

Research Article

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PCR Typing of *Mycobacterium ulcerans* Lineages Based on Large Sequence Polymorphisms

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Abstract

Genetic fine typing of *Mycobacterium ulcerans*, the causative agent of the neglected tropical disease Buruli ulcer, has long been hampered by the remarkable genetic homogeneity of patient isolates. However, large sequence polymorphisms (LSPs), mainly originating from insertional-deletional genomic events, allow the differentiation between major lineages of *M. ulcerans* and other mycolactone producing mycobacteria (MPMs). Here, we describe an LSP-based rapid PCR typing scheme for these mycobacteria. Application of this approach to a selection of mycobacterial strains isolated from Buruli ulcer patients in Japan shows that they belong to the *M. ulcerans* asian haplotype.

Keywords: Genetic diversity; Large sequence polymorphisms; Bacterial phylogeny; Epidemiology; Buruli ulcer; *Mycobacterium ulcerans*

Introduction

Evolutionary affiliation of bacterial strains can be accomplished by various genetic typing methods [1]. Choice of the most suitable method depends on the population structure of the analysed species [2-4]. Large sequence polymorphisms (LSPs) are labour-intensive to identify in mycobacteria but constitute very reliable indicators for kinship and evolutionary order [3,5,6]. In particular, insertions/deletions (InDels) with identical sequence breakpoints, sequence signatures, and insertional gene content show unequivocal common ancestry and witness highly reliably bacterial phylogeny [7-9,6,10].

Analysis of LSPs has enabled the genetic differentiation of major lineages in a world-wide collection of *M. ulcerans* strains [11,8]. Since *M. ulcerans* is undergoing reductive evolution [12] it has been suspected that ongoing genome reduction would allow the performance of genetic fine typing of local *M. ulcerans* populations for micro-epidemiological studies. However, a lack of further diversification of African *M. ulcerans* isolates was observed [13]. Still, in a worldwide collection of isolates, LSP analyses revealed an intra-species evolutionary scenario with two distinct phylogenetic lineages. *M. ulcerans* haplotypes from Asia, South America, and Mexico belong to the ancestral lineage, whereas the classical lineage includes haplotypes from Africa, Australia, and South East Asia. Altogether seven haplotypes based on LSPs have been described [8,9] and a panel of LSPs has been identified to characterize the Asian *M. ulcerans* InDel haplotype [8,14]. Furthermore, studies including diverse “mycolactone producing mycobacteria” (MPMs) elucidated strain relatedness by InDel analysis within well defined regions of difference (RDs) [7].

M. ulcerans is the cause of Buruli ulcer, a chronic necrotizing skin disease, with a focus in West-Africa and Australia, but is found in tropical regions throughout the world [15-17]. In Japan and China sporadic cases of clinical Buruli ulcer (like) disease were reported. These were associated with pathogens that were given the (sub-) species names *M. shinshuense* or *M. ulcerans* ssp. *Shinshuense* [18-25]. Sequence analysis of the 16S rRNA already suspected a close genetic relationship to *M. Ulcerans* [26,25] albeit the position of *M. shinshuense* among pathogenic mycobacteria remained unclear.

The acquisition of the virulence plasmid pMUM001, encoding enzymes required for the synthesis of the cytotoxic polyketide mycolactone, is a key factor in the pathogenesis of Buruli ulcer [27-29]. Compared to Buruli ulcer endemic areas of West Africa and Australia, where the classical lineage is found, the disease occurs only very sporadically in endemic regions where the ancestral lineage is distributed [30,14,31]. Genomic studies imply that strains belonging to the classical lineage have undergone patho-adaptive loss of genes [29,12,27,4,14,32]. Yet, it remains unclear at which point of evolution, in which geographic region, and by which selective pressure(s) enhanced virulence has emerged.

Here, we present a rapid PCR-based genotyping scheme for allocation of new *M. ulcerans* isolates into the previously defined InDel haplotypes. This analysis is easy to perform since it is based on amplification products (amplicons) obtained by conventional PCR designed for haplotype specific LSPs. As an example for the application of the method we have characterized a selection of Japanese mycobacterial strains associated with Buruli ulcer (like) clinical lesions, that have been given different (sub-)species names. Also, application of LSP analysis to RD6, which is highly variable between the lineages, indicates closer relatedness of the *M. ulcerans* classical lineage to the South American InDel haplotype than to other members of the ancestral lineage.

Materials and Methods

Mycobacterial strains

Strains used in this study are: *M. marinum* M (ATCC BAA-535) and ATCC927, origin of both strains as described in [33]; *M. ulcerans*

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Ghana IFIK 1066089 (own collection); Malaysia ITM 941328, Australia ITM 9549, Australia ITM 5142, China ITM 980912, Japan 882/ITM 8756 [18] (ATCC 33728), Surinam ITM 842, origin of ITM strains as described in [11]; *M. shinshuense* 753 [21], strain kindly provided by P. Small, University of Tennessee, USA.

Genomic DNA extraction

DNA was extracted as described earlier [34,35]. Bacterial pellets of 20 mg (wet weight) were heat inactivated for 1 hour at 95°C in 500 µl PBS, resuspended in lysis buffer (15% sucrose, 0.05 M Tris (pH 8.5), 0.05 M EDTA), and sequentially treated with lysozyme (10 mg/ml, 1 hr, 37°C) and SDS (4%) and proteinase K (0.2 M proteinase K)(1 hr, 37°C). After enzyme deactivation and adding of 0.1 mm diameter zirconia beads, samples were homogenized using the Precellys 24 homogenizer (Bertin Technologies, Montigny-le Bretonneux, France) at conditions of 3x30 s at 6800 rpm. Supernatants were subjected to phenol-chloroform extraction and ethanol precipitation, and DNA concentration was determined using the NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE) and adjusted to retrieve equal starting material.

DNA amplification

PCR was performed using FirePol 10x BD buffer and 0.5 µl FirePolTaq-Polymerase (Solis BioDyne, Tartu, Estonia), 5 ng genomic DNA or the corresponding volume of nuclease free water as a negative control, 0.6 µM forward and reverse primers each, 1.7 mM MgCl₂ and 0.3 mM of each dNTP in a total volume of 30 µl. Long-range PCR polymerase mix (Fermentas, St. Leon-Rot, Germany) was applied according to the manufacturer's protocol to retrieve PCR products longer than 3 kb. PCR reactions were run in a GeneAmp PCR System 9700 PCR machine. The thermal profile for PCR amplification of mycobacterial genomic DNA included an initial denaturation step of 95-98°C for 3 min, followed by 32 cycles of 95°C for 20 sec, annealing at 58-65°C for 20 sec, and elongation at 72°C for 30 sec upto 4 min. The PCR reactions were finalized by an extension step at 72°C for 10 min. PCR products were analyzed on 1% agarose gels by gel electrophoresis using ethidium bromide staining and the AlphaImager illuminator (Alpha Innotech, San Leandro, CA, USA). Primers (Sigma-Aldrich, Steinheim, Germany) were selected on the genome sequences of *M. ulcerans* Agy99 (Genbank accession number CP000325) and *M. marinum* M (Genbank accession number CP000854 and CP000895) using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Results and Discussion

LSP based haplotyping

LSPs differentiating between *M. ulcerans* and *M. marinum* lineages were used to design primer pairs that allow easy and reliable amplification of haplotype-specific DNA sequences (Figure 1). In the case of RD12, such an amplicon (369 bp) clearly distinguishes the patient isolate *M. marinum* M from other mycobacteria due to the presence of a sequence feature (insertion InsRD12A containing the gene M_MAR3972) in that particular strain (Fig 1A), [34]. Other *M. marinum* strains and MPMs yield a PCR product with different size (315 bp) in a duplex PCR reaction due to the absence of that particular insertion (Figure 1A). Using a set of selected (monoplex or duplex) PCR reactions targeting sequences in earlier defined RDs, PCR products specific for the established LSP haplotypes allow the positioning of

new mycobacterial strains into the current genealogy (Figure 1B and C). With this amplicon typing scheme, all *M. ulcerans* haplotypes of the ancestral lineage can be determined, the two major lineages can be distinguished from each other, and two haplotypes can be assigned among the Australian *M. ulcerans* strains (Figure 1B and C).

Affiliation of *M. shinshuense*

We applied the above described PCR scheme to type a panel of Asian mycobacterial isolates. Mycobacterial strains from Asia (Japan, China, Malaysia) were tested for the presence of specific LSPs within six different RDs featuring unique characteristics for the Asian InDel haplotype (Figure 2). These InDel regions were earlier identified and characterized using isolates from Japan (ITM 8756) and China (ITM 980912) [8,14]. DNA from the Malaysian *M. ulcerans* strain showed features of both the classical and the ancestral lineage in the investigated RDs but may have an additional specific genetic signature in RD11. As expected [36,25], isolates of Japanese origin, irrespective of their (sub-) species designations, showed in all RDs genetic traits of the common *M. ulcerans* Asian InDel haplotype (Figure 2).

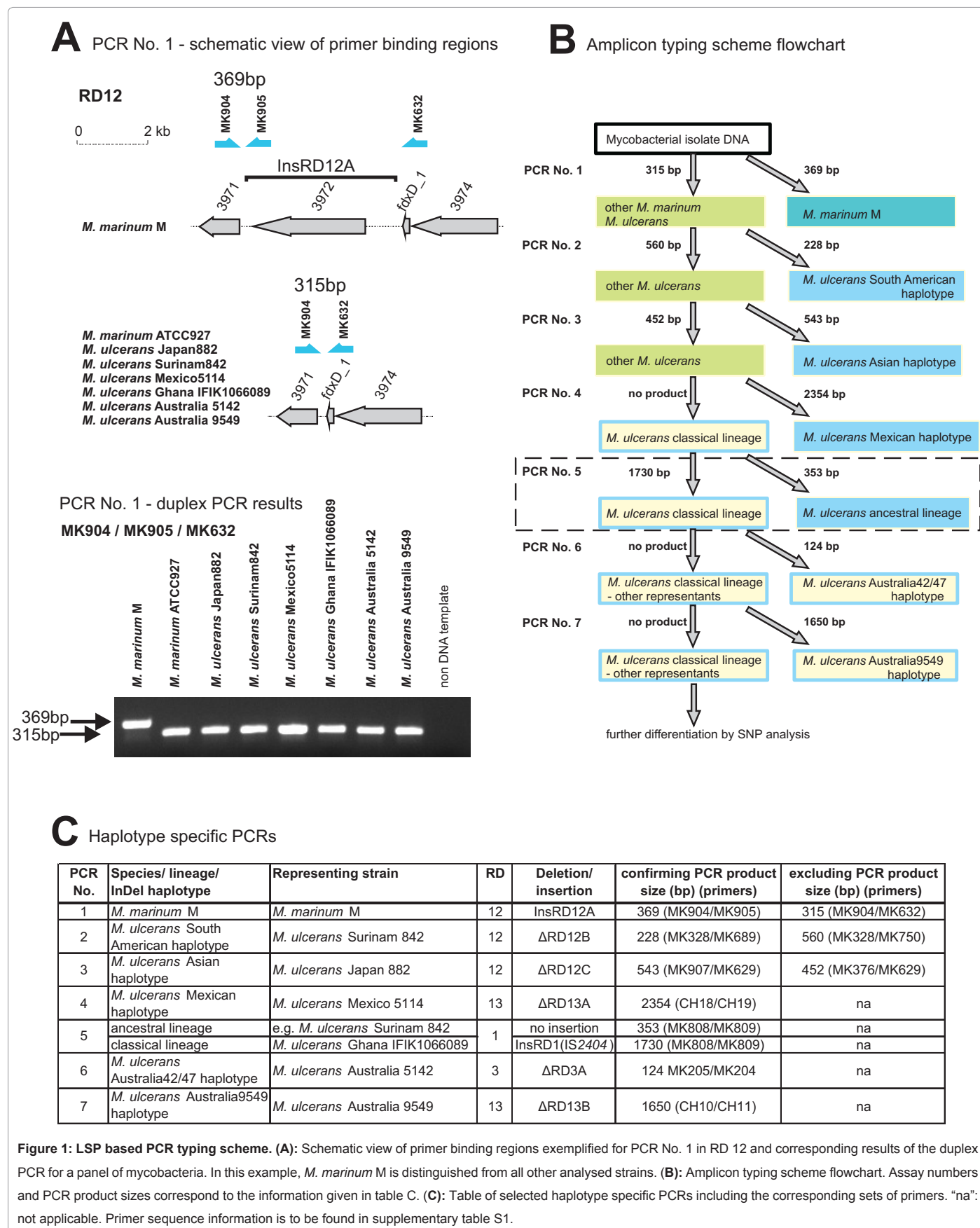
Relationship between South American isolates and the classical lineage

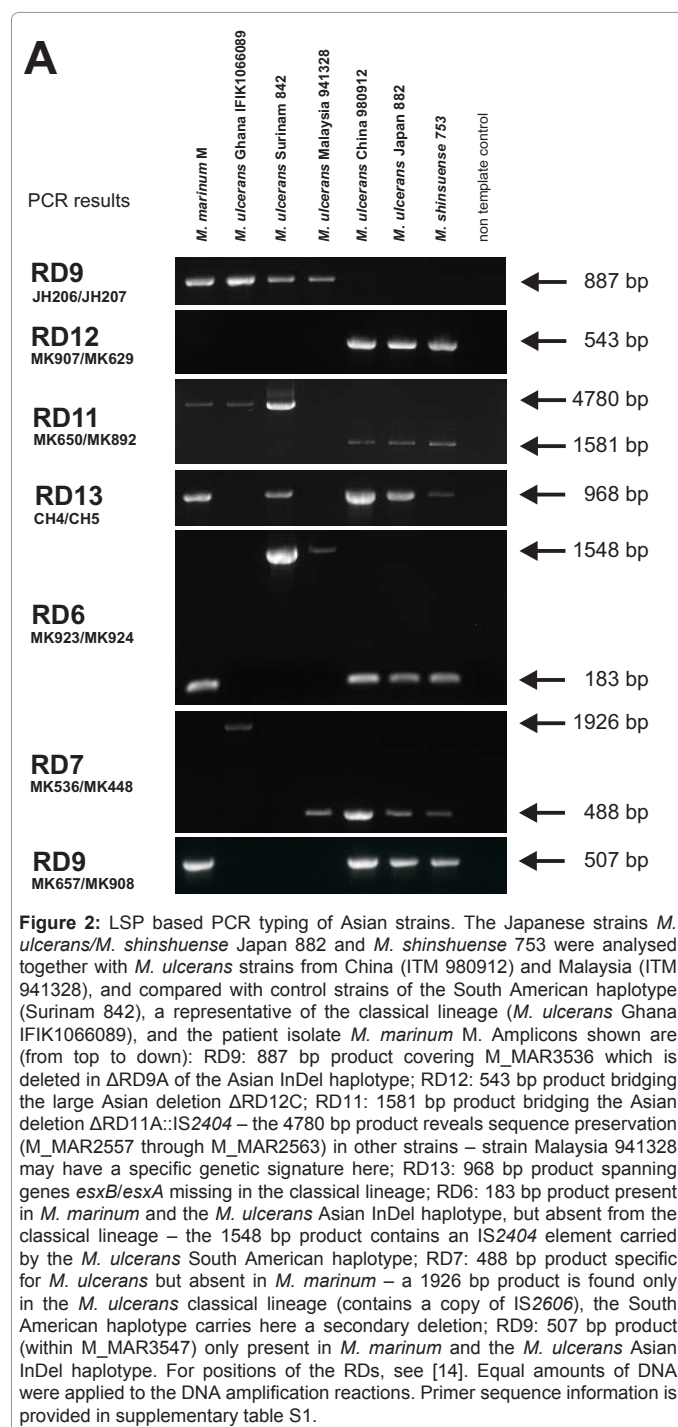
RD6 is a complex region with regard to LSP based genetic variations and therefore particularly suitable to differentiate between lineages. Gene segments from three different regions of an *M. marinum*-like genome were shuffled together in the *M. ulcerans* ancestral lineage, thereby inverting one segment (a 1 kb piece comprising M_MUL0504/0505) and forming the sequence situation found in the Asian haplotype in RD6 (Figure 2). Within this shuffled region, the South American haplotype shows a subsequent insertion of 21 kb through lateral gene transfer (containing 21 coding sequences of "insertion sequences and phages", M_MUL0508 through M_MUL0529, with 57-83% identity over between 600-1900 bps, revealed by nucleotide fasta blasts, to genes of Actinobacteria other than *M. marinum*). This insertion left genetic marks at the edges that testify the genome shuffling in the order of evolutionary events from the *M. marinum* sequence via the Asian to the South American InDel haplotype sequences. Interestingly, this very sequence situation of the South American haplotype is shared by the *M. ulcerans* classical lineage which only shows yet additional copies of the insertion sequence elements (ISEs) IS2404 and IS2606 within this 21 kb insertion stretch. Hence, LSP analysis in this RD provides evidence for the classical lineage to show closer genetic kinship to the South American haplotype than to any other member of the ancestral lineage.

Conclusion

In this study, we demonstrate that a simple amplicon typing scheme based on InDels allows the distinction between major lineages of *M. ulcerans*. For instance, patient isolates from Japan that have been given different (sub-)species names, exhibited identical traits characteristic for the Asian *M. ulcerans* lineage. This supports the recent suggestion to give all MPMs, including *M. shinshuense*, the species name *M. Ulcerans* [36].

At some point in the *M. ulcerans* evolution, the mycolactone producing ancestor of the classical lineage has been equipped with particular virulence, most likely through ISE-associated genomic changes and at least in part through loss of highly immunogenic proteins [12,14,32]. The strength of LSP analyses to show unequivocal details of genetic signatures is very helpful in the development of





evolutionary scenarios. Application of next generation genome sequencing technologies will allow the refinement of these scenarios and to further elucidate the relationship between different lineages of *M. ulcerans* and other MPMs. While LSP analyses are not suitable to fine-type local *M. ulcerans* populations [13], whole genome SNP typing greatly enables microepidemiological studies to address open questions concerning the transmission of *M. ulcerans* [12,13,37,38].

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