Comparative genomics confirms a rare melioidosis human-to-human transmission event and reveals incorrect phylogenetic reconstruction due to polyclonality

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Abstract

Human-to-human transmission of the melioidosis bacterium, Burkholderia pseudomallei, is exceedingly rare, with only a handful of suspected cases documented to date. Here, we used whole-genome sequencing (WGS) to characterize one such unusual B. pseudomallei transmission event, which occurred between a breastfeeding mother with mastitis and her child. Two strains corresponding to multilocus sequence types (STs)-259 and -261 were identified in the mother’s sputum from both the primary culture sweep and in purified colonies, confirming an unusual polyclonal infection in this patient. In contrast, primary culture sweeps of the mother’s breast milk and the child’s cerebrospinal fluid and blood samples contained only ST-259, indicating monoclonal transmission to the child. Analysis of purified ST-259 isolates showed no genetic variation between mother and baby isolates, providing the strongest possible evidence of B. pseudomallei human-to-human transmission, probably via breastfeeding. Next, phylogenomic analysis of all isolates, including the mother’s mixed ST-259/ST-261 sputum sample, was performed to investigate the effects of mixtures on phylogenetic inference. Inclusion of this mixture caused a dramatic reduction in the number of informative SNPs, resulting in branch collapse of ST-259 and ST-261 isolates, and several instances of incorrect topology in a global B. pseudomallei phylogeny, resulting in phylogenetic incongruence. Although phylogenomics can provide clues about the presence of mixtures within WGS datasets, our results demonstrate that this methodology can lead to phylogenetic misinterpretation if mixed genomes are not correctly identified and omitted. Using current bioinformatic tools, we demonstrate a robust method for bacterial mixture identification and strain parsing that avoids these pitfalls.

DATA SUMMARY

1. Whole-genome sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) and GenBank under BioProject accession number PRJNA559002.

2. The GenBank accession number for the MSHR0643 assembly is VXLH00000000.1.

3. The SRA accession numbers for all raw sequence data are listed in Table 1.

INTRODUCTION

Burkholderia pseudomallei, a Gram-negative environmental bacterium found in soil and water in mostly tropical regions, is the causative agent of melioidosis [1]. This underreported and historically neglected disease is increasingly being recognized as endemic in diverse tropical regions globally, and is hyperendemic in northern Australia and Southeast Asia [2]. B. pseudomallei is an opportunistic bacterium that most commonly affects people who are in regular contact with soil and water, with percutaneous inoculation and inhalation the main routes of infection, and infection by ingestion uncommon [1, 3]. The high mortality rate of melioidosis (10–40%) even with antibiotic...
treatment [4], combined with the intrinsic resistance of *B. pseudomallei* towards a wide range of antibiotics [5], highlight the significant public-health importance of this bacterium [1]. Increasing awareness and detection of melioidosis in new locales and the lack of a vaccine against *B. pseudomallei* have further increased the global public-health significance of this pathogen [6]. Due to these factors, *B. pseudomallei* is considered a Tier 1 Select Agent pathogen due to its potential for misuse as a biological warfare agent [7].

Multilocus sequence typing (MLST) is a commonly used genotyping method for determining the population structure, geography, source attribution and transmission patterns of many bacterial pathogens, including *B. pseudomallei* [8–14]. With the advent of whole-genome sequencing (WGS), simultaneous genomic characterization, phylogeography, multilocus sequence type (ST) determination, antibiotic-resistance profiling and fine-scale resolution of *B. pseudomallei* population structure, evolution and transmission profiles have become possible [15]. WGS has also assisted with the identification of polyclonal *B. pseudomallei* infections, including one reported instance of a polyclonal infection with the same ST [16].

Although rare, a handful of suspected cases of human-to-human *B. pseudomallei* transmission have been documented, including between siblings with cystic fibrosis [17], between siblings with diabetes [18], between an American Vietnam veteran diagnosed with *B. pseudomallei*-associated prostatitis and his spouse (although supported only by serology) [19], and three cases between mother and child [3, 20]. In one of the mother–to-child transmission cases, a mother with *B. pseudomallei*-associated mastitis in her left breast was suspected to have transmitted this pathogen to her breastfed infant [3]. Mother–to-child *B. pseudomallei* transmission via transplacental, breast or perinatal routes has been suspected in a handful of other human cases [3, 20, 21], and in animals [22]. However, no human–to-human transmissions reported to date have been confirmed using WGS, which is essential for ruling out concomitant environmental sources of infection. In the current study, WGS was used to understand the dynamics of this suspected human-to-human transmission event, which was also characterized by a polyclonal infection detected in the mother’s sputum. Using comparative genomics, we provide the strongest possible evidence for human-to-human *B. pseudomallei* transmission between mother and child. We next examined the impact of the strain mixture identified in the mother’s sputum sample on phylogenetic interpretations. We observed confounding phylogenomic results when the mixed genome was included in analyses of both single-ST and highly diverse global strain phylogenies, a finding that has implications for fine-scale phylogenomic investigation of outbreak, source attribution and host transmission studies.

**METHODS**

**Case history and bacterial culture**

The clinical history of the mother-to-child transmission case has been described elsewhere [3]. Briefly, a 7-month-old breast-feeding child from a remote region in northern Australia was hospitalized in 2003 with acute cough, fever and tachypnoea. Four days after admission, the mother was observed to have a fever and pleuritic chest pain, and was subsequently diagnosed with mastitis in the left breast. Upon *B. pseudomallei* culture confirmation in the child’s cerebrospinal fluid (CSF), blood, and nasal and throat swabs, the mother was also tested for *B. pseudomallei* infection in blood, sputum and multiple breast milk specimens, and from nasal, throat and rectal swabs. Of these, *B. pseudomallei* was isolated from the mother’s breast milk and sputum (Table 1) [3]. All clinical specimens were cultured on Ashdown’s media as described elsewhere [13]. DNA extractions were performed as previously described [23] on a sweep of the primary culture streak (herein referred to as primary culture sweeps) of each *B. pseudomallei*-positive clinical specimen in an effort to capture potential strain mixtures in these original specimens, and subsequently from individually purified colonies derived from these specimens.

**WGS and in silico MLST**

As part of the ongoing Darwin Prospective Melioidosis Study (DPMS), which commenced in 1989 [24], all mother and child primary culture sweeps and purified colonies (i.e. isolates) were subjected to WGS using the Illumina HiSeq2500 Next examined the impact of the strain mixture identified in the mother’s sputum sample on phylogenetic interpretations. We observed confounding phylogenomic results when the mixed genome was included in analyses of both single-ST and highly diverse global strain phylogenies, a finding that has implications for fine-scale phylogenomic investigation of outbreak, source attribution and host transmission studies.
Table 1. Summary of ST-259 and ST-261 B. pseudomallei isolates

| Isolate ID* | Sample type | Patient | Year of isolation | Multilocus ST | NCBI accession number | Genome coverage |
|-------------|-------------|---------|-------------------|---------------|-----------------------|-----------------|
| MSHR1574    | CSF         | Child   | 2003              | ST-259        | SRR9959037            | 134×            |
| MSHR1574_Sweep | CSF     | Child   | 2003              | ST-259        | SRR9959038            | 94×             |
| MSHR1580    | Blood       | Child   | 2003              | ST-259        | SRR9959039            | 109×            |
| MSHR1580_Sweep | Blood   | Child   | 2003              | ST-259        | SRR9959040            | 63×             |
| MSHR1583    | Breast milk | Mother  | 2003              | ST-259        | SRR9959042            | 125×            |
| MSHR1583_Sweep | Breast milk | Mother | 2003              | ST-259        | SRR9959036            | 68×             |
| MSHR1631    | Sputum      | Mother  | 2003              | ST-259 and ST-261 | SRR9959043         | 60×             |
| MSHR1631_Mixed | Sputum | Mother  | 2003              | ST-259 and ST-261 | SRR9959045         | 131×            |
| MSHR1581    | Sputum      | Mother  | 2003              | ST-261        | SRR9959041            | 61×             |
| MSHR0120    | Blood       | Other†  | 1992              | ST-259        | SRR2975709             | 31×             |
| MSHR0669    | Blood       | Other†  | 1998              | ST-259        | SRR9959034            | 111×            |
| MSHR1224    | Blood       | Other†  | 2001              | ST-259        | SRR9959035            | 64×             |
| MSHR1328    | Sputum      | Other†  | 2001              | ST-259        | SRR10134763            | 123×            |
| MSHR1357    | Abscess     | Other†  | 2002              | ST-259        | SRR10134764            | 139×            |
| MSHR3509    | Blood       | Other†  | 2009              | ST-259        | SRR10134763            | 97×             |
| MSHR0643    | Urine       | Other†  | 1998              | ST-259        | SRR9959033            | 122×            |

*Isolates with the '_Sweep' suffix were obtained from primary culture sweeps to capture B. pseudomallei population diversity. MSHR1631_Mixed was the only sample found to contain a mixture of two genotypes. Isolates without the '_Sweep' suffix were obtained from purified single colonies derived from the '_Sweep' culture.

†Temporally or geographically distinct clinical ST-259 isolates obtained between 1992 and 2009 from other patients living in the Top End region of the Northern Territory, Australia.

Comparative genomics

Comparative genomic analysis was performed with the default settings of SPANDx v3.2 (https://github.com/dsarow/SPANDx) [29], which wraps bwa (Burrows–Wheeler Aligner) [30], SAMtools [31], the Genome Analysis Toolkit (gatk v3.2–2) [32], BEDTools [33] and SNPEff [34] into a single pipeline. Mapping was carried out using the closed Australian genome MSHR1153 [26] as the reference, with the SPANDx -i flag enabled to provide indel variant identification. In addition, the SPANDx -a and -v flags were enabled to permit both the annotation of all SNP and indel variants, and the rapid identification of putative mixtures based on ambiguous (i.e. '?' variant calls in the 'All_SNPs_annotated.txt' and 'All_indels_annotated.txt' outputs generated by SPANDx.

Mixture analysis

‘Heterozygous’ SNPs in each isolate were enumerated from the gatk v4.1 HaplotypeCaller [35] VCF output following alignment of reads using bwa [30]. For each heterozygous SNP identified in MSHR1631_Mixed, the depth (i.e. number platform and Illumina Nextera XT chemistry to generate 2×100bp read data (Australian Genome Research Facility, Melbourne, Australia). WGS was performed on primary culture sweeps and isolates from the mother’s (n=6) and child’s (n=4) specimens. Reference-assisted draft genome assemblies were performed using MGAP v1.0 (default settings) [25], with the closed Australian MSHR1153 genome (CP009271.1 and CP009272.1 for chromosomes 1 and 2, respectively) [26] used as the reference for read mapping and variant calling. We used a reference-assisted assembly approach as, in our experience, doing so results in assemblies with less fragmentation and fewer SNP and small insertion-deletion (indel) errors that require subsequent manual correction. In silico MLST was performed by BIGSdb [27], which is embedded within the PubMLST B. pseudomallei database available at http://pubmlst.org/bpsuemallei/. For the mixed-strain sample (MSHR1631_Mixed), manual allele assignment was performed by inspecting alignment files using Tablet [28] and parsing SNPs corresponding to the different strain ‘haplotypes’ based on allele abundance.
of reads) supporting each allele was extracted from the VCF file and normalized by the total read depth at each SNP position. One sweep culture, MSHR1631_Mixed, exhibited a substantial number of heterozygous SNPs when compared with all other isolates and sweep cultures, so was further investigated as a possible mixture. Variant identification in MSHR1631_Mixed was determined using gatk v4.1 Haplo-typeCaller due to its ability to natively handle polyploid samples. Variant filtering was performed using the parameters described in SPANDx v3.2 [29]. Additionally, to ensure robust variant calling and to assess mixture composition, we tested multiple ploidy settings (n=2, 3, 4 and 5).

**Phylogenomic analyses**

A maximum parsimony (MP) phylogenetic tree representing a global snapshot of *B. pseudomallei* isolates was reconstructed using orthologous, biallelic, core-genome SNPs identified across 145 publicly available genomes [36] using the default settings of SPANDx and MSHR1153 as the reference genome. The new isolates/sweep cultures sequenced as part of this study were also included in phylogenomic analyses, both with and without the inclusion of MSHR1631_Mixed. The global trees were rooted with MSHR0668 [37], as this strain is the most ancestral *B. pseudomallei* strain according to a large *Burkholderia* spp. phylogeny [15].

To investigate *B. pseudomallei* transmission from mother to child, a combined SNP–indel [11] MP tree containing all available ST-259 isolates was first reconstructed, with the mgap-assembled ST-259 genome MSHR0643 (GenBank ref: VXLH00000000.1) used as the reference for SPANDx analysis. MSHR0643 was chosen as the reference genome as it had the fewest contigs (n=93) of any ST-259 strain. Also included in this analysis were seven temporally or geographically distinct ST-259 isolates that were not epidemiologically linked to the mother–baby cases (Table 1). To further investigate putative mutations among mother and baby ST-259 isolates, SPANDx analyses were also performed using the baby's CSF isolate, MSHR1574, and the mother's sputum isolate, MSHR1631, as reference genomes, with these comparisons performed using only mother–baby ST-259 isolates. MP phylogenetic tree reconstruction and bootstrapping (300 replicates) were performed using paup* v4.0a165 and visualized with iTOL v4 [38].

**PFGE**

PFGE with SpeI-digested DNA was performed on mother and child isolates as previously described [39].

**RESULTS AND DISCUSSION**

*B. pseudomallei* causes melioidosis, a life-threatening disease with a predicted global incidence of ~165 000 cases annually [2]. Almost all *B. pseudomallei* infections occur via contact with contaminated water or soil, with human-to-human transmission events considered exceedingly rare [40]. Here, we used genomics to examine, in high resolution, one such human-to-human transmission event where a nursing mother with culture-confirmed melioidosis mastitis was suspected to have transmitted *B. pseudomallei* to her child through contaminated breast milk [3]. PFGE analysis on isolates retrieved from the mother and her child shortly after diagnosis identified two pulsotypes in the mother's sputum isolates (Fig. 1), suggesting a potential polyclonal infection. Consistent with the PFGE findings, *in silico* MLST data showed strains from the mother's sputum and breast milk matched the CSF- and blood-derived isolates retrieved from the child, with all isolates being ST-259. To further understand this unusual case, WGS was performed on all available specimens from these cases to elucidate transmission dynamics from mother to child, to investigate the potential presence of within-host strain mixtures in the mother and, finally, to examine the effects of strain mixtures on downstream phylogenomic interpretations.

Prior studies have relied upon epidemiological and clinical observations [3, 17, 19, 20], often alongside gel electrophoresis-based genotyping methods [3, 17, 18], to examine cases of suspected *B. pseudomallei* transmission between human hosts. However, these genotyping methods lack the necessary resolution for definitive confirmation of such transmission events, as they only assess a small fraction of the genome. As such, infections arising from independent environmental sources, or even from a single environmental point source as

![Fig. 1. PFGE analysis of mother and child isolates using SpeI digestion. *Isolate not subjected to WGS in this study due to culture destruction.*](image-url)
observed in outbreak scenarios [11, 41], cannot be ruled out using such lower-resolution methods.

To obtain the most epidemiologically robust information from our WGS data, phylogenomic analysis of all mother-child ST-259 isolates was performed using a combined SNP–indel approach, which we have previously shown provides both higher resolution and a better fit with outbreak chronology compared with phylogenomic reconstruction using just SNPs [11]. This approach identified no SNP nor indel differences between the mother and child ST-259 isolates (Fig. 2a). Further comparative genomic analyses examining copy-number variants or larger deletions also failed to find any other genetic variation among the mother–child ST-259 isolates using three different ST-259 reference genomes (MSHR0643 from an unrelated patient 5 years prior to the mother–baby case; the MSHR1631 sputum isolate from the mother; and the MSHR1574 CSF isolate from the baby). Although there will always remain the possibility that the mother and child were infected from a single environmental point source, or that the child infected the mother prior to either becoming symptomatic, our collective clinical, epidemiological and genomic findings point strongly to ST-259 B. pseudomallei transmission from mother to child, with breastfeeding being the most likely route of infection. Our findings provide the strongest evidence presented to date that B. pseudomallei can transmit between human hosts. This finding raises clinical and biowarfare concerns, particularly in cases where a B. pseudomallei strain has developed acquired antimicrobial resistance (AMR) in one human host who subsequently transmits the strain to another. Although acquired AMR in B. pseudomallei is relatively uncommon, there are myriad chromosomal mutations that can lead to clinically relevant AMR in B. pseudomallei [42], leading to more challenging pathogen eradication [43]. While this phenomenon has not yet been documented, our study demonstrates that human-to-human transfer of an AMR B. pseudomallei strain is possible.

To further understand ST-259 diversity on a broader scale, the ST-259 mother–child isolates were compared with seven
temporally or geographically distinct clinical ST-259 isolates obtained between 1992 and 2009 from patients living in the Top End region of the Northern Territory, Australia. The mother–child clade was most closely related to MSHR0120, differing by seven variants (Fig. 2). MSHR0120 was retrieved from a patient diagnosed with melioidosis 11 years prior, who lived at the same remote locale as the mother and child. Additionally, minimal differences (between 36 and 45 variants) were observed between the mother–child clade and other ST-259 isolates, suggesting close relatedness of strains within this ST, but a clear difference between the mother–child cases and all other documented ST-259 cases in the Top End region. Taken together, these results provide further evidence for human-to-human *B. pseudomallei* transmission between mother and child.

Simultaneous infections with multiple *B. pseudomallei* strains have previously been reported [16, 44, 45]; however, the true rate of polyclonal *B. pseudomallei* infections is unknown. Polyclonality may increase the risk of neurological disease when one or more strains encode a *Burkholderia mallei* bimA (*bimA_Bm*) genetic variant [46], and may cause issues with accurate point-source attribution in epidemiological investigations if polyclonality is not taken into account. Most clinical microbiological laboratories typically only select a single bacterial pathogen colony for further genotypic and phenotypic characterization, which results in a considerable genetic bottleneck and the loss of strain mixtures from polyclonal clinical specimens. This shortcoming can be overcome using more time-intensive methods, such as the selection of multiple colonies for genetic analysis, sequencing of a ‘sweep’ of primary culture growth for further genetic characterization or by total metagenomic sequencing of the clinical specimen. Due to inherent ethical and technical challenges with metagenomic sequencing of clinical specimens, we chose to genome-sequence culture sweeps and the individual colonies purified from them to identify putative *B. pseudomallei* strain mixtures in the mother and child clinical specimens. Consistent with the PFGE findings, *in silico* MLST and gatk HaplotypeCaller analysis of mother–child sweeps revealed that two distinct strains (ST-259 and ST-261) were found in one of the two sputa retrieved from the mother (MSHR1631_Mixed; Fig. 3), but not in other primary sweep specimens from this patient [1× sputum (MSHR1581_Sweep); 1× breast milk (MSHR1583_Sweep)], nor in the samples obtained from the child [1× CSF (MSHR1574_Sweep); 1× blood (MSHR1580_Sweep)]. WGS of single purified colonies from MSHR1631_Mixed and MSHR1581_Sweep confirmed that both ST-259 and ST-261 were present in this patient’s sputum specimens. Collectively, these results confirm that the mother had a simultaneous infection with two strains, adding to the documented polyclonal *B. pseudomallei* cases.

![Fig. 3. Example of ‘heterozygous’ (i.e. strain mixture) SNP calls at the sequence read level according to gatk HaplotypeCaller. Heterozygous SNP calls in MSHR1631_Mixed (ST-259 and ST-261) were parsed apart by comparing against homozygous SNP calls from MSHR1574 (ST-259) and MSHR1581 (ST-261). Horizontal bars represent forward (red) and reverse (blue) reads aligned against the MSHR1153 reference genome. Coloured boxes represent ‘heterozygous’ SNPs (also indicated by asterisks at the top).](image)
To better understand this polyclonal infection from a bioinformatic standpoint, we first performed a high-throughput analysis of putative mixtures in our isolate dataset (Table 1) using the SPANDx ‘All_SNPs_annotated.txt’ and ‘All_indels_annotated.txt’ outputs, which incorporates a '?' for ambiguous variant calls according to GATK v3.2–2. This approach readily flagged MSHR1631_Mixed as a probable mixture based on its very high number of ambiguous SNP (18885 of 41438 total SNPs; 45.6 %) and indel (1643 of 3328 total indels; 49.4 %) characters compared with all other genomes [SNPs, range 426–518 (1.0–1.3%); indels, range 165–217 (5.0–6.5%)]. In other words, MSHR1631_Mixed encoded 40× more ambiguous SNPs and 9× more ambiguous indels than non-mixed strains using this approach. Next, we quantified the number of high-quality heterozygous SNPs in MSHR1631_Mixed using GATK v4.1. Haploid genomes such as bacterial genomes do not encode heterozygous SNPs; therefore, heterozygous SNPs are typically ignored by bacterial genome variant-calling software. The inclusion of heterozygous SNPs in an analysis of the mother–child isolates amongst a global dataset of *B. pseudomallei* genomes showed that MSHR1631_Mixed contained 12× the mean number of heterozygous SNPs compared with all other mother–child samples (Fig. 4). In total, 34567 SNPs were identified in this sample, 47.8% of which were ‘heterozygous’. In contrast, a mean of 29914 SNPs were identified in the other nine mother–baby samples, of which only 5.2% were ‘heterozygous’. Next, homozygous SNPs identified in representative pure isolates (MSHR1574 for ST-259; MSHR1581 for ST-261) were used to identify the strain origin of each heterozygous allele from MSHR1631_Mixed SNPs. Using this method, 96% of heterozygous SNPs were matched to the correct strain. ST-259 was the dominant clone (averaging 87% of reads at each variant) and ST-261 was present as a minor allelic component (averaging 13% of reads at each variant). No evidence of a tertiary strain was observed in MSHR1631_Mixed when different ploidy settings were tested, indicating that no other strains were present.

![Graph](image_url)

**Fig. 4.** Quantification of ‘heterozygous’ (i.e. strain mixture) SNP calls across all mother–child isolates and a global *B. pseudomallei* genome set. MSHR1631_Mixed contained 12× the mean number of ‘heterozygous’ calls according to the GATK UnifiedGenotyper, indicating the presence of a *B. pseudomallei* strain mixture in this sample. No other analysed genomes contained detectable mixtures.
The utility of SNP data derived from WGS to identify and study mixtures has been demonstrated in different diploid and polyploid organisms [47–49]. Current approaches for identifying mixtures in bacterial organisms include proportion estimates against a database of known STs or species-specific marker genes [50, 51], which requires prior knowledge of the specific bacterial population, or long-read sequencing [52], which is currently costly and error-prone when used in isolation. Other studies have identified mixtures in bacterial genomes using the UnifiedGenotyper function of gatk v3 to detect ambiguous variants in a similar fashion to our SPANDx analysis [53], or genotype binning based on differences in haplotype depth of coverage in five cases of mixed Mycobacterium tuberculosis infections, including one case where three genotypes were suspected in a single patient [54]. Bioinformatic solutions are available for ploidy inference of eukaryotic organisms [47–49, 55], which rely on the depth ratio of the two most abundant alleles sequenced for all heterozygous SNP positions across the genome (also referred to as ‘allele balance’). Such approaches assume SNP allele balances remain relative to each other; for example in a diploid sample, 50% of reads would support one allele and the other 50% would support the other allele [47]. However, the allele balance assumption does not hold in bacterial mixtures, which may contain mixed ratios of any proportion. Despite this shortcoming, we demonstrated the feasibility of using SNP and read depth data to parse apart bacterial mixtures without any prior knowledge of the mixture composition. This approach relies on sequencing at a depth of ≥50x to ensure adequate sampling of a minor allelic component present at a~5–10% proportion [42]. However, in our view, such an approach is only suited for parsing apart mixtures of two strains. Although the major strain is potentially identifiable in ≥3-strain mixtures, parsing apart minor components is a complex problem that remains largely unresolved using short-read data.

Finally, we investigated the effects of strain mixtures on phylogenomic reconstruction to determine whether the inclusion of even one mixture had undesirable effects on tree topology and phylogenetic inference. Phylogenomic analyses were performed with the ST-259 (Fig. 2) and global (Fig. 5) datasets, both with (Figs 2b and 5b) and without (Figs 2a and 5a) MSHR1631_Mixed inclusion. Tree

![Fig. 5.](https://example.com/fig5.png) Global MP phylogenetic analyses demonstrating the effects of strain mixtures on tree topology. Branch colours denote geographical origin of *B. pseudomallei* strains: red, Australian isolates; blue, Asian isolates; pink, African isolates; lime green, Papua New Guinean isolates; gold, South American isolates. Isolate names in dark blue text, ST-259 mother–child isolates; light blue text, temporally or geographically distinct ST-259 isolates; green text, ST-261 mother isolates; purple text, MSHR1631_Mixed sample. (a) Exclusion of the mixed genome, MSHR1631_Mixed, results in correct topology and separation of ST-259 and ST-261 according to previous global *B. pseudomallei* phylogenies [13, 15, 36]; these two STs differ by >20000 SNPs. (b) Inclusion of MSHR1631_Mixed greatly alters topology, leading to incorrect isolate and clade clustering, and collapsed branches in the clade containing MSHR1631_Mixed. Specifically, the ST-261 isolates (indicated by asterisks) cluster incorrectly with ST-259, with branch collapse observed in this clade. The Papua New Guinean isolates are also incorrectly placed in this phylogeny (indicated by black arrows). The number of characters used to reconstruct each tree differs by 14503 SNPs (a, 207 209 SNPs; b, 192 706 SNPs), resulting in branch collapse.
comparisons identified two confounding issues in the trees containing MSHR1631_Mixed: phylogenetic incongruence [56] in the global dataset, which resulted in multiple instances of incorrect clade placement, and branch collapse in both the ST-259 and global datasets, which was caused by the removal of informative characters for phylogenetic reconstruction due to the presence of ambiguous characters in MSHR1631_Mixed. In the ST-259 tree, the number of SNP–indel characters separating isolates decreased from 35 to 21 variants (Fig. 2b). In turn, the inferred relatedness between the mother–child ST-259 isolates and other ST-259 isolates was exaggerated due to this branch collapse (Fig. 2b; red arrow). In the global dataset, branch collapse reduced the total number of informative characters available for tree reconstruction by 18051 SNPs when compared with the non-mixed phylogeny. Phylogenetic incongruence was also evident in the global tree, whereby ST-261 isolates (MSHR1581_Sweep and MSHR1581; green text) incorrectly resided in the same clade as ST-259 (Fig. 5b; asterisk). In contrast, the non-mixed dataset separated these two STs by approximately 20000 SNPs, with clear separation of these clades (Fig. 5a). Surprisingly, branches across both trees had very high bootstrap support values at the ST-261 and ST-259 clades despite branch collapse and phylogenetic incongruence in the mixed dataset. Of further concern, the phylogeny containing MSHR1631_Mixed caused incorrect geographical assignment of the Papua New Guinean clade, unexpectedly shifting its known grouping with Australian strains [15, 57] to the Asian clade; this incorrect placement received very high bootstrap support (Fig. 5b).

The negative effects of strain mixtures on phylogenomic inference highlights the importance of strict quality controls throughout each stage of the experiment, especially during computational analysis. Bioinformatically, bacterial mixtures can be readily detected, as demonstrated in this study. However, standard practice in microbial variant calling pipelines is to report only homozygous variants for downstream analysis, with heterozygous SNPs typically ignored. Additionally, most phylogenetic reconstruction software treat heterozygous SNPs as missing or non-informative characters, even when encoded with IUPAC-ambiguous characters [58]. Our results provide unequivocal evidence that caution is needed in phylogenomic interpretation when dealing with potential strain mixtures. As these mixtures are not easily identifiable from phylogenetic analysis, it is prudent that all microbial genomics studies include a mixture screening assessment of all genomes prior to variant calling and phylogenomic reconstruction to avoid removing phylogenetic informative characters, which we show can readily result in branch collapse and phylogenetic incongruence. Based on our findings, it is our strong recommendation that any sample demonstrating evidence of a mixture should be entirely omitted from a dataset, particularly if the purpose is to examine fine-scale differences between or among closely related samples, such as in the current study. This criterion may be relaxed if a given study’s aims can tolerate branch collapse or phylogenetic incongruence; however, the effects of both mixture inclusion and exclusion should be investigated in such cases to understand the potentially confounding impact of the mixture(s) on the dataset under investigation. In such instances where a mixed genome is essential for a study, additional laboratory passage to purge the mixture(s) should be considered. In cases where this is not possible, longer-read sequencing may assist with parsing apart allele mixtures.

In conclusion, we demonstrate the utility of comparative genomics to both confirm human-to-human *B. pseudomallei* transmission and to identify simultaneous infection with multiple *B. pseudomallei* strains. Using a naturally occurring mixed genome comprising two strains at an 87%:13% ratio, we describe an effective method to accurately identify and quantify such mixtures from WGS data, and highlight the confounding effects that even a single mixed genome can place on accurate phylogenomic interpretations for both closely related (e.g. single ST) and species-wide phylogenies. Our findings demonstrate the essentiality of assessing all microbial genome datasets for the presence of strain mixtures as a routine part of sequence data quality control. We strongly recommend that such mixtures be removed prior to phylogenomic analysis to avoid erroneous misinterpretations of strain relatedness.

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**Author contributions**

B.J.C. identified the transmission event; M.M. conducted specimen sample processing, PFGE and DNA extractions. A.A. performed bioinformatic analysis with assistance and supervision from D.S.S. and E.P.P. A.A. wrote the initial manuscript draft. D.S.S. and E.P.P. critically reviewed and edited the manuscript. B.J.C., M.M., D.S.S. and E.P.P. conceived of the study and obtained funding. All authors reviewed and approved the final manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

Ethics approval for this study was obtained from the Human Research Ethics Committee of the Northern Territory Department of Health and Families, Australia, and the Menzies School of Health Research, Australia (HREC 02/38). Informed written consent was obtained from all participants or, where under 18 years of age, their legal guardians.

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