MinION sequencing enables rapid whole genome assembly of *Rickettsia typhi* in a resource-limited setting

Ivo Elliott[1,2], Damien Ming[1], Matthew T. Robinson[1,2], Pruksa Nawtaisong[1], Paul N. Newton[1,2], Mariateresa de Cesare[3], Rory Bowden[3], Elizabeth M. Batty[2,3,4]

Affiliations:

1. Laos-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao PDR
2. Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom
3. Wellcome Centre for Human Genetics, University of Oxford, Oxford, United Kingdom
4. Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Corresponding author: Ivo Elliott ivo@tropmedres.ac

Correspondence address: Ivo Elliott, Laos-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao PDR.

Abstract

The infrastructure and costs associated with performing next-generation sequencing have been barriers to its introduction into resource-limited settings. Oxford Nanopore Technologies' (ONT) portable MinION sequencer overcomes many of these challenges to allow small scale sequencing projects with minimal laboratory and computing infrastructure. We performed whole-genome sequencing (WGS) of *Rickettsia typhi*, an important and neglected cause of fever across much of the tropics and subtropics. Sequencing libraries were prepared from extracted DNA following standard ONT protocols and sequenced on a

laptop in our laboratory in Vientiane, Laos. Basecalling was performed subsequently using ONT's Albacore software and assembled locally using a bioinformatics pipeline. This generated a single contig assembly of 1,078,916bp. Frequent SNP and indel error rates, commonly seen with ONT sequencing data, resulted in multiple gene annotation errors. As a result, DNA was also sequenced in the United Kingdom using the Illumina platform to generate a polished genome. Only 3 previous *R. typhi* whole genome sequences exist. Comparison with these shows evidence of reductive genome evolution with no rearrangements and few differences between strains collected over a 90-year time period. This is the first WGS to be performed in Laos and the first WGS of an *R. typhi* sample collected in the last 50 years. We demonstrate the utility of portable sequencing platforms such as the MinION for carrying out next-generation sequencing projects in resource-limited settings.

Introduction

Until recently, next-generation sequencing (NGS) has been the preserve of high-income settings. Although the costs of NGS have dramatically decreased over the past decade[1], initial investment into sequencing platforms and the technical expertise to run and maintain them have prevented their introduction into lower income settings. Downstream processing of sequencing data is frequently hampered by a lack of computational infrastructure and poor Internet connectivity.

Oxford Nanopore Technologies' (ONT) MinION sequencer is a portable real-time device for DNA and RNA sequencing. The MinION weighs under 100g and plugs directly into a laptop via a USB, with no additional computing infrastructure required[2]. Minimal laboratory facilities are required to extract DNA and prepare sequencing libraries. The MinION has been used to sequence and assemble a number of bacterial[3,4] and eukaryotic[5-8] genomes, either alone or in conjunction with Illumina short-read data to improve the assemblies[9].

The Lao People's Democratic Republic (Laos) is a landlinked country in Southeast Asia of ~7 million people. Laos is ranked 135th in the world on the Human Development Index (HDI), a composite statistic of life expectancy,

education and per capita income[10]. There are very few functioning molecular laboratories in Laos and no previous published NGS projects.

Rickettsia typhi is an obligate intracellular, Gram-negative bacterium in the family Rickettsiaceae that causes the disease murine typhus. The pathogen is transmitted to humans primarily by flea faecal contamination of the bite of the Oriental rat flea *Xenopsylla cheopis* and has a worldwide tropical and sub-tropical distribution. The disease is an important and grossly under-recognized global cause of febrile illness. Typically those infected suffer from high fever, headache, myalgia, arthralgia, nausea, vomiting and may have a macular rash. Complications are unusual but include myocarditis, meningoencephalitis, seizures and renal failure[11]. Mortality is low, around 1-2% with antibiotic treatment[12]. Once diagnosed, recovery is typically rapid following the introduction of doxycycline.

Despite its importance as a pathogen, to date there have been just three whole-genome sequences published for *R. typhi*. The type-strain, Wilmington, was isolated from a patient in North Carolina in 1928 and the sequence published in 2004[13,14]. Two further sequences were published online in 2014, originating from a patient in Northern Thailand collected in 1965 and the other from a bandicoot rat, collected in Myanmar in the 1970s[15-17] (Table 1).

We report the successful whole-genome sequencing (WGS) of a human isolate of *R. typhi* using the MinION platform in Laos. We discuss the challenges of undertaking NGS in resource-limited settings and compare the sequence generated to those previously published.

Materials and Methods

R. typhi culture and DNA extraction

Frozen L929 mouse fibroblast cells (ATCC CCL-1), infected with *R. typhi* isolated from a patient, TM2540, from Pakkading district, Bolikhamxay province, Laos with suspected acute murine typhus infection presenting in 2012 to Mahosot Hospital, Vientiane were reanimated. Frozen aliquots were briefly thawed at room temperature before being transferred to 25 cm^2 cell culture flasks

containing a L929 cell monolayer at 80% confluence in RPMI 1640 media (Gibco), supplemented with 10% foetal calf serum (Sigma). Flasks were incubated at 35 °C in 5% CO_2 atmosphere for seven days. Cells were subsequently mechanically detached and transferred to a 75 cm^2 flask, and again incubated at 35 °C in 5% CO_2 atmosphere.

For DNA extraction, cells from three 75 cm^2 flasks were mechanically detached. Suspended cells were transferred to a 50 ml conical centrifuge tube and centrifuged at 3,220xg for 10 minutes. Supernatant was discarded and pellet was re-suspended in 3ml fresh media and transferred to 1.5 ml micro-centrifuge tubes. Tubes were vortexed for one minute, then centrifuged at 300×*g* for 3 minutes. The supernatant was passed through a 2 µm filter (Corning, USA), mixed with 10 µl/ml DNAase (1.4 µg/µl) and incubated at room temperature for 30 minutes. The mixture was centrifuged at 18,188xg for 10 minutes and washed twice with 0.3 M sucrose (Sigma-Aldrich, USA). DNA extraction was then carried out on the *R. typhi* pellet using the DNeasy Blood & Tissue kit (Qiagen, USA) and eluted products stored immediately at -20 °C.

DNA was quantified using Qubit dsDNA High Sensitivity assay kit (Thermofisher, USA) following manufacturer's protocols. Presence of *R. typhi* was confirmed through quantitative polymerase chain reaction (qPCR) targeting the 17kDa outer membrane antigen[18].

Preparation of MinION sequencing libraries

The ONT 1D Genomic DNA by ligation (SQK-LSK108) protocol was followed. Briefly, 1.4 µg of DNA was fragmented in a Covaris g-TUBE (Covaris Ltd., Brighton, U.K.) by centrifugation. Sheared DNA was repaired using NEBNext FFPE repair mix (New England Biolabs, MA, USA). End-repair and dA-tailing was performed with the NEBNext Ultra II End Repair/dA-tailing module. Adapter ligation was done using NEB Blunt/TA Ligase Master Mix, and final purification of the library was carried out using Agencourt AMPure XP beads (Beckman Coulter Inc., CA, USA).

MinION sequencing

MinION libraries were run on an ONT MinION R9.5 flow cell after priming and loading 75µl of library. The sequencing was run for 48 hours without live base calling, due to poor Internet connectivity, on a Dell Latitude E5470 XCTO laptop with 256GB SATA Class 20 Solid State Drive. Base calling was subsequently performed on fast5 files using the ONT Albacore module.

Illumina sequencing

An Illumina sequencing library was generated from the same extracted DNA using the Nextera library preparation method. The library was sequenced on the Illumina MiSeq with 2×260bp reads. A total of 1,201,068 paired reads were generated, giving 312Mb of total sequence coverage.

Bioinformatic analysis

The species composition of the MinION reads was determined using Centrifuge[19] testing against the prebuilt non-redundant database and keeping only the best hit per read, and the results were visualized with Pavian[20].
Multiple assembly strategies were assessed using MinION data alone, or in combination with Illumina short reads. A draft genome assembly was generated from the MinION reads using Canu[21] with the suggested parameters for ONT sequencing "correctedErrorRate=0.120 -nanopore-raw" and an estimated genome size of 1.1Mb. The Canu draft assembly was polished using Nanopolish[3]. The Illumina reads were mapped to the Canu+Nanopolish assembly using Stampy v1.26[22], and the mapped reads were used to error correct the assembly using Pilon v1.22[23]. The Pilon output was used to determine the number and type of assembly errors which were corrected. Racon was also used to improve the initial Canu assembly, before Pilon polishing but this gave more errors than Canu alone or Canu+Nanopolish and was not analysed further. After Pilon polishing, Snippy was used to call variants against the polished genome using the Illumina data. A further 49 errors were corrected that were not detected by Pilon. Prokka v1.14[24] was used to annotate both the new assemblies and the

existing *R. typhi* genomes to give consistent data for comparison. The new genome was rotated to being with the *yqfL* gene for consistency with other *R. typhi* genomes.

The core genome of *R. typhi* was obtained using Roary[25] with identity set at 90%. The concatenated core gene alignments produced with MAFFT[26] were used as input to RAxML[27] and a tree was drawn using FigTree.

Data availability

The three existing *R. typhi* genomes were downloaded from RefSeq under the accession numbers NC_006142.1, NC_017066.1 and NC_017062.1. The sequence data is available at the ENA under project PRJEB2567. The new strain is named TM2540 and the assembly is available at the ENA under accession ERZ497871.

Results

MinION flow cells and reagents were shipped using an international courier from Oxford, United Kingdom to Laos at +4°C. On dispatch 1,242 active pores were available and on receipt after 72hrs travel, 1,103 active pores were available. Sequencing using the MinKNOW platform was performed for 48 hours and generated approximately 250,000 reads, with a total fast5 file size of 20GB. Basecalling using Albacore software was performed after the sequencing was complete on the laptop. 222,848 reads passed quality filters and were used in further analysis.
The species composition of the reads was checked using Centrifuge. As the *Rickettsia typhi* sample was grown in L929 fibroblast cells, we expected to see some contaminating reads from the mouse genome. 134,802 (61%) of the reads were classified as belonging to the genus *Rickettsia*. Of those, 126,447 reads classified as *Rickettsia typhi* at the species level and 41,428 (19%) were classified as belonging to the genus *Mus* (Figure 1).

The FASTQ files produced by Albacore were assembled using Canu. This produced a single contig assembly of 1,078,916bp. A first round of polishing was carried out using Nanopolish, producing a new assembly of 1,099,322bp. To correct errors in the assembly produced from ONT long reads alone, we polished the genome by mapping Illumina short read sequencing to the Canu+Nanopolish assembly and using Pilon to correct errors. After this process, a total of 5,842 errors were corrected, with over 95% (5,594) being small insertions with the result that 12kb was added to the genome during polishing. In comparison, when Pilon was run on the Canu draft genome before Nanopolish polishing, 19,970 errors were corrected, confirming that Nanopolish polishing improves the draft genome using MinION reads alone. Using Snippy detected a further 49 errors which were not corrected using Pilon. This work was performed using a non-Laos server and took longer than expected due to slow Internet speeds.

The polished genome assembly is 1,111,798 bp, extremely similar to the three reference genomes, which are 1,111,496-1,112,957bp in length (Table 1). Alignment of the four whole genomes in Mauve shows a single continuous block, indicating that the genomes are co-linear and that no rearrangements have taken place between strains (Supplementary Figure 1).

After annotation, there were 864 predicted genes in the TM2540 strain, compared to the 864-865 genes predicted in all other strains. Roary was used to define the core genome of the four *R. typhi* strains, which included 848 genes. Of the remaining nine gene groups not found in all samples, five were found in three of the four strains, and four were unique to a single strain. Eight of these gene groups are annotated as hypothetical proteins and the one remaining gene is *rnpA* which encodes the ribonuclease P protein component and is found only in the Wilmington strain. Due to the high co-linearity of these genomes, it is possible to look for genes not present in a strain by finding the flanking genes and searching for a possible pseudogene. In two instances where the gene was not detected in one of the four strains, we were able to find a pseudogene, caused by the loss of a start codon in one case, and a frameshift mutation in a second

case. In another gene group, a 150bp insertion in the fourth strain has caused the gene to cluster separately from the other three strains.

Using the core gene alignments as input (with a total concatenated length of 839649bp), a phylogenetic tree was constructed (Figure 2) and a pairwise difference matrix was generated (Table 2). This suggests that the TM2540 strain is most closely related to the TH1527 strain from northern Thailand.

Discussion

The cost of Next Generation Sequencing has fallen significantly over the past decade. The many techniques that have evolved are becoming increasingly important tools in both research and clinical settings. Despite the falling costs, the initial investment in equipment, the technical expertise to run and maintain it and associated computing infrastructure and bioinformatics support remain a barrier to its introduction into resource-poor settings. Laos has very few clinical or research laboratories and relatively slow Internet connectivity. Although our research laboratory can perform molecular diagnostics and cell culture, to date there has been no NGS capacity. This frequently necessitates the costly shipment of samples to other countries to undertake NGS projects. The portable nature of the MinION sequencer, improved stability of newer versions of the flow cells and reagents has allowed the first successful WGS to be performed in Laos.

Murine typhus is an important and severely neglected tropical and subtropical disease of worldwide distribution. In Vientiane an estimated 10% of non-malarial fevers in adult inpatients is caused by *R. typhi*[28]. Surprisingly, for a disease to which many millions of people are potentially exposed, there are only three published whole genome sequences, and these isolates from infected humans and a bandicoot rat date back more than 50 years. We therefore selected this pathogen to attempt the first WGS in Laos.

The *R. typhi* genome is 1.1Mb and contains almost no repeats, which makes genome assembly for this species relatively simple. Running Canu with the

default ONT parameters was sufficient to assemble the genome in a single contig and we polished the assemblies using both Nanopolish, to utilize the signal-level data from ONT sequencing, and Pilon and Snippy to use the extra information available from Illumina sequencing with its lower error rate. While the synteny with other genomes suggests that there are no large misassemblies in our genome, all rounds of polishing corrected many small SNP and indel errors in the Canu draft genome. The Canu+Nanopolish assembly was an improvement over the initial draft assembly, but Illumina sequencing corrected many short deletions in our assembly that could not be corrected using ONT sequencing alone, and it is possible that errors still remain in the sequence. Even for relatively simple bacterial genomes, a combination of ONT with data from another technology with a low error rate is currently necessary to produce an accurate sequence. ONT data alone may suffice for applications which can work with sequences which still retain some base-pair level errors, such as determining large-scale genome rearrangements[29], or determining the species of an unknown sample[30,31]. Increases in sequencing accuracy, combined with bioinformatics advances in assembly and polishing software, may allow for the future use of ONT data alone to give complete and accurate sequences, increasing the applications for this device.

Comparative analysis of the four available *R. typhi* strains shows a closed pan-genome, with almost no genes which are variably present between strains, and no genome rearrangements. As *R. typhi* is an intracellular pathogen, it may have few opportunities for horizontal gene transfer from other bacteria. While the gene content is very similar between strains, we did see evidence of ongoing pseudogenisation in the genome, which is not unexpected as pseudogenes and degraded genes are common in Rickettisiales[32]. Although these four samples were collected from both humans and a rodent across a long timeframe, we see only 3,402 mutations between the two most divergent strains. The TM2540 strain is more closely related to the TH1527 and B9991CWPP strains than the Wilmington strain, which would be expected as those strains are more closely related geographically and temporally, but more strains are needed to determine whether *R. typhi* populations show geographic structure.

Several studies have demonstrated the ability of MinION to perform in remote sites with little laboratory infrastructure. The MinION was used to detect and monitor nosocomial tuberculosis infection in a university teaching hospital in Zambia[33], to sequence DNA from amphibians in montane rainforest in Tanzania[34], and for outbreak surveillance of both Ebola and Zika virus in Guinea and Brazil respectively[35,36]. There have also been several reports of off-line use of MinION in more extreme conditions, including the high Arctic, Antarctic dry valleys and aboard the International Space Station[37-39]. Here we demonstrate the current applicability of the MinION to resource-poor settings where laboratory infrastructure exists, but NGS capacity is unavailable. With concerns in some countries about the export of biological samples for WGS in other countries, MinION systems could facilitate countries without current WGS facilities to undertake such work.

Some limitations remain on the use of ONT's MinION platform. Currently individual flow cells remain costly at approximately USD900 per flow cell[40]. However, the cost per sample can be reduced as multiplex sequencing now allows for multiple bacterial strains to be sequenced on the same flow cell[9], and purchasing flow cells in larger quantities also reduces costs. The flow cell chemistry is constantly updating to improve flow cell reliability and accuracy, and increased throughput will allow for increases in multiplexing, further decreasing sequencing costs. The release of simple to use basecalling devices such as the MinIT will also improve access to sequencing when computational resources and support are limited.

Conclusions

The MinION and future ONT developments are likely to advance the capability of NGS in resource-poor settings, particularly for smaller scale projects, where local infrastructure and cost limit the opportunities to develop NGS capacity. Laos is one such setting and we here demonstrate that with limited infrastructure WGS can be successfully performed locally. We also provide the first human *R. typhi*

sequence for an isolate collected within the last 50 years and compare existing strains of this important and neglected tropical disease.

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Strain	Source	Accession	Genome length (bp)	Predicted genes	GC percentage
Wilmington	Human, North Carolina, USA. 1928	NC_006142.1	1111496	865	0.289
B9991CWPP	Bandicoot rat, Myanmar. 1970s	NC_017062.1	1112957	864	0.289
TH1527	Human, Chiang Rai, Thailand. 1965	NC_017066.1	1112372	864	0.289
TM2540	Human, Bolikhamxay, Laos. 2012	ERZ497871	1111798	864	0.289

Table 1. Strain information. The Wilmington, B991CWPP, and TH1527 strains are previously assembled strains of R. typhi; TM2540 is the assembly produced in this study.

	TM2540	B9991CWPP	TH1527	Wilmington
TM2540	-	2633	2434	3402
B9991CWPP	2633	-	505	1473
TH1527	2434	505	-	974
Wilmington	3402	1473	974	-

Table 2. Pairwise differences between strains based on the core gene alignment.

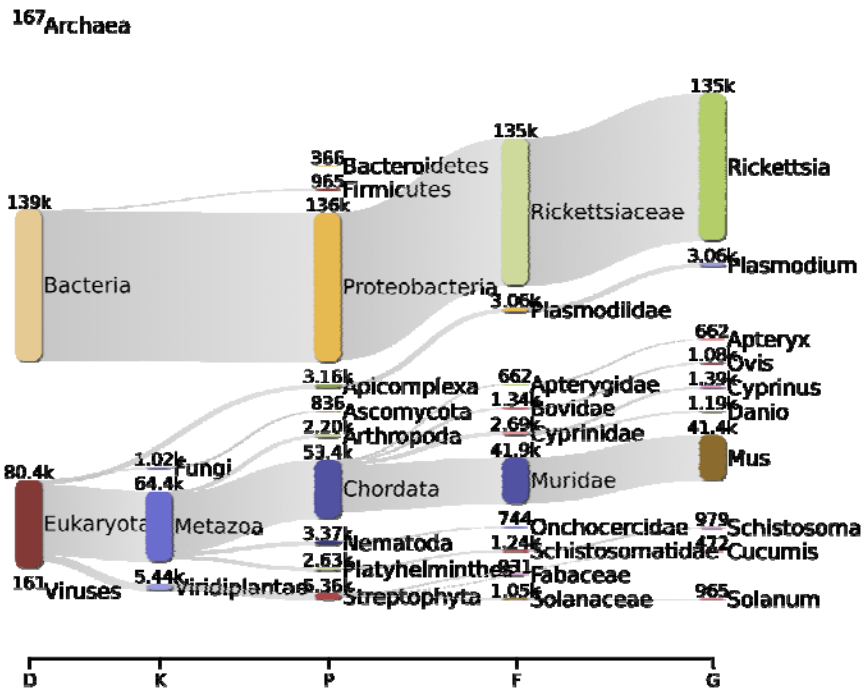


Figure 1. Pavian visualization showing the proportions of ONT reads assigned at the genus level.

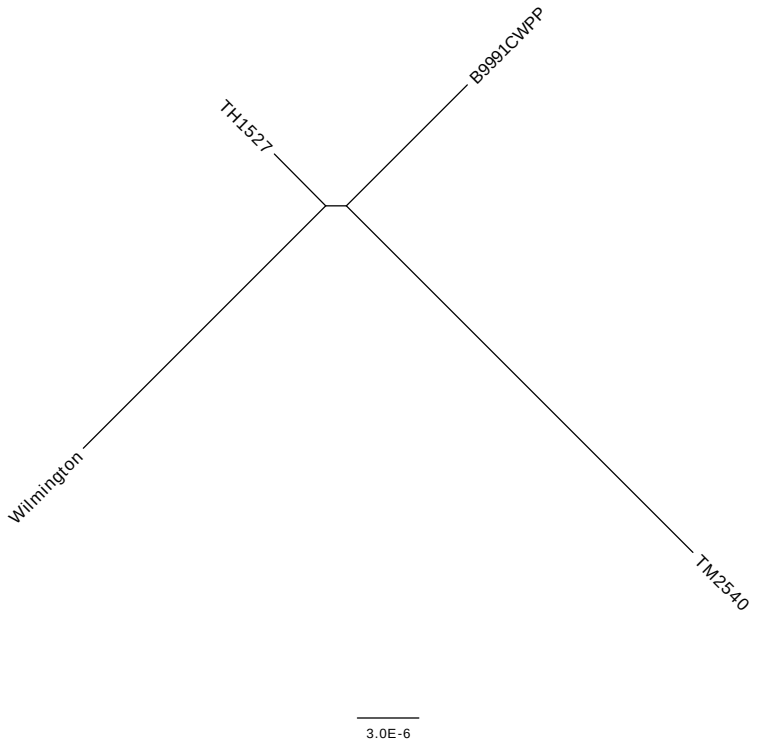
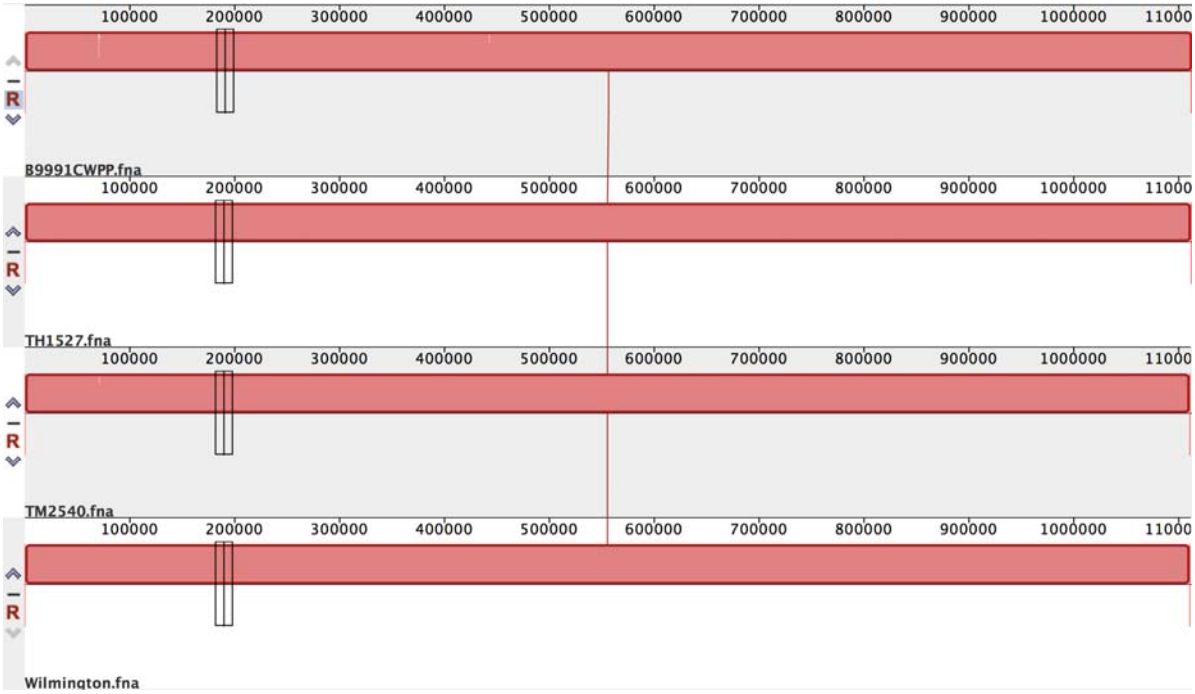


Figure 2. Phylogeny produced using core gene alignments in RAxML and visualized using FigTree.

Supplementary Information



Supplementary Figure 1. Alignment of the four strains using progressiveMauve. The genomes are one collinear block with no rearrangements.