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Screening methods for detection of ancient Mycobacterium tuberculosis complex fingerprints in NGS data derived from skeletal samples

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Abstract:	Background Recent advances in ancient DNA (aDNA) studies, especially in increasing isolated DNA yields and quality, opened the possibility of analysis of ancient host microbiome. However, this analysis could lead to numerous pitfalls, including spurious identification of pathogens based on fragmentary data or environmental contamination, leading to incorrect epidemiological conclusions. Within the Mycobacterium genus, MTBC (Mycobacterium tuberculosis complex) members responsible for tuberculosis share up to ~99% genomic sequence identity, while other more distantly related MOTT (Mycobacteria other than tuberculosis) can be causative agents for pulmonary diseases or soil dwellers. Therefore, reliable determination of species complex is highly relevant for interpretation of sequencing results. Results Here we present a novel bioinformatical approach, used for screening of ancient tuberculosis in sequencing data, derived from 28 individuals (dated 4400 - 4000 BC and 3100 - 2900 BC) from Central Poland. We demonstrate that cost effective next generation screening sequencing data (c.a 20M reads per sample) could yield enough information to provide statistically supported identification of probable ancient disease cases. Conclusions Application of appropriate bioinformatic tools, including an unbiased selection of genomic alignment targets for species specificity, makes it possible to extract valid data from full-sample sequencing results (without subjective targeted enrichment procedures). This approach broadens the potential scope of paleoepidemiology both to older, suboptimally preserved samples and to pathogens with difficult intrageneric taxonomy.
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Response to Reviewers:	Lodz, 7th of April, 2019 Dear GigaScience Editor, We appreciate the insightful and detailed comments of the reviewers. We included all the minor language-related corrections in the resubmitted manuscript. Following is a point-by-point reply to the major points raised by the reviewers. Reviewer 1 1. Our statistical approach, as we state directly in the manuscript, is aimed at finding positive outliers in a pool of samples with unknown presence of mycobacterial sequences. When applied to a dataset like Kay et al., which consists exclusively of individuals with previously confirmed ancient mycobacterial infection, its outcome is therefore necessarily limited to identifying the outliers with highest microbial load (in the case of tuberculosis, potentially those individuals who died during the active phase of the disease) - these outliers being by definition always a minority of analysed samples. This explanation is provided in the text of the manuscript. 2. We have performed the MapDamage analysis suggested by the reviewer and it indeed yielded a positive result - we thank the reviewer for this suggestion as this strengthened our conclusions significantly. We have now included a new Supplementary Fig. 4, and we have reworded both the legend to Supplementary Fig. 3 and the sentences in the manuscript that refer to it. 3. Libraries were build using Meyer et al. (2010) protocol with modifications proposed by Gamba et al. (2014). We have performed mapDamage analysis in the way which fit to double stranded libraries. Information about different Meyer protocol and single stranded libraries was incorrectly added to previous version of the manuscript. 4. We have expanded Supplementary Tab. 2 to include the absolute numbers of reads - both total and aligning to each alignment target. Reviewer 2 1. We have now expanded the analysis of state of the art in biochemical detection of ancient mycobacteria by citing recent articles mentioning improvements in cell wall component analysis. 2. Indeed, Pott’s disease is usually regarded as pathognomonic signature of TB. When we said that many pathological conditions of the spine can mimic Pott’s disease thought that they can be diagnosed mistakenly as tuberculosis, especially in practice with poorly preserved skeletons. The present text has been appropriately modified (both in the Introduction and Discussion) to clarify this statement. Moreover, according to the Reviewer’s suggestion, the list of pathological conditions has been replaced with the Table S1, which include a short description of basic differences between these lesions and bone tuberculosis. Dear Editor, regarding to your comment: “As your revised manuscript focuses more on a method, it may be more suitable as a "Technical Note" rather than a "research article" “ We would like to proceed this manuscript as research paper. In our work we present original dataset which allow us to present a novel bioinformatical approach, used for screening of ancient tuberculosis in sequencing data, derived from 28 individuals (dated 4400 - 4000 BC and 3100 - 2900 BC) from Central Poland. That dataset was not previously published elsewhere. Sincerely, Dominik Strapagiel

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Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	Yes
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Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the "Availability of Data and Materials" section of your manuscript.	Yes

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Screening methods for detection of ancient *Mycobacterium tuberculosis* complex fingerprints in NGS data derived from skeletal samples

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Abstract

Background: Recent advances in ancient DNA (aDNA) studies, especially in increasing isolated DNA yields and quality, opened the possibility of analysis of ancient host microbiome. However, this analysis could lead to numerous pitfalls, including spurious identification of pathogens based on fragmentary data or environmental contamination, leading to incorrect epidemiological conclusions. Within the *Mycobacterium* genus, MTBC (*Mycobacterium tuberculosis complex*) members responsible for tuberculosis share up to ~99% genomic sequence identity, while other more distantly related MOTT (*Mycobacteria* other than *tuberculosis*) can be causative agents for pulmonary diseases or soil dwellers. Therefore, reliable determination of species complex is highly relevant for interpretation of sequencing results.

Results: Here we present a novel bioinformatical approach, used for screening of ancient tuberculosis in sequencing data, derived from 28 individuals (dated 4400 - 4000 BC and 3100 - 2900 BC) from Central Poland. We demonstrate that cost effective next generation screening sequencing data (c.a 20M reads per sample) could yield enough information to provide statistically supported identification of probable ancient disease cases.

Conclusions: Application of appropriate bioinformatic tools, including an unbiased selection of genomic alignment targets for species specificity, makes it possible to extract valid data from full-sample sequencing results (without subjective targeted enrichment procedures). This approach broadens the potential scope of paleoepidemiology both to older, suboptimally preserved samples and to pathogens with difficult intrageneric taxonomy.

Keywords

ancient DNA, aTB, ancient tuberculosis, NGS

Background

A rapid population growth initiated in the Neolithic period, connected with domestication of animals and increase of human sedentism, played a key role in pathogen transmission within the so-called first epidemiological transition[1-4]. The identification of infectious diseases and selection of unique fingerprints of their causative agents, especially those derived from skeletal elements, are still of the greatest interest for paleopathologists and anthropologists, which is evidenced by the range of available analysis methods. Members of the *Mycobacterium tuberculosis* complex (MTBC) are genetically very closely related and are causative agents for one of the oldest human infectious diseases – tuberculosis (TB). It is a disease that may leave lesions on patients' bones, enabling a diagnosis based on bone morphology [5]. The main problem of paleopathological diagnoses based solely on dry bones is that TB-related bone changes are often nonspecific. The most reliable skeletal indicator of TB are destructive lesions in thoracic and lumbar spine sections, which can lead to destruction and collapse of vertebral bodies, resulting in spinal kyphosis, or gibbus, known as Pott's disease [5-7]. However, there are several pathological conditions which could mimic TB in dry bone leading to erroneous diagnosis, especially when they affect the spine (Supplementary Tab. 1). Although their differential diagnosis from TB is well known in paleopathology it could be problematic to use it in analysis of often poorly preserved archaeological human remains[8, 9]. Diagnoses based on bone lesions in other region of the skeleton are even more tentative; these are primarily based on osteomyelitis of the joints (most commonly the hip and knee, but also ankle and elbow) and periosteal reactive lesions (mainly in the ribs or diaphysis of the long bones, including tubular bones of the hands and feet in children [6, 9]. Bone lesions from TB in nonspinal locations may be indistinguishable from those of other etiologies[5, 6] . Lastly, morphological studies of bones do not permit detection of many individuals affected with TB in past human populations: data from the pre-antibiotic era show that bone changes occur only in about 3–7% of individuals with active TB [9].

Since the 1990s, new possibilities to diagnose TB in archaeological specimens have arisen, offered by the detection and analysis of mycobacterial DNA and other biomolecules specific to MTBC at the molecular level [10-21]. A common complication in molecular studies for ancient MTBC detection is the presence of DNA and other metabolites from the whole microbiome of the individual whose remains are being analysed as well as from environmental bacteria that have colonised the skeleton *post-mortem* [22, 23]. These contaminants might include Mycobacteria other than *M. tuberculosis* (MOTT), some of which are prevalent in the environment, while others are associated with clinical cases of non-tuberculosis diseases [22, 24-26]. It should be emphasized that members of *Mycobacterium tuberculosis* complex themselves are characterized by a particular high sequence similarity [27, 28], which leads to often unsurmountable difficulties in distinguishing them on the molecular level.

Detection of cell wall components such as mycolic, mycocerosic and mycolipenic acids [13, 15, 18, 19] with matrix-assisted laser desorption/ionization tandem time of flight (MALDI-TOF) which present profiles specific for MTBC is considered a reliable method to identify ancient causative agents in human archaeological samples. Initial attempts to use mass spectrometry to detect cell wall lipids were shown to be erroneous in some cases [15, 29, 30]. In more recent studies, the combination of cell wall lipid analysis with genetic markers showed significant improvement in discriminative ability for ancient mycobacteria [31, 32]. Polymerase chain reaction, followed by gel electrophoresis, is still a popular method for detection of MTBC ancient DNA in human samples such as bones and teeth [32-34], mummified soft tissues [35, 36], or calcified pleura [10]. Known cases of tuberculosis disease proven on the basis of ancient DNA derived from human material are old as 9000 BC [37], through Iron Age [38] and up to modern times [39]. However, PCR-based methods have not been without controversy due to the possibility of cross-contamination as well as limitations of selection of proper sequences. While repetitive insertion sequences, e.g. IS6110 and IS1081, are widely used and sometimes considered as a biomarker specific to MTBC bacteria [34], the current consensus recommends strong caution in their use due to their presence in MOTT bacteria. Those commonly used markers have even been found to occur in soil mycobacteria [40-45], and even weak homology can cause false-positive PCR results for unrelated microbes [40, 46].

Recently, next generation sequencing (NGS) methods were introduced for detection of causative agents of ancient diseases [47, 48], including MTBC, with or without pre-enrichment of MTBC aDNA [49-52]. The increasing quantity of data generated by NGS and efficiency of non-Sanger-based sequencing platforms requires a new approach in processing tools: suitable bioinformatic pipelines are required for reliable DNA analysis of ancient causative agents. Similar to PCR, where the use of only short conserved regions considered as specific for MTBC may lead to false positive results, improper analysis of NGS data can misinterpret sequences from modern known or unknown environmental *Mycobacteria* which are present in ancient human skeletons [26]. New analytical tools for more unequivocal answers to questions of identification and differentiation of *ante-mortem* causative from *post-mortem* non-causative microbial agents are urgently needed. Application of specifically designed *in silico* (bioinformatical approach) verification methods for improved downstream processing of molecular fingerprint data from ancient samples is necessary for drawing conclusions on clinical prevalence and epidemiology of pathogenic mycobacteria in history. Here we present an improved strategy for specific identification of bacteria from the *M. tuberculosis* complex in ancient non-enriched NGS data. The main purpose of this study was to design an unbiased genomic marker alignment query composed of sequences belonging strictly to MTBC members. Therefore, we present a workflow including appropriate bioinformatic alignment algorithms and statistical tools that allowed the identification of tuberculosis causative agents, using fragment length variation to balance selectivity (species specificity) with sensitivity of detection.

Sample Description

Ancient bone samples come from skeletal remains of 28 individuals representing two Neolithic populations from the Kujawy region in Central Poland: the Middle Neolithic Brześć Kujawski Group of the Lengyel culture (BKG), dated to ca. 4400-4000 BC (26 individuals) and the Late Neolithic Globular Amphora culture (GAC), dated to ca. 3100-2900 BC (2 individuals), previously described in [18, 53] (Supplementary Tab. 2). The skeletons come from two archaeological sites, BK 3 and BK 4, which represent relics of a settlement and cemetery of the BKG culture with some secondary objects within them, like the GAC grave. Both sites overlap each other, thus soil conditions and diagenetic agents were similar for all skeletal remains analyzed. Bone material was taken mainly from vertebral bodies of individuals with well-preserved skeletons. One of two individuals belonging to the GAC revealed bone lesions consistent with Pott’s disease. BKG samples provided more ambiguous evidence of skeletal lesions. One individual showed destructive lesions of the thoracic and lumbar vertebrae with central collapse of the vertebral bodies which may indicate tuberculous spondylodiscitis. Three other individuals of this population revealed only relatively mild and nonspecific inflammatory bone changes in the postcranial skeleton which were located on the internal surface of the ribs, tibia and femur shafts, as well as foot bones.

Analyses

Reference target construction (alignment target)

As our main reference sequence, we used the most commonly applied modern laboratory strain of *M. tuberculosis* (MTB), H37Rv, for which the whole genomic sequence is available. In order to select a subset of this reference sequence as an alignment target providing enhanced specificity for tuberculosis-causing agents (MTBC members), we first derived a set of all protein-coding sequences (CDS) from the H37Rv genome using the RAST tool [54]. These 4,360 sequences were screened using the BLAST tool (Megablast) at the National Library of Medicine sequentially against 12 available genomic sequences of selected MOTT: *M. kansasii, M. avium subsp. paratuberculosis, M. ulcerans, M. smegmatis, M. fortuitum, M. haemophilum, M. marinum, M. simiae, M. asiaticum, M. xenopi, M. phlei, M. abscessus*. Any detected similarities (gapless alignments >10 bp) between a H37Rv CDS and any MOTT genomic sequences resulted in the exclusion of this CDS from the result dataset, which was therefore restricted to sequences fully specific for MTBC, having no homologs in any MOTT genome. The resulting set of sequences was subsequently called the Borówka et al. alignment target and consisted of 1,534 coding sequences with total sequence length of 0.814 Mbp. Since no sequences from other MTBC species were used at this stage, and it is known that they exhibit up to 99.9% nucleotide sequence similarity [55], the constructed alignment target cannot be considered specific only for *M. tuberculosis*, but rather for the whole MTBC; this is justified in epidemiological studies on ancient samples by the need to include all clinically equivalent causative agents for the same disease entity: tuberculosis. For comparison purposes, we prepared and used two literature-derived, knowledge-based H37Rv sequence subsets as alternative alignment targets: the c. 0.046 Mbp sequence used for capture enrichment in Bouwman et al. (2012) [52] for sequencing mycobacterial samples from a 19th century skeleton, subsequently called the Bouwman et al. alignment target, and the two genes (*katG* and *mpt40*, total length 0.004 Mbp) listed as MTBC-specific among the capture enrichment probes used by Bos et al. (2014) [50] for sequencing mycobacterial samples from 11th-13th century Peruvian skeletons, subsequently called the Bos et al. alignment target. All the reference sequences were prepared for alignment by indexing with the suffix array - induced sorting algorithm, implemented in the BWA software package (BWA).

Since the construction of the Borówka et al. alignment target was based on elimination of sequences similar to other mycobacterial species, we reasoned that the performance of an alignment target is directly linked to the number of similarities between the MTB genome and other potentially interfering mycobacterial species (both ancient and environmental) present in the ancient host-derived sample. In order to quantify this, we subjected the publicly available genome sequences of *Mycobacterium* species to an *in-silico* procedure to generate collections of short sequences broadly analogous to authentic NGS reads. Including reads below a certain length in similarity analysis of ancient microbial DNA leads to non-specific matches (for both evolutionary and statistical reasons); this threshold is usually arbitrarily set to around 30 bp, but a broader analysis might make it easier to construct a reliable algorithm for detection of specific ancient pathogens. Therefore, in our further analysis both of reference and authentic ancient NGS sequences we extracted groups (bins) of non-human sequences over several length thresholds: ≥20bp, ≥25bp, ≥30bp and ≥35bp, to enable a thorough analysis of specificity gain upon increase in minimal sequence length. For reference *Mycbacterium* genomes, k-mers of specified length (corresponding to the lower limit of read length for NGS bins: 20, 25, 30 or 35) were filtered against the human genome assembly hg19, and the resulting "short read" collections were aligned to the full MTB reference genome or its selected subsets (Borówka et al., Bouwman et al. and Bos et al. alignment targets). Table 1 shows the respective number of genomic k-mers from MTB complex and MOTT species which match the MTBC alignment targets as well as the total lengths of assayed genomes for comparison. Since the various subsets of the MTB genome differ in length and thus the probability of random match increases with target length, we standardised the obtained data by presenting it as a percentage of k-mers from a given mycobaterial genome that match the alignment target, divided by the ratio of target length to the full MTB genome length (genomic coverage of the target). These values, which are an inverse measure of alignment target specificity (they increase if more "reads" from a species which is not MTB or MTBC can be mistaken for MTBC), are shown in Table 1. As a reference, the MTB genome itself was also subjected to this procedure - obviously, the match percentage values are almost 100% here. Several conclusions can be drawn from these data: firstly, it is obvious that selecting longer reads (in this case longer k-mers) for comparison increases specificity, with reads 30 bp long or longer optimal for specific identification of the MTB complex, reflecting a common consensus in the field. However, it is important to note that shorter reads still add important information to the analysis, as the rate of specificity increase (decrease in matching read percentage with increase in read length) varies between species (i.e. some species have longer stretches of highly similar sequence to MTB). For example, while *M. smegmatis* has a very high match percentage to the Borowka et al. alignment target at low read length, this is rapidly lost at longer (more genuine) read lengths; the opposite is true e.g. for *M. marinum*. It is a derivation of the evolutionary history of the genus, but in this case also a practical caveat for further interpretation of sequence matches in actual aDNA samples. Moreover, the specificity of various alignment targets varies, with the Borówka et al. target being consistently the most specific (for longer k-mers) for distinguishing MOTT, while it is (by design) not well suited to distinguishing other members of the MTB complex from MTB itself.

Since we intended to develop a highly specific screening test (based on low depth sequencing strategy) for verification of MTBC infection in Neolithic samples with *a priori* relatively low degree of aDNA preservation, we decided on a statistical approach. Since any preserved ancient mycobacterial DNA would be only a fraction of total aDNA, and it in turn would only be a fraction of total reads (the balance being the modern environmental metagenome), a balance between sensitivity and specificity in verifying this very low number of reads must be struck. In sedentary, communal populations MTBC infection tends to be epidemic in character, but in most individuals with latent infection the microbial load (and thus the probability of DNA survival in ancient samples) is relatively low and constant. Any similarity analysis based on sequence alignment will also invariably generate false positive alignment hits, thus, it would be impossible to construct a test with sufficient statistical power to distinguish individuals genuinely free of ancient MTBC and those with average/modest latent infection. Therefore, we concentrated on the detection of outlier individuals with high microbial load (which may be later selected for enrichment-based further genetic analysis, such as phylogenetic studies or genome reconstruction), measured by the positive read ratio (the intrinsically very low ratio of reads matching the MTBC alignment target to all eligible reads). Based on the epidemiology of MTBC infection, we assumed a quasi-normal distribution of positive read ratios in a randomly selected sample of ancient individuals, with outliers as candidates for active tuberculosis and for selection for more in-depth studies. Thus, our method was based on standardising read ratio values to normal distribution parameters (arithmetic mean and standard deviation) and, as a further step in the detection algorithm for ancient tuberculosis (aTB), we applied a typical cutoff value of 1.5xSD to detect outliers.

As a first stage of testing our screening approach on actual NGS data from ancient material, we used a control dataset based on published NGS results of confirmed tuberculosis-infected individuals - 18th/19th-century mummified bodies from a crypt in Vác, Hungary, described by Kay et al. (2015) [48]. The aim of that study was to reconstruct and analyse historical genome sequences of *M. tuberculosis*, which resulted in sequencing results with high coverage. Since all these samples (26 bodies) were previously demonstrated by PCR to come from infected individuals [56], application of our screening procedure did not aim at distinguishing "positive" from "negative" samples, but at validating the selection of individuals with highest microbial load (especially since some of them were sampled from 1-3 different parts of the body), at the same time enhancing specificity (vs. MOTT). We used the Kay et al. dataset for verification of specificity of all applied alignment targets: Borówka et al., Bouwman et al., Bos et al. and the whole genome sequence of *M. tuberculosis* H37Rv, with our algorithm aimed at detection of strongest aTB outliers. While application of the Borówka et al. target sequence (with 30 bp read length cutoff) detected four samples as outliers, they turned out to belong only to two individuals (bodies 68 and 92) (Supplementary Tab. 3). This validated our approach as a suitable method for selecting ancient samples with highest MTBC genetic material content, especially since, despite our alignment target consisting only of sequences specific exclusively for MTBC, it turned out that those four samples were also those that showed the highest ratio of aligned reads to the full *M. tuberculosis* reference sequence (and thus the highest number of reads used to reconstruct the ancient genome) in the original study by Kay et al. (shown there in Supplementary Tab. 3). Moreover, only the two alignment targets prepared with both specificity and sensitivity in mind (Borówka et al. and Bouwman et al.) led to identification of all three samples from body 68 as outliers.

Subsequently, we applied the full statistical approach (with all four NGS read length bins) and the four selected genomic alignment targets: full reference *Mycobacterium tuberculosis* H37Rv genome (broadest possible target), two published targets consisting of rationally selected genes (applied previously to enrichment-based sequencing: Bouwman et al. and Bos et al.) as well as the novel specificity-tailored target (Borówka et al.), to the Neolithic samples from Brześć Kujawski. Table 2 presents the number of reads in each read length bin used for alignment with targets and statistical analysis, while Supplementary Tables 4-7 show the alignment results as numbers and ratios of matching reads. Fig. 1 presents the results of statistical analysis as outlying standardised ratio values in different read length bins. Overall, the expected population structure of majority of individuals with few positive reads and outlier individuals with an exceptional number of positive reads is confirmed. However, it is immediately obvious that the composition of outlier individuals depends strongly not only on the genomic alignment target, but also on minimum length of reads used for the alignment. There are individuals who remain positive (with a high relative ratio of reads aligning to the respective target) for all four length bins (e.g. 4_BK4 for the *Mycobacterium tuberculosis* H37Rv target), i.e. the share of putative MTBC-derived sequences remains constant despite the decrease in number of analysed sequences and increase in sequence complexity. There are individuals who, despite being outliers for the bins including shorter reads, lose this status for the more restrictive bins (e.g. 55_BK4 for the Borówka et al. target), i.e. the majority of their MTBC-like sequences were of low complexity. Contrastingly, in some individuals the share of MTBC-like sequences increases above the cut off value only for bins with longer reads (e.g. 31_BK4 for the Borówka et al. target), i.e. most specifically aligned fragments are relatively long. It is again apparent that since most of this change concerns reads between 20 and 29 bp in length, the optimal threshold for read aligning to a genomic target for specificity towards MTBC is ≥30 bp. Thus, the three individuals which exceed the threshold of 1.5xSD for the MTBC-specific Borówka et al. target (17_BK4, 29_BK4 and 31_BK4) are considered with high probability to be ancient cases of MTBC infection and merit selection for further in-depth studies by a more cost-intensive approach.

Since the cut off-based detection algorithm, while robust for the presented dataset, may be less suitable for other, less homogenous groups of ancient individuals, we also set out to construct an objective, parametric testing-based outlier detection algorithm. Since the main objective of our overall study is specificity of MTBC detection, we applied this algorithm to the original Borówka et al. genomic alignment target. Based on the observation that positive read ratio tends to depend monotonically on read length bin – either consistently increasing or decreasing for outlier individuals – we decided to calculate a monotonicity parameter. We first standardised positive read ratios as percentage of average positive read ratio (without assumptions towards normal distribution, Supplementary Fig. 1) and then calculated ratios of these values for adjoining read length bins (≥25bp/≥20bp, ≥30bp/≥25bp and ≥35bp/≥30bp). The arithmetic mean of these values (Supplementary Tab. 8) depended on monotonicity of the studied relationship and had a normal distribution among individuals in our study. For outlier detection, we applied a one-tailed critical z value test on both tails on the sample. We consider the positive outliers (individuals with consistently increasing share of positively aligned reads with increasing read length) to be potential individuals with high MTBC loads, suitable for further analysis both by virtue of good mycobacterial genomic material preservation and high certainty of this material belonging to ancient MTBC. On the other hand, negative outliers may either be individuals with ancient MOTT infection (we suggest this as highly probable for 4_BK4) or samples with high proportion of short, non-specific alignments, probably due to environmental contamination (most probably 55_BK4) - to distinguish these two groups, a comparison with the more Mycobacterium-generic whole-genome alignment target is necessary (see below). This approach, while retaining the strong specificity of the cut off approach, gains increased sensitivity due to inclusion of individuals with high background of environmental sequences (low initial positive alignments in the short-read bin) which nevertheless retain specific long positively aligned sequences upon read length restriction, e.g. 21_BK4.

An immediately obvious result of our analysis was that the comparison of alignment targets constructed with different assumptions leads to surprisingly large differences in assignation of individuals. Aligning aDNA sequences versus the whole MTB genome results in identification of two strong outliers (4_BK4 and 32_BK4). The same two individuals are identified, albeit with a smaller divergence, by using the enrichment bait sequence set uses by Bouwman et al. as alignment target. Since this subset of genomic sequences was originally selected for enrichment of lineage-distinguishing polymorphisms rather than for MTB complex specificity, this result is expected and confirms the efficiency of the outlier detection method and ≥30bp as optimal read length. On the other hand, our Borówka et al. genomic subset selected on the basis of MTB complex specificity led to identification of three different individuals as outliers (17_BK4, 29_BK4 and 31_BK4), while 4_BK4 and 32_BK4 had positive read values close to average. This is even more conspicuous when positive ratio values for the two different alignment targets (whole genome and specific subset Borówka et al.) are plotted against each other (Fig. 2). In our opinion this points to the broadly recognized risk of mistakenly identifying ancient infections caused by MOTT as tuberculosis based on the extensive similarity between the respective mycobacterial genomes. While restricting the alignment target leads to loss of sensitivity due to unavoidable decrease of absolute number of aligned reads, which is a significant problem for ancient DNA, it is offset by the increase in specificity of detection. This distinction is crucial for epidemiological hypotheses where elimination of false positives is of paramount importance. We further show this by aligning our reads to the purportedly MTBC-specific target sequences selected by Bos et al. (sequences of only two *M. tuberculosis* specific genes), where increase of specificity leads to detection of the 29_BK4 individual, but the extreme loss of sensitivity linked to minuscule absolute number of reads (the highest number of positive reads in the ≥30bp bin is 13 – see Supplementary Tab. 7) leads to high experimental noise and low reliability of assignment of individuals, and it is not recommended.

Since for two individuals which were strongly enriched in mycobacterial sequences (4_BK4 and 32_BK4) we posit the existence of an ancient MOTT infection (as they do not score highly in comparison with the specific Borówka et al. alignment target), we decided to verify if this assumption is supported by aligning the optimal read bin (≥30bp) to full genomes of other mycobacterial species as targets. Indeed, as seen in Supplementary Fig. 2, those two individuals are also strong outliers in read ratio values after aligning to the *M. marinum* genome - moreover, when plotted against read ratio values for the MTB genome, it is apparent that they show higher similarity to *M. marinum*, since they are located on the *M. marinum* side of the read ratio regression line. This finding validates our workflow in that it corroborates the usefulness of read length binning while further demonstrating the advantages of read aligning to targets selected for species discrimination (like the Borówka et al. target) which allow for immediate flagging of suspicious samples with spuriously high absolute similarity to the MTB genome. We have also attempted to verify the possibility of distinguishing samples with predominantly ancient mycobacterial sequences from samples with recent environmental MOTT contamination by performing mapDamage analysis. MapDamage analysis shows that the low absolute number of reads that map to all *M. tuberculosis* alignment targets (including the full MTB genome) in the case of our samples prevents us from drawing meaningful conclusions in this regard (even for the samples with highest read numbers - 4_BK4, 32_BK4, 17_BK4, 29_BK4, 31_BK4). For general confirmation of ancient status of analysed reads, MapDamage analysis was performed for human sequences (aligning to the human genome build 37) and is presented in Supplementary Fig. 3 for all 6 individuals with potential MOTT and MTBC infections. Since the samples with potential MOTT infection (4_BK4, 32_BK4) included a substantial number of reads that aligned to the *M. marinum* genome (Supplementary Tab. 9), we were also able to perform MapDamage analysis for these reads (Supplementary Fig. 4), confirming the ancient character of mycobacterial sequences.

Discussion

The evolutionary and ecological complexity of mycobacteria, including the existence of a group of closely related pathogens known as the *Mycobacterium tuberculosis* complex, consists of a large number of more distantly related human and animal pathogens causing diseases other than tuberculosis, and an abundance of free-living (including soil- and water-borne) mycobacterial species in the environment. These all contribute to the difficulty in the unequivocal determination of ancient tuberculosis on the basis of MTBC aDNA. Present-day paleoepidemiology uses tools of classical biological anthropology as well as modern clinical diagnostics at the molecular level. Morphological diagnosis of tuberculosis is based on certain bone changes, especially those described as Pott's disease. This approach is not optimal from the point of view of sensitivity, since bone lesions are present only in 2% of all cases of tuberculosis infection and 10-20% of cases of extrapulmonary tuberculosis [41, 57]. The specificity of this tool is also relatively low: even in the case of Pott’s disease, which is regarded by paleopathologists as the pathognomonic skeletal signature of TB, there are several lesions that may be difficult to differentiate from TB in archaeological skeletal remains. . In spite of that limitations, osteological analysis is often the main starting point of a study and cannot be disregarded. However, in our study the occurrence of bone lesions that could be linked in any way with tuberculosis did not correlate with the results of our genetic analyses. There are two possible explanations for this fact. First, the bone changes were not caused by tuberculosis, which is in accordance with a lack of pathognomonic characteristics of the disease on the skeleton alone, as was clarified before; it applies primarily to the graves 12_BK4, 18_BK4, 47_BK4, and 73_BK4. It may also be that the preservation of MTBC aDNA was too poor to pass the sensitivity/specificity threshold of the method proposed here.

Among molecular techniques which are used for diagnosis of ancient tuberculosis cases, both biochemical methods based on mass spectrometry and PCR amplification of marker sequences have been successfully used in literature, e.g. for preliminary description of the Hungarian mummies used subsequently to reconstruct aTB genomes [48, 56]. However, both these groups of methods suffer from a number of drawbacks which make them less useful in an ancient epidemiological context than in a contemporary one: environmental contamination from modern soil mycobacteria can overwhelm both traces of ancient MTBC mycolic acids and less specific PCR amplicons, while strong care must be taken to prevent in-lab cross-contamination with genuine MTBC samples. Therefore, NGS has a number of advantages in diagnosis of ancient tuberculosis, having the potential to be both highly sensitive and highly specific; but the balance between sensitivity and specificity depends on the selection of reference genomic sequences and crucially on the method of alignment. A large quantity of generated data allows potentially to detect ancient mycobacteria selectively, unequivocally and semi-quantitatively, while making possible additional analyses such as preservation period-related DNA damage pattern detection (e.g. mapDamage [58, 59], phylogenetic analysis of genetic kinship [50] or even full genome reconstruction [48]). Due to small absolute amounts of actual ancient pathogen DNA in most types of human body samples, a common approach is to use pre-sequencing enrichment (usually using probe capture, e.g. [50]). Only in bodies preserved in exceptional, isolated conditions, such as the Hungarian mummies from a 18th century crypt, was a non-enriched metagenomics approach used [48]. Use of enrichment techniques strongly increases sensitivity, but comes with its own drawbacks (apart from increased cost), the most relevant of which is the need to pre-design a set of sequences (probes or primers) that will define and limit the scope of subsequently obtained NGS data. A full metagenome approach is often more relevant when dealing with a highly ancient sample like in the present study, when neither the infection prevalence nor the pathogen identity are known to any precision and a preliminary NGS study is needed for formulation of specific hypotheses and pre-selection of individuals for further analysis.

However, in the case of ancient MTBC (especially samples more than a thousand years old), specificity is a more important consideration than sensitivity. While modern MTBC contamination in the laboratory is a risk factor, it would not mask ancient data in a semi-quantitative study and would be obvious if DNA damage analysis were performed. A more important consideration is the possible presence of ancient MOTT which can be unpredictably genetically similar to MTBC. The sources of these MOTT can be either soil contamination (including dead animals) which could have happened at any time since inhumation (preventing reliable elimination by DNA damage analysis), or actual ancient MOTT which were pathogenic/infectious/commensal to ancient humans. Thus, the design of sequencing analysis workflow has to take into account the necessity to filter out unknown related sequences that are not derived from MTBC - this was the main rationale behind the design of our study. While contamination with mycobacterial sequences within the laboratory (amplicons, genuine Mycobacterium DNA) can be prevented by correct workflow (separation of pre- and post-PCR areas etc.), equipment and strict procedures, contamination by environmental DNA is inescapable and has to be taken into account in the case of archaeological bone samples preserved by inhumation. Since for ancient samples direct contact of bones with the environment has lasted for a very long time (unlike more recent samples from vault inhumation), mycobacterial DNA derived from environmental (soil) MOTT can have undergone accretion in bones throughout this period, with some of it ancient enough to be indistinguishable in terms of location and state of preservation from DNA of infectious microbes buried with the body. All MTBC are obligate pathogens and thus are an unlikely source of environmental contamination of ancient samples. Therefore, for preliminary identification of potentially interesting samples in ancient inhumated bones, specificity in methods of detection of ancient infectious agents from this group should be developed towards exclusion of MOTT, with distinction between members of MTBC as a secondary, much less important goal. Since MTBC also share a very high proportion of coding sequences, achieving specificity for *M. tuberculosis* s.s. could occur only by drastically limiting the size of the reference marker sequence, thus leading to very low sensitivity, especially for usually highly degraded aDNA. Moreover, the division of MTBC into lineages is not entirely concordant with classical taxonomic division into species, so attempting an artificial distinction between some lineage groups based on accumulated NGS data would not be recommended. Our approach is designed as a relatively low-cost, first-pass classification of ancient samples based on whole-metagenome NGS data. When a highly specific method like the one we propose is used to identify likely ancient MTBC infection, potential lineage determination or any other phylogenetic studies (in pre-selected samples) should proceed by other methods developed specifically for this purpose, based on the presence of lineage-specific polymorphisms (with the caveat that enrichment for specificity-related sequences before NGS will certainly lead to loss of the majority of phylogenetically important loci, so a full metagenomic sequencing round with sufficient coverage is inevitable).

We postulate that a combination of read length-based genomic alignment analysis and a careful knowledge-based selection of the alignment target makes it possible to achieve relatively high specificity of aTB detection against all potential false positive sources. Therefore, a robust tool for specifically identifying NGS-derived sequences that belong to ancient MTBC with high confidence is a priority task in molecular paleoanthropology. Even more relevant to paleoanthropological studies, confusion between MOTT and MTBC can lead to spurious identification of ancient individuals as tuberculosis sufferers or carriers, invalidating conclusions relevant to paleoepidemiology. We demonstrate that read length selection is not only highly relevant (as has been shown before and by us, only reads above ca. 30 bp can be used with high confidence), but when a statistics-based approach to multiple length thresholds is used, it can yield a substantial increase in specificity of MTBC identification. At the same time, selection of pre-filtered alignment target, with combined knowledge-based (selection of transcribed sequences) and automated (exclusion of sequences aligning with MOTT genomes) delineation of MTBC-specific sequences (which we call the Borówka et al. target), makes it possible to perform in-depth specificity analysis by comparing the alignments of *in silico* fragmented mycobacterial genomes (mimicking actual NGS data). Combining the novel alignment target and the read length binning approach, we were able to select with high confidence three ancient individuals with probable ancient MTBC infection and two further individuals with highly probably ancient mycobacteriosis caused by MOTT (which would be misidentified as tuberculosis if another alignment target or to short reads were taken into account). Of course the limitations of our data make these identifications preliminary and another round of directed (e.g. enrichment-based) sequencing would be required both for positive identification of the infectious agent and for potential phylogenetical analysis of its spatial and/or temporal kinship. However, in our case read length analysis allowed us to suggest *M. marinum* as the potential ancient infectious agent based on statistical analysis; obviously, positive confirmation of this diagnosis would require tools that are currently unavailable such as proven *M. marinum*-specific enrichment probes as well as a much better sequence coverage that could be achieved in a preliminary study (Supplementary Fig. 2). Still, this possible pathogen identification is not at odds with the archeological context as the inhumation site is next to a lake (Smetowo) and within a geographical region rich in post-glacial lakes (Kujawy), so some individuals could have had routine professional contact with fish. Our combined procedures used robust tools but cannot be treated as definite proof. Our samples are relatively old (in comparison to most other ancient tuberculosis cases studied by molecular means before) and thus the absolute read numbers from an unbiased NGS approach is low. We demonstrate that this disadvantage makes it relatively difficult to perform DNA damage analysis (except for samples with a very high absolute number of reads). However, we provide a consistent proof of concept for a tool which allows relatively cheap and unbiased selection of samples (e.g. individuals) for further analysis, e.g. by enrichment capture NGS. Thus, we suggest that it is possible to use global NGS results from ancient samples as an economical pre-screening tool for more complex methods, while applying bioinformatic tools to maximise the number of reliable conclusions that can be drawn from a limited dataset.

Methods

Ancient DNA extractions

A dedicated ancient DNA sample preparation facility at the University of Lodz was used, taking standard precautions to avoid any contamination. All disposable materials, buffers, water, clean room surfaces and bone material, were UV-irradiated for at least 30 minutes before any subsequent steps were taken. The fragments of bone material were isolated using Dremel disks, (USA), surface-cleaned, UV-irradiated for

7.5 minutes on each side, and ground into a fine powder, further used for DNA extraction procedures following the protocol of Dabney et al. with modifications [60-62]. Ancient DNA was successfully isolated from all bone samples (See Supplementary Fig. 3). Illumina libraries were prepared in separate facility, according to Meyer et al. protocol [63] with modifications proposed by Gamba et al. [60] without UDG treatment of the samples. All libraries were subjected to the screening next-generation sequencing on the Illumina Nextseq 500 platform (100bp single-end sequencing), yielding between 2.2 and 33.9 million reads per individual (median number of reads after incomplete and truncated read trimming – 16.9 million reads per individual, Tab. 2). This dataset contains ancient human sequences from the deceased individuals, ancient microbial sequences from parasites, pathogens, commensals or symbionts of the deceased individuals, as well as genomic sequences from environmental organisms (mainly microbes, but also potentially higher Eukaryotes), to which the skeletal remains were exposed *post-mortem*.

Bioinformatical procedures

Raw NGS reads were subjected to standard quality processing such as trimming and adapter sequence removal (-q 30 --phred33 --illumina --length 20), using the Trim Galore! software package [64]. Since the predominant expected type of sequence in skeletal samples is ancient human genomic DNA and its presence would unnecessarily complicate our analysis, the read datasets were subsequently subjected to filtering by alignment to the standard (hg19) human genome reference sequence. This alignment was performed using the BWA_aln algorithm (-n 0.04, -l 1000), with duplicate removal, using the AGAT software tool - ocwrapper3mt.py script [65]. Any read which aligned without gaps within the default mismatch rate (dependent on sequence length, e.g. 2 mismatches per 17 bp) was eliminated from the sample dataset. Subsequently, separate sub-datasets (bins) of reads were generated on the basis of (trimmed) read length: minimal read length threshold ~~-~~≥20bp, ≥25bp, ≥30bp and ≥35bp. These datasets were used for alignment to reference targets. These procedures were applied also to the Kay et al dataset, used for the Borówka et al. method verification.

Estimation of terminal base deamination damage pattern was done by using mapDamage2.0 analysis with specifying a length (-l) of 75 bp (Supplementary Fig.3 and Supplementary Fig. 4).

Query sequence preparation

Selected 18 reference *Mycobacterial* genomes, including 5 of *M. tuberculosis* complex (underlined): *M. abscessus*, *M. africanum*, *M. asiaticum*, *M. avium*, *M. bovis*, *M. caprae*, *M. fortuitum*, *M. haemophilum*, *M. kansasii*, *M. leprae*, *M. marinum*, *M. microti*, *M. phlei*, *M. simiae*, *M. smegmati*, *M. tuberculosis*, *M. ulcerans*, *M. xenopi* were used. Nucleotide sequences of each organism have been subjected to fragmentation with FA_TOOL script (small_tool.py) [66] respectively for 20 bp, 25 bp, 30 bp and 35 bp-long fragments and allocated in same manner to length bins. Further, fragmented genomes were used for specificity testing of each constructed target which allowed to overcome the problem of very short and non-specific fragments with threshold estimation.

Verification of specificity and sensitivity of NGS screening method

Due to the lack of available NGS data of positive M. tuberculosis cases, we tested in-silico methods by using the Kay et al. (2015) dataset (PRJEB7454), derived from Hungarian mummies tissue microbiome sequencing. SRA files for each sample were identified and downloaded, further fastq files passed through trimming with deprivation of the adapter sequences [65]. Raw sequencing files were conducted to human genome reference sequence (hg19) filtration in spite the fact that host DNA material could be dominant in the sample. Alignment was performed to the tested targets M. tuberculosis H37Rv, Borówka et al., Bos et al., and Bouwman et al. using the AGAT software tool [65]. Statistics for each individual are presented in Supplementary Table 3. Summarized results of aTB cases from Brześć Kujawski are included in Supplementary Tables 4-7.

Statistical processing and parametric testing-based outlier detection algorithm

Collected unmapped sequences from the original dataset, as well as from the Kay et al. dataset, were aligned to constructed marker sequences: *M. tuberculosis H37Rv*, Borówka et al. **(**Supplementary Table 10**)**, Bos et al., and Bouwman et al. with application of experimentally determined minimal read length threshold ≥17 bp, ≥20bp, ≥25bp, ≥30bp and ≥35bp for detection of potential ancient MTBC cases. For detection of outlier individuals with high microbial load/positive read ratio, we standardised read ratio values to normal distribution parameters (arithmetic mean and standard deviation) and, as a further step in the aTB detection algorithm, applied a typical cut off value of 1.5xSD to detect outliers, postulating these to be candidates for active tuberculosis.

Based on the observation that positive read ratio tends to depend monotonically on read length bin – either consistently increasing or decreasing for outlier individuals – we decided to calculate a monotonicity parameter. We first standardised positive read ratios as percentage of average positive read ratio and then calculated ratios of these values for adjoining read length bins (≥25bp/≥20bp, ≥30bp/≥25bp and ≥35bp/≥30bp). For outlier detection, we applied a one-tailed critical z value test on both tails of the sample. We consider the positive outliers (individuals with consistently increasing share of positively aligned reads with increasing read length) to be confirmed ancient tuberculosis sufferers (See Supplementary tables 3-7).

Availability of supporting data and materials

The datasets supporting the conclusions of this article are available under the NCBI repository project "Identification of ancient tuberculosis in human archaeological remains" (acc. num. PRJNA422903) including Biosamples and related Sequence Read Archive (SRA). Other supporting data are available via the Gigascience database, GigaDB [67].

Additional files

Borówka_et_al_Supplemetary_Tables.xls

Borówka_et_al_Supplementary_Tables_legends.doc

Borówka_et_al_Supplementary_Figures.pdf

Declarations

Abbreviations

aDNA – Ancient DNA

aTB – Ancient tuberculosis

NGS – Next Generation Sequencing

MTBC – *Mycobacterium Tuberculosis* Complex

MOTT – Mycobacteria other than tuberculosis

SRA - Sequence Read Archive

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

P.B. and D.S. conceived the study, were responsible for extraction of aDNA, preparation of NGS libraries and Next Generation Sequencing of samples. P.B, D.S and Ł.P analyzed the data, discussed the results, and wrote the manuscript. Ł.P. participated in the statistical analysis and figure preparation. B.M wrote and ran AGAT primary analysis. B.B-S. precipitated in sample selection and preparation for laboratory phase. J.D., WL analyzed the samples for pathological changes, participated in the study design, analyzed and discussed the data, and participated in drafting the manuscript. E.Ż. participated in the study design, analyzed and discussed the data, and participated in drafting the manuscript. D.S. coordinated studies and was responsible for the final version of the manuscript; all authors read and approved the final manuscript.

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Tables and Figure legends

Table 1. Number of genomic k-mers from MTBC and MOTT members after initial hg19 clearing step matching selected targets, with k-mer length distinction (≥20bp, ≥25bp, ≥30bp, ≥35bp). with estimation of percentage of k-mers from a given mycobaterial genomes matching the *M. tuberculosis* genome for query length ≥30 and ≥35.

	k-mer length		**Query length ≥20**					**Query length ≥25**					**Query length ≥30**					**Query length ≥35**				
	Alignment target	**Genome length (bp)**	**% of sequences mapped to M. tuberculosis genome**	**Full genome**	**Borowka et al.**	**Bos et al.**	**Bouwman et al.**	**% of sequences mapped to M. tuberculosis genome**	**Full genome**	**Borowka et al.**	**Bos et al.**	**Bouwman et al.**	**% of sequences mapped to M. tuberculosis genome**	**Full genome**	**Borowka et al.**	**Bos et al.**	**Bouwman et al.**	**% of sequences mapped to M. tuberculosis genome**	**Full genome**	**Borowka et al.**	**Bos et al.**	**Bouwman et al.**
Species group																						
	M. leprae	3268203	3.19%	140922	19736	101	1683	4.88%	215257	4349	85	2240	2.61%	115138	1430	26	1201	1.45%	63860	543	6	715
MOTT	*M. abscessus*	5067172	5.26%	232228	46530	158	2915	2.87%	126769	2816	103	1890	1.39%	61160	283	46	1065	0.75%	33175	62	14	644
	M. smegmatis	6988209	11.19%	493570	107339	543	6917	5.68%	250537	7793	340	2919	2.88%	127219	1187	162	1610	1.64%	72286	262	65	944
	M. fortuitum	6254616	8.48%	374030	77785	291	5208	5.22%	230382	5940	131	2774	2.69%	118483	958	40	1534	1.53%	67463	236	16	916
	M. phlei	5349645	8.98%	396255	88582	391	5909	6.57%	289788	9912	157	3597	3.45%	152331	1665	97	1951	2.03%	89593	377	56	1176
	M. simiae	5938797	9.33%	411677	80142	339	5414	9.51%	419641	12734	197	4578	5.35%	235800	3904	93	2702	3.15%	139050	1450	33	1575
	M. asiaticum	5910436	9.00%	396854	76829	413	5597	10.69%	471493	19780	392	5022	0.00%	265638	5366	186	2806	3.54%	156188	1531	71	1706
	M. xenopi	4434836	7.14%	314850	60482	262	4336	8.17%	360395	11534	207	4105	0.00%	200395	3233	120	2126	2.62%	115687	1060	68	1235
	M. marinum	6660144	9.48%	418304	82499	466	5715	14.08%	621166	52438	707	6366	7.88%	347459	16301	266	3465	4.49%	198076	4208	88	2046
	M. ulcerans	5805761	8.26%	364492	71682	339	4800	12.26%	540893	36626	448	5543	6.94%	306075	10994	160	3094	4.04%	178217	3088	61	1886
	M. kansasii	6402301	10.51%	463445	89051	472	6353	15.93%	702577	39990	596	7181	9.54%	420814	13458	278	4032	5.82%	256893	4132	129	2373
	M. avium	4829781	8.07%	356159	71620	322	5128	12.08%	532953	16610	194	5331	7.31%	322606	4752	110	3271	4.58%	202232	1475	65	2095
	M. haemophilum	4235765	7.08%	312375	52214	274	4137	13.05%	575862	22641	540	6284	7.98%	352034	8023	374	3703	4.94%	217744	2893	254	2322
MTBC	*M. caprae*	4288871	17.53%	773238	181627	598	9935	94.85%	4184378	734742	2306	37814	96.27%	4245996	725608	2253	35935	96.21%	4244109	713211	2214	34394
	M. microti	4370115	17.81%	785606	188016	825	10498	96.71%	4266542	772527	3989	40576	98.17%	4330722	771950	3873	38507	98.12%	4328596	758572	3785	36841
	M. africanum	4389314	17.87%	788161	186939	850	10494	97.15%	4285645	764554	4038	40740	98.63%	4350937	764150	3893	38685	98.60%	4349503	751103	3796	37018
	M. bovis	4345492	17.72%	781857	184148	592	10161	96.31%	4248729	750458	2304	39042	97.79%	4313964	749050	2252	36990	97.76%	4312566	735993	2213	35367
	M. tuberculosis	4411532	18.07%	797099	192022	833	10844	98.41%	4341179	791071	3947	42253	99.97%	4410355	792717	3851	40180	100.00%	4411458	779771	3777	38435

Table 2. Number of reads (per individual) used for alignment and statistical processing.

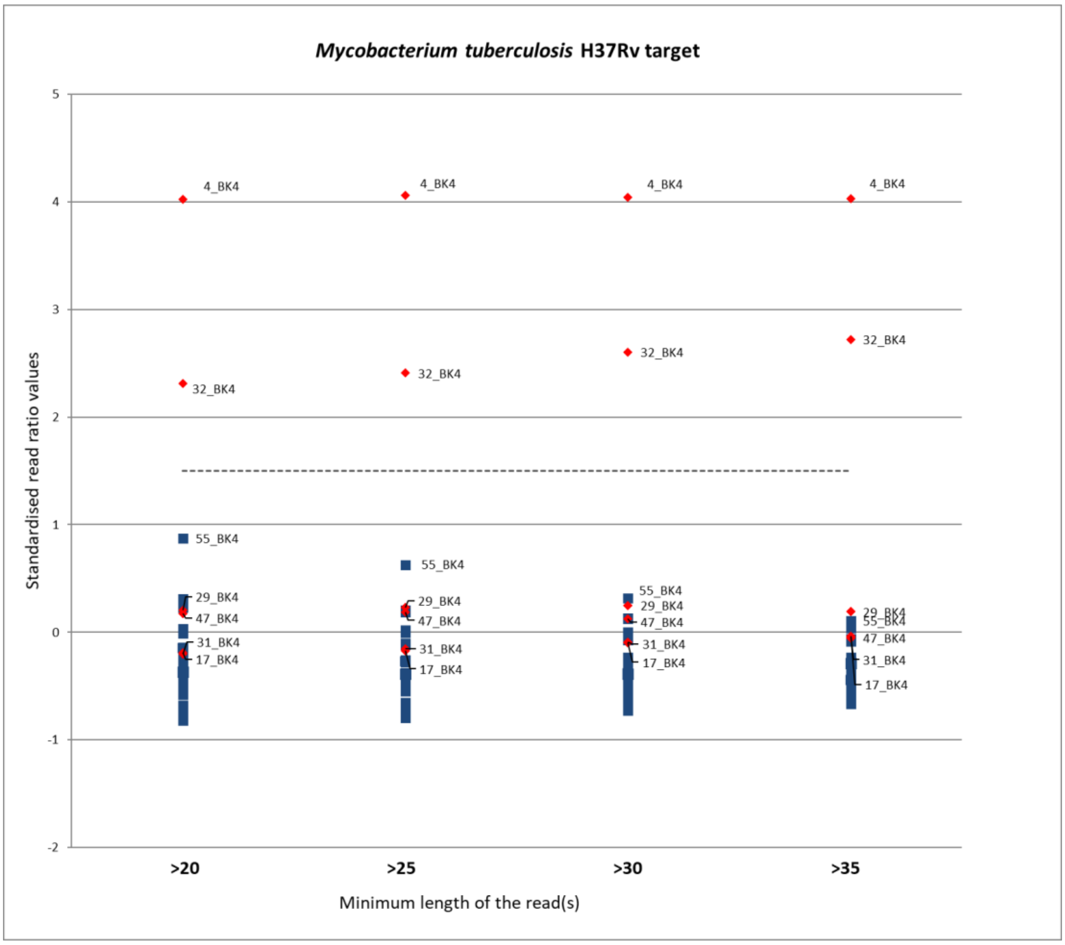
Sample ID	**Raw reads**	**Trimmed reads**	**Average read length**	**Non-human reads**	**Non-human reads**	**Non-human reads**	**Non-human reads**

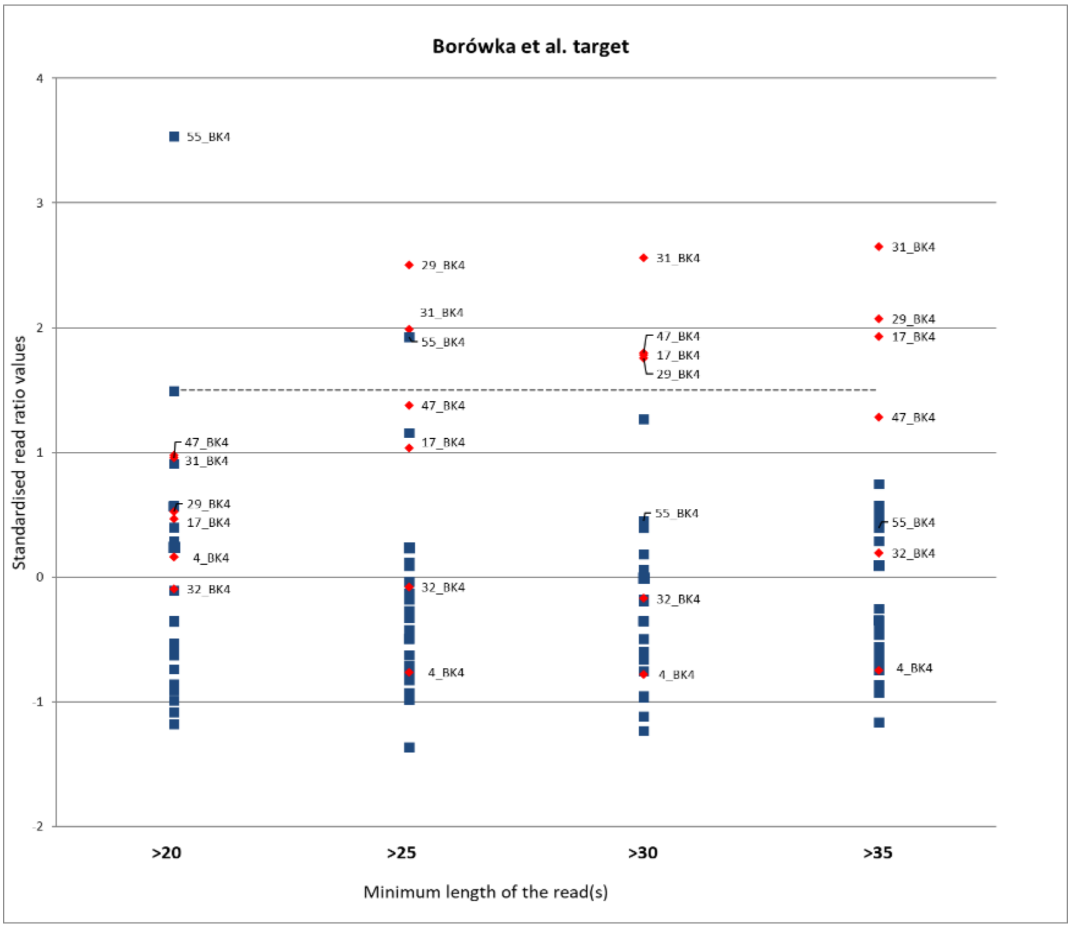
				(>20)	(>25)	(>30)	(>35)
1_BK4	17507911	17038725	57.6	16977024	16902603	16378765	15191086
4_BK4	18816573	18215498	51.7	18095660	17960494	17086604	15246279
6_BK4	16322105	15815995	55.0	15551094	15427193	14682610	13220243
7_BK4	2231650	2160395	59.7	2102955	2095297	2047913	1936435
9_BK4	14974057	14503433	53.5	14240738	14085752	13149549	11600503
11A_BK4	16432267	16000777	58.0	15766313	15695767	15172161	14034604
11B_BK4	18522995	18078222	55.7	725913	718941	674747	597601
12_BK4	23116936	22273434	55.6	21272850	21151065	20156692	18073071
14_BK4	17849685	17383629	58.8	17310864	17235014	16752835	15595926
15_BK4	16062102	15607381	58.2	15539859	15460941	14915585	13881414
17_BK4	14980797	14496468	58.1	14426404	14372805	14078235	13247545
18_BK4	24217412	23575201	59.1	23370869	23281268	22704123	21306454
21_BK4	11890953	11500254	60.1	11271958	11237968	11021676	10439448
22_BK4	17996717	17498339	58.8	17417850	17365274	17013067	16007094
25_BK4	17560698	16997518	57.7	16888515	16816770	16375850	15237575
29_BK4	8994172	8724285	58.1	8683928	8642230	8393680	7800006
31_BK4	20427813	19941632	58.4	19741741	19684774	19309226	18187574
32_BK4	35100769	33926405	54.9	33754943	33623260	32780233	30194531
33_BK4	24501712	23719299	58.3	21669095	21595959	21031538	19569420
34_BK4	16453473	16047224	57.3	14901123	14842998	14421402	13376818
47_BK4	18736966	18155651	55.6	17998648	17903991	17174561	15478180
55_BK4	17435264	16904284	48.0	16768595	16530082	14886541	12170210
65_BK3	17465925	16921732	50.6	16735483	16587671	15466034	13185810
71_BK4	17919758	17434181	50.4	17086979	17017135	16549441	15441174
72_BK4	16355009	15952974	57.9	15874302	15812384	15444022	14541576
73_BK4	17050731	16578547	57.8	16270896	16212738	15778509	14632126
77_BK4	14044420	13478859	56.0	13390126	13322735	12866845	11763625
78_BK4	17004599	16352717	60.1	16250859	16164585	15758397	15027226

Fig 1. Changes in standardised ratio values in different read length bins and targets (red diamonds - outliers in *Mycobacterium tuberculosis* H37Rv and Borówka et al. targets in bin of reads equal or longer than 30).

Fig 2. Comparison of alignment targets constructed with different assumptions (red diamonds indicate outliers in *Mycobacterium tuberculosis* H37Rv and Borówka et al. targets in bin of reads equal or longer than 35).

Fig 1


Mycobacterium tuberculosis H37Rv target
Standardised read ratio values
5
4
3
2
1
0
-1
-2
4_BK4
32_BK4
55_BK4
29_BK4
47_BK4
31_BK4
17_BK4
>20
>25
>30
>35
Minimum length of the read(s)


Borówka et al. target
Standardised read ratio values
4
3
2
1
0
-1
-2
55_BK4
29_BK4
31_BK4
47_BK4
17_BK4
4_BK4
32_BK4
>20
>25
>30
>35
Minimum length of the read(s)

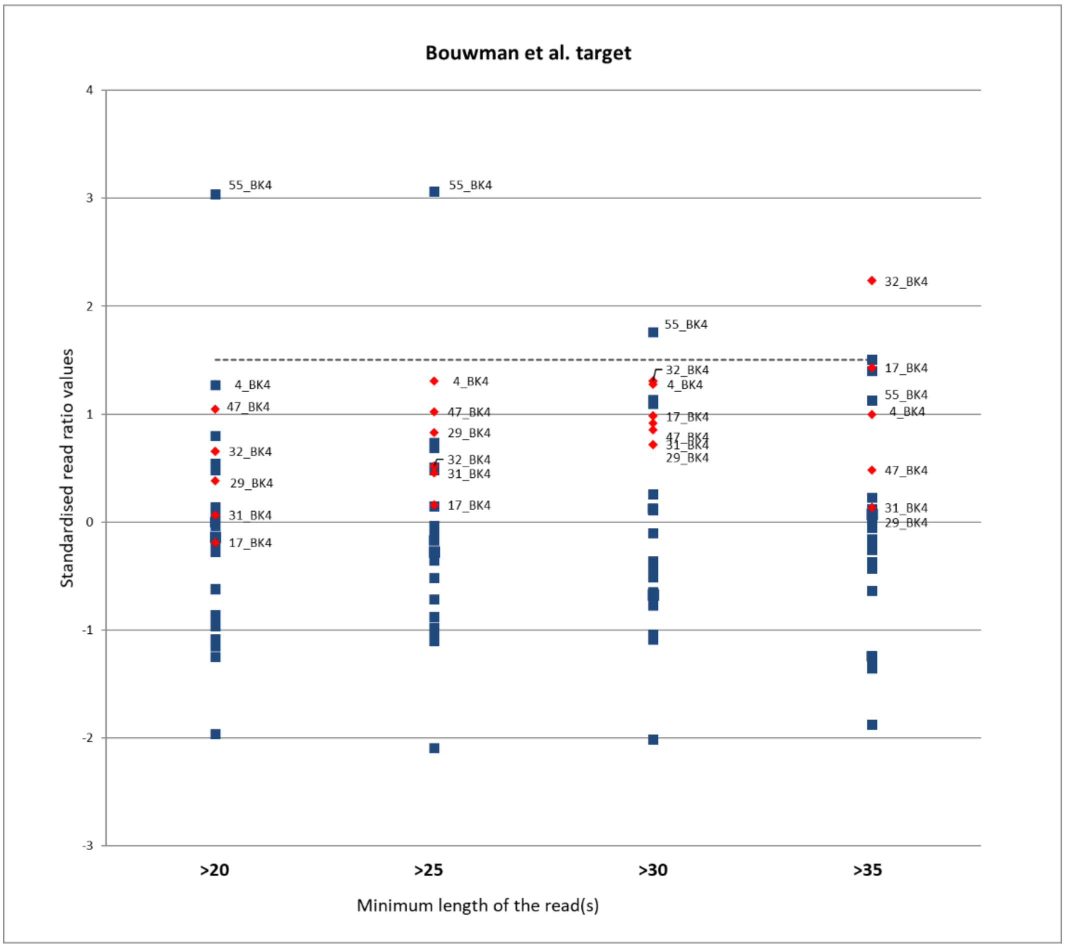


Fig 1. Changes in standardized ratio values in different read length bins and targets (red diamonds – outliers in *Mycobacterium tuberculosis* H37Rv and Borówka et al. targets in bin of reads equal or longer than 30). Dotted line present cutoff values based on 1.5×SD.

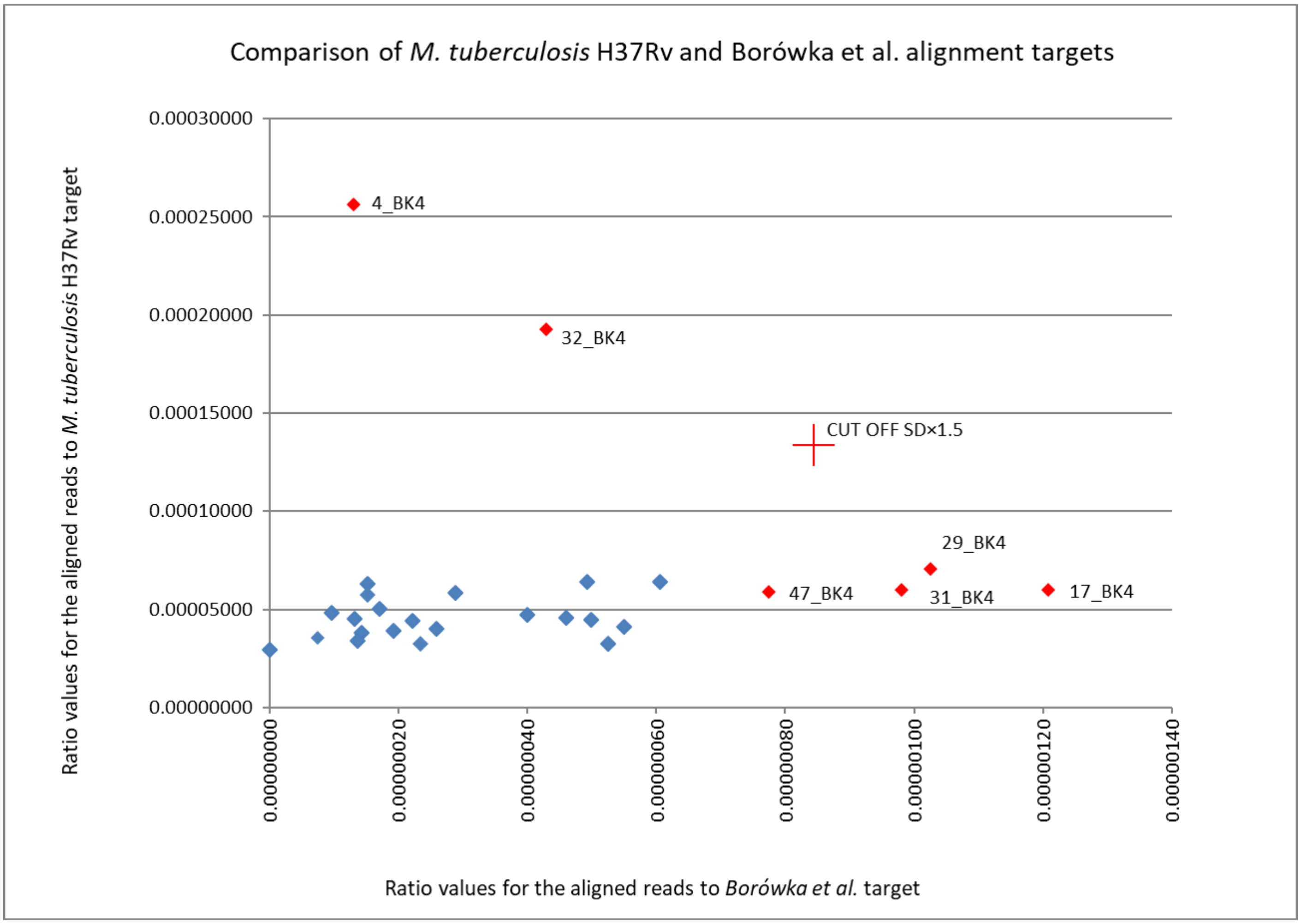


Fig 2. Comparison of alignment targets constructed with different assumptions (red diamonds indicate outliers in *Mycobacterium tuberculosis* H37Rv and Borówka et al. targets in bin of reads equal or longer than 35).

Supplementary figures

Supplementary tables

Supplementary Tables Legend

Lodz, 7th of April, 2019

Dear GigaScience Editor,

We appreciate the insightful and detailed comments of the reviewers. We included all the minor language-related corrections in the resubmitted manuscript. Following is a point-by-point reply to the major points raised by the reviewers.

Reviewer 1

1. Our statistical approach, as we state directly in the manuscript, is aimed at finding positive outliers in a pool of samples with unknown presence of mycobacterial sequences. When applied to a dataset like Kay et al., which consists exclusively of individuals with previously confirmed ancient mycobacterial infection, its outcome is therefore necessarily limited to identifying the outliers with highest microbial load (in the case of tuberculosis, potentially those individuals who died during the active phase of the disease) - these outliers being by definition always a minority of analysed samples. This explanation is provided in the text of the manuscript.

2. We have performed the MapDamage analysis suggested by the reviewer and it indeed yielded a positive result - we thank the reviewer for this suggestion as this strengthened our conclusions significantly. We have now included a new Supplementary Fig. 4, and we have reworded both the legend to Supplementary Fig. 3 and the sentences in the manuscript that refer to it.

3. Libraries were build using Meyer et al. (2010) protocol with modifications proposed by Gamba et al. (2014). We have performed mapDamage analysis in the way which fit to double stranded libraries. Information about different Meyer protocol and single stranded libraries was incorrectly added to previous version of the manuscript.

4. We have expanded Supplementary Tab. 2 to include the absolute numbers of reads - both total and aligning to each alignment target.

Reviewer 2

1. We have now expanded the analysis of state of the art in biochemical detection of ancient mycobacteria by citing recent articles mentioning improvements in cell wall component analysis.

2. Indeed, Pott’s disease is usually regarded as pathognomonic signature of TB. When we said that many pathological conditions of the spine can mimic Pott’s disease thought that they can be diagnosed mistakenly as tuberculosis, especially in practice with poorly preserved skeletons. The present text has been appropriately modified (both in the Introduction and Discussion) to clarify this statement. Moreover, according to the Reviewer’s suggestion, the list of pathological conditions has been replaced with the Table S1, which include a short description of basic differences between these lesions and bone tuberculosis.

Dear Editor, regarding to your comment:

“As your revised manuscript focuses more on a method, it may be more suitable as a "Technical Note" rather than a "research article" “

We would like to proceed this manuscript as research paper. In our work we present original dataset which allow us to present a novel bioinformatical approach, used for screening of ancient tuberculosis in sequencing data, derived from 28 individuals (dated 4400 - 4000 BC and 3100 - 2900 BC) from Central Poland. That dataset was not previously published elsewhere.

Sincerely,

Dominik Strapagiel