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Short communication

High-resolution melting PCR analysis for rapid genotyping of *Burkholderia mallei*

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ABSTRACT

Burkholderia (*B.*) *mallei* is the causative agent of glanders. A previous work conducted on single-nucleotide polymorphisms (SNP) extracted from the whole genome sequences of 45 *B. mallei* isolates identified 3 lineages for this species. In this study, we designed a high-resolution melting (HRM) method for the screening of 15 phylogenetically informative SNPs within the genome of *B. mallei* that subtype the species into 3 lineages and 12 branches/sub-branches/groups. The present results demonstrate that SNP-based genotyping represent an interesting approach for the molecular epidemiology analysis of *B. mallei.*

1. Short communication

Burkholderia (B.) mallei is the causative agent of glanders in equids and camels, a disease recently qualified as a re-emergent due to the increased number of cases reported in several parts of the world during the last 20 years (Khan et al., 2013). *B. mallei* is a genetically homogenous species that is very closely related to the much more diverse species *B. pseudomallei* from which it recently evolved. Its genome is thought to be continuously evolving through random insertion sequence-mediated recombination events (Losada et al., 2010). Due to a lack of diversity, only molecular characterization techniques with high discrimination power could enhance genetic differentiation at strain level. Most of the genotyping methods applied to *B. mallei* were initially developed for *B. pseudomallei*. Whereas multilocus sequence typing based on 7 housekeeping genes failed to differentiate *B. mallei* strains which match the ST40 sequence type for nearly all of them (Godoy et al., 2003; Losada et al., 2010), a 23-loci Multiple Locus Variable Number of Tandem repeats Analysis (MLVA) method derived from the 32-loci MLVA method for *B. pseudomallei* (U'Ren et al., 2007) succeeded to distinguish *B. mallei* outbreak isolates for example in Pakistan and Emirates (Hornstra et al., 2009; Scholz et al., 2014). However, this method requires the analysis of 23 loci which is technically demanding, expensive and time-consuming. Given that variable number of tandem repeats are inappropriate for determining deep levels of evolutionary relatedness (Hornstra et al., 2009) and given the increasing availability of whole genome sequences (WGS), it is now possible to interrogate nearly every base of the genome and to identify specific single nucleotide polymorphisms (SNPs) able to discriminate *B. mallei* isolates at strain level. The post- real-time PCR high resolution melting (HRM) analysis offers the possibility to discriminate different amplicons based on their melting temperature (Tm) and allows the detection of genetic variations such as SNPs (Tamburro and Ripabelli, 2017).

In a previous work, a minimum spanning tree based on 2296 *B. mallei* specific SNPs extracted from the WGS of 45 strains identified 3 lineages for *B. mallei* (Laroucau et al., 2018). In this study, inside these 3 lineages (L1 to L3), subdivisions into branches (Br, up to 3), sub-

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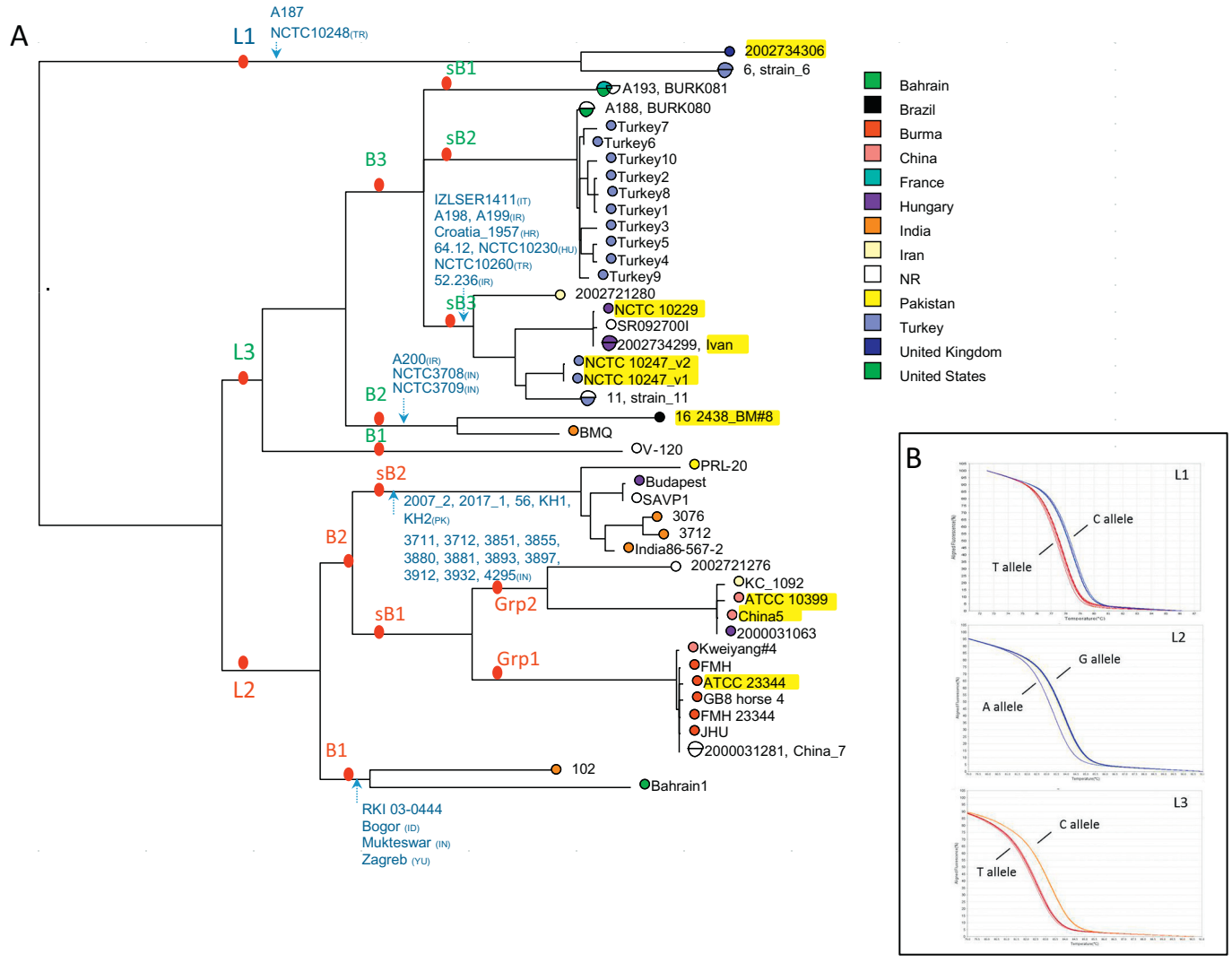

Fig. 1. A. SNP-based tree determined from 45 *B. mallei* publicly available whole genome sequences. Whole-genome sequences of 45 *B. mallei* strains present in public databases were aligned and mapped against the reference sequence ATCC 23344 using the BWA algorithm implemented in BioNumerics with 90% parameter identity. Strain-specific SNPs were identified using the BioNumericswgSNP module and then filtered using fixedconditions (minimum 30× coverage, removal of repeated elements, contiguous SNPs, ambiguous and non-informative bases, removal of gaps)(Laroucau et al., 2018). A tree was generatedin BioNumerics using the filtered SNP matrix as input and using the maximum parsimony algorithm (BioNumerics 7.6.1) (Applied Maths). DNA preparations from8 fully sequenced strains initially tested with the panel of SNPs markers are highlighted in yellow.PCR-HRM clustering results for the *B. mallei* DNAs included in this study, without preliminary WGS information, are shown in blue. B. Normalized melting curves obtained for three SNP markers (L1, L2 and L3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

branches (sB, up to 3) and even groups (Gp) could be identified, as illustrated in Fig. 1. A first set of 15 SNPs specific for each of these lineages, branches, sub-branches and groups was identified *in silico* using BioNumerics 7.6.1 (Applied Maths) and PCR primers targeting these SNPs were designed using Primer 3web version 4.0.0 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 1). Singleplex PCR amplifications were conducted on a ViiA7™ Real-Time PCR instrument (Life Technologies) using the LightCycler® 480 High Resolution Melting Master Mix (Roche Diagnostics). Reaction mixtures consisted of 10 ng DNA, 0.2 µM of each primer, 10 µL LightCycler® 480 HRM master mix and 2.5 mM $MgCl_2$ in 20 µL final volume. The following amplification parameters were used: 10 min at 95 °C followed by 40 cycles consisting in 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. Samples were next heated to 95 °C for 30 s, cooled down to 65 °C for 1 min and heated from 65 °C to 95 °C at a rate of 0.025 °C/s with 25 fluorescence acquisitions/°C. HRM data were analyzed by the ViiA7™ Software version 1.2.1. For each of the SNPs, synthetic DNA oligonucleotides carrying either the C/G or A/T substitutions (Eurofins, Germany) were included as controls (Table 1, Supplementary material 1). All yielded amplicons produced a single melting peak. Each peak was characterized by a melting curve, with Tm values depending on the SNP carried by the amplicon. On average, differences in *Tm* values of about 0.4 to 1.1 °C were observed between the two allelic states (Table 1).

This panel of 15 SNPs was first validated on 8 DNA preparations from the 45 fully sequenced strains used for the phylogenetic clustering (Laroucau et al., 2018). As determined from WGS data, all strains clustered within their predicted lineage, branch, sub-branch and/or group, namely L1 for NCTC120/2002734306, L2B2sB1Gp1 for ATCC23344, L2B2sB1Gp2 for NCTC10245/ATCC10399 and China 5, L3B2 for 16-2438_BM#8, and L3B3sB3 for NCTC10229, Ivan and NCTC10247 (Table 2).

The developed PCR-HRM method was further applied to 33 DNA preparations from either ancient or contemporary *B. mallei* strains isolated in Croatia (Croatia_1957), Hungary (64.12 and NCTC10230), India (Mukteswar, NCTC3708, NCTC3709, 3711, 3712, 3851, 3855,