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Single Nucleotide Polymorphisms on the Road to Strain Differentiation in *Mycobacterium ulcerans*▽

Michael Käser,* Julia Hauser, and Gerd Pluschke

Swiss Tropical Institute, Molecular Immunology, Socinstrasse 57, 4002 Basel, Switzerland

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The genomic fine-typing of strains of *Mycobacterium ulcerans*, the causative agent of the emerging human disease Buruli ulcer, is difficult due to the clonal population structure of geographical lineages. Although large sequence polymorphisms (LSPs) resulted in the clustering of patient isolates originating from across the globe, differentiation of strains within continents using conventional typing methods is very limited. In this study, we analyzed *M. ulcerans* LSP haplotype-specific insertion sequence elements among 83 *M. ulcerans* strains and identified single nucleotide polymorphisms (SNPs) that differentiate between regional strains. This is the first genetic discrimination based on SNPs of *M. ulcerans* strains from African countries where Buruli ulcer is endemic, resulting in the highest geographic resolution of genotyping so far. The findings support the concept of genome-wide SNP analyses as tools to study the epidemiology and evolution of *M. ulcerans* at a local level.

Mycobacterium ulcerans causes the devastating cutaneous disease Buruli ulcer (BU). More than 30 countries worldwide have reported this emerging disease, reaching epidemic proportions in some areas, and children between the ages of 5 and 15 in the rural wetlands of West Africa are most affected (38). Although proximity to marshes and wetlands is a risk factor, the mode of transmission remains an enigma (10, 26, 27, 36, 37). Discrimination of genetic variants has become an indispensable tool to unravel the evolution, epidemiology, and transmission of pathogenic organisms and to gain insight into host-pathogen interactions (6, 13, 24). In *M. ulcerans*, such elucidation is impossible due to a remarkable lack of genetic diversity on a local geographic scale (22). Conventional genetic differentiation tools commonly used for phylogenetic profiling in *Mycobacterium tuberculosis*, such as restriction fragment length polymorphism, amplified fragment length polymorphism, variable-number tandem repeats (VNTR), and multilocus sequence typing, could distinguish between continental lineages only when applied to *M. ulcerans* (1–4, 7, 8, 15, 18, 29, 31, 34, 35). However, two publications using VNTRs reported the first discrimination of strains between and within African countries (16, 33). The identification of regions of difference (RDs) in a worldwide collection of *M. ulcerans* isolates led to an evolutionary scheme on the continental level, with two distinct genetic lineages that can be subgrouped into six haplotypes (20, 28). Strains of the "ancestral" lineage are genetically closer to *Mycobacterium marinum*, the progenitor of *M. ulcerans*, whereas the "classical" lineage accounts for the majority of BU cases and represents the most virulent genotype. Characterization of the large sequence polymorphisms (LSPs) showed that insertion sequence (IS) element (ISE) expansion is associated with the observed genome instability (17, 19, 40). ISs are compact mobile DNA segments capable of inserting at multiple sites in a target molecule, usually by a recombinase that is encoded by a coding sequence (CDS) contained within the ISE itself (23). Thus, uncontrolled duplications and insertions of ISEs occur at relatively high frequency in replicating bacteria, leading to genomic insertions, deletions, and rearrangements that have the potential for molecular epidemiological applications. In *M. tuberculosis*, until recently, IS-mediated insertions/deletions (InDels) used to be the principal source of genome plasticity (6) and are widely used as evolutionary markers in epidemiological studies. For *M. ulcerans*, two ISEs were defined, IS*2404* and IS*2606* (30, 32). Earlier, site-specific IS*2404* elements were identified to be unique for and confined to distinct *M. ulcerans* haplotypes (19). Here, we specifically amplified such unique ISEs and compared their sequences for a collection of 83 *M. ulcerans* isolates including 67 derived from Africa. We aimed at the detection of single nucleotide polymorphisms (SNPs) in these ISEs that made genetic distinction within haplotypes and on a regional level possible.

MATERIALS AND METHODS

Bacterial strains. Isolates used for SNP identification with their country origin are listed in Table 1.

Genomic DNA preparation. Genomic DNA from clinical and environmental isolates was extracted from bacterial pellets using an optimized method for mycobacterial DNA preparation (21). Bacterial pellets of about 20 mg (wet weight) were heat inactivated for 1 h at 95°C, followed by cell wall disruption and digestion. DNA was extracted from the supernatants by phenol-chloroform (Fluka, Buchs, Switzerland) extraction and subjected to ethanol precipitation as described previously (21). DNA was measured by the optical density at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher, Waltham, MA).

Amplification, sequencing, and alignment of haplotype-specific IS*2404* elements. Versions of IS*2404* were selected that are haplotype specific. For the African/Australian haplotypes, the primer pair MK323 (GCGGTACAAGCTT CCCAAAG) and MK814 (AGCCAGAGCTTTGGATTTGA) was applied to yield a PCR product of 2 kb comprising IS*2404* (MUL_3871) in RD12, and the pair MK809 (GGTGCTTAACGAAACGTGCT) and MK808 (ACGAAATCG AATTCCTCGTG) was used to yield a PCR product of 2 kb comprising IS*2404* (MUL_2990) in RD1. Primers MK808 and MK809 amplified a 360-bp PCR fragment of *glnA3* lacking IS*2404* in the South American and Asian haplotypes. The primer pair MK382 (GATCCTCGATCCGGTGTTC) and MK410 (GGAT CTCCACCTTCGTCAAC) amplified a specific IS*2404* element within RD9 con-

* Corresponding author. Present address: Ghanaian-German Centre for Health Research, University of Ghana, School of Public Health, P.O. Box LG 13, Legon, Accra, Ghana. Phone: 233 277291546. Fax: 41 61 2848 101. E-mail: m.kaeser@unibas.ch.

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TABLE 1. *M. ulcerans* strains used in this study

Geographic region[a]	Strain identifier[b]	Year of isolation[a]	Geographic region[a]	Strain identifier[b]	Year of isolation[a]
Ghana, Greater Accra region	Agy99	1999	Ghana, Greater Accra region	NM97/03	2003
Ghana, Greater Accra region	NM14/02	2002	Ghana, Greater Accra region	NM102/03	2003
Ghana, Greater Accra region	NM18/02	2002	Ghana, Greater Accra region	NM103/03	2003
Ghana, Greater Accra region	NM19/02	2002	Ghana, Ashanti region	ITM GH970321	1997
Ghana, Greater Accra region	NM20/02	2002	Ghana, Ashanti region	ITM GH970359	1997
Ghana, Greater Accra region	NM21/02	2002	Ghana, Eastern region	NM53/02	2002
Ghana, Greater Accra region	NM22/02	2002	Ghana, region ND	NM01/03	2003
Ghana, Greater Accra region	NM23/02	2002	Ghana, region ND	NM06/03	2003
Ghana, Greater Accra region	NM27/02	2002	Ghana, region ND	NM94/03	2003
Ghana, Greater Accra region	NM28/02	2002	Ghana, region ND	NM95/03	2003
Ghana, Greater Accra region	NM30/02	2002	Ghana, region ND	NM98/03	2003
Ghana, Greater Accra region	NM32/02	2002	Ghana, region ND	ITM GH970483	1997
Ghana, Greater Accra region	NM33/02	2002	Angola	ITM AN960658	1996
Ghana, Greater Accra region	NM34/02	2002	Ivory Coast	ITM IC940511	1994
Ghana, Greater Accra region	NM37/02	2002	Ivory Coast	ITM IC940815	1994
Ghana, Greater Accra region	NM38/02	2002	Ivory Coast	ITM IC940662	1994
Ghana, Greater Accra region	NM40/02	2002	Democratic Republic of Congo	ITM DRC5150	1962
Ghana, Greater Accra region	NM41/02	2002	Democratic Republic of Congo	ITM DRC5151	ND
Ghana, Greater Accra region	NM43/02	2002	Democratic Republic of Congo	ITM DRC5155	1976
Ghana, Greater Accra region	NM44/02	2002	Togo	ITM TOGO970680	1997
Ghana, Greater Accra region	NM46/02	2002	Benin	ITM BEN970104	1997
Ghana, Greater Accra region	NM47/02	2002	Benin	ITM BEN970111	1997
Ghana, Greater Accra region	NM48/02	2002	Benin	ITM BEN940512	1994
Ghana, Greater Accra region	NM49/02	2002	Benin	ITM BEN940886	1994
Ghana, Greater Accra region	NM50/02	2002	Benin	ITM BEN001441	2000
Ghana, Greater Accra region	NM51/02	2002	Papua New Guinea	ITM PNG9357	ND
Ghana, Greater Accra region	NM52/02	2002	Papua New Guinea	ITM PNG941331	1994
Ghana, Greater Accra region	NM54/02	2002	Malaysia	ITM MALAY941328	1994
Ghana, Greater Accra region	NM56/02	2002	Australia, Queensland	ITM AU941324	1994
Ghana, Greater Accra region	NM59/02	2002	Australia, Victoria	ITM AU5142	1967
Ghana, Greater Accra region	NM60/02	2002	Australia, Victoria	ITM AU5147	ND
Ghana, Greater Accra region	NM61/02	2002	Australia, Victoria	ITM AU9550	ND
Ghana, Greater Accra region	NM62/02	2002	Australia, Victoria	ITM AU940339	1994
Ghana, Greater Accra region	NM68/02	2002	Australia, region ND	ITM AU8849	ND
Ghana, Greater Accra region	NM69/02	2002	Australia, region ND	ITM AU9549	ND
Ghana, Greater Accra region	NM72/03	2003	Australia, region ND	ITM AU941325	1994
Ghana, Greater Accra region	NM74/03	2003	Australia, region ND	ITM AU941327	1994
Ghana, Greater Accra region	NM76/03	2003	Surinam	ITM SU842	1986
Ghana, Greater Accra region	NM77/03	2003	French Guiana	ITM FG7922	1990
Ghana, Greater Accra region	NM78/03	2003	China	ITM CH980912	1998
Ghana, Greater Accra region	NM89/03	2003	Japan	ITM JP9756	1998
Ghana, Greater Accra region	NM90/03	2003			

[a] ND, not defined.
[b] ITM, Institute of Tropical Medicine, Antwerp, Belgium (4,16); NM, Noguchi Memorial Institute for Medical Research, Accra, Ghana (16) (isolated as outlined earlier [39]). All strains are isolates from human BU patients, with the exception of the environmental strain ITM BEN001441 (25).

fined to the South American haplotype, and primer pair MK892 (GCAATGTG ATGCACAACCTC) and MK650 (CGTTCGATTTCACCTCACC) amplified a specific IS*2404* element within RD11 unique for the Asian haplotype. Sequencing of the respective PCR products was done using the primers used for the PCR and the IS*2404*-specific internal primers MK661 (GATTGGTGCTCGGTCAA CTC), MK662 (TCAGGTAGTGCGACTTCAAGG), MK663 CAGCGTGGA GGTGGTCTATG), and SR685 (AGGCCAACACATCGAGAAAC) to cover the entire amplicon. PCR was performed using the FirePol 10× BD buffer and 0.5 μl of FirePol *Taq* polymerase (Solis BioDyne, Tartu, Estonia) with 5 ng of genomic DNA, 0.6 μM (each) of forward and reverse primer, 1.7 mM $MgCl_2$, and a 0.3 mM concentration of each deoxynucleoside triphosphate in a total volume of 30 μl. PCRs were run in a GeneAmp PCR System 9700 PCR machine. The thermal profile for PCR amplification of *Escherichia coli* plasmids and *M. ulcerans* genomic DNA included an initial denaturation step of 95 to 98°C for 3 min, followed by 32 cycles of 95°C for 20 s, annealing at 58 to 65°C for 20 s, and elongation at 72°C for 30 s up to 2 min. The PCRs 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 an AlphaImager illuminator (Alpha Innotech, San Leandro, CA). PCR amplicons were purified using a NucleoSpin purification kit (Machery-Nagel, Düren, Germany) and subjected to direct sequencing by Macrogen, Seoul, South Korea. Primers (Sigma-Aldrich, Steinheim, Germany) were designed using Primer3 software, version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All sequences were subjected to multiple sequence alignments using the ClustalW2 tool of the European Molecular Biology Library-European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/index.html) for phylogenetic analysis.

RESULTS

We selected copies of four IS*2404* elements that were earlier identified to be confined to one specific haplotype only (19). In RD9, an IS*2404* element was inserted in between orthologues of the partial CDSs, MMAR_3539 and MMAR_3559, in only the South American haplotype (Fig. 1). In RD11, an IS*2404* element was inserted in between the orthologues of the CDSs, MMAR_2557 and MMAR_2563 in only the Asian haplotype (Fig. 1). The two IS*2404* elements in RD1 (MUL_2990) and RD12 (MUL_3871) were confined to the classical lineage (Fig. 1), represented by the

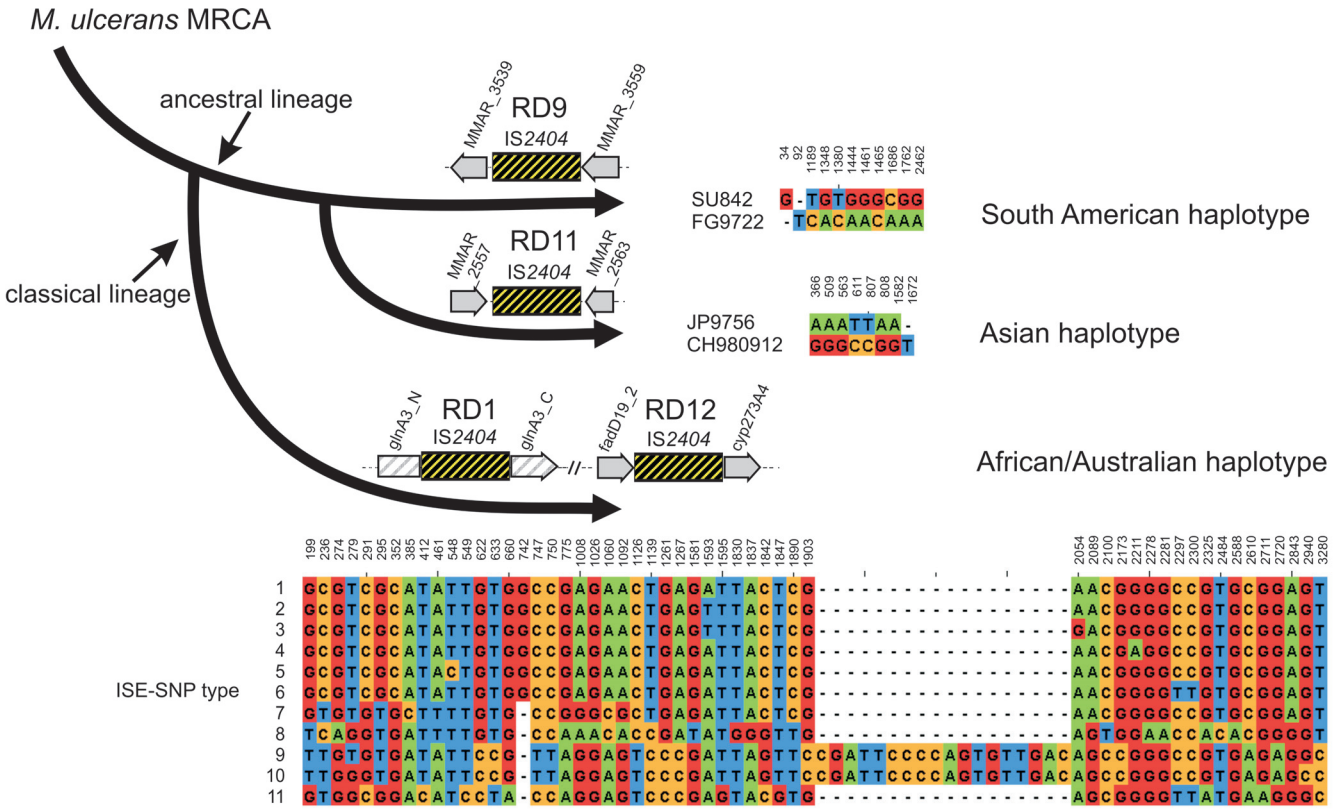

FIG. 1. Sequence variation in haplotype-specific IS*2404* elements. IS*2404* elements that are unique for, and confined to, the shown haplotypes were amplified with site-specific primers located outside of the ISEs, and SNPs were determined by sequencing. Only sites that differ in the aligned nucleotides (European Bioinformatics Institute ClustalW2) are shown in the sequence panels (JalView, version 2.4) (9) from the 1,362-bp (RD9), 1,366-bp, and adjacent regions (RD11) and from 1,497 bp (RD1) combined with 1,822 bp (RD12) to yield 3,319 bp of concatenated chromosomal ISE fragments, including their flanking regions. SNP position numbers in RD1 and RD12 are given according to the *M. ulcerans* Agy99 sequence, with 1 corresponding to position 3313231 in RD1 and 1498 to position 4326896 in RD12. SNP position numbers in RD9 and RD11 are given according to the *M. marinum* M sequence, with 1 corresponding to position 4348015 in RD9 and 1 corresponding to position 3108032 in RD11. For the 79 sequenced *M. ulcerans* isolates of the African/Australian haplotype, 11 various ISE-SNP types (1 to 11) were identified, as indicated in the sequence panel. MRCA, most recent common ancestor.

African/Australian isolates. Primers flanking the respective ISEs and specific for the haplotype-specific constellation of the respective RDs were used to specifically amplify these four IS*2404* element-containing loci without contamination by the many other copies of ISEs present in the *M. ulcerans* genomes. The RD9-associated IS*2404* was amplified for two South American strains, and the RD11-associated IS*2404* was amplified for two Asian strains, each belonging to the ancestral lineage. The two IS*2404* versions associated with RD1 and RD12 were amplified for 79 *M. ulcerans* strains belonging to the classical lineage (Fig. 1). Fifty-four of these strains originated from Ghana, and 13 more were from other West and Central African countries; 12 strains derived from Australia, Malaysia, and Papua New Guinea. The chromosomal context within each haplotype was identical for all strains (data not shown), i.e., the nucleotide composition at the breakpoints, the adjacent regions, and the deleted DNA stretches associated with the ISE insertion (in RD9 and RD11). The IS*2404* elements were between 1,362 and 1,367 bp long.

Within the South American haplotype, the patient isolate originating from Surinam could be distinguished by 11 SNPs (including one gap) from the one from French Guiana (Fig. 1). The two strains from Japan and China, belonging to the Asian haplotype, could be distinguished by eight SNPs (including one gap) from each other (Fig. 1). Within the African/Australian haplotype for the two RD9- and RD11-associated ISEs, altogether 72 variable nucleotide positions were found that cluster the 79 strains into 11 groups that we designated ISE-SNP types (Fig. 1). When these SNPs are linked to epidemiological data (Fig. 2), we found that the West African region from the Ivory Coast to Togo harbors a mixture of ISE-SNP types. All four patient isolates from Benin showed identical nucleotide sequences defined by one common SNP (T548C). This polymorphism is shared by the environmental strain BEN001441 (25). We found four genotypes represented within southern Ghana, with a majority having ISE-SNP type 2. The ISE-SNP type 1 cluster contains the sequenced reference strain Agy99 along with another strain from Ghana as well as isolates from the Democratic Republic of Congo, the Ivory Coast, and Angola. ISE-SNP type 3 from within the Greater Accra region in Ghana comprises four identical strains that differ in one SNP (T-A) from other *M. ulcerans* isolates of the same area. Two strains from Ghana and one from the Ivory Coast (forming

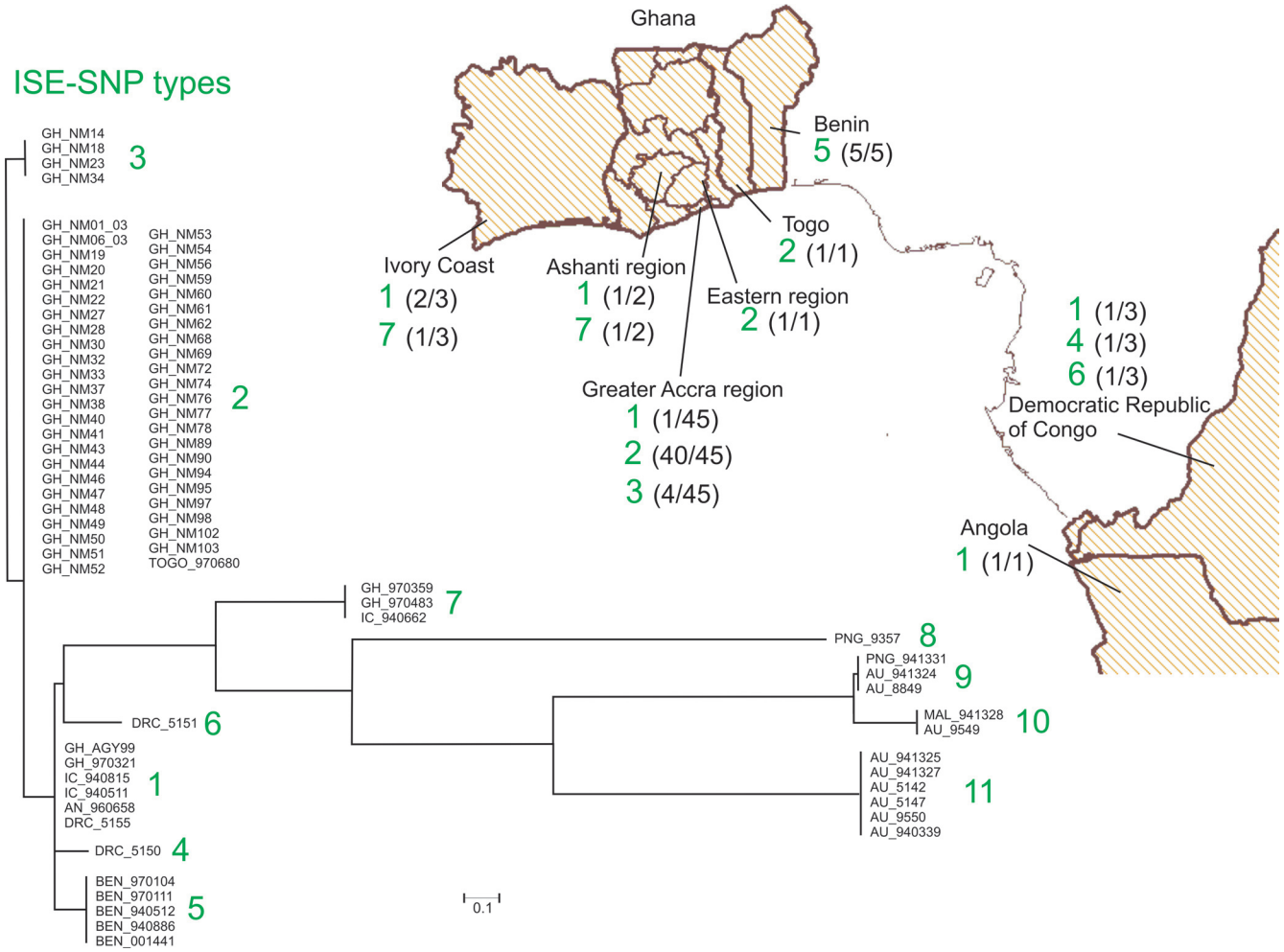

FIG. 2. Phylogenetic and epidemiological patterns of the ISE-SNP types in the classical lineage. Identified SNPs were used to create a neighbor-joining tree (using percent identity) with JalView, version 2.4 (9), for visualization of their phylogenetic relationships. Clusters were assigned ISE-SNP type numbers (1 to 11, corresponding to Fig. 1). The geographic origins of the African strains are shown on the map (created using HealthMapper, version 4.3.1, software [http://www.who.int/health_mappping/tools/healthmapper/en/index html] with the permission of the WHO Geographic Information Systems department). Frequencies of the respective ISE-SNP type per geographic sample size are indicated in brackets for each country or region. Genetic distances (legend) are reflected by the lengths of the branches. GH, Ghana; IC, Ivory Coast; DRC, Democratic Republic of Congo; AN, Angola; BEN, Benin; PNG, Papua New Guinea; AU, Australia; MAL, Malaysia.

ISE-SNP type 7) are, with respect to the ISE-SNP type, quite distinct from the remainder of the African isolates but were found in closer genetic proximity to strains from Southeast Asia and Australia (ISE-SNP types 8 to 11) (Fig. 2). Within the Australian isolates, the insertion of 18 nucleotides in five strains (Fig. 1, ISE-SNP types 9 and 10) has probably emerged through homologous recombination of an internal part of another IS*2404* fragment in a common progenitor, grouping these strains originating from Papua New Guinea, Australia, and Malaysia together. However, other *M. ulcerans* isolates from southern Australia, i.e., Victoria (ISE-SNP type 11), do not have this small insert, resulting in two different clusters within Australia.

Here, we defined ISE-SNP types in Africa that seem to be either geographically clustered (e.g., ISE-SNP types 5 and 6) or more widespread (e.g., ISE-SNP types 1 and 7). These genotypes unveil a clearer picture of *M. ulcerans* dispersal and epidemiology in Africa and on a worldwide scale.

DISCUSSION

Characterization of InDel diversity among a worldwide collection of *M. ulcerans* strains by comparative genomic hybridization analysis (20) has yielded markers for the investigation of the phylogeography of *M. ulcerans* patient isolates on a global scale. Continental haplotypes with unique constellations in particular RDs were defined (19, 20). Here, we combine the strength of lineage-specific unequivocal genetic InDel markers with the high-resolution power of SNPs. We now determined the nucleotide sequence of RD-associated haplotype-specific copies of IS*2404* and identified SNPs, allowing further subdivision of continental lineages. In particular, within the classical lineage, sequence analysis of the RD9- and RD11-associated IS*2404* elements yielded 11 SNP types (ISE-SNP types) across a panel of 79 *M. ulcerans* strains. Since the two selected ISEs are identical in their chromosomal context across the tested classical lineage strains, the haplotype-specific insertions in

RD9 and RD11 must have occurred in a common ancestor, and accumulation of SNPs represents secondary events. Since IS*2404* is highly redundant in *M. ulcerans*, the occurrence of point mutations, whether synonymous or nonsynonymous in nature, is irrelevant for the microbe's biology.

The resolution of ISE-SNP typing is higher than that achieved with other DNA fingerprinting techniques: ISE-SNP types correlated with the more limited VNTR/ mycobacterial interspersed repetitive unit-VNTR fine-typing and, in particular, enhanced the resolution within the Atlantic African genotype (16, 33). Some ISE-SNP types seem to be widespread across West African countries (e.g., ISE-SNP types 1, 2, 3, and 7 in the Ivory Coast, Ghana, Togo, the Democratic Republic of the Congo, and Angola). Others appear more delimited (such as ISE-SNP type 5 in Benin and types 4 and 6 in the Democratic Republic of the Congo). Among *M. ulcerans* isolates from Ghana, four ISE-SNP types (1, 2, 3, and 7) were identified. The retrieved phylogenetic tree (Fig. 2) depicts the highest resolution of *M. ulcerans* phylogeny within and between continents. The ISE-SNP type analysis revealed genetic relatedness of a subgroup of African strains (ISE-SNP type 7 from Ghana and the Ivory Coast) to the Southeast Asian/Australian clusters (ISE-SNP types 8 through 11). The latter indicates the possible link of an origin from common ancestors of ISE-SNP type 7 to Australian *M. ulcerans* strains. Interestingly, the only *M. ulcerans* isolate ever cultivated from the environment and originating from Benin (25) showed the same ISE-SNP type as the patient isolates coming from the same country, supporting the current hypothesis that infection with BU disease results from environmental exposure.

Within the *M. tuberculosis* complex, and even within *M. ulcerans*-related mycolactone-producing mycobacteria, analysis of LSPs represents a valuable approach for genetic fingerprinting (5, 6, 12, 18). However, with the increasing availability of multiple whole-genome sequences, SNP identification adds considerably to phylogeographic analyses (11, 13, 14). We conclude that also for *M. ulcerans*, SNP typing rather than analysis of LSPs will yield sufficient resolution for microepidemiological studies. The resolution obtained here for the classical lineage is thus far based on only two copies of IS*2404*. Analysis of a larger number of ISE copies or of the entire genome of a collection of isolates may yield a large enough number of SNPs to resolve the spatial and temporal dispersal of genetic *M. ulcerans* variants on the regional level.

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ADDENDUM IN PROOF

A subsequent study that applied next-generation sequencing to two additional genomes of *M. ulcerans* strains from Ghana confirmed our conclusion in revealing 68 SNP loci that led to the differentiation of a collection of 54 strains from this region of endimicity into 13 SNP haplotypes (W. Qi, M. Käser, K. Röltgen, D. Yeboah-Manu, and G. Pluschke, PLoS Pathog. **5:**e1000580, 2009).

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