Aligner optimization increases accuracy and decreases compute times in multi-species sequence data

Kelly M. Robinson, Aziah S. Hawkins, Ivette Santana-Cruz, Ricky S. Adkins, Amol C. Shetty, Sushma Nagaraj, Lisa Sadzewicz, Luke J. Tallon, David A. Rasko, Claire M. Fraser, Anup Mahurkar, Joana C. Silva and Julie C. Dunning Hotopp

Abstract
As sequencing technologies have evolved, the tools to analyze these sequences have made similar advances. However, for multi-species samples, we observed important and adverse differences in alignment specificity and computation time for BWA-MEM (Burrows-Wheeler aligner-maximum exact matches) relative to BWA-ALN. Therefore, we sought to optimize BWA-MEM for alignment of data from multi-species samples in order to reduce alignment time and increase the specificity of alignments. In the multi-species cases examined, there was one majority member (i.e. Plasmodium falciparum or Brugia malayi) and one minority member (i.e. human or the Wolbachia endosymbiont wBm) of the sequence data. Increasing BWA-MEM seed length from the default value reduced the number of read pairs from the majority sequence member that incorrectly aligned to the reference genome of the minority sequence member. Combining both source genomes into a single reference genome increased the specificity of mapping, while also reducing the central processing unit (CPU) time. In Plasmodium, at a seed length of 18 nt, 24.1 % of reads mapped to the human genome using 1.7±0.1 CPU hours, while 83.6 % of reads mapped to the Plasmodium genome using 0.2±0.0 CPU hours (total: 107.7 % reads mapping; in 1.9±0.1 CPU hours). In contrast, 97.1 % of the reads mapped to a combined Plasmodium-human reference in only 0.7±0.0 CPU hours. Overall, the results suggest that combining all references into a single reference database and using a 23 nt seed length reduces the computational time, while maximizing specificity. Similar results were found for simulated sequence reads from a mock metagenomic data set. We found similar improvements to computation time in a publicly available human-only data set.

DATA SUMMARY
The human dataset analyzed during the current study is available from the 1000 Genomes Project in the Sequence Read Archive (SRA) (http://www.internationalgenome.org, accession number ERR022446), the three Plasmodium–human datasets are available from the Wellcome Trust Sanger Institute in the SRA (accession numbers ERR015379 https://www.ncbi.nlm.nih.gov/sra/?term=ERR015379, ERR012739 https://www.ncbi.nlm.nih.gov/sra/?term=ERR012739, and ERR015360 https://www.ncbi.nlm.nih.gov/sra/?term=ERR015360), the Brugia–Wolbachia dataset is available from the University of Maryland Institute for Genome Sciences in the SRA (accession number SRR5188379 https://www.ncbi.nlm.nih.gov/sra/?term=SRR5188379) and the simulated data from the mock metagenome dataset is available from the University of Maryland Institute for Genome Sciences in Dryad doi:10.5061/dryad.m1m0p. Tables S1, S2, and S3 are available in the online Supplementary Material.

INTRODUCTION
One of the most popular tools for aligning genome sequencing reads to a reference genome is the Burrows–Wheeler aligner (BWA). The BWA software package allows users to align highly similar sequences to a large reference genome based on searches for exact matches with the Burrows–Wheeler transform [1]. There are three different BWA algorithms: BWA-ALN (or BWA-backtrack), BWA-SW (Smith–Waterman Alignment) and BWA-MEM (maximum exact matches). BWA-ALN was the original BWA algorithm and was
developed for 36–100 bp reads [1]. **BWA-ALN** allows for gaps and mismatches, facilitating the identification of insertions and deletions [1]. When originally implemented, **BWA-ALN** was 10–20× faster than the leading alignment tool, without sacrificing accuracy [1].

**BWA-SW** was originally used for mapping longer reads, like those originating from the 454 platform, but was updated for use with 200–1 000 000 bp sequencing reads [1]. As the length of sequencing reads increases, so does the error rate and the potential for structural variation within the reads. Therefore, alignment algorithms for longer reads must switch from global matches to local matches to accommodate this error rate and potential structural variation [1]. **BWA** also implemented a Smith–Waterman-like dynamic program to quickly align the query sequence to all suffix trees of the reference [1]. In addition to this seed-and-extend approach, heuristics were added to further increase the speed of **BWA-SW** [1]. However, as the amount of data generated by large-scale genomics projects increased and read length decreased, **BWA-SW** lacked the ability to analyze the data [1].

The most recent addition to the **BWA** suite is **BWA-MEM** [2]. **BWA-MEM** was implemented to increase the speed of aligning 100–1000 bp sequences to a large reference genome, while maintaining accuracy [2]. **BWA-MEM** has the ability to perform chimeric alignments, handle sequencing errors, and can choose between local or global/end-to-end alignments [2]. It is ideal for sequencing reads >70 bp and can handle reads up to 1 Mbp [2]. **BWA-MEM** uses a seed-and-extend approach and extends the seed with an affine-gap Smith–Waterman algorithm, but also implements re-seeding to reduce incorrect mappings from a missing seed [2]. **BWA-MEM** also employs heuristics that prevent it from extending an alignment through a poorly mapping area [2]. Another significant improvement of **BWA-MEM** is that larger reference databases can be used. With **BWA-ALN**, the complete reference index was limited to around the length of the human genome [1], but **BWA-MEM** can now handle longer references [2]. **BWA-MEM** is reported to be much faster than other aligners, and provides accurate alignments for both short and long reads [2].

Due to the speed and accuracy of **BWA**, it has been the aligner of choice for many different types of analyses from various organisms. **BWA** has been used for the analysis of all phases of the 1000 Genomes Project, studying multiple types of genetic variation in thousands of individuals across the globe [3–5]. In addition to studying this genetic variation, **BWA** has also been used to align cancer genome sequences to the human genome, including ovarian cancer [6], stomach cancer [7], prostate cancer [8], renal-cell cancers [9–11], cutaneous melanoma [12], head and neck squamous cell carcinoma [13], lung cancers [14, 15], papillary thyroid cancer [16], urothelial bladder carcinoma [17], acute myeloid leukemia [18], colon and rectal cancer [19], breast cancer [20], endometrial cancer [21], and glioblastoma [22].

### IMPACT STATEMENT

As most organisms do not live in isolation, it can be informative to study combinations of organisms in co-culture. This has been greatly facilitated by ever improving sequencing data and tools. However, many of these tools were not developed for multi-species datasets, and it is not immediately apparent that optimization is needed or what optimization is needed. How multi-species datasets are handled varies even between generations of the same aligner, like the Burrows–Wheeler aligner (**BWA-ALN** and **BWA-MEM** [maximum exact matches]). In this case, optimization could be obtained with **BWA-MEM** by using a combined reference, containing all of the genomes of the species present, and changing the seed stringency. There are many uses for aligners for multi-species datasets that will benefit from this optimization including: (a) use in dual-species RNASeq datasets where mis-mapping leads to inappropriate gene expression calculations, (b) use in identifying discordant read pairs that suggest horizontal/lateral gene transfer, and (c) use to cull datasets, for instance removing human reads from clinical pathogen data sets prior to data deposition. All of these cases will benefit from the increased specificity of the optimization here with little loss to sensitivity.

While **BWA** was initially designed for single nucleotide polymorphism (SNP)-based analysis [2], it has also been used to identify structural variation. The Mobile Element Locator Tool (**MELT**) [5, 23], which identifies mobile elements in eukaryotic sequencing samples, relies on **BWA**-generated alignments. **MELT** was used in the 1000 Genomes Project to identify structural variants and LINE element insertion and deletion events in these samples [5, 23]. **LGTSeek** relies on **BWA-ALN** to align read pairs to identify lateral gene transfers (**LGTs**) from a donor organism to a recipient organism [24]. **LGTSeek** can align sequencing reads to the recipient reference, then align the unmapped reads to the potential donor references, identifying pairs where one read maps to the host and the other read maps to the donor [24]. This approach relies on the speed and accuracy of **BWA-ALN** to accurately identify these categories of reads.

While **BWA-MEM** aligns the sequencing reads to the reference more quickly than **BWA-ALN** [1], we have observed instances of low stringency/specificity of these alignments when using **LGTSeek**. This decreased specificity can result in the double mapping of sequencing reads to multiple references. For example, this was observed while examining dual-species transcriptome data [25] from *Wolbachia* endosymbionts and *Drosophila ananassae*, and was solved by combining the reference genomes. In this case, in preliminary work, **BWA-ALN** and **BWA-MEM** were both used for the alignments, but the counts were wildly different. More specifically, **BWA-MEM** mapped bona fide *Wolbachia* sequencing reads to the *D. ananassae* reference genome, when only a very small
portions of the read matched the *Drosophila* reference genome. This likely results from the fact that *BWA* was designed with the intent of mapping sequence data from a single organism to a single reference genome. However, as more tools use *BWA* for multi-species comparisons and more researchers use it in host–pathogen samples, these misalignments can significantly confound analyses.

Here, we sought to optimize *BWA-MEM* in eukaryote/eukaryote and eukaryote/prokaryote dual-species datasets by altering various options to prevent incorrect mappings. We identified appreciable effects upon altering the seed length of *BWA-MEM* alignments, which determines the minimum base pair length of an exact match, and by default is 19 nt. Since *BWA-MEM* can handle larger reference databases, we also separately analyzed the effect of combining host–pathogen references into one larger database. Both methods decrease computational time, while increasing or maintaining specificity of the alignments. During this research, we also observed that the computational time spent on human genome sequence alignments, in general, could be decreased by increasing the seed length, which we attribute to the time spent attempting to align poor quality, off-target, non-human contaminant sequences to the reference genome.

**METHODS**

**Sample description**

**Human dataset**

The 100 bp paired-end Illumina human dataset is available from the 1000 Genomes Project in the Sequence Read Archive (SRA) (http://www.internationalgenome.org, ERR022446). The data is from a Colombian male and the sample (HG01133) of B-lymphocytes was collected from the peripheral vein [5].

**Plasmodium–human dataset**

The 76 bp paired-end Illumina *Plasmodium*–human data-sets are available from the Wellcome Trust Sanger Institute in the SRA (ERR015379, ERR012739 and ERR015360) from a study that extracted DNA from leukocyte-depleted blood samples or after short-term *in vitro* culture [26]. These samples (PK0055-C, PK0076-C and PK0044-C) all originated in Burkina Faso in 2008 [26].

**Brugia–Wolbachia dataset**

The 101 bp paired-end *Brugia–Wolbachia* dataset is available from the University of Maryland Institute for Genome Sciences in the SRA (SRR5188379) from a currently unpublished study designed to test DNA amplification methods on low input DNA samples. DNA was extracted from a population of *Brugia malayi* worms. Qiagen Repli-G (Qiagen) was used to amplify 2 ng genomic DNA with tetramethylammonium chloride (TMAC) [27]. A genomic DNA library was constructed for sequencing on the Illumina platform using the KAPA Hyper Prep kit (Kapa Biosystems) according to the manufacturer’s protocol. DNA was fragmented with the Covaris E210. The DNA was purified between enzymatic reactions and the size selection of the library was performed with SPRI-select beads (Beckman Coulter Genomics). The PCR amplification step was performed with primers containing a 7 nt index sequence. The indexed libraries were pooled and sequenced on a on a Hiseq2500 sequencer (Illumina).

**Simulated metagenomic dataset**

A dataset of 101 bp paired-end sequencing reads from a mock metagenomic community was simulated with ARK v. 2.3.7 [28]. An even 8× sequencing depth with a HiSeq2500 error profile, a minimum fragment length of 300 bp and sd of 105 bp was generated across the reference genomes: *Acinetobacter baumanii* ATCC17978; *Actinomyces odontolyticus* ATCC17982; *Bacillus cereus* ATCC10987; *Bacteroides vulgatus* ATCC8482; *Clostridium beijerinckii* ATCC51743; *Deinococcus radiodurans* DSM20539; *Enterococcus faecalis* ATCC47077; *Escherichia coli* ATCC700926; *Helicobacter pylori* ATCC700392; *Lactobacillus gasseri* DSM 20243; *Listeria monocytogenes* ATCCBAA-679; *Methanobrevibacter smithii* ATCC53061; *Neisseria meningitidis* ATCCBAA-335; *Propionibacterium acnes* DSM16379; *Pseudomonas aeruginosa* ATCC47085; *Rhodobacter sphaeroides* ATCC17023; *Staphylococcus aureus* ATCCBAA-1718; *Staphylococcus epidermidis* ATCC12228; *Streptococcus agalactiae* ATCCBAA-611; *Streptococcus mutans* ATCC700610; and *Streptococcus pneumoniae* ATCCBAA-334.

**BWA alignments**

All alignments were written as *SAM* output files using *BWA* v. 0.7.12. The `qsub -m` function, as implemented in GE 6.2u5 lx24-amd64 dated December 1, 2009, was used to generate a summary report from each experiment including the central processing unit (CPU) time for each script that was executed. To reduce variation in CPU times, all jobs were executed on a single multi-core node ensuring that <40% of the total number of cores and <75% of total memory were used. Each job was executed in triplicate, and no replicate jobs were running simultaneously. For *BWA-ALN/SAMPE* alignments, the script contained *ALN* commands for each FASTQ file executed sequentially followed by the *SAMPE* command, which were all executed with the default parameters. For *BWA-MEM* alignments, the script contained a single command to align the sequencing read pairs to their respective references; default parameters were used except where seed length was altered. The Ensembl GRCCh37 human reference containing the decoy sequence was used for alignments to the human genome. The *B. malayi* genome [29] version 4 was obtained from WormBase (www.wormbase.org) and was used as the combined reference for *Brugia* and *Wolbachia*. The *Wolbachia* wBm genome [30] reference was obtained by pulling the Bm_006 contig from the combined reference and the *Brugia* reference was made by using all contigs except Bm_006. The *Plasmodium* reference was used as version 3.0 of high quality *Plasmodium falciparum* 3D7 draft genome released by PlasmoDB-9.3 [31]. The combined human–*Plasmodium* reference was created by adding the *Plasmodium* contigs to the GRCCh37 FASTA file. *SAMTOOLS* *FLAGSTAT* v.1.1 was used to determine the number of total
reads, secondary reads, and mapped reads in each sample. To correct for the number of secondary reads, the percentage of reads mapping was calculated as (mapped reads – secondary reads)/(paired in sequencing reads) × 100.

RESULTS

Datasets

Four types of datasets were used for this study: eukaryote/prokaryote, eukaryote/eukaryote, eukaryote only and simulated metagenomic. The first dataset contained 101 bp paired-end Illumina sequencing reads from a population of B. malayi worms and their Wolbachia endosymbiont strain wBm from an unpublished study examining DNA amplification methods; it will be referred to as the Brugia–Wolbachia dataset (Table 1). Using the Perl 5.8.8 rand() and srand () functions, 250 000 first reads were randomly selected and the taxonomy of best hits for these reads was generated with a MEGA-BLAST (v. 2.2.17) search against the NCBI nucleotide database NT. We typically find that in MEGA-BLAST searches of this nature that some percentage of reads have no match, and that the best criterion to use is the percentage of reads with a best match relative to the number of reads that have any match, and this criterion was used in all other instances. Based on this criterion, this dataset was estimated to contain 79.7 % reads with matches having best matches to B. malayi and 14.5 % reads with best matches to Wolbachia wBm. However, the version of NT used for these searches does not have the B. malayi genome, so most reads did not have matches, making this measurement skewed in this case. If we instead focus on all reads, 14.1 % of reads had a best match to B. malayi and 2.6 % of reads had a best match to Wolbachia wBm, which is likely a better reflection of the composition of this data.

The second dataset that we initially focused on contained 76 bp paired-end Illumina sequence reads from P. falciparum sequenced from a human sample from the SRA (ERR015379) [26]; it will be referred to as the Plasmodium–human dataset (Table 1). Parsing the taxonomy of best hits of 250 000 randomly selected first reads generated with a MEGA-BLAST search against NT, this dataset was estimated to contain 87.5 % reads with best matches to P. falciparum reads and 7.4 % reads with best matches to human sequences.

The third dataset contained 101 bp paired-end Illumina sequence reads that have been simulated from a mock metagenomic community (Dryad doi:10.5061/dryad.m1m0p); it will be referred to as the mock metagenomic dataset (Table 1). The final dataset examined was 100 bp Illumina paired-end human sequencing reads from the 1000 Genomes Project (ERR022446) [5]; it will be referred to as the 1000 Genomes dataset (Table 1). Parsing the taxonomy of best hits of 250 000 randomly selected first reads generated with a MEGA-BLAST search against NT, this dataset was estimated to contain 94.7 % of reads with matches having best matches to human sequences.

| Table 1. Total number of reads for each dataset |
|-----------------------------------------------|
| The datasets are listed that were used in this study, with the total number of reads. |

| Dataset            | Accession no. | Total no. of reads |
|--------------------|---------------|--------------------|
| Brugia–Wolbachia   | SRR5188379    | 31 494 916         |
| Plasmodium–human   | ERR015379     | 6 051 406          |
| Plasmodium–human   | ERR012739     | 8 080 550          |
| Plasmodium–human   | ERR015360     | 25 867 194         |
| 1000 Genomes       | ERR022446     | 219 493 146        |
| Simulated metagenome| Dryad acc. (doi:10.5061/dryad.m1m0p) | 5 490 726 |

In the Plasmodium–human dataset, the aggregate number of read pairs aligning to each reference was >100 % until the seed length was set to >29 nt, when mapping drastically declined due to a likely over-stringent seed length. The aggregate number of read pairs decreased from 107.8 to 102.2 % when the seed length was increased from 18 to 23 nt (Fig. 1, Table 2). This problem of read pairs aligning to both references was resolved, meaning they were mapped to the best position, by combining the Brugia and Wolbachia references into a single reference sequence (Fig. 1, Table 2). The percentage of reads that mapped to Brugia only varied by 0.87 % across all seed lengths (Fig. 1, Table 2). However, the extensive amount of recent LGT to B. malayi from its Wolbachia endosymbiont [32] confounds this analysis such that we sought to evaluate this phenomenon with several subsequent datasets.

In the Plasmodium–human dataset, the aggregate number of read pairs aligning to each reference was >100 % until the seed length was set to >29 nt, when mapping drastically declined due to a likely over-stringent seed length. The aggregate number of read pairs decreased from 107.8 to 102.2 % when the seed length was increased from 18 to 23 nt (Fig. 1, Table 2). This problem of read pairs aligning to both references was resolved by combining the human and Plasmodium reference into one file (Fig. 1, Table 2). For all seed lengths mapped against this combined reference, the Plasmodium–human dataset consistently had 94.6–97.1 % of read pairs aligning (Fig. 1, Table 2). Aligning the Plasmodium–human data to the Plasmodium reference or the
Plasmodium–human combined reference led to a consistent percentage of read pairs mapping across seed length up to 30 nt, indicating that increasing the seed length did not dramatically decrease the number of read pairs that were aligned for these seed lengths (Fig. 1, Table 2). Increasing the seed length beyond 30 nt led to a substantial decrease in the number of reads aligned, as expected (Table S1, available in the online Supplementary Material), which was only tested with this dataset.

Two further Plasmodium–human datasets were tested, ERR012739 and ERR015360, to ensure these observations were not limited to the original dataset selected. Parsing the taxonomy of best hits of 250,000 randomly selected first reads generated with a MEGA-BLAST search against NT, ERR012739 was estimated to contain 73 % Plasmodium reads and 18 % human reads, while ERR015360 was estimated to contain 60 % Plasmodium reads and 3 % human reads. All three datasets showed the same trends as the original dataset examined (Fig. 2), namely over-mapping of reads when mapped to separate references that could be largely eliminated by mapping to an aggregate reference and/or increasing the seed length to 23 nt.

Similar over-mapping of reads was again observed with a simulated dataset of a 21-member mock metagenomic community (Fig. 3). When mapped to the composite reference, 100 % of the reads mapped, as expected for a simulated dataset. However, even at a seed length of 30 nt, the aggregate percentage of reads mapping to each individual reference was still >104 % (Fig. 3), which may be due in part to multi-mapping of reads to the many highly similar rRNAs. Mapping to an aggregate reference and/or increasing the seed length to 23 nt improved the mapping specificity (Fig. 3). However, it is less clear that 23 nt was the ideal seed length as substantial improvements could be observed beyond a 23 nt seed length (Fig. 3). This difference may be due to using simulated sequence data, the high degree of similarity in regions of the genomes like the rRNA, or the complexity of the population. We investigated using real sequencing data from a mock
community sequenced by the human microbiome project [33, 34], but only single end reads were available.

CPU times and trends

The CPU time of the alignments was also interrogated, by measuring in triplicate using a single grid node with 48 CPUs and 95.6 GB memory, controlling for variation by limiting CPU usage to <40% of the maximum and memory usage to <75% of the maximum (Fig. 4). We could not control for other factors like caching and network traffic, but saw little variation in CPU time across replicates, which were not run simultaneously on the node. The alignment of the reads to the combined Brugia–Wolbachia reference database took similar CPU time relative to aligning to only the Brugia reference (Fig. 4, Table 3) demonstrating the benefit of using a combined reference when possible.

Alignments to the human reference genome had the most dramatic decrease in CPU time with increasing seed lengths for both the human–Plasmodium dataset and the 1000 Genomes dataset (Fig. 4). In the case of the 1000 Genomes dataset, the CPU time at a seed length of 18 nt was 21.1 ±1.7 h (Table 3). Increasing the seed length to 23 nt reduced the CPU time to 16.8±1.0 h, resulting in a 1.3-fold reduction or 4.3 h decrease in CPU time (Fig. 4, Table 3).

The Plasmodium–human dataset showed even more substantial improvements when aligning to the combined dataset, but most substantially between 18 and 20 nt seed lengths. For example, at a seed length of 18 nt, the alignment of this dataset to the human reference took 1.7±0.1 h, while the alignment to the combined reference only took 0.7 ±0 h, a 2.4-fold decrease (Table 3, Fig. 4). The seed length also plays a role as observed by the decrease in CPU hours with increasing seed length up to ~23 nt. An increase in seed length to 23 nt reduced the Plasmodium–human CPU time 3.4-fold in the alignments to just the human reference (Fig. 4, Table 3). Overall, improvements to the CPU time for both host–pathogen datasets plateaued by seed length of 23 nt (Fig. 4, Table 3). The decreasing CPU time needed as a function of seed length, combined with the over-mapping of reads against a single reference compared to a combined reference, suggests that using BWA-MEM to generate poor alignments to the incorrect reference can lead to excessive use of computational resources. Increasing the seed length beyond 30 nt led to a sizable decrease in the CPU hours (Table S2), but this is likely a result of the undesirable decrease in the number of reads aligned (Table S1).

Comparing BWA-MEM to BWA-ALN

We also sought to compare the percentage of mapped read pairs aligned with BWA-ALN and BWA-MEM. We expected that BWA-MEM would map more reads, since it could map reads with more sequencing errors, as well as greater sequence variation. Consistent with these expectations, in all cases tested, BWA-ALN mapped a lower percentage of reads relative to BWA-MEM (Table 2). Also, as expected, given the underlying algorithm, BWA-MEM computed the alignments faster than BWA-ALN when the majority of reads map to the reference genome (i.e. Plasmodium–human against the Plasmodium genome, Plasmodium–human against the combined reference, Brugia–Wolbachia against the Brugia genome, Brugia–Wolbachia against the combined reference, and 1000 Genomes against the human genome). Conversely, we anticipated that when the vast majority of reads did not map to the reference, BWA-MEM would spend large amounts of compute time trying to incorrectly align them to the genome (i.e. Plasmodium–human against the human genome and Brugia–Wolbachia against the Wolbachia genome). As expected, these latter two cases were aligned more quickly with BWA-ALN than BWA-MEM at the lower seed lengths tested (Table 3).
The increase in the percentage of reads mapping with BWA-MEM at a seed length of 18 nt led to an increase in both the sequencing read depths, as well as the coverage. There are 12,127,496 positions in the human genome that had 1/C2 sequencing depth with a seed length of 18 nt using BWA-MEM with a maximum sequencing depth of 7990/C2 with the default parameters of SAMTOOLS Mpileup, which would discard positions with >8000/C2 sequencing depth. In contrast, 5,378,722 positions in the human genome had 1/C2 sequencing depth with BWA-ALN/SAMPE with a maximum sequencing depth 919/C2. Therefore, there was >8-fold higher maximum sequencing depth and >2-fold more positions with coverage.

The Plasmodium reads mapping to the human genome with a seed length of 18 nt were not evenly distributed throughout the human genome. While reads mapped to all of the chromosomes evenly, three non-chromosomal FASTA entries had reads that were significantly over-represented relative to the length of the sequence (\(\chi^2, P<0.00005\)), including the unplaced genomic contig GL000220.1 with 69,056 reads in 161,802 bp, the unplaced genomic contig GL000226.1 with 16,735 reads in 15,008 bp, and the decoy sequence contig hs37d5 with 569,969 reads in 35,477,943 bp.

We further wanted to examine whether high-quality matches of Plasmodium reads to the human genome reference enabled alignment of related poorly mapping reads.
The CPU time reported is the sum of the CPU time for aligning read 1, the CPU time for aligning read 2 and the CPU time for running the algorithm on those two alignments.

BWA mapped with and their mates were removed from the dataset. The CPU time in hours is provided for a subset of datasets for BWA-Aln chaining, and seed extension. We used the 10.2% of Plasmodium reads aligning to the human reference with BWA as our proxy for high-quality matches. Those reads and their mates were removed from the FASTQ files and these modified human–Plasmodium FASTQ files were realigned to the human genome using BWA-MEM for all seed lengths tested. We found that the percentage of reads mapping to the human reference with BWA-MEM was the same from both the filtered FASTQ files and the initial FASTQ files after accounting for the removal of the 10.2% of the reads that correctly aligned to Plasmodium (Table S3).

There was no substantial difference between mapping to the Plasmodium genome with BWA-Aln/SAMPE and BWA-MEM. There were 16 995 093 Plasmodium positions with >5× coverage in the BWA-MEM alignment with a seed length of 18 nt, while there were 16 698 169 Plasmodium positions with >5× coverage in the BWA-Aln/SAMPE alignment. When modified FASTQ files were made by removing reads identified as human using BWA-MEM and those modified files were mapped with BWA-Aln/SAMPE back to the Plasmodium genome, 16 698 614 Plasmodium positions still had >5× coverage, which is remarkably similar to the 16 698 169 Plasmodium positions with >5× coverage when mapping all reads to the Plasmodium genome without filtering. However, when modified FASTQ files were made after removing reads identified as human using BWA-MEM and those modified files were mapped with BWA-Aln/SAMPE back to the Plasmodium genome, only 14 214 800 Plasmodium positions still had >5× coverage, which is only 85% of the positions with coverage in the Plasmodium genome using all reads and BWA-Aln/SAMPE. Therefore, the excess removal of reads based on their alignment with BWA-MEM at a seed length of 18 nt may result in a loss of coverage of ~2 000 000 Plasmodium positions that could lead to an inability to call SNPs and other important genetic variants.

### DISCUSSION

**Effects on multi-species genome sequencing datasets**

New algorithms are making remarkable advances to expand the complexity of alignments to best capture all the genomic

![Fig. 4. CPU time for all BWA-MEM datasets. The mean and SD of CPU time in hours for each of three replicate alignments is plotted against each seed length for a subset of datasets. In the two dual-species data sets, the sequencing reads were aligned to the reference genome of each species separately, as well as a combined reference of both genomes. The 1000 Genomes dataset was only aligned to the human reference genome.](image-url)
variation in a single organism [1, 2, 35–38]. These improved alignments are essential for better identification of SNPs, copy number variants and instances of structural variation. However, there is the potential for problems to arise when these algorithms are applied in untested scenarios, like multi-species sequencing projects. In these cases, one of the vital steps is properly attributing the reads to each organism.

Numerous studies have reported transcriptomics-based gene expression differences of two organisms within a single sample [39–50]. For the gene expression differences to be accurately obtained, the reads from both organisms need to map uniquely to the appropriate reference. The results presented here for *Brugia–Wolbachia* and *Plasmodium–human* read mappings suggests this may not occur all the time. *Plasmodium* reads map to the human genome and *Brugia* reads map to the *Wolbachia* genome, which could result in transcripts appearing to be transcribed that are not. It also could result in erroneous assessment of the level of differential gene expression.

This may also present problems in mapping-based metagenomics analyses, and our results suggest that using a combined reference and increasing the seed length may lead to improvements. The exact seed length is not clear, and further work using real sequencing data from mock communities, as opposed to simulated data, may be necessary to resolve the matter.

In parasite and pathogen sequencing projects that require the direct isolation of nucleic acids from a human patient, sequencing reads are often screened prior to deposition in public repositories, like the SRA [51], to remove human sequences. Theoretically, mapping-based algorithms, like BWA, provide fast and efficient mechanisms for removing such human reads from parasite and pathogen sequencing projects. However, the results presented here suggest that too many sequences may be erroneously targeted for removal. Specifically, only 85% of the positions have coverage in the *Plasmodium* genome after removing ‘human’ reads with BWA-MEM with a seed length of 18 nt relative to removing ‘human’ reads with BWA-ALN/SAMPE.

Lastly, pipelines like PathSeq [52] and LGTSeek [24] use aligners to identify the putative provenance of reads, assigning a taxonomy for reads and/or read pairs. PathSeq identifies microbial reads in samples, in order to identify novel microbe–disease associations [52]. LGTSeek identifies read pairs with discordant taxonomy, like bacteria-eukaryote read pairs in animal and human genomes that may indicate bacteria-animal LGT [24]. In this case, a stringent mapping assignment is needed in order to assign the most appropriate taxonomy. The results from both the *Brugia–Wolbachia* and the *Plasmodium–human* datasets suggest that this may not be the case with BWA-MEM without further optimization. While we focus on differences between BWA-ALN/SAMPE and BWA-MEM, it is important that any aligner be thoroughly tested when used in these scenarios for the particular organisms under study.

**Solutions**

Given the results presented here, one solution that should be employed whenever possible is to include the genomes of all expected organisms in a single reference database. Doing so increased the specificity of the alignments and decreased computation times. This strategy has already been used in human genome sequencing projects that include the Broad Institute’s ‘decoy’ genome sequence in their reference genome. The ‘decoy’ genome sequence contains sequences frequently encountered in human whole-genome sequencing projects that are otherwise not in the reference [5].

In some studies, however, not all reference organisms are known. For example, when running algorithms like PathSeq [52] or LGTSeek [24] to identify instances of ‘foreign’ DNA, the potential sources are unknown and are the focus of the study. Additionally, if a sequencing library or run is contaminated, the researcher may not be aware of the presence of sequence reads from additional organisms that could be leading to an increase in computation times. In these cases, the specificity would be improved and the CPU times decreased by increasing the seed length. In this study, a seed length of 23 nt was ideal since lower seed lengths increased computation times and off-target mappings, while higher seed lengths could reduce sensitivity with no improvement to computation times or off-target mappings. It is not clear if this is the universal ideal seed length that would be applicable for all scenarios. The ideal seed length may be a function of the evolutionary distances and similarity between the organisms being sequenced concomitantly. However, we have demonstrated that the use of simulated datasets constructed from the reference datasets may be useful in informing these decisions and identifying what further experimental controls may be needed.

While much of our analysis focuses on a seed length of 18 nt, it is important to remember that the default seed length for BWA-MEM is 19 nt. We initially identified this problem with a seed length of 19 nt; however, it is much more apparent and easier to interrogate at a seed length of 18 nt. Lastly, there may be scenarios that warrant the use of legacy aligners, like BWA-ALN, which may be less sensitive, but have a higher degree of specificity.

**Computational resource optimization**

Analysis of genomic data can be computationally intensive, leading to long CPU times and high analysis costs. Our analysis suggests that almost all datasets can benefit from an increase in the seed length to reduce CPU time and associated costs. However, this decrease in computation time may affect the ability to identify ‘split reads’, where a read matches two different locations in the genome, suggesting structural variation in genomics projects and splicing in transcriptomics projects. As such, further work may be needed by specialists in a field (e.g. structural genomic scientists and annotation specialists) to demonstrate how changing the seed length may or may not affect the validity of structural variation and gene structure predictions. Split
reads in RNAseq projects of dual-species projects need to be handled with care, balancing the sensitivity of detecting splice junctions with the specificity of correct mapping. At a minimum, this can be dealt with in multi-species datasets by aligning to a combined reference that contains all known members of the consortium sequenced.

Conclusion
As sequencing technologies continue to advance and sequencing becomes less expensive, the tools to analyze these sequences need to be optimized for the intended study. Here, BWA-MEM was optimized to reduce alignment time and increase the specificity of alignments. While this optimization might not be necessary for all datasets, we found it to be particularly helpful in sequencing data obtained simultaneously from multiple organisms. In these cases, there was one dominant sequence source (i.e. Plasmodium or Brugia) and one minority sequence source (i.e. human or Wolbachia). Increasing the seed length reduced the number of read pairs aligning, incorrectly, to the reference genome from the minority sequence source (human or Wolbachia). Overall, increasing the seed length to 23 nt and combining both organisms into one reference genome can reduce the CPU time, sometimes quite substantially, with more specificity in the mapping, as was the case with the Plasmodium–human dataset. In some situations, the use of legacy aligners may also be warranted. Less substantial, but still possibly significant, improvements to CPU time upon changing the seed length were found in all cases. This suggests using a 23 nt seed length by default may be desirable.

Funding information
This work was funded by the National Institute of Allergy and Infectious Diseases U19AI110820 to CMF, DAR, JCS, and JCDH (U19AI110820), the National Institutes of Health through an NIH Director’s Transformative Research Award (1-R01-CA206188) to JCDH, and the National Science Foundation Advances in Biological Informatics (ABI-1457957) to JCDH.

Acknowledgements
The results published here are in part based upon data generated by the 1000 Genomes Project. Information about the 1000 Genomes Project can be found at “http://www.internationalgenome.org/”. We would like to thank Dr. Jeremy Foster and Dr. Silvia Libro at New England Biolabs for kindly providing the Brugia malayi DNA used for sequencing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Data bibliography
1. 1000 Genomes Project. Wellcome Trust Sanger Institute. Sequence Read Archive – ERR022446 (2017).
2. Wellcome Trust Sanger Institute. Sequence Read Archive – ERR015379 (2017).
3. Wellcome Trust Sanger Institute. Sequence Read Archive – ERR012739 (2017).
4. Wellcome Trust Sanger Institute. Sequence Read Archive – ERR015360 (2017).
5. University of Maryland Institute for Genome Sciences. Sequence Read Archive – SRR5188379 (2017).

References
1. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1774–1780.
2. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arxiv 2013;3:13033997.
3. Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM et al. A map of human genome variation from population-scale sequencing. Nature 2010;467:1061–1073.
4. Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM et al. An integrated map of genetic variation from 1,092 human genomes. Nature 2012;491:56–65.
5. Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM et al. A global reference for human genetic variation. Nature 2015;526:68–74.
6. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature 2011;474:609–615.
7. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014;513:202–209.
8. Abeshouse A, Ahn J, Akbani R, Ally A, Amin S et al. The molecular taxonomy of primary prostate cancer. Cell 2015;163:1011–1025.
9. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 2013;499:43–49.
10. Linehan WM, Spellman PT, Ricketts CJ, Creighton CJ, Fei SS et al. Comprehensive molecular characterization of papillary renal-cell carcinoma. N Engl J Med 2016;374:135–145.
11. Davis CF, Ricketts CJ, Wang M, Yang L, Cherniack AD et al. The somatic genomic landscape of chromophobe renal cell carcinoma. Cancer Cell 2014;26:319–330.
12. Akbani R, Aksamir Kadir C, Aksoy BA, Albert M, Ally A et al. Genomic classification of cutaneous melanoma. Cell 2015;161:1681–1696.
13. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015;517:576–582.
14. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519–525.
15. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014;511:543–550.
16. Agrawal N, Akbani R, Aksoy BA, Ally A, Arachchi H et al. Integrated genomic characterization of papillary thyroid carcinoma. Cell 2014;159:676–690.
17. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315–322.
18. Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 2013;368:2099–2074.
19. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–337.
20. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.
21. Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y et al. Integrated genomic characterization of endometrial carcinoma. Nature 2013;497:67–73.
22. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H et al. The somatic genomic landscape of glioblastoma. Cell 2013;155:462–477.
23. Sudmant PH, Rausch T, Gardner EJ, Handsaker RE, Abyzov A et al. An integrated map of structural variation in 2,504 human genomes. Nature 2015;526:75–81.

24. Riley DR, Sieber KB, Robinson KM, White JR, Ganesan A et al. Bacteria-human somatic cell lateral gene transfer is enriched in cancer samples. PLoS Comput Biol 2013;9:e1003107.

25. Kumar N, Lin M, Zhao X, Ott S, Santana-Cruz I et al. Efficient enrichment of bacterial mRNA from host-bacteria total RNA samples. Sci Rep 2016;6:34850.

26. Manske M, Miotto O, Campino S, Auburn S, Almagro-Garcia J et al. Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. Nature 2012;487:375–379.

27. Oyoa SO, Manske M, Campino S, Claessens A, Hamilton WL et al. Optimized whole-genome amplification strategy for extremely AT-biased template. DNA Res 2014;21:661–671.

28. Huang W, Li L, Myers JR, Marth GT. ART: a next-generation sequencing read simulator. Bioinformatics 2012;28:593–594.

29. Gheedin E, Wang S, Spiro D, Caler E, Zhao Q et al. Draft genome of the filarial nematode parasite Brugia malayi. Science 2007;317:1756–1760.

30. Foster J, Ganatra M, Kamal I, Ware J, Makarova K et al. The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLoS Bio 2005;3:e121.

31. Gardner MJ, Hall N, Fung E, White O, Berriman M et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 2002;419:498–511.

32. Ioannidis P, Johnston KL, Riley DR, Kumar N, White JR et al. Extensively duplicated and transcriptionally active recent lateral gene transfer from a bacterial Wolbachia endosymbiont to its host filarial nematode Brugia malayi. BMC Genomics 2013;14:639.

33. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature 2012;486:207–214.

34. Human Microbiome Project Consortium. A framework for human microbiome research. Nature 2012;486:215–221.

35. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Meth 2015;12:357–360.

36. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.

37. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Bio 2009;10:R25.

38. Lee WP, Stromberg MP, Ward A, Stewart C, Garrison EP et al. MOSAIK: a hash-based algorithm for accurate next-generation sequencing short-read mapping. PLoS One 2014;9:e90581.

39. Humphrys MS, Creasy T, Sun Y, Shetty AC, Chibucos MC et al. Simultaneous transcriptional profiling of bacteria and their host cells. PLoS One 2013;8:e80597.

40. Vannucci FA, Foster DN, Gebhart CJ. Laser microdissection coupled with RNA-seq analysis of porcine enterocytes infected with an obligate intracellular pathogen (Lawsonia intracellularis). BMC Genomics 2013;14:421.

41. Rienksma RA, Suarez-Diez M, Mollenkopf HJ, Dolganov GM, Dorhoi A et al. Comprehensive insights into transcriptional adaptation of intracellular mycobacteria by microbe-enriched dual RNA sequencing. BMC Genomics 2015;16:34.

42. Avraham R, Haseley N, Brown D, Penaranda C, Jijon HB et al. Pathogen cell-to-cell variability drives heterogeneity in host immune responses. Cell 2015;162:1309–1321.

43. Mavromatis CH, Bokil NJ, Totsika M, Kakkanat A, Schaele K et al. The co-transcriptome of uropathogenic Escherichia coli-infected mouse macrophages reveals new insights into host-pathogen interactions. Cell Microbiol 2015;17:730–746.

44. Aprianto R, Slager J, Holsappel S, Veening JW. Time-resolved dual RNA-seq reveals extensive rewiring of lung epithelial and pneumococcal transcriptomes during early infection. Genome Biol 2016;17:198.

45. Westermann AJ, Förstner KU, Amman F, Barquist L, Chao Y et al. Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature 2016;529:496–501.

46. Baddal B, Muzzi A, Censini S, Calogero RA, Torricelli G et al. Dual RNA-seq of nontypeable Haemophilus influenzae and host cell transcriptomes reveals novel insights into host-pathogen cross talk. MBio 2015;6:e01765-15.

47. Tierney L, Linde J, Müller S, Brunke S, Molina JC et al. An interspecies regulatory Network inferred from simultaneous RNA-seq of Candida albicans invading innate immune cells. Front Microbiol 2012;3:85.

48. Chibucos MC, Soliman S, Gabremariam T, Lee H, Daugherty S et al. An integrated genomic and transcriptomic survey of mucormycosis-causing fungi. Nat Commun 2016;7:12218.

49. Bruno VM, Shetty AC, Yano J, Fidel PL, Noverr MC et al. Transcriptomic analysis of vulvovaginal candidiasis identifies a role for the NLRP3 inflammasome. MBio 2015;6:e00182-15.

50. Liu Y, Shetty AC, Schwartz JA, Bradford LL, Xu W et al. New signaling pathways govern the host response to C. albicans infection in various niches. Genome Res 2015;25:679–689.

51. National Center for Biotechnology Information. Sequence Read Archive Overview [updated January 10 2017]; 2017. https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement.

52. Kostic AD, Ojesina AI, Pedamallu CS, Jung J, Verhaak RG et al. PathSeq: software to identify or discover microbes by deep sequencing of paired-end Illumina reads. Nat Biotechnol 2011;29:393–396.

---

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.