned bam files to the hg19 reference genome. EHdn was run in a case- control mode as opposed to outlier analysis. Samples that harbour the same

mutations were treated as cases and samples with alternate expansions used as controls. For STRetch, samples were used as a control to each other and the depth normalised read counts compared to identify significantly expanded loci in each sample relative to the rest. The tools were run using the hg19 reference genome, and the hg19 reference panels containing STR-disease loci (https://stripy.org/expansionhunter-catalog-creator). GangSTR was run with the same parameters described above. However, for Fragile X Syndrome and Spinal and Bulbar Muscular Atrophy, GangSTR was run by specifying the ploidy of the X chromosome for male and female. Our results were compared to those of ExpansionHunter's and PCR validation experiments previously reported for these samples (Dolzhenko et., al 2017, Supplementary table 1).

Data availability

All STR catalogues and code for analyses are available at https://doi.org/10.25392/leicester.data.22041020.

Genotype call vcf files for GangSTR and HipSTR are available for the Genome In a bottle samples are at https://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/

Genotype call vcf files for GangSTR and HipSTR are available for the 1000 Genomes samples used are at https://doi.org/10.25392/leicester.data.19804360.v1

Figure 1 - Flowchart of analyses and samples used in this study

a) Genome in a bottle (GIAB) samples

b) 1000 Genomes project samples

c) Known clinical samples available from Coriell

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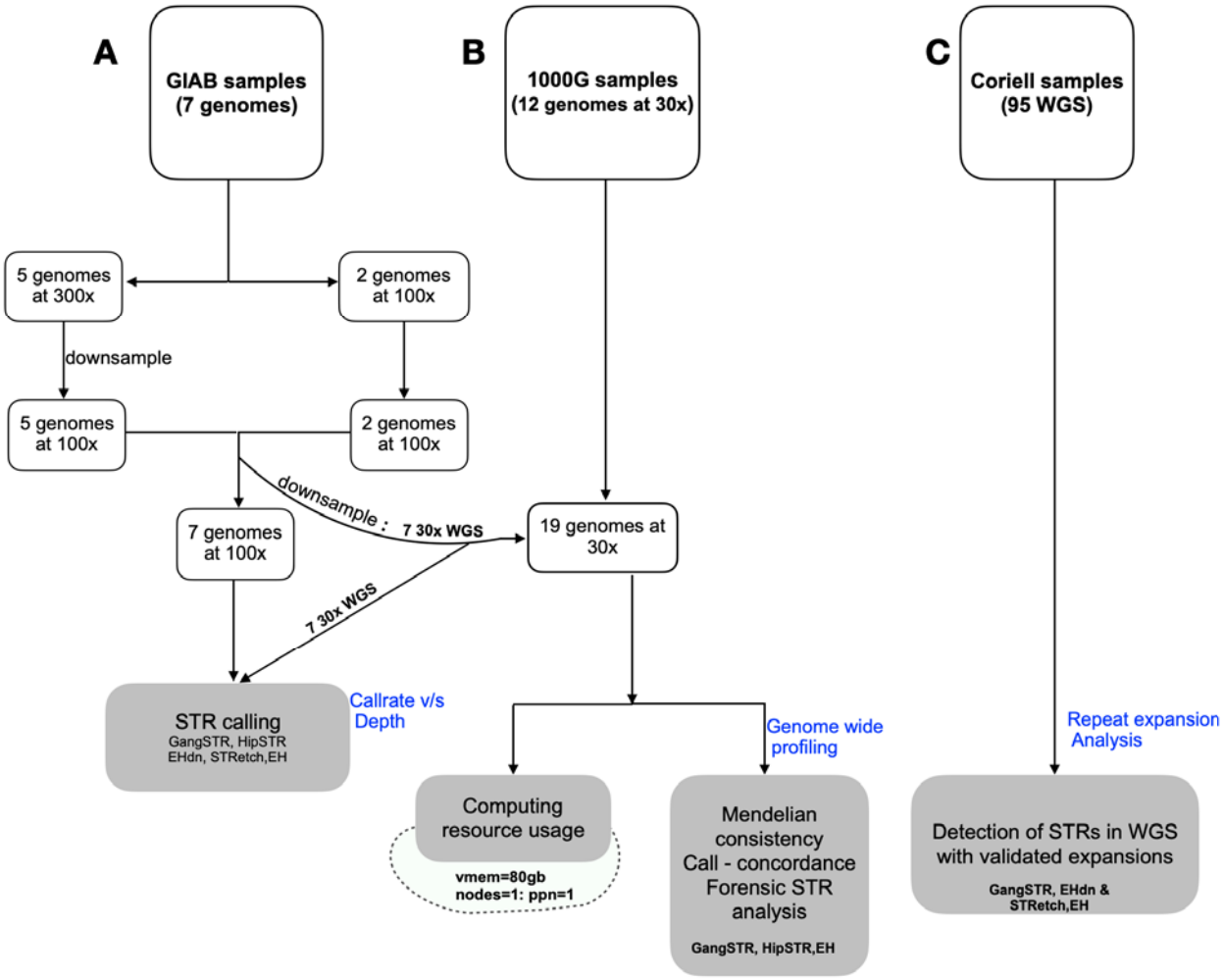


Results

Computing resource usage

For large scale studies involving thousands of samples, the relative processing time of different software tools can become an important aspect of software choice. To evaluate this, we recorded the time and memory taken to process each bam file from five samples sequenced at 30x coverage, using a single core of an Intel Xeon Skylake CPU running at 2.6GHz clock speed with 80Gb RAM available (Table 2). GangSTR, HipSTR and STRetch were run on the same STR panel containing about ~811,899 loci. GangSTR took between 10 and 11 hours, by far the longest time per sample, considerably longer than its comparator software HipSTR, despite calling fewer STRs. This is presumably due to the extra computational time needed to incorporate mate pair distances into the genotype calls. We were unable to run ExpansionHunter (EH) on this custom panel as it ran into errors. The current release of EH is sensitive to the presence of unambiguous nucleotides in regions surrounding the STR in the refence genome and cannot genotype repeats close to the edge of the chromosome (https://github.com/Illumina/ExpansionHunter/issues/136).

STRetch took between 7 and ~9 hours to process a single bam file, compared with EHdn which took much less time, around 30 minutes. However, STRetch is the only software tested with a multithreading option, and increasing the core number from 1 to 28 reduces the time to about 1.5 hours per sample. STRetch also was notable for its high memory usage – typically around 10Gb – in contrast to the other three software tools using ~1Gb or less.

Table 2. Comparison of processing times and memory usage of STR-calling software

	CpuTime (hours:minutes:seconds)				RAM (Gb)			
Sample	gang STR	HipSTR	STRetch	EHdn	gangSTR	HipSTR	STRetch	EHdn
NA18485	11:06:20	3:17:28	7:46:34	00:30:50	0.05	0.39	10.85	0.57
NA18487	11:07:22	2:56:32	7:39:35	00:30:09	0.06	0.45	10.24	0.48
NA06984	10:33:59	3:07:29	7:24:40	00:30:33	0.07	0.37	10.19	0.58
NA06989	9:04:34	2:39:39	6:50:14	00:27:24	0.11	0.38	10.17	0.57
NA12329	10:38:08	2:53:43	8:01:12	00:29:49	0.09	0.39	10.23	0.51

Number of common STR loci genotyped

We compared GangSTR, EH and HipSTR, the three software tools that aim to genotype all STRs across the genome. They all rely on a reference panel of STR loci provided by the user, and the number of the loci in that reference panel provide the upper limit on the number of called STRs. Therefore, our results are presented as a percentage of STRs called using that particular reference panel.

Table 3 shows several broad patterns common to both GangSTR, EH and HipSTR. Firstly, all software tools call a high proportion of the STRs in their respective catalogues across all samples, with GangSTR and EH calling a higher proportion than HipSTR, although the different sizes of their catalogues must be taken into account. Secondly, more calls are made from 100x coverage data than 30x. The increase is small across most samples but is large (over 10 percentage points) for HG005 for HipSTR and GangSTR, where both tools call fewer than 90% of STRs in the 30x coverage data.

Taken together, 30x 150bp is not only sufficient but appears optimal for STR calling, at least using the measures in table 3, there is little benefit in increasing sequencing depth and increasing read length seems slightly detrimental, at least for GangSTR and HipSTR.

Table 3 – Number of loci called as percentage of total in catalogue for GangSTR and HipSTR and EH, and call concordance as function of sequence depth

		GangSTR					HipSTR					ExpansionHunter				
Sample	Read length	Called at 30x (%)	Called at 100x (%)	Calls in both (%)	Identical by allele length (%)	Identical by allele sequence (%)	Called at 30x (%)	Called at 100x (%)	Calls in both (%)	Identical by allele length (%)	Identical by allele sequence (%)	Called at 30x (%)	Called at 100x (%)	Calls in both (%)	Identical by allele length (%)	Identical by allele sequence (%)
HG002	2x150	95.5	98.2	90.7	99.9	NA	87.9	89.5	84.8	99.6	99.3	94.8	99.7	94.8	98.5	NA
HG003	2x150	95.4	98.2	90.7	99.9	NA	87.8	89.4	84.7	99.5	99.2	94.8	99.7	94.8	98.4	NA
HG004	2x150	95.7	97.6	90.6	99.9	NA	87.5	89.0	84.4	99.5	99.2	99.2	99.7	99.1	98.4	NA
HG005	2x250	86.6	98.2	85.3	99.9	NA	52.6	66.2	50.5	99.7	99.3	95	99.7	95	95.8	NA
HG006	2x150	95.1	98.2	90.5	99.9	NA	86.9	89.4	84.1	99.4	99.0	94.8	99.7	94.8	98.3	NA
HG007	2x150	95.6	97.5	90.4	99.9	NA	87.2	89.0	84.3	99.4	99.0	99.2	99.7	99.1	98.3	NA
NA12878	2x150	95.9	97.6	90.7	99.9	NA	72.5	88.3	71.0	99.5	99.1	99.2	99.7	99.2	98.3	NA
HG002	2x250	93.3	NA	NA	NA	NA	69.8	NA	NA	NA	NA	99.6	NA	NA	NA	NA
HG003	2x250	93.1	NA	NA	NA	NA	68.7	NA	NA	NA	NA	99.6	NA	NA	NA	NA
HG004	2x250	94.1	NA	NA	NA	NA	69.4	NA	NA	NA	NA	99.7	NA	NA	NA	NA

N/B: minimum call quality => 0.9

Genotype call concordance of common STRs

To more fairly compare the number of STRs genotyped and accuracy of HipSTR and GangSTR, we called STRs using both software tools and the same STR catalogue of 811899 STRs, included in both reference panels. Over 90% of STRs in the catalogue were called by both GangSTR and HipSTR, with GangSTR calling slightly more STRs (Figure 2a). We compared the actual genotype calls made on the STRs called by both HipSTR vs GangSTR by plotting each allele call for each on a scatterplot, normalised against the allele in the reference genome (Figure 2b). This shows that 87.6% of the calls were identical between HipSTR and GangSTR, and of those calls 95.0% were identical with the reference genome. There was no strong bias in under- or over-calling repeat number made by HipSTR or GangSTR.

Accuracy of common STR calls assessed using Mendelian inheritance

Accuracy of the genotypes made by GangSTR and HipSTR can be measured using the expectation of Mendelian inheritance of alleles, whereby we would expect to see one allele of a child's genotype in their mother and one allele in their father. Any allele in a child not observed in one of the parents is due either to a genotyping error in the child or the parent, or a de novo mutation in the child. Assuming a rate of de novo mutations at STR loci as 10^{-4} per generation, (Willems et al., 2016), the contribution to observed inheritance errors of de novo mutation is minimal and therefore differences in inconsistencies can be attributed to differences in STR genotyping accuracy.

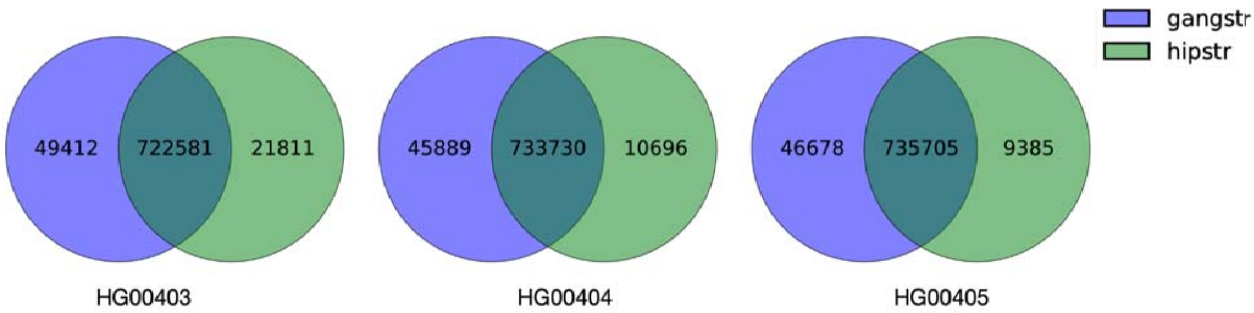
Analysis of Mendelian inconsistencies using bcftools software across five mother-father-child trios shows very little difference in genotyping accuracy between HipSTR and GangSTR (table 4). Comparison between the Ashkenazi trio sequenced at 100x coverage and 30x coverage shows that increased sequencing coverage does not improve genotyping accuracy.

Figure 2 – Comparison of STR calls from HipSTR and GangSTR

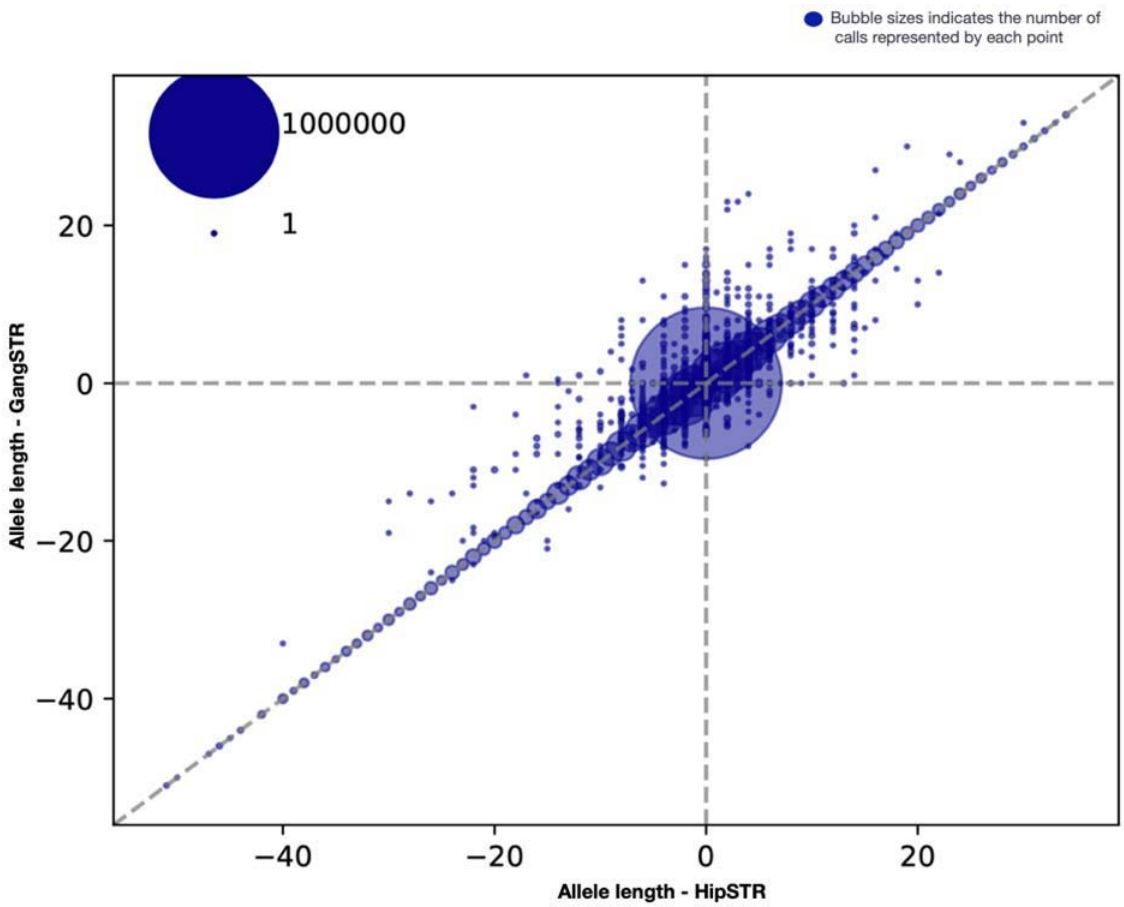
d) Comparison of the numbers of loci called by HipSTR (green) and GangSTR (blue) on three individuals of the CHS trio sequenced at 30x coverage.

e) Comparison of the genotypes of the STRs called by both GangSTR and HipSTR. X axis represents the call made by GangSTR in comparison to the reference sequence (0=same as reference sequence, -20 represents 20 fewer repeat units than the reference sequence. The Y axis represents the call made by HipSTR.

a)



b)


Bubble sizes indicates the number of calls represented by each point
1000000
1
20
0
−20
−40
Allele length - GangSTR
−40
−20
0
20
Allele length - HipSTR

328

Table 4 – Mendelian inheritance of HipSTR and GangSTR

Parent-offspring Trio	**Coverage**	**GangSTR Mendelian consistent (%)**	**HipSTR Mendelian consistent (%)**
Ashkenazi HG002,HG003,HG004	100x	99.0	99.0
Ashkenazi HG002,HG003,HG004	30x	99.3	99.1
YRI: NA18485, NA18489, NA18487	30x	99.1	99.1
CEU: NA06984, NA06989, NA12329	30x	99.8	99.9
CHS: HG00403, HG00404, HG00405	30x	99.9	99.9
IBS: HG01500, HG01501, HG01502	30x	99.2	96.6

In order to investigate the possible reasons for erroneous genotype calls, we stratified the genotypes that showed Mendelian inconsistencies by repeat unit size (figure 3). By comparing against the distribution of STRs stratified by repeat unit size in the reference list, It is clear that, for both HipSTR and GangSTR, there is overrepresentation of 2 bp repeat unit STRs in the incorrect genotype calls. Therefore for both approaches, 2 bp repeat STR genotypes have the highest error rate, over twice the error rate of other STRs.

Genotyping of known forensic STR loci

Thirteen STRs, known as core Combined DNA Index System (CODIS) STRs, are long STRs that show extensive variation between individuals and are used across many different forensic STR panels for identification of individuals from genomic DNA (Butler, 2006; Brearley et al., 2020). Analysis of the performance of GangSTR, EH and HipSTR in calling these STRs in comparison to capillary electrophoresis (CE) data is useful both for practical forensic analysis,

but also as a measure of the error rate in the STR calling of the software, if we accept that capillary electrophoresis can be regarded as a gold standard. Matched capillary electrophoresis data for the 13 core STRs, generated using Promega Powerplex Fusion 24 assay, has been published (Kim et al., 2019), and our calls for NA12878 were compared against these data (table 5). EH calls matched CE data at 10/13 loci, called one allele incorrectly at THO1 locus and two alleles at both FGA and D21s11 loci. GangSTR showed identical genotypes with CE data for 11/13 loci, with one locus (D21S11) not called and one (TH01) calling one allele incorrectly. HipSTR showed the same results as GangSTR, except that, in addition, it incorrectly called one allele at D13S317.The three tools were also consistent across 19/22 forensic STRs analysed for Mendelian inheritance errors across the 6 trios (Supplementary table 2).

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Figure 3 - Repeat unit size distribution of Mendelian inconsistencies

a) HipSTR calls that are Mendelian inconsistent, b) all STRs in the reference panel used by both GangSTR and HipSTR and c) GangSTR calls that are Mendelian inconsistent.

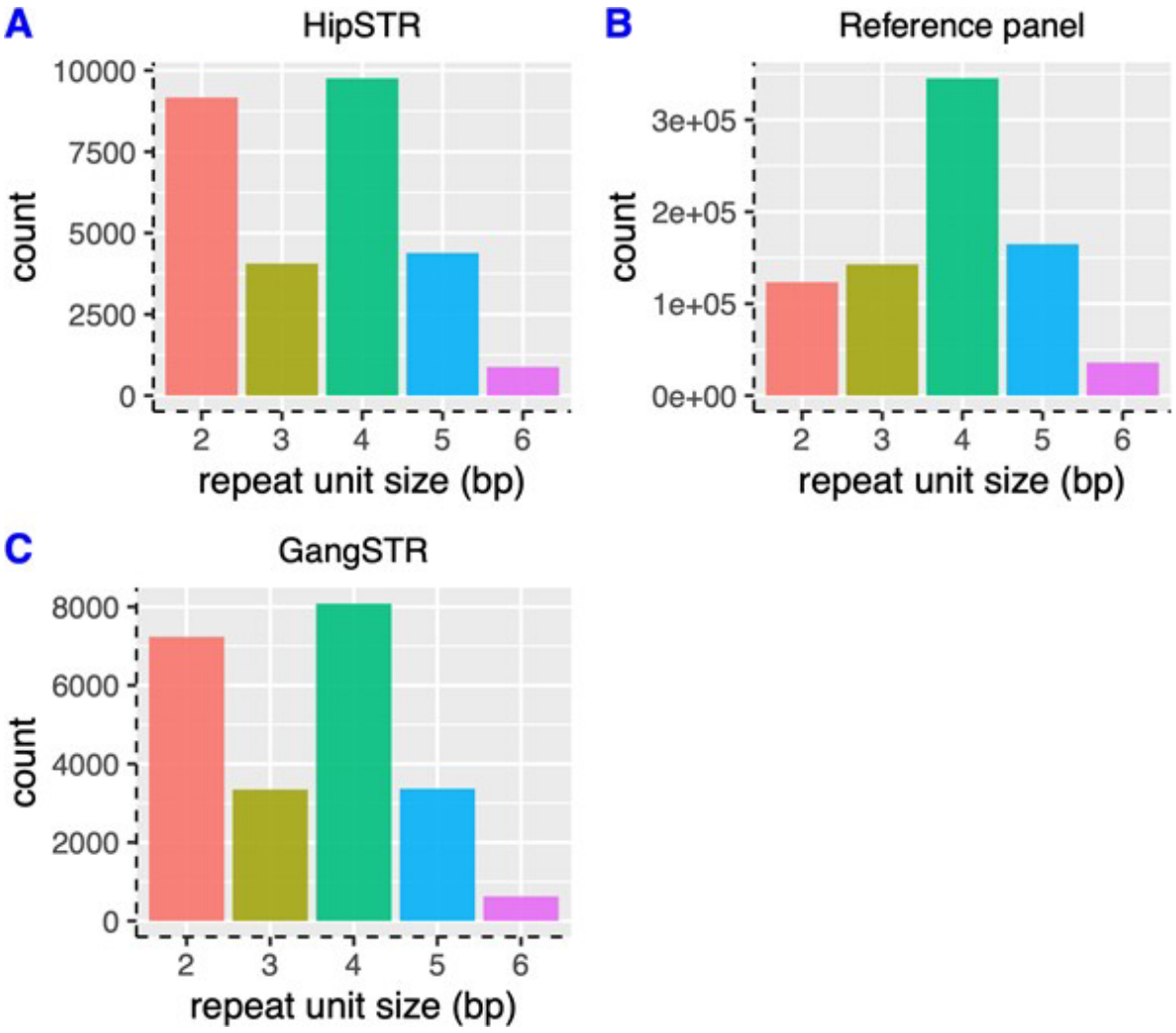

Table 5 - Comparison of STR genotype calls at core forensic loci for NA12878

Gene	Chr	Start	End	Motif	GangSTR 100x	GangSTR 30x	HipSTR 100x	HipSTR 30x	CE
CSF1PO	5	150076324	150076375	ATCT	10,11	10,11	10,11	10,11	10,11
D5S818	5	123775556	123775599	ATCT	12,12	12,12	12,12	12,12	12
D7S820	7	84160226	84160277	TATC	8,10	8,10	8,10	8,10	8,10
D13S317	13	82148025	82148068	TATC	11,12	11,12	11.3,12.3	11.3,12.3	11,12
D16S539	16	86352702	86352745	GATA	10,11	10,11	10,11	10,11	10,11
D21S11	21	19181973	19182099	TCTA	F	F	F	F	30
TH01	11	2171088	2171115	AATG	7,8	7,7	7,9.8	7,9.8	7,9.3
TPOX	2	1489653	1489684	AATG	8,8	8,8	8,8	8,8	8
vWA	12	5983977	5984044	AGAT	15,17	15,15	15,17	15,17	15,17
D3S1358	3	45540739	45540802	TCTA	16,17	16,17	16,17	16,17	16,17
D8S1179	8	124894865	124894916	TATC	12,12	12,12	12,12	12,12	12
D18S51	18	63281667	63281738	AGAA	16,17	16,17	16,17	16,17	16,17
FGA	4	154587736	154587823	GGAA	22,24	22,24	22,24	22,24	22,24

N/B: F=failed QC; CE=capillary electrophoresis genotype

Detection of STR expansions at known clinical loci

To assess the reliability in calling expanded STRs in a real-world situation, we compared STRetch, EHdn and GangSTR performance in calling expanded STRs in clinical samples with known STR expansions (Table 6). All three methods showed high sensitivity, with EHdn (96% sensitivity) and GangSTR (88% sensitivity) outperforming STRetch (68% sensitivity). Although STRetch and GangSTR were able to flag repeat expansions in most of the genes analysed, the two tools underestimated the repeat lengths in comparison to Southern blot experiment at DMPK, FMRI and FXN loci, similar to results were reported for ExpansionHunter (Dolzhenko et al., 2017). These loci were characterised by longer and or double expansions in both alleles ranging between 50 to 1000 repeat units in the samples that were screened. STRetch performed particularly poorly at the FMR1 locus(Table 6). To assess specificity, 21 samples

with known normal allele length at FMR1, with GangSTR calling one of these as expanded. This is a small dataset to robustly test false positive rate, but suggests overall high specificity for the three software tools used.

Table 6 - Detection of STR expansions at known clinical loci

Disease	Gene	Total analysed	Identified using EHDN	Identified using STRetch	Identified using GangSTR
Spinal-bulbar muscular atrophy	*AR*	1	0	0	0
Myotonic dystrophy type 1	*DMPK*	16	16	16	16
Fragile X	*FMR1*	35	35	19	32
Friedreich's ataxia	*FXN*	25	25	11	19
Huntington's disease	*HTT*	13	13	13	13
Dentatorubral-pallidoluysian atrophy	*ATN1*	2	2	2	1
Spinocerebellar ataxia type 1	*ATXN1*	3	0	3	3
Spinocerebellar ataxia type 3	*ATXN3*	1	0	1	1
Total		96	91 (96%)	65 (68%)	85 (88%)
Fragile X (Normal)	*FMR1*	21	0	0	1

Discussion

Genomewide analysis of STRs using short read sequencing has lagged behind studies of other variations, including single nucleotide variation and, to a certain extent, structural variation. It is likely that STRs underly some undiscovered genetic associations with complex disease (Hannan 2010). Several software tools have been developed with the aim of accurately calling STR genotypes genome-wide, but with different ultimate aims. Some have been developed to detect and genotype large repeat expansions that are outliers from the population distribution of allele lengths for thay STR. Development of these was motivated by well- established repeat expansions causing a variety of Mendelian diseases, and more recent discoveries showing that large repeat expansions can underlie a large amount of complex disease, such as ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Other software tools aim to genotype STRs genome-wide irrespective of their alleles, using a catalogue of known STRs.

We selected the most recent software tools most appropriate to our needs in detecting both STR expansions and genome-wide STR genotypes in a population-based sample. A previous study describes detection of six disease-causing STR expansions from whole exome sequences using several STR-calling software tools, including GangSTR and STRetch but not EHdn (Rajan-Babu et al., 2021). Expansion Hunter (Dolzhenko et al., 2019), STRetch and exSTRa (Tankard et al., 2018) were used to detect clinically-related STR expansions in those 6 genes with high specificity. Other reports include those focused on forensic STR detection (Valle-Silva et al., 2022), or are focused on genotyping a particular, or small number, of STR loci (Budiš et al., 2019).

Our aim was to assess the feasibility of applying these software tools, both in terms of quality of the resulting data and practicality of using the software, for large cohorts of genomes. We assessed this in a variety of ways. Processor and memory usage, in conjunction with processing time, was measured so that we could assess the feasibility of scaling up analysis to thousands of genomes given current computing resources. For the software using a catalogue to genotype known STRs (GangSTR, HipSTR and ExpansionHunter), we examined the effect of increasing sequencing depth on the proportion of STRs genotyped, and accuracy. We then examined the concordance between GangSTR and HipSTR using common STR catalogues, and the relative number of Mendelian inconsistencies in trios to assess STR genotyping accuracy. We found sequencing depth to have no effect on the number or quality of STR

genotypes called by HipSTR, but higher sequencing depth had a modest positive effect on GangSTR STR genotype calling. Quality of STR calls for both GangSTR and HipSTR was very similar when given the same STR catalogue, and GangSTR used considerably less memory than HipSTR, but took between 3x and 4x more CPU time. Both GangSTR and HipSTR performed very well, but not perfectly, in genotyping STR loci used for forensics.

The software tools STRetch, EHdn and GangSTR can all call expanded repeats, with only EHdn not requiring a predefined catalogue of STR loci. Because expanded repeats are expected to be rare, and underlie several clinical conditions, we used a previous dataset to test the ability of STRetch, EHdn and GangSTR to genotype known STR expansions. EHdn and GangSTR show higher sensitivity than STRetch, at least under the conditions tested. EHdn used the least resource, analysing a genome in about half an hour using about half a Gb of memory under our conditions.

For case-control studies where thousands of genomes have been sequenced at ~30x coverage for both large expansions and known STRs genomewide, we have decided to use GangSTR and EHdn. EHdn takes fewer computing resources compared to its rivals, and is the only STR genotyper that can genotype expansions without a prior defined catalogue, broadening its scope to identify previously unknown expansions. GangSTR and HipSTR are similar, with the increased computing resources needed by GangSTR offset in our view by its ability to detect larger STR expansions longer than the read length, to support and extend EHdn calls.

We note that, although the clinical repeat expansions and the forensic STRs have been validated by orthogonal data and methods, this is not the case with the other STR genotypes. For STRs that are associated with disease in subsequent analyses, it will be important to validate those particular STRs on a subset of samples using alternative methods, such as capillary electrophoresis. STR genotype calling from high throughput sequencing remains an area of active development, and we hope that further progress can be made in reliably calling STRs from both short read and long read sequencing data.

Our study had some limitations inherent to different tools. First, we did not analyse the 1bp repeat unit motifs. Both GangSTR and EHdn have been optimised to genotype 2-20bp repeat motifs, of which EHdn only genotypes expanded repeats. 1bp repeat motifs present a challenge to many sequencing technologies due to PCR stutter noise and may introduce artefacts in sequence, however, a detailed assessment of accuracy of 1bp repeat motif STR genotyping in PCR-free sequencing is needed. Secondly, apart from EHdn, the rest of the tools require

defined STR coordinates built from reference genomes. Therefore STRs not assembled in the reference genome, including the highly complex regions of the genome that are often hard to sequence and assemble, cannot be genotyped. However, with improvements in PCR free protocols and long read sequencing, combined with new genome assemblies, the ability to genotype these regions will improve.

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Supplementary Materials description

Supplementary table 1

STR genotypes of genomes from samples with a known STR expansion disease

Supplementary table 2

STR genotypes of known forensic STR loci called using GangSTR, ExpansionHunter, and HipSTR