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 and are highly polymorphic. Some cause Mendelian disease, and others affect gene expression. 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 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. We find that HipSTR, ExpansionHunter and GangSTR perform well in genotyping common STRs, including the CODIS 13 core STRs used for forensic analysis. GangSTR and ExpansionHunter outperform HipSTR for genotyping call rate and memory usage. ExpansionHunter denovo (EHdn), STRling and GangSTR outperformed STRetch for detecting expanded STRs, and EHdn and STRling 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.

Particular 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 genomewide association signals detected using SNPs. Linkage disequilibrium between STRs and SNPs varies, but is expected to be 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 multi-allelic 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 sequencing reads that completely span 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 STR. 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).

This study aimed to compare software, suitable for biobank-scale data analysis, that genotypes common STRs and identifies rarer STR expansions genome-wide, with a view to identifying an approach to investigate the role of genomic STR variants in polygenic human diseases. 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. Three recent pieces of software are focused on profiling large repeat expansions: Expansion Hunter De Novo (EHdn) (Dolzhenko et al., 2020), STRetch (Dashnow et al., 2018) and STRling (Dashnow et al., 2022). They all use sequence reads that span STRs and mate pair distance information to identify large expansions, however, unlike STRetch, EHdn and STRling do not require a predefined catalogue of STRs to genotype an STR locus. Therefore EHdn and STRling can also identify novel STR loci not assembled in the reference genome. STRetch, STRling and EHdn can all detect and genotype large repeat expansions in a sample that are outliers from the population distribution of allele lengths. Some of the key differences between these tools are summarised in Table 1. Here we assess the ability of STRetch, STRling and EHdn to identity rarer STR expansions by running them on 116 PCR free WGS containing clinically-validated trinucleotide repeat expansions, normal repeat sizes and or pre-mutations (Dolzhenko et al., 2017).

We also compare three software tools that focus on genome-wide genotyping of both short and expanded (longer than sequencing read length) repeat arrays given a reference catalogue of

STR genomic coordinates: ExpansionHunter,GangSTR and HipSTR. ExpansionHunter and GangSTR use sequence mate-pair distance information together with STR-spanning sequence reads. Although HipSTR does not use mate-pair distance information and therefore limited by sequence read length, a previous study showed that it was shown to outperform its counterparts (Willems et al., 2017), and therefore was included in this study. These three tools report diploid allele lengths. In addition, HipSTR genotypes the allele sequence. HipSTR was compared to both GangSTR and ExpansionHunter to genotyping common STR genome wide. 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 genome wide. We use both Mendelian inheritance in parent-offspring trios and comparison with capillary electrophoresis-based genotypes of forensically-relevant STRs to estimate the genotyping error rate of different approaches. Both GangSTR and ExpansionHunter can also identify large STR expansions and were compared against EHdn, STRling and STRetch to assess their ability to call rarer large STR expansions.
Table 1 in methods summarises difference/similarities of the tools analysed in this study. These tools have been partially compared in literature by the authors but there is still lack of comprehensive comparison of the STR calling tools. Two additional tools that call STR expansions but are excluded from this study are exSTRa and Tredparse. exSTRa does not estimate the allele length and Tredparse cannot estimate repeat lengths longer than sequencing mate-pair length (Bahlo et al., 2018, Mousavi et al., 2019)

The genome data analysed here includes those from the Genome in a Bottle consortium and the 1000 Genomes project, which are collections of publicly-available samples. Our aim in this study is to benchmark different STR-calling software tools primarily using these reference samples. In particular, the Genome in a Bottle consortium provides key reference standard samples analysed by many different methods, so as new technical approaches become available we encourage others to use these same reference samples to facilitate fair comparison of software tools between studies (Zook et al., 2016).

Methods

Samples and datasets

An overview of the approaches used in this paper is shown in Figure 1, and the different features of the software tools are shown in Table 1.

Table 1: Software for genotyping tandem repeats compared

Software	Repeat unit size range (bp)	STR catalogue required?	Genomewide?	Approach	Estimates repeat length and/or sequence	Reference
HipSTR v.0.6.2	1-6	Yes	Yes; only < read length	Individual calling	Both	Willems et al., 2017
GangSTR v.2.5.4	1-20 ;20+	Yes	Yes	Individual calling	Only length	Mousavi et al., 2019
ExpansionHunter v.5.0.0	1-6 ;6+	Yes	Yes	Individual calling	Only length	Dolzhenko et., 2017
STRetch	1-6	Yes	yes, only expansions	Case/control - outlier	Only length	Dashnow et al., 2018
STRling	1-6	No	yes, only expansions	Individual calling & Case/control - outlier	Both	Dashnow et al., 2022
ExpansionHunter Denovo	2-20; 20+	No	yes, only expansions	Case/control -outlier	Both	Dolzhenko et al., 2020

The six tools were benchmarked by analysing three different datasets. Firstly, ten gold-standard high coverage human genome sequences from the Genome in a Bottle consortium (https://github.com/genome-in-a-bottle/giab_data_indexes) consisting of two child-parent trios. Secondly, four child-parent trios randomly selected from the 1000 Genomes project (https://www.internationalgenome.org/data) and thirdly, 116 samples with known trinucleotide repeat mutations obtained from the Coriell Institute (EGA accession number EGAD00001003562). The accession numbers for samples obtained from the Genome in a Bottle (GIAB) and the 1000 Genomes Project are listed in Table 2.

The 116 whole genomes including previously validated disease-causing STR expansions obtained (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 had been sequenced at 2x150 bp reads on Illumina HiSeqX and repeat expansions previously detected using ExpansionHunter and standard PCR techniques (Dolzhenko et., al 2017). All samples had been Illumina sequenced using PCR-free methods. The sequencing methods for these data sets have been described in Dolzhenko et al., 2017, https://doi.org/10.1038/sdata.2016.25 and Byrska-Bishop et al., 2022.

Bam file preparation

The seven GIAB bam files were down-sampled 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 down-sampled to 100x and all samples were down-sampled further to ~30x genome coverage using Picard v2.6.0 software. The final 30x coverage was chosen to assess the applicability of STR genotyping tools on large cohorts of genomes sequenced at 30x genome coverage, which is a standard coverage adopted by the 1000 Genomes project, for example. 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 twelve 30x genomes 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 2). These sequences had been aligned to the GRCh38 human genome assembly (Zook et al., 2016). All the bam files were sorted and indexed using samtools v1.9 (Li et., 2009) before calling for STR genotypes.

Table 2 - Samples used in this study

Sample ID	Population	Trio	Collection	Average fold sequence coverage	Paired-end read length (bp)	Accession number
HG002	Ashkanazi	Son	GIAB	30, 100, 300	150, 250	NA24385
HG003	Ashkanazi	Father	GIAB	30, 100, 300	150, 250	NA24149
HG004	Ashkanazi	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

Computing memory and run time evaluation.

To evaluate compute resource usage, 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. GangSTR, HipSTR, STRetch and ExpansionHunter were run on a custom STR catalogue containing 811899 STRs of 2-6 bp repeat units. This catalogue was built from GangSTR's catalogue (hg38_v13.bed) available at https://github.com/gymreklab/GangSTR, limited to 2-6 bp repeat units. Because ExpansionHunter is sensitive to the presence of unambiguous 'NNs' in a reference genome around the STR, some of the loci were dropped leaving a total of 790661 loci. This custom catalogue used is available at : https://doi.org/10.25392/leicester.data.22041020. GangSTR, HipSTR, and ExpansionHunter were run at default parameters using this custom catalogue. STRetch was run using whole genome sequencing pipeline starting from mapped bam files (Dashnow et al., 2018). STRling and ExpansionHunter Denovo (EHdn) do not require a reference catalogue. STRling was run using single sample pipeline (Dashnow et al., 2022). Both EHdn and STRetch require a control set of samples. To explore STR expansion profiles in each sample, for EHdn, the sample to be examined was treated as a case sample and the rest of the samples as controls and performed outlier analysis. For STRetch, we build a control set from the remaining subset of the genomes analysed in this study using STRetch pipeline (Dashnow et al., 2018).

Comparison of software for genotyping STRs

HipSTR, GangSTR and ExpansionHunter performance was compared genome-wide by assessing: (a) the proportion of STRs genotyped (b) accuracy of the calls made by analysing Mendelian inheritance patterns in 6 child-parent trios and sample call-concordance compared across varying sequence depths and sequence read lengths. First, each tool was run using its own STR catalogue published and tested on the respective tool. These catalogues are of different sizes , with GangSTR listing 832,380 STR loci, HipSTR listing 1,638,945 loci and ExpansionHunter listing 174,293 loci. These catalogues are available at: https://github.com/HipSTR-Tool/HipSTR, https://github.com/gymreklab/GangSTR and https://github.com/Illumina/RepeatCatalogs. HipSTR and ExpansionHunter catalogues consist of STRs of 2-6 bp units while GangSTR consist of 2-20bp repeat units. GangSTR was run using default parameters. HipSTR was run using some non-default parameters : --min-reads 10 and --def-stutter-model. The parameters were used to set the minimum total reads required to genotype a locus from 100 and allow a default stutter model, recommended for running few samples (Willems et al., 2017). ExpansionHunter was run at default parameters, see codes used in data availability section. For comparisons between the three software tools, they were all run using the custom catalogue of 790661 STRs described above. The raw variant calling files (VCF) were filtered using dumpSTR tool to remove calls with read coverage

below 10 and those with abnormally high coverage by setting the max call depth for all tools to 1000 (Mousavi et al., 2021). Additional parameters were used for GangSTR: --filter- spanbound-only and --filter-badCI, to filter out calls where only spanning or bounding reads were found, or calls with the maximum likelihood genotype estimates outside of the 95% bootstrap confidence interval as recommended by the GangSTR authors (Mousavi et al 2019). The performance of the three methods was further assed by comparing the genotype calls to CE data (published in Kim et al., 2019 for sample NA12878) across 13 forensic STRs, known as core Combined DNA Index System (CODIS), supplementary file 1. This panel was further extended to include 9 additional forensic STRs and assessed the accuracy of the 3 software by assessing the mendelian inheritance patterns in the 5 parent-child trios.

Comparison of software for detecting and genotyping STR expansions

To assess the sensitivity of GangSTR, EHdn, STRling and STRetch at detecting STR expansions, the four tools were run on samples with known clinical STR expansions that had been analysed by ExpansionHunter in a previous publication (Dolzhenko, et. al 2017). EHdn was run in a case- control mode as opposed to outlier analysis (Dolzhenko et al 2020). For each clinical STR expansion locus, the samples with those STR expansions were treated as cases, and samples with different STR expansions or normal allele sizes 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. Both STRetch and GangSTR were run using STR catalogues containing 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. STRling was run using joint calling pipeline (Dashnow et al., 2022). Our results were compared to the previously reported validated calls for these samples (Dolzhenko et., al 2017).

Data availability

Code for analyses, and the full set of genotype calls at the clinical and forensic loci are available at https://doi.org/10.25392/leicester.data.22041020.

Genotype call vcf files for GangSTR and HipSTR and ExpansionHunter are available for the Genome In a bottle samples are at https://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/ and https://doi.org/10.25392/leicester.data.22041020

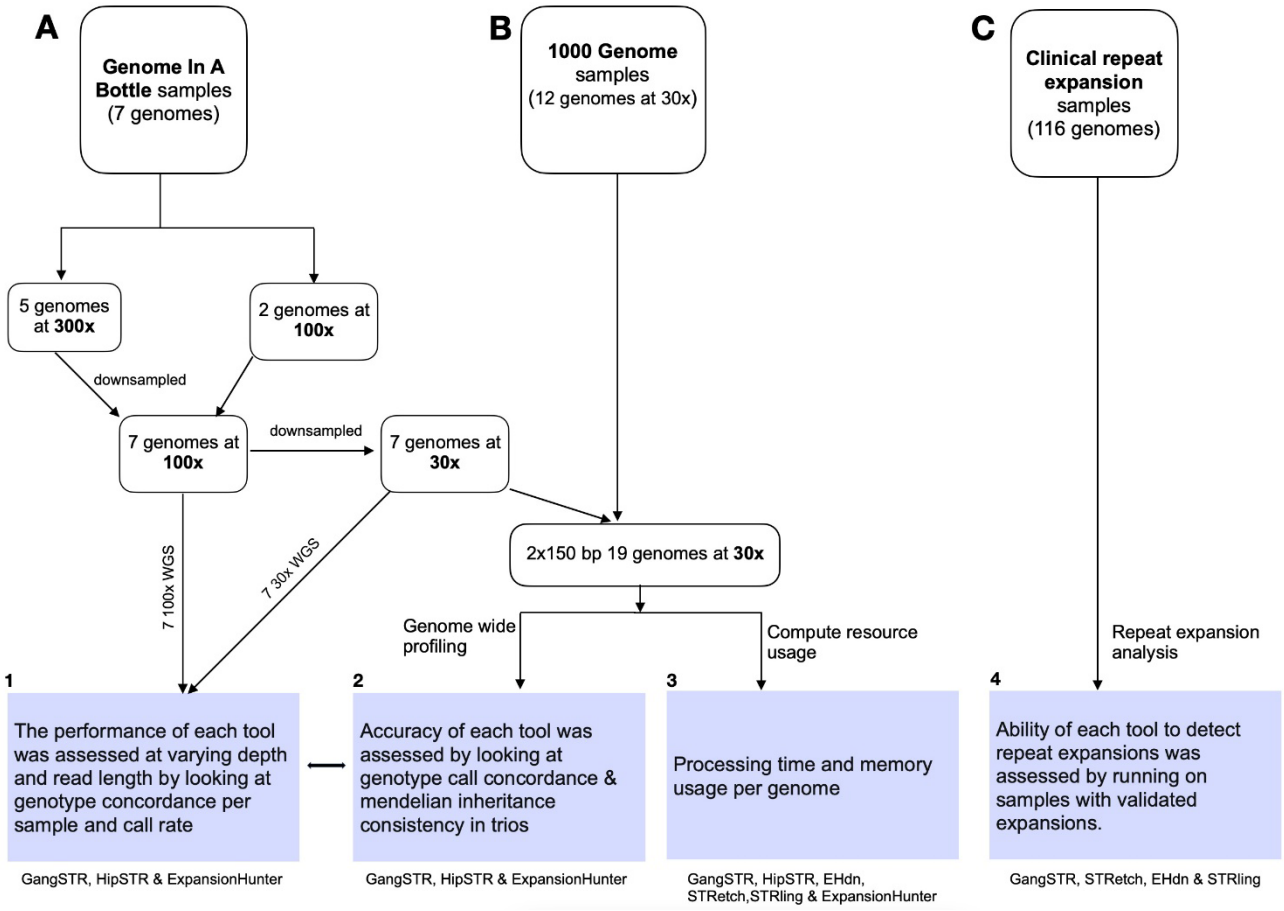
Genotype call vcf files for GangSTR, ExpansionHunter and HipSTR are available for the 1000 Genomes samples used are at https://doi.org/10.25392/leicester.data.22041020

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. We first compared the software tools that use a STR catalogue (Table 3). GangSTR, HipSTR, ExpansionHunter and STRetch were run on the same STR catalogue containing 790661 loci. GangSTR took an average of 10 hours per diploid genome across five samples, STRetch took about 7 hours and HipSTR took about 3 hours. ExpansionHunter default mode (seeking mode) needed more than 7 days and was not run to completion. For the software tools that do not require a software catalogue, EHdn and STRling took around 30 minutes per diploid genome.

STRetch and ExpansionHunter are the only software tools tested with a multithreading option. For STRetch increasing the core number from 1 to 28 reduces the time from 7 hours to about 1.5 hours per diploid genome. Using ExpansionHunter's streaming mode (that is recommended for large genomic catalogs) with at least 16 threads noticeably improved its performance, reducing its run time to an average of 1.5 hours per diploid genome (Table 3). ExpansionHunter was notable for its high memory usage – typically around 70Gb in contrast to the other 5 software tools using either ~17 Gb or less than 1GB (Table 3).

Table 3. Comparison of processing times and memory usage of STR-calling software

	CPU Time (hours:minutes:seconds)							**RAM in Gb**						
Sample	GangSTR	HipSTR	STRetch	EH (default mode)	EH (streaming mode)	EHdn	STRling	GangSTR	HipSTR	STRetch	EH (default mode)	EH (streaming mode)	EHdn	STRling
NA18485	10:33:01	3:58:53	06:39:38	> 168 h	01:33:22	00:30:50	00:24:33	0.04	0.50	17.63	n/a	69.80	0.57	1.16
NA18487	10:27:03	3:00:32	07:10:47	> 168 h	01:28:28	00:30:09	00:24:03	0.03	0.49	17.60	n/a	72.56	0.48	0.87
NA06984	10:13:42	2:47:50	06:21:20	> 168 h	01:23:40	00:30:33	00:24:19	0.04	0.40	17.42	n/a	70.11	0.58	1.16
NA06989	8:41:14	2:34:05	05:57:05	> 168 h	01:17:33	00:27:24	00:22:11	0.04	0.42	17.51	n/a	69.82	0.57	0.82
NA12329	10:24:04	2:50:49	07:15:59	> 168 h	01:19:26	00:29:49	00:23:42	0.05	0.34	17.40	n/a	72.57	0.51	0.76
Average	10:03:48	03:02:25	06:40:57	n/a	01:24:29	00:29:45	00:23:45	0.04	0.43	17.51	n/a	70.97	0.54	0.96

Number of common STR loci genotyped and genotype call concordance of common STRs.

We compared GangSTR, ExpansionHunter and HipSTR, which are the three software tools that aim to genotype all STRs across the genome. They all rely on a catalogue of STR loci provided by the user, and the number of the loci in that catalogue provide the upper limit on the number of genotyped STRs. Therefore, our results are presented as a percentage of STRs called using that particular catalogue. We first used the different catalogues provided with each software tool, and found several patterns common to both GangSTR, ExpansionHunter and HipSTR (Supplementary table 1). Firstly, all software tools call a high proportion of the STRs in their respective catalogues across all samples, with GangSTR and ExpansionHunter 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 is not only sufficient but appears optimal for STR calling, with little benefit in increasing sequencing depth. For read length, 150bp paired-end is optimal and increased read length seems slightly detrimental, at least for HipSTR, which made fewer calls across the four samples that were sequenced at 2x250bp.

To ensure a fair comparison across tools, we repeated our analysis using the same STR catalogue containing 790661 loci (Table 4). For all software tools and samples, 90% of STRs in this catalogue were called, with most calling >99%. We compared the actual genotype calls made on the STRs called by three tools by plotting each allele call for each on a scatterplot, normalised against the allele in the reference genome (Figure 2). This shows that over 85% of the calls were identical between the three methods, and of those calls, at least 89.0% were identical with the reference genome. There was no strong bias in under- or over-calling repeat number made by HipSTR, ExpansionHunter or GangSTR. There was high concordance of the genotype calls made at 2x150bp compared to 2x250bp for each tool (Supplementary table 1).

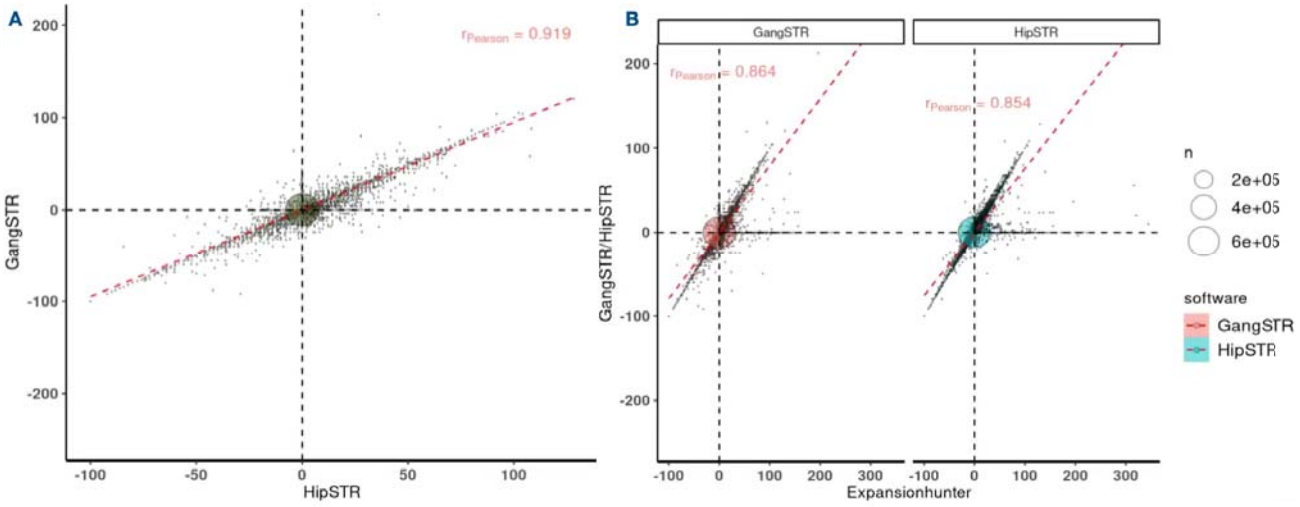
Table 4 – Percentage of loci called in a shared STR catalogue by GangSTR and HipSTR and ExpansionHunter

		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	99.7	99.7	99.6	99.4	NA	96.2	96.0	95.5	99.8	99.9	99.8	99.9	99.8	98.9	NA
HG003	2x150	99.7	99.7	99.6	99.4	NA	96.1	96.0	95.5	99.8	99.9	99.8	99.9	99.8	99.8	NA
HG004	2x150	99.2	99.2	99.1	99.4	NA	95.8	95.5	95.2	99.8	99.9	99.3	99.5	99.3	98.9	NA
HG005	2x250	98.5	99.6	98.4	99.6	NA	93.9	94.3	92.9	99.7	99.1	99.8	99.9	99.8	98.4	NA
HG006	2x150	99.4	99.5	99.3	99.3	NA	96.0	95.9	95.0	99.8	99.9	99.7	99.9	99.7	98.7	NA
HG007	2x150	99.0	99.1	98.9	99.3	NA	95.7	95.4	95.0	99.8	99.8	99.3	99.5	99.3	98.7	NA
NA12878	2x150	99.1	99.1	98.9	99.3	NA	95.7	95.4	95.0	99.8	99.8	99.3	99.5	99.3	98.8	NA
HG002	2x250	96.1	NA	NA	NA	NA	94.1	NA	NA	NA	NA	99.3	NA	NA	NA	NA
HG003	2x250	95.8	NA	NA	NA	NA	93.9	NA	NA	NA	NA	99.8	NA	NA	NA	NA
HG004	2x250	96.8	NA	NA	NA	NA	93.9	NA	NA	NA	NA	99.4	NA	NA	NA	NA

NA: not analysed

Figure 2 – Comparison of STR calls from ExpansionHunter, HipSTR and GangSTR

A). Comparison of the HG002 genotypes of the STRs called by both GangSTR and HipSTR. The x-axis represents the call made by GangSTR in comparison to the reference sequence (0=same as reference sequence, -100 represents 100 fewer repeat units than the reference sequence. The y-axis represents the call made by HipSTR. B) Comparison of the genotypes of the STRs called by either GangSTR or HipSTR compared to ExpansionHunter (x-axis). The dotted red lines shows correlation between the calls compared.



Accuracy of common STR calls assessed using Mendelian inheritance

Accuracy of the genotypes made by GangSTR, ExpansionHunter 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 the five mother-father-child trios with 150bp sequence reads shows very little difference in genotyping accuracy across the three methods. GangSTR and HipSTR showed the highest genotyping accuracy (table 5). Comparison between the HG002,HG003,HG004 trio sequenced at 100x coverage and 30x coverage shows that increased sequencing coverage does not improve genotyping accuracy.

Table 5 – Mendelian inheritance of STR alleles

Parent-offspring Trio	Coverage	GangSTR Mendelian consistent (%)	HipSTR Mendelian consistent (%)	ExpansionHunter Mendelian consistent (%)
HG002,HG003,HG004	100x	99.5	99.6	99.2
HG002,HG003,HG004	30x	99.7	99.5	98.7
NA18485,NA18489,NA18487	30x	99.6	99.4	98.5
NA06984,NA06989,NA12329	30x	99.8	99.9	98.9
HG00403,HG00404,HG00405	30x	99.9	99.9	99.0
HG01500,HG01501,HG01502	30x	99.7	99.6	98.7

In order to investigate the possible reasons for erroneous genotype calls, we stratified the genotypes that showed Mendelian inconsistencies by repeat unit size normalised against the repeat-unit counts in the reference list (Figure 3). It is clear that for the three tools; ExpansionHunter, HipSTR and GangSTR, there is overrepresentation of 2 bp repeat unit STRs in the incorrect genotype calls. Therefore, for these methods 2 bp repeat STR genotypes have the highest error rate, over twice the error rate of other STRs (supplementary figure 1).

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, ExpansionHunter 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 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 6). ExpansionHunter calls matched CE data at 10/13 loci, called one allele incorrectly at the THO1 locus and two alleles at both FGA and D21S11 loci. GangSTR genotypes matched CE data for 11/13 loci, with one locus (D21S11) not called and one (TH01) called 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 six trios (Supplementary file 1).

Detection of STR expansions at known clinical loci

To assess the reliability in calling expanded STRs in a clinical situation, we compared STRetch, EHdn and GangSTR performance in calling expanded STRs in samples with known STR expansions (Table 7). All four methods showed high sensitivity, with EHdn (95% sensitivity), GangSTR (89% sensitivity) and STRling (94% sensitivity) outperforming STRetch (68% sensitivity). Although STRetch, STRling and GangSTR were able to flag repeat expansions in most of the genes analysed, the three tools underestimated the repeat lengths in comparison to Southern blot results at *DMPK*, *FMR1* and *FXN* loci (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 (supplementary file 1). Both STRetch and STRling performed poorly at the *FMR1* locus (Table 7). Of the samples identified as expanded ($p<=0.05$; adjusted for multiple testing) at the *FMR1* locus by STRetch, all had reported repeat lengths below the expected *FMR1* premutation ranges (Hannan et al., 2018). For STRling, half the samples identified had reported repeat lengths below the expected *FMR1* premutation ranges. To assess specificity, 21 samples with known non-pathogenic allele length at *FMR1* were analysed, with STRling called two of these as expanded and GangSTR identifying one sample with an expanded allele length (supplementary file 2). This is a small dataset to robustly test false positive rate, but suggests overall high specificity for the three software tools used.

Figure 3 – Repeat unit size distribution of Mendelian inconsistencies

a) ExpansionHunter, GangSTR and HipSTR calls that are Mendelian inconsistent as a proportion of motif counts in the catalogue, b) all STRs in the catalogue used by the three software tools.

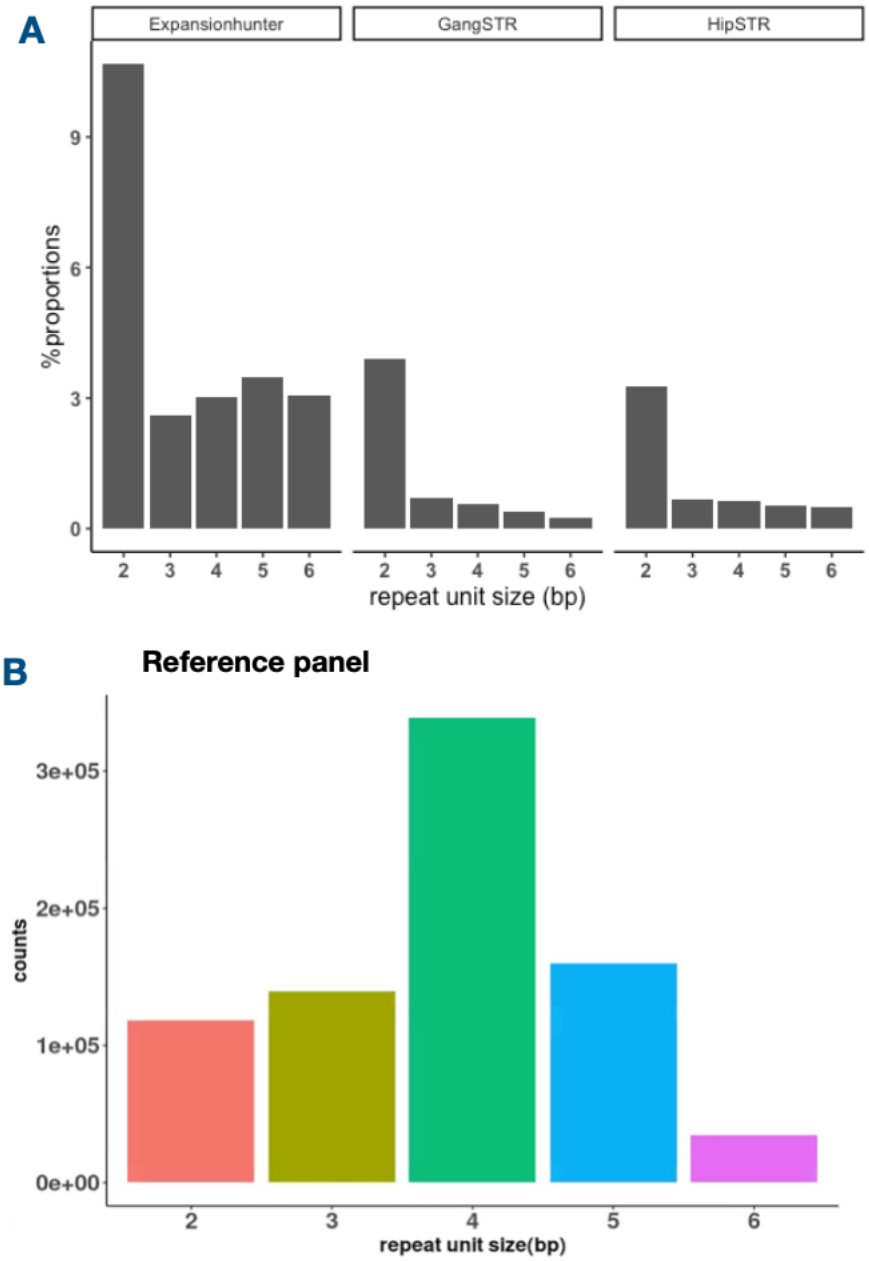


Table 6 - Comparison of STR genotype calls at core forensic loci for NA12878

Locus	Chrom	Start	End	Motif	GangSTR 100x	GangSTR 30x	HipSTR 100x	HipSTR 30x	ExpansionHunter 100x	ExpansionHunter 30x	CE
CSF1PO	5	150076324	150076375	ATCT	10,11	10,11	10,11	10,11	10,11	10,11	10,11
D5S818	5	123775556	123775599	ATCT	12,12	12,12	12,12	12,12	12,12	12,12	12,12
D7S820	7	84160226	84160277	TATC	8,10	8,10	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	11,12	11,12
D16S539	16	86352702	86352745	GATA	10,11	10,11	10,11	10,11	10,12	10,11	10,11
D21S11	21	19181973	19182099	TCTA	-	-	-	-	18,34	34,34	30,30
TH01	11	2171088	2171115	AATG	7,8	7,7	7,9.8	7,9.8	7,10	7,10	7,9.3
TPOX	2	1489653	1489684	AATG	8,8	8,8	8,8	8,8	8,8	8,8	8,8
vWA	12	5983977	5984044	AGAT	15,17	15,15	15,17	15,17	15,17	15,17	15,17
D3S1358	3	45540739	45540802	TCTA	16,17	16,17	16,17	16,17	16,17	16,17	16,17
D8S1179	8	124894865	124894916	TATC	12,12	12,12	12,12	12,12	12,13	12,13	12,12
D18S51	18	63281667	63281738	AGAA	16,17	16,17	16,17	16,17	16,17	16,17	16,17
FGA	4	154587736	154587823	GGAA	22,24	22,24	22,24	22,24	23,25	23,25	22,24

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

Table 7 - Detection of STR expansions at known clinical loci

Disease	Gene	Total analysed	Identified using EHdn	Identified using STRetch	Identified using GangSTR	Identified using STRling
Spinal-bulbar muscular atrophy	*AR*	1	0	0	0	0
Myotonic dystrophy type 1	*DMPK*	16	16	16	16	16
Fragile X	*FMR1*	34	34	19	32	33
Friedreich's ataxia	*FXN*	25	25	11	19	24
Huntington's disease	*HTT*	13	13	13	13	12
Dentatorubral-pallidoluysian atrophy	*ATN1*	2	2	2	1	2
Spinocerebellar ataxia type 1	*ATXN1*	3	0	3	3	1
Spinocerebellar ataxia type 3	*ATXN3*	1	0	1	1	1
Total		95	90 (95%)	65 (68%)	85 (89%)	89 (94%)
Unaffected		21	0	0	1	2

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 that particular 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 and STRling (Rajan-Babu et al., 2021). ExpansionHunter (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, ExpansionHunter 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 made by Expansionhunter, GangSTR and HipSTR was very similar

but slightly higher between GangSTR and HipSTR when given the same STR catalogue. Both GangSTR and HipSTR used less memory than Expansionhunter, but GangSTR took about 3x more CPU time. Both GangSTR and HipSTR performed very well, but not perfectly, in genotyping STR loci used for forensics.

The software tools STRetch, STRling, ExpansionHunter, EHdn and GangSTR can all call expanded repeats, with only EHdn and STRling 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, STRling, EHdn and GangSTR to genotype known STR expansions. EHdn, STRling and GangSTR show higher sensitivity than STRetch, at least under the conditions tested. EHdn and STRling 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 for known STRs and EHdn for large expansions. Although STRling used similar time and memory as EHdn when run in a single sample mode, its resource usage can increase linearly in joint calling mode which is appropriate for case-control outlier analysis (Dashnow *et al*, 2022). EHdn and STRling are the only STR genotypers that can genotype expansions without a prior defined catalogue, broadening their 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. ExpansionHunter default mode was unusable at genotyping a catalog of 790661 STR loci as it needed more than 7 days but efficient in streaming mode albeit higher memory requirements about 70 Gb per genome using 16 cores.

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 and STRling, 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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Ethics statement

All sequencing data used in this study are from previous studies, from fully consented individuals. Sample collections are from the 1000 Genomes project (https://www.internationalgenome.org/sample_collection_principles/) , Personal Genome Project Canada (https://personalgenomes.ca/) , or NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research.

Authorship statement

All authors conceived and designed the study. JWO sourced data and carried out the analysis. EJH and JWO wrote the paper, with input from all authors.

Conflict of Interest statement

The authors declare no conflict of interest.

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