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Insertion Sequence Element Single Nucleotide Polymorphism Typing Provides Insights into the Population Structure and Evolution of *Mycobacterium ulcerans* across Africa

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Buruli ulcer is an indolent, slowly progressing necrotizing disease of the skin caused by infection with *Mycobacterium ulcerans*. In the present study, we applied a redesigned technique to a vast panel of *M. ulcerans* disease isolates and clinical samples originating from multiple African disease foci in order to (i) gain fundamental insights into the population structure and evolutionary history of the pathogen and (ii) disentangle the phylogeographic relationships within the genetically conserved cluster of African *M. ulcerans*. Our analyses identified 23 different African insertion sequence element single nucleotide polymorphism (ISE-SNP) types that dominate in different areas where Buruli ulcer is endemic. These ISE-SNP types appear to be the initial stages of clonal diversification from a common, possibly ancestral ISE-SNP type. ISE-SNP types were found unevenly distributed over the greater West African hydrological drainage basins. Our findings suggest that geographical barriers bordering the basins to some extent prevented bacterial gene flow between basins and that this resulted in independent focal transmission clusters associated with the hydrological drainage areas. Different phylogenetic methods yielded two well-supported sister clades within the African ISE-SNP types. The ISE-SNP types from the "pan-African clade" were found to be widespread throughout Africa, while the ISE-SNP types of the "Gabonese/Cameroonian clade" were much rarer and found in a more restricted area, which suggested that the latter clade evolved more recently. Additionally, the Gabonese/Cameroonian clade was found to form a strongly supported monophyletic group with Papua New Guinean ISE-SNP type 8, which is unrelated to other Southeast Asian ISE-SNP types.

Buruli ulcer (BU) is a slowly progressing necrotizing disease of the skin and subcutaneous tissue that is caused by infection with *Mycobacterium ulcerans* (1). BU is the third most common mycobacterial disease in humans, after tuberculosis and leprosy, and the least understood of the three (2). Even though the infection affects all age groups, at least half of all cases occur in children under age 15 years (3). More than 30 countries worldwide have reported (but not always confirmed) this emerging disease, with the highest incidence in West and Central Africa, where the disease occurs in foci among people living in rural marshes, wetlands, and riverine areas (1, 4). As proximity to these slow-flowing or stagnant water bodies is a known risk factor for *M. ulcerans* infection (5) and as *M. ulcerans* DNA has been detected in a variety of aquatic specimens (6, 7), it is generally believed that *M. ulcerans* is an environmental mycobacterium that can initiate infection after microtraumata of the skin (8). However, the exact mode of transmission and the environmental reservoir(s) of *M. ulcerans* remain largely unknown (9), as (i) culturing the slow-growing mycobacterium from an environmental source is particularly difficult (10) and (ii) the significance of the detection of *M. ulcerans* DNA by PCR in environmental samples remains unclear in the disease ecology of BU (6, 7, 11–16).

Multilocus sequence typing analyses (17) and subsequent whole-genome comparisons (18) have proved that *M. ulcerans* recently evolved from a *Mycobacterium marinum* progenitor by acquisition of the virulence plasmid pMUM001. This plasmid harbors genes required for the synthesis of the macrocyclic polyketide toxin mycolactone (19), which has cytotoxic and immunosuppressive properties that cause chronic ulcerative skin lesions with limited inflammation and thus plays a key role in the pathogenesis of BU (20). Both the acquisition of the plasmid and a reductive evolution (21, 22) led the generalist *M. marinum* to become a highly specialized mycobacterium that is more adapted to a restricted environment, such as that of a vertebrate host. Analysis of the genome sequence suggests that this new niche is likely to an obscure, aerated, osmotically stable, extracellular environment where slow growth, the loss of several immunogenic proteins, and

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production of mycolactone provided selective advantages (18, 22). Many of the changes in this evolutionary process were mediated by two insertion sequence elements (ISE), IS*2404* and IS*2606*, that are present in the *M. ulcerans* genome in ≈200 and ≈90 copies, respectively (22). These short, mobile genetic DNA elements promote genetic rearrangements by modifying gene expression and sequestering genes, profoundly affecting mycobacterial genome plasticity (23). Increased ISE numbers are expected, as the aforementioned lifestyle shift causes many loci to become excessive, as they are no longer essential for survival in the new environment (24). Subsequent whole-genome comparisons (18) have shown furthermore that the resulting niche-adapted genomic signature was established in an *M. ulcerans* progenitor before its intercontinental dispersal.

Deciphering the structure of pathogenic bacterial populations is instrumental for the understanding of the epidemiology, global spread, and evolutionary history of bacterial infectious diseases. Moreover, understanding the population structure allows for studying meaningful bacterial differences that can affect disease control, including public health interventions, such as vaccination programs (25). Differences in the ratio of genetic variation caused by *de novo* mutations relative to recombination bring about a spectrum of different bacterial population structures, ranging from "clonal" (no recombination) to "nonclonal" (where a lot of recombination of alleles prevents the emergence of stable clones) (26). Because of the clonal population structure of *M. ulcerans*, conventional genetic fingerprinting methods have largely failed to genetically differentiate clinical disease isolates, complicating molecular analyses on the elucidation of the disease ecology and the population structure and evolutionary history of the pathogen (27). However, in 2009, Käser et al. (28) identified single nucleotide polymorphisms (SNPs) within *M. ulcerans* haplotype-specific IS*2404* elements MUL_2990 and MUL_3871, which are located in region of difference 1 (RD1) and RD12, respectively (29). The identified SNPs differentiated multiple genotypes among isolates originating from one region in Ghana, resulting in the highest geographical resolution of genotyping achieved to date without the use of whole-genome sequencing. Given the apparent rarity of recombination in *M. ulcerans*, ISE-SNP types should contain sufficient phylogenetic signal to reconstruct recent evolutionary events on a continental scale. Hence, in the present study, we applied a redesigned form of the ISE-SNP typing technique as described by Käser et al. (28) to a vast panel of *M. ulcerans* isolates originating from multiple African disease foci to gain deeper insights into the population structure and evolutionary history of the pathogen and to continue to disentangle the phylogeographic relationships within the genetically conserved cluster of African *M. ulcerans*.

MATERIALS AND METHODS

A panel ($n = 171$) of 157 *M. ulcerans* clinical isolates and 14 clinical specimens with a quantification cycle C_q (IS*2404*) of ≤32 originating from disease foci in 11 different African countries was selected to assess the polymorphisms in the RD1- and RD12-associated haplotype-specific copies of IS*2404* (Tables 1 and 2). Clinical specimens consisted of tissue fragments and swabs originating from ulcerated and nonulcerated BU lesions. These surplus samples had been collected for routine diagnostic purposes and for rechecking for quality control. All isolates and specimens were selected from the comprehensive mycobacterial collection of the Institute of Tropical Medicine (ITM) and were chosen to maximize temporal and spatial diversity within countries in which more than 20 isolates/specimens were available. Isolates and specimens were processed and analyzed for bacterial polymorphisms without use of any patient identifiers, except for country and village of origin if this information was available.

Based on conventional phenotypic and genotypic methods, bacterial isolates had previously been assigned to the species *M. ulcerans*. They had all tested positive for IS*2404* via primers that amplify all copies of IS*2404* routinely used for diagnostic PCR (30). Mycobacterial isolates were maintained for prolonged storage at ≤−70°C in Dubos broth enriched with growth supplement and glycerol. They were recultured on solid Löwenstein-Jensen medium. DNA was obtained by scraping 1 to 2 loopfuls of colonies into 400 μl of Tris-EDTA followed by heat inactivation at 100°C for 5 min and subsequent centrifugation to remove cellular debris. Clinical specimens were maintained (after decontamination) at ≤−18°C. The modified Boom DNA extraction procedure was carried out on all clinical specimens as previously described (31).

As the original ISE-SNP typing method described by Käser et al. (28) resulted in aspecific bands, short sequence reads, and high background signals, we redesigned and optimized primers and conditions for PCR and sequencing for application directly on clinical specimens. Primer pair RD1_SENSE (GGTGCTTAACGAAACGTGCTG) and RD1_ANTI_SENSE (ACGGGCTATCTGGAGAACGA) was designed to amplify a fragment of 1,431 bp in RD1 that comprises IS*2404* (MUL_2990), while the primer pair RD12_SENSE (CGTTGGCGCGGTACAAGCTTCCCAA) and RD12_ANTI_SENSE (GATGGTCGCGGTGCTGCTTGCCCT) was used to amplify a 1,871-bp PCR product in RD12 that comprises IS*2404* (MUL_3871). Primers were designed with Primer Premier 6 (Premier Biosoft, CA) and evaluated *in silico* with Amplify 3.1.4 (Bill Engels, University of Wisconsin). The PCR design was challenging, as only haplotype-specific copies of IS*2404* (MUL_2990 and MUL_3871) were to be amplified and because the relevant regions were of considerable size (1,730 bp for RD1 and 1,905 bp for RD12). Although we reduced the size of the amplicons in both assays (by 299 bp for RD1 and by 48 bp for RD12), they still contained all the variable nucleotide positions described by Käser et al. (28). PCR mixtures contained 1.0 U of HotStarTaq polymerase (Qiagen, Hilden, Germany), 3.0 μl 10× PCR buffer, 6.0 μl Qsolution, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, and 0.5 μM each primer in a total volume of 30 μl. PCRs were carried out on a Biometra TProfessional thermal cycler under the following conditions: an initial denaturation step of 15 min at 95°C, followed by 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 65°C (RD1) or 70°C (RD12), and elongation for 2 min at 72°C, and ending with a final elongation step of 10 min at 72°C. PCR products were visualized with ethidium bromide on 1% agarose gels by electrophoresis (30 min, 100 V). PCR products were purified by automated gel excision. Bidirectional sequencing was performed at the Genetic Service Facility of the Flanders Institute for Biotechnology (GSF-VIB) on an Applied Biosystems 3730 DNA analyzer capillary sequencer with the ABI Prism BigDye Terminator cycle sequencing v3.1 kit and the PCR primers.

We estimated the bacterial load by a quantitative PCR (qPCR) for IS*2404* as described by Fyfe et al. (32) on a set of 122 clinical specimens to determine the C_q (for IS*2404*) below which the optimized genotyping PCRs were always successful.

The sequences of RD1 and RD12 were concatenated to yield a 3,278-bp fragment and aligned using Clustal X v2.1 (33). Sequences were trimmed to an equal length, and all currently known ISE-SNP types (including 5 ISE-SNP types from Papua New Guinea, Australia, and Malaysia) (28) were added to this data set. We mapped SNPs according to the Agy99 bacterial reference chromosome (GenBank accession no. NC_008611). We constructed a neighbor-joining (NJ) tree based on p distances between ISE-SNP types (34) in MEGA v5 (35). Maximum parsimony (MP) and maximum likelihood (ML) trees were estimated in the same program by using a heuristic search with the tree bisection-reconnection branch-swapping algorithm and random addition of taxa. Relative branch support was evaluated with 1,000 bootstrap replicates (36) for

TABLE 1 Isolates used in this study[a]

ISE-SNP type	Culture no.	Country of origin	Administration division			Source	YOI	Remark
			First level	Second level	Third level			
1	ITM_940511	Ivory Coast	Moyen-Cavally	Duékoué	Niambli	ITM	1994	
1	ITM_000483	Ivory Coast	Moyen-Cavally	Duékoué	Niambli	ITM	2000	
1	ITM_000870	Ivory Coast	Dix-Huit Montagnes	Zouan-Hounien	Ouyatouo	ITM	2000	
1	ITM_063519	DRC	Bas-Congo	Cataractes/Songololo	Luima/Cité Songololo	IME	2006	
1	ITM_071924	Congo	Kouilou	Madingo-Kayes	Loukouala	ITM	2007	Originated from same patient as 071925
1	ITM_071925	Congo	Kouilou	Madingo-Kayes	Loukouala	ITM	2007	Originated from same patient as 071924
1	ITM_072398	DRC	Bas-Congo	Cataractes/Songololo	Bamboma/Mbanza-Manteke	IME	2007	
1	ITM_072401	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2007	
1	ITM_072732	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2007	
1	ITM_072733	DRC	Bas-Congo	Cataractes/Songololo	Luima-Mayanga/Ngombe	IME	2007	
1	ITM_072734	DRC	Bas-Congo	Cataractes/Songololo	Bamboma/Mbanza-Manteke	IME	2007	
1	ITM_072735	DRC	Bas-Congo	Cataractes/Songololo	Luima/Luvuvamu	IME	2007	
1	ITM_072840	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2007	Originated from same patient as 072841
1	ITM_072841	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2007	Originated from same patient as 072840
1	ITM_073453	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2007	
1	ITM_073459	Benin	Kouffo	Lalo	Ahojinako	CDTUB Lalo	2007	
1	ITM_073463	DRC	Bas-Congo	Cataractes/Songololo	Luima/Kisonga	IME	2007	
1	ITM_073477	DRC	Bas-Congo	Cataractes/Songololo	Luima/Cité Songololo	IME	2007	
1	ITM_073478	Angola	Malanje	Marimba	Kafufu/Luremo (Kwango River)	IME	2007	
1	ITM_073479	DRC	Bas-Congo	Cataractes/Songololo	Luima/Kisonga	IME	2007	
1	ITM_082600	DRC	Bas-Congo	Cataractes/Songololo	Kilueka/Nzundu	IME	2008	
1	ITM_100140	DRC	Bas-Congo	Cataractes/Songololo	Lovo/Tole	IME	2010	
1	ITM_100141	DRC	Bas-Congo	Cataractes/Songololo	Mayanga/Mpelo	IME	2010	Originated from same patient as 100141
1	ITM_100142	DRC	Bas-Congo	Cataractes/Songololo	Luima/Luvuvamu	IME	2010	Originated from same patient as 100142
1	ITM_100832	DRC	Bas-Congo	Cataractes/Songololo	Palabala/Nkamuna	IME	2010	
1	ITM_100833	DRC	Bas-Congo	Cataractes/Songololo	Mayanga/Mpelo	IME	2010	
1	ITM_032481	DRC	Bas-Congo	Cataractes/Songololo	Luima/Nkondo-Kiomba	IME	2003	
1	ITM_040149	Ghana	Ashanti	Asante Akim North	Agogo Presbyterian Hospital	ITM	2003	
1	ITM_991591	Togo	Maritime	Vo	Anagali	ITM	1999	
1	ITM_050303	Congo	Kouilou			ITM	1979	Originated from same patient as 050304
1	ITM_050304	Congo	Kouilou			ITM	1979	Originated from same patient as 050303
1	ITM_960658	Angola	Bengo	Dande	Caxito	ITM	1996	Originated from same patient as 960657
1	ITM_960657	Angola	Bengo	Dande	Caxito	ITM	1996	Originated from same patient as 960658
1	ITM_072662	Ghana	Ashanti	Asante Akim North	Ananekrom	KCCR	2007	
1	ITM_072646	Ghana	Ashanti	Atwima Mponua	Abofrom	KCCR	2007	
1	ITM_072651	Ghana	Ashanti	KMA	Kaase	KCCR	2007	
1	ITM_120140	Cameroon	Adamawa Region	Maya-Banyo	Bankim/Mbondji II	CPC	2011	
1	ITM_030950	Benin	Kouffo	Lalo	Adoukandji	CDTUB Lalo	2003	
1	ITM_030716	Benin	Kouffo	Lalo	Tchito/Village Aboeti	CDTUB Lalo	2003	
1	ITM_102686	Nigeria	Oyo State	Ibadan	Ibadan	ITM	2010	
1	ITM_083232	Angola	Lunda Norte	Xa-Muteba	Kwango River	ITM	2008	
1	ITM_000869	Ivory Coast	Moyen-Cavally	Duékoué	Guezon	ITM	2000	
1	ITM_990007	Ivory Coast	Haut-Sassandra	Issia	Guetuzon II	ITM	1998	
1	ITM_991633	Ivory Coast	Moyen-Cavally	Duékoué	Guezon	ITM	1999	
2	ITM_030791	Benin	Kouffo	Lalo	Tchito/Gare	CDTUB Zagnanado	2003	
2	ITM_970680	Benin	Mono	Houéyogbé	Sahoué	CDTUB Lalo	1997	
2	ITM_022876	Benin	Kouffo	Lalo	Tohou	CDTUB Lalo	2002	
2	ITM_021434	Benin	Kouffo	Klouékanmè	Adjassagon	CDTUB Lalo	2002	
2	ITM_012596	Benin	Mono	Bopa	Lobogo	CDTUB Lalo	2001	
2	ITM_071938	Benin	Kouffo	Lalo	Tandji	CDTUB Lalo	2007	
4	ITM_5150	DRC	Bandundu	Kwilu		ITM	1962	
5	ITM_940512	Benin	Zou	Ouinhi	Ouokon	CDTUB Zagnanado	1994	
5	ITM_010157	Benin	Zou	Zogbodomè	Domè-Houandougon	CDTUB Zagnanado	2001	
5	ITM_000951	Benin	Zou	Zogbodomè	Domè-Houandougon	CDTUB Zagnanado	2000	
5	ITM_970435	Benin	Ouémé	Bonou	Bonou	CDTUB Zagnanado	1997	Originated from same patient as 970301

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TABLE 1 (Continued)

ISE-SNP type	Culture no.	Country of origin	Administration division			Source	YOI	Remark
			First level	Second level	Third level			
5	ITM_970301	Benin	Ouémé	Bonou	Bonou	CDTUB Zagnanado	1997	Originated from same patient as 970435
5	ITM_000479	Benin	Zou	Zagnanado	Zagnanado/Doga	CDTUB Zagnanado	2000	
5	ITM_092100	Benin	Zou	Zagnanado	Doga-Domè	CDTUB Zagnanado	2009	
5	ITM_083865	Benin	Zou	Ouinhi	Tohoue/Hounnoumè	CDTUB Zagnanado	2008	
5	ITM_093013	Benin	Zou	Ouinhi	Ouinhi/Monzoungoudo	CDTUB Zagnanado	2009	
5	ITM_093695	Benin	Zou	Ouinhi	Ouinhi/Monzoungoudo	CDTUB Zagnanado	2009	
5	ITM_101300	Benin	Zou	Ouinhi	Sagon/Adamè	CDTUB Zagnanado	2010	
5	ITM_101302	Benin	Zou	Ouinhi	Dasso/Bossa	CDTUB Zagnanado	2010	
5	ITM_102554	Benin	Zou	Ouinhi	Dasso/Agonkon	CDTUB Zagnanado	2010	
5	ITM_081919	Benin	Zou	Ouinhi	Dasso/Yaago and Akantomè	CDTUB Zagnanado	2008	
5	ITM_092997	Benin	Zou	Djidja	Oungbègame	CDTUB Zagnanado	2009	
5	ITM_080066	Benin	Zou	Ouinhi	Sagon/Ayizè	CDTUB Zagnanado	2008	
5	ITM_070381	Benin	Zou	Ouinhi	Dasso/Yaago	CDTUB Zagnanado	2007	
5	ITM_073151	Benin	Zou	Ouinhi	Ouinhi/Monzoungoudo	CDTUB Zagnanado	2007	
5	ITM_070131	Benin	Zou	Zagnanado	Dovi-Dove/Tévedji	CDTUB Zagnanado	2007	
5	ITM_092473	Benin	Zou	Ouinhi	Tohoue/Midjannangon	CDTUB Zagnanado	2009	
5	ITM_082549	Benin	Zou	Ouinhi	Tohoue/Akassa	CDTUB Zagnanado	2008	
5	ITM_090149	Benin	Zou	Zagnanado	Dovi-Dove/Tévedji	CDTUB Zagnanado	2009	
5	ITM_091800	Benin	Zou	Ouinhi	Tohoue/Gangban	CDTUB Zagnanado	2009	
5	ITM_083584	Benin	Zou	Ouinhi	Ouinhi/Ahicon	CDTUB Zagnanado	2008	
5	ITM_9146	Benin	Zou	Zagnanado	Kpedekpo/Loko-Alankpe	CDTUB Zagnanado	1992	
5	ITM_991721	Benin	Atlantique	Toffo	Séhoué	CDTUB Zagnanado	1999	
5	ITM_092472	Benin	Atlantique	Toffo	Séhoué/Agaga	CDTUB Zagnanado	2009	
5	ITM_070383	Benin	Ouémé	Dangbo	Dékin	CDTUB Zagnanado	2007	
5	ITM_070625	Nigeria	Ogun State	Yewa North	Odja Odan	CDTUB Zagnanado	2007	
5	ITM_061509	Benin	Zou	Zagnanado	Zagnanado	CDTUB Zagnanado	2006	
5	ITM_081676	Benin	Plateau	Adja-Ouere	Tatonnoukon	CDTUB Zagnanado	2008	
5	ITM_081681	Benin	Plateau	Issaba	Onigbolo	CDTUB Zagnanado	2008	
5	ITM_082696	Benin	Ouémé	Adjohoun	Abato	CDTUB Zagnanado	2008	
5	ITM_091801	Benin	Zou	Zogbodomè	Kpokissa/Hinzounmè	CDTUB Zagnanado	2009	
5	ITM_092101	Benin	Ouémé	Dangbo	Gbéko	CDTUB Zagnanado	2009	
5	ITM_093694	Benin	Ouémé	Dangbo	Gbéko	CDTUB Zagnanado	2009	
5	ITM_100126	Benin	Zou	Zogbodomè	Kpokissa	CDTUB Zagnanado	2010	
5	ITM_951009	Benin	Zou	Zagnanado		CDTUB Zagnanado	1995	
6	ITM_5151	DRC	Maniema	Kasongo		ITM	1972	
7	ITM_970359	Ghana	Ashanti	Amansie West	Manso-Afraso	ITM	1997	
7	ITM_970606	Ghana	Ashanti	Amansie West	Yaw Kasakrom	ITM	1997	
7	ITM_970677	Ghana	Ashanti	Amansie West	Manso Dominase	ITM	1997	
7	ITM_970678	Ghana	Ashanti	Asante Akim North	Afrisre	ITM	1997	
7	ITM_970959	Ghana	Ashanti	Amansie West	Manso-Afraso	ITM	1997	
7	ITM_970964	Ghana	Ashanti	Amansie West	Offinho Asaman	ITM	1997	
7	ITM_971351	Ghana	Ashanti	Atwima Mponua	Achiase	ITM	1997	
7	ITM_980063	Ghana	Ashanti	Atwima Mponua	Achiase	ITM	1998	
7	ITM_940662	Ivory Coast	Moyen-Cavally	Duékoué	Nanandi	ITM	1994	
7	ITM_990006	Ivory Coast	Haut-Sassandra	Issia	Guetuzon I	ITM	1998	
7	ITM_990734	Ivory Coast	Moyen-Cavally	Duékoué	Duékoué	ITM	1999	
7	ITM_991632	Ivory Coast	Haut-Sassandra	Issia	Bediegbeu	ITM	1999	
7	ITM_072634	Ghana	Ashanti	Asante Akim North	Adoniem	KCCR	2007	
7	ITM_072652	Ghana	Ashanti	Atwima Mponua	Achiase	KCCR	2007	
7	ITM_072654	Ghana	Ashanti	Atwima Mponua	Achiase	KCCR	2007	
7	ITM_072657	Ghana	Ashanti	Atwima Mponua	Achiase	KCCR	2007	
7	ITM_072658	Ghana	Western Region	Wassa West	Owusukrom	KCCR	2007	
7	ITM_072650	Ghana	Ashanti	Atwima Nwabiagya	Kyereyase	KCCR	2007	
7	ITM_072630	Ghana	Central	Upper Denkyira	Nkotumso	KCCR	2007	
7	ITM_072656	Ghana	Ashanti	Atwima Mponua	Abompe	KCCR	2007	
7	ITM_072655	Ghana	Ashanti	Atwima Mponua	Sireso	KCCR	2007	
7	ITM_072653	Ghana	Ashanti	Atwima Mponua	Amadaa	KCCR	2007	
7	ITM_072645	Ghana	Ashanti	Atwima Mponua	Achiase	KCCR	2007	
12	ITM_072814	Benin	Ouémé	Dangbo	Gbéko	CDTUB Zagnanado	2007	
13	ITM_021433	Benin	Kouffo	Lalo	Gnizoumè/Hangbanou	CDTUB Lalo	2002	
13	ITM_022045	Benin	Kouffo	Lalo	Adoukandji/Yamontouhoué	CDTUB Lalo	2002	
13	ITM_022287	Benin	Zou	Agbangnizoun	Kpota	CDTUB Lalo	2002	
13	ITM_022875	Benin	Kouffo	Lalo	Gnizoumè	CDTUB Lalo	2002	
13	ITM_030717	Benin	Kouffo	Lalo	Ahomadégbé	CDTUB Lalo	2003	
13	ITM_030718	Benin	Kouffo	Lalo	Lalo	CDTUB Lalo	2003	

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TABLE 1 (Continued)

ISE-SNP type	Culture no.	Country of origin	Administration division			Source	YOI	Remark
			First level	Second level	Third level			
13	ITM_031892	Benin	Kouffo	Lalo	Hlassamè	CDTUB Lalo	2003	
13	ITM_071804	Benin	Kouffo	Lalo	Zalli	CDTUB Lalo	2007	
14	ITM_991590	Togo	Maritime	Vo	Tchekpo Deve	ITM	1999	Originated from same patient as 000909
14	ITM_000909	Togo	Maritime	Vo	Tchekpo Deve	ITM	2000	Originated from same patient as 991590
14	ITM_993354	Togo	Maritime	Vo	Tchekpo Deve	ITM	1999	
14	ITM_042407	Togo	Maritime	Vo	Kodji Kopé	ITM	2004	
15	ITM_070404	DRC	Bas-Congo	Cataractes/Songololo	Kimpese/Cité-Kimpese	IME	2007	Originated from same patient as 070123
15	ITM_070123	DRC	Bas-Congo	Cataractes/Songololo	Kimpese/Cité-Kimpese	IME	2007	Originated from same patient as 070404
15	ITM_092479	DRC	Bas-Congo	Cataractes/Songololo	Kimpese/Cité-Kimpese	IME	2009	
16	ITM_990008	Ivory Coast	Haut-Sassandra	Issia	Zakogbeu	ITM	1998	
18	ITM_070386	Nigeria	Anambra State	Ayamelum	Ifite Ogwari	ITM	2007	
19	ITM_001211	Ivory Coast	Dix-Huit Montagnes	Zouan-Hounien	Zouan-Hounien	ITM	2000	
20	ITM_020279	Cameroon	Centre Region	Nyong-et-Mfoumou	Ayos	CPC	2002	
20	ITM_091067	Gabon	Moyen-Ogooué	Ogooue et des Lacs	Junkville	ITM	2009	
20	ITM_110450	Gabon	Moyen-Ogooué	Ogooue et des Lacs	Gravier	ITM	2011	
22	ITM_020280	Cameroon	Centre Region	Nyong-et-Mfoumou	Akolo	CPC	2002	
22	ITM_120542	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
23	ITM_021081	Cameroon	Centre Region	Nyong-et-Mfoumou	Obis	CPC	2002	
23	ITM_9102	Cameroon	Centre Region			ITM	1970	
23	ITM_9103	Cameroon	Centre Region			ITM	1970	
23	ITM_101500	Gabon	Moyen-Ogooué	Ogooue et des Lacs	Lambaréné/Adaghe	ITM	2010	
23	ITM_110893	Gabon	Moyen-Ogooué	Ogooue et des Lacs	Issac	ITM	2011	
23	ITM_120138	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2010	
23	ITM_120139	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga/Wouma	CPC	2011	
23	ITM_120141	Cameroon	Centre Region	Nyong-et-Mfoumou	Medjap	CPC	2011	
23	ITM_120142	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga/Ekolman	CPC	2011	
23	ITM_120534	Cameroon	Centre Region	Nyong-Et-Soo	Bembé	CPC	2011	
23	ITM_120535	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
23	ITM_120536	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga/Djo'o	CPC	2011	
23	ITM_120538	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
23	ITM_120539	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
23	ITM_120543	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
23	ITM_120143	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	
24	ITM_051459	Uganda	Northern Region	Adjumani	Adjumani	NCTC	2005	
25	ITM_120537	Cameroon	Centre Region	Nyong-Et-Soo	Edjom	CPC	2011	
26	ITM_120540	Cameroon	Centre Region	Nyong-et-Mfoumou	Akam-Engali	CPC	2011	
27	ITM_120541	Cameroon	Centre Region	Nyong-et-Mfoumou	Akonolinga	CPC	2011	

[a] Abbreviations: CDTUB, Centre de Dépistage et de Traitement de l'Ulcère de Buruli; CPC, Centre Pasteur du Cameroun; DRC, Democratic Republic of Congo; IME, Institut Médical Evangélique; KCCR, Kumasi Centre for Collaborative Research in Tropical Medicine; NCTC, National Collection of Type Cultures; PNLUB, Programme National de Lutte contre l'Ulcère de Buruli; YOI, year of isolation.

the NJ and MP tree and 200 for the ML tree. Phylogenetic trees for ML analysis were inferred with the nucleotide substitution model selected within jModelTest v0.1.1 (37). Phylogenetic relationships were inferred with ISE-SNP 28 (strain ITM_030524) from Papua New Guinea as the outgroup, isolated from a patient who had never traveled outside of the region around Yarapos in the East Sepik Province (J. Taylor, personal communication). Trees were drawn using FigTree software (38).

A haplotype network was derived using the median joining algorithm after processing the data with the reduced median method as implemented within Network v4.6.1.0 with the default settings (39).

The open source geographic information system Quantum GIS (QGIS) (40) was used to generate the illustration of the geographical distribution of African *M. ulcerans*. The geographical locations of the residences of BU patients at the time of clinical visit were rendered as points. In the case where residence information was missing, we used the location of the hospital supplying the sample. A modification of the QGIS Python plugin Shift Points was used to modify this point shape file, in which point features with the same position overlapped. Point displacement rendered such features in a circle around the original "real" position. The river layer was translated from the River-Surface Water Body Network data set of the African Water Resource database of the Food and Agriculture Organization (FAO) of the United Nations (41). The administrative borders of countries were rendered from the Global Administrative Unit Layers data set of FAO (http://www.fao.org/geonetwork/srv/en/metadata.show?id=12691).

All statistical testing was performed in R v2.15.2 (42). The correlation between the number of isolates per country and the number of ISE-SNP types per country was checked by using Spearman's rank order correlation coefficient. To examine the relation between ISE-SNP types and the greater West African hydrological drainage basins, the Fisher exact test was used.

RESULTS

Primers and conditions for PCR and sequencing were redesigned and optimized from those described by Käser et al. (28) for application directly on clinical specimens (see Fig. S1 in the supplemental material). Isolates ITM_5150, ITM_5151 ITM_940511, ITM_940512, ITM_960658, ITM_940662, ITM_970359, and ITM_970680 were included in the panel to validate the redesigned

TABLE 2 Clinical specimens used in this study[a]

ISE-SNP type	Sample no.	Country of origin	Administration division			Source	YOI
			First level	Second level	Third level		
1	BK121032	Nigeria	Cross River State	Ogoja	TBL Hospital Monaiya	ITM	2012
1	BK120888	DRC	Maniema	Kibombo	Likeri	PNLUB	2012
1	BK120890	DRC	Maniema	Kasongo	Samba/Malela	PNLUB	2012
1	BK120891	DRC	Maniema	Kasongo	Kankumba	PNLUB	2012
1	BK065361	Nigeria	Enugu State	Igbo Eze North	Nkpo Hamida	ITM	2006
5	BK121025	Nigeria	Ogun State	Abeokuta North	Abeokuta/Ijaye State Hospital	ITM	2012
5	BK121026	Nigeria	Ogun State	Abeokuta North	Abeokuta/Ijaye State Hospital	ITM	2012
5	BK121031	Nigeria	Ogun State	Yewa South	Oke-Odan/PHC Oke-Odan	ITM	2012
17	BK065369	Nigeria	Ebonyi State	Ohaozora	Iburu	ITM	2006
20	BK105250	Gabon	Nyanga	Douigni	Moussamou Kougou	ITM	2010
21	BK101660	Gabon	Moyen-Ogooué	Ogooue et des Lacs Department	Lambaréné/Point V	ITM	2010
23	BK100901	Gabon	Moyen-Ogooué	Ogooue et des Lacs Department	Lambaréné/Bellevue	ITM	2010
23	BK100900	Gabon	Moyen-Ogooué	Ogooue et des Lacs Department	Lambaréné/Isaac	ITM	2010

[a] Abbreviations: DRC, Democratic Republic of Congo; PNLUB, Programme National de Lutte contre l'Ulcère de Buruli; YOI, year of isolation.

assays. These isolates were also included in the panel of Käser et al. (28) and gave identical genotypes as with the redesigned assays.

As our collection of African *M. ulcerans* isolates did not represent all countries and their different regions where BU is endemic to the same extent, owing to the low sensitivity of culture, we fine-tuned the technique for application directly on clinical specimens by adjusting individual PCR component concentrations and optimizing the thermal PCR profile. We were thus able to deduce sequence information of clinical samples with a modest bacterial load corresponding to a C_q (IS*2404*) of ≤32. Failure of PCR amplification for specimens with C_q (IS*2404*) values of >32 was caused by a low mycobacterial DNA concentration.

Amplification and sequencing of IS*2404* in MUL_2990 (RD1) and MUL_3871 (RD12) was successful for the entire collection of 157 (100%) clinical isolates (Tables 1 and 2). The optimized method also proved successful for all 14 clinical specimens analyzed, with a C_q (IS*2404*) of ≤32. A total of 75 (31 in MUL_2990 [RD1] and 44 in MUL_3871 [RD12]) variable nucleotide positions were identified, including four insertions/deletions (indels) (Fig. 1). This resulted in 28 ISE-SNP types, of which 23 were found on the African continent. Sixteen of these were newly identified types, while the other seven corresponded to the ISE-SNP types described by Käser et al. (28). The Papua New Guinean ISE-SNP type 28, used in the phylogenetic analyses as an outgroup, was also a novel type.

The Spearman's rank correlation coefficient showed a significant relationship between the number of isolates per country and number of ISE-SNP types identified per country ($r[9] = 0.79$; $P < 0.01$). We were able to identify all African ISE-SNP types described by Käser et al. (28) except for ISE-SNP type 3, which was found in the Greater Accra Region of Ghana, a region not covered by our panel. Some ISE-SNP types were common (types 1, 2, 5, 7, and 23), while others were represented by only one isolate/clinical specimen (types 4, 6, 12, 16, 17, 18, 19, 21, 24, 25, 26, and 27). Our panel included a number of linked isolates originating from the same patient. In all eight occurrences (Table 1), these linked isolates revealed the same ISE-SNP type. The geographical distribution of all African ISE-SNP types is shown in Fig. 2A. Most ISE-SNP types had a distinct restricted geographical localization. For example, all 41 isolates of ISE-SNP type 5 were recovered from an area in West Africa with a 60-km radius. Other African ISE-SNP types were more widely dispersed. ISE-SNP type 1, for instance,

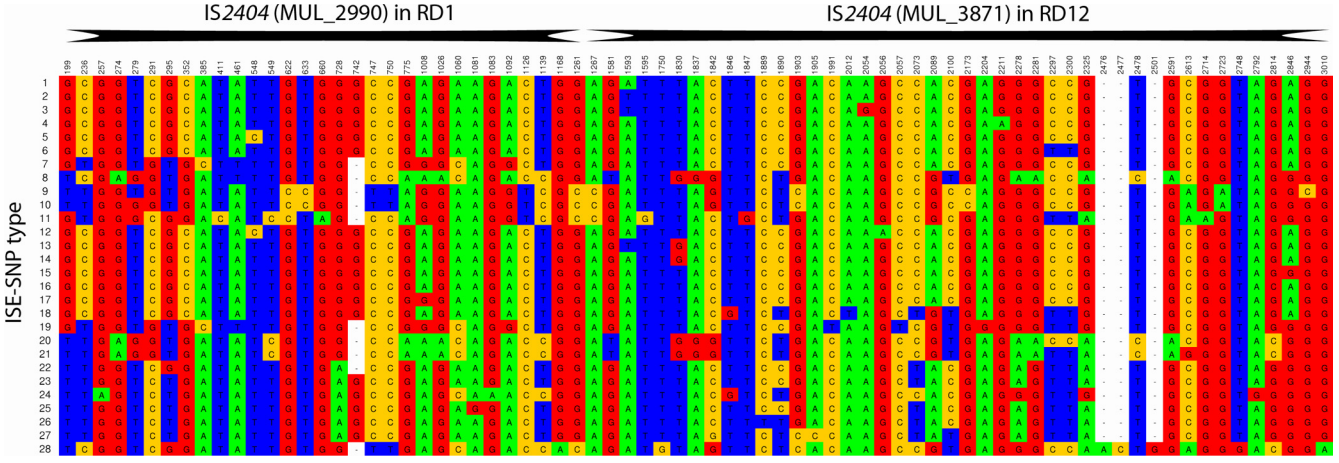

FIG 1 Sequence variation in two haplotype-specific concatenated IS*2404* elements: MUL_2990 (RD1) and MUL_3871 (RD12). Only variable nucleotides in the aligned sequences are shown for all 28 ISE-SNP types. SNP position numbers are given according to the scheme described by Käser et al. (28), with position 1 corresponding to position 3313231 in RD1 and 1498 to position 4326896 in RD12, according to the Agy99 bacterial reference chromosome.

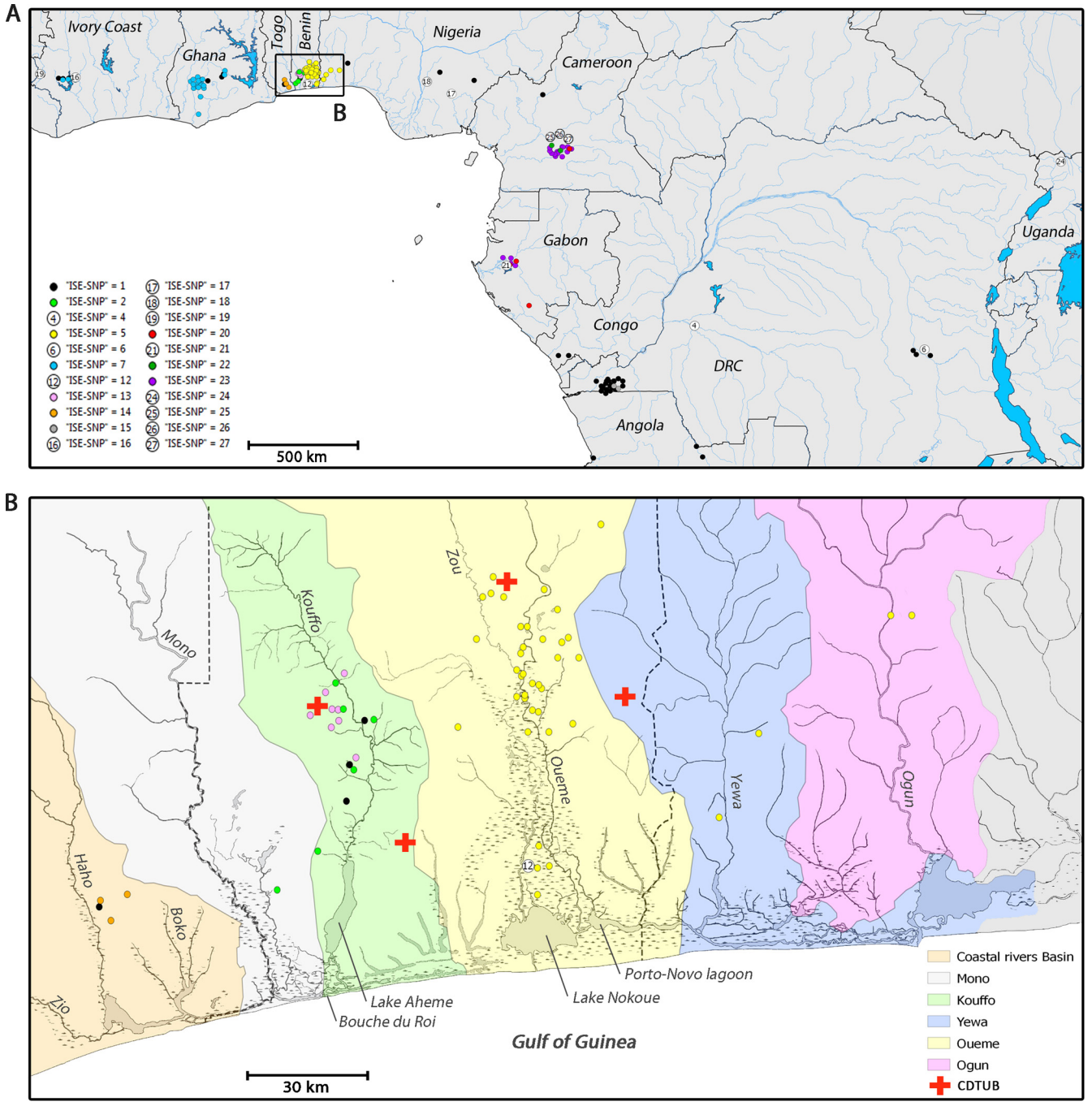

FIG 2 (A) The geographical distribution of African *M. ulcerans*. The location of residence of the individual BU patients at the time of clinical visit was retrospectively correlated with the ISE-SNP typing results. ISE-SNP types represented by only one clinical isolate or specimen are depicted as numbers, while more common ISE-SNP types are color coded. (B) The uneven distribution of ISE-SNP types over the different greater hydrological drainage basins of West Africa.

although also emerging in clusters in Central Africa, was identified throughout Central and West Africa. Furthermore, some regions harbored a multitude of different ISE-SNP types, while other regions yielded just one. In southern Benin, for example, the greatest variety of allelic patterns was found to have as many as five ISE-SNP types (*viz.* types 1, 2, 5, 12, and 13) circulating.

We found a strong relationship (Fisher's exact test, $P < 0.0001$) between the distribution of ISE-SNP types and the greater West African hydrological drainage basins (Table 3; Fig. 2B). Hydrologically, this region can be divided into separate main drainage areas: the Mono, the Kouffo, the Oueme, the Yewa, the Ogun, and the Togolese Coastal Rivers Basin. The main rivers of these border-crossing basins all arise on the central west-African plateau and form broad fertile richly inundated plains when they reach the lowlands of the coastal regions, where areas where BU is endemic are concentrated. Here, the basins can be divided into an inland

TABLE 3 Distribution of ISE-SNP types over the hydrological drainage basins of southern Benin, southern Togo, and southwestern Nigeria

ISE-SNP type	No. of times the type was found in basin					
	Coastal Rivers Basin	Mono	Kouffo	Oueme	Yewa	Ogun
1	1	0	3	0	0	0
2	0	1	5	0	0	0
5	0	0	0	37	2	2
12	0	0	0	1	0	0
13	0	0	8	0	0	0
14	3	0	0	0	0	0

region drained by a network of freshwater rivers and streams that discharges into a region of extensive brackish-water swamps interconnected with lakes, narrow lagoons, and streams parallel to the coastline. Haplotype ISE-SNP 5 dominates in the areas of BU endemicity of the Oueme, the Yewa, and the Ogun basins. The haplotype is best represented along the Oueme and its last tributary, the Zou River, where most Beninese BU cases are reported (43). After the confluence, the Oueme traverses over 1,500 km^2 of floodplains, after which the river discharges into Lake Nokoue, Porto-Novo Lagoon, and the coastal lagoons of Nigeria which are all interconnected by the numerous channels of the deltaic fan of the Oueme River. Two other drainage units, the Ogun and the Yewa, discharge in this same system of lagoons and streams. So, although the basins are separate drainage systems, they discharge into this collective interconnected system, which could potentially explain the observed shared distribution of haplotype ISE-SNP 5. Haplotypes ISE-SNP 2 and 13 dominate in the areas of BU endemicity of the Kouffo Basin (44, 45). After draining the regions of high BU endemicity of the commune of Lalo, the Kouffo discharges via Lake Aheme in the "western lagoonal complex," which is in contact with the Gulf of Guinea at Bouche du Roi. Although this system is part of a semicontinuous line of narrow lagoons that runs behind the dunes along the entire coastal strip until the Ghanaian border, it is not in contact with the interconnecting drainage system of the Oueme Delta. The lower course of the Mono River forms the border between Togo and Benin and discharges in the same western lagoonal system as the Kouffo River. The Mono River basin, however, has no known areas of BU endemicity, despite similar riverine habitats. Even further west, in southern Togo, three small coastal rivers (the Boko, Haho, and Zio) form a third small basin. The basin encompasses a couple of regions of BU endemicity in which the Togolese haplotype ISE-SNP 14 is represented.

The NJ method yielded two well-supported sister clades within the African ISE-SNP types (Fig. 3A). The first clade comprised ISE-SNP types 20 and 21, which circulate in different regions of BU endemicity of Cameroon and Gabon; this clade also had high bootstrap support for the MP and ML analyses. A second pan-African clade comprised all other African ISE-SNP types (Fig. 3B). Support for other nodes within the pan-African clade was very low (bootstrap values of <70%), except in the NJ analysis for (i) a clade of ISE-SNP types 7 and 19 which circulate in Ghana and Ivory Coast, (ii) a clade of ISE-SNP types 22, 23, 25, 26, and 27, which all circulate in Cameroon and neighboring Gabon, and (iii) modest support in the NJ analysis for a clade of ISE-SNP types 1, 2, 3, 4, 5, 12, 13, 14, 15, 16, and 17, found throughout the continent. *Mycobacterium ulcerans* haplotypes from Australia and Southeast Asia were also included in the analysis, as these, together with African haplotypes, belong to the more virulent and distinct "classic" phylogenetic lineage (29, 46), relative to *M. ulcerans* isolates elsewhere. It is of particular interest that ISE-SNP type 8 from Papua New Guinea forms a strongly supported monophyletic group with ISE-SNP types 20 and 21 from Cameroon and Gabon and is distinctly unrelated to other Southeast Asian clinical isolates, which belong to ISE-SNP types 9, 10, and 28 (Fig. 3A). In contrast, other ISE-SNP types found in Papua New Guinea are related to Malaysian and Australian clinical isolates.

The phylogenetic network (Fig. 4) showed that a number of African ISE-SNP types are closely related and only differ in a single, or a few, mutational steps. However, other African ISE-SNP types are more distantly related and even differ in their number of mutational steps, which is similar to the number of mutational steps between African and non-African types. Analogous with the phylogenetic tree analysis, the pan-African clade is divided into two major clusters. The first major cluster comprises the common Central and West African ISE-SNP type 1 and several, closely related yet rarer ISE-SNP types. The second cluster comprises ISE-SNP types 22, 23, 25, 26, and 27, which are circulating in Cameroon and neighboring Gabon. The network also showed several more distantly related African haplotypes, of which most are relatively rare (types 18, 19, 20, 21, and 24) and one is common (type 7).

DISCUSSION

In this study, we applied an optimized ISE-SNP genotyping technique to a comprehensive panel of isolates from all African countries that ever yielded culture-confirmed BU cases. This analysis, unparalleled in size and scope, allowed us to assess the diversity and population structure across regions of BU endemicity on a continental scale and to explore the phylogenetic and phylogeographic relationships within the genetically conserved cluster of African *M. ulcerans* ISE-SNP types.

Analysis of polymorphisms in the RD1 and RD12 genomic regions, which have been determined to be among the most variable of the *M. ulcerans* bacterial chromosome (46), over our comprehensive sample panel spanning 11 African countries where *M. ulcerans* BU is endemic, identified 23 different African ISE-SNP types. The observed low level of polymorphisms (47), together with the characteristic geographical restriction of most ISE-SNP types, suggest a highly clonal population structure of African *M. ulcerans*. This is in agreement with the findings of Doig et al. (18), who found that clinical isolates from Ghana and Benin were only separated by an average pairwise distance of 160 SNPs over the entire 5.6-Mbp sequenced bacterial chromosome. This low sequence diversity is in strong contrast to that of other pathogens, like *Helicobacter pylori*, for which microevolution can be observed even within serial bacterial isolates from individual humans with prolonged infection (48). The genetic conservation among African *M. ulcerans* might reflect a short evolutionary history since its intracontinental dispersal, but it might also be explained by a low mutation rate. Reliable estimates of mutation rates are required to resolve these issues (47).

Closely related ISE-SNP types dominate in different areas of BU endemicity. The identified SNPs describe a phylogenetic path wherein these individual ISE-SNP types document the sequential accumulation of mutations from a common root. If we assume

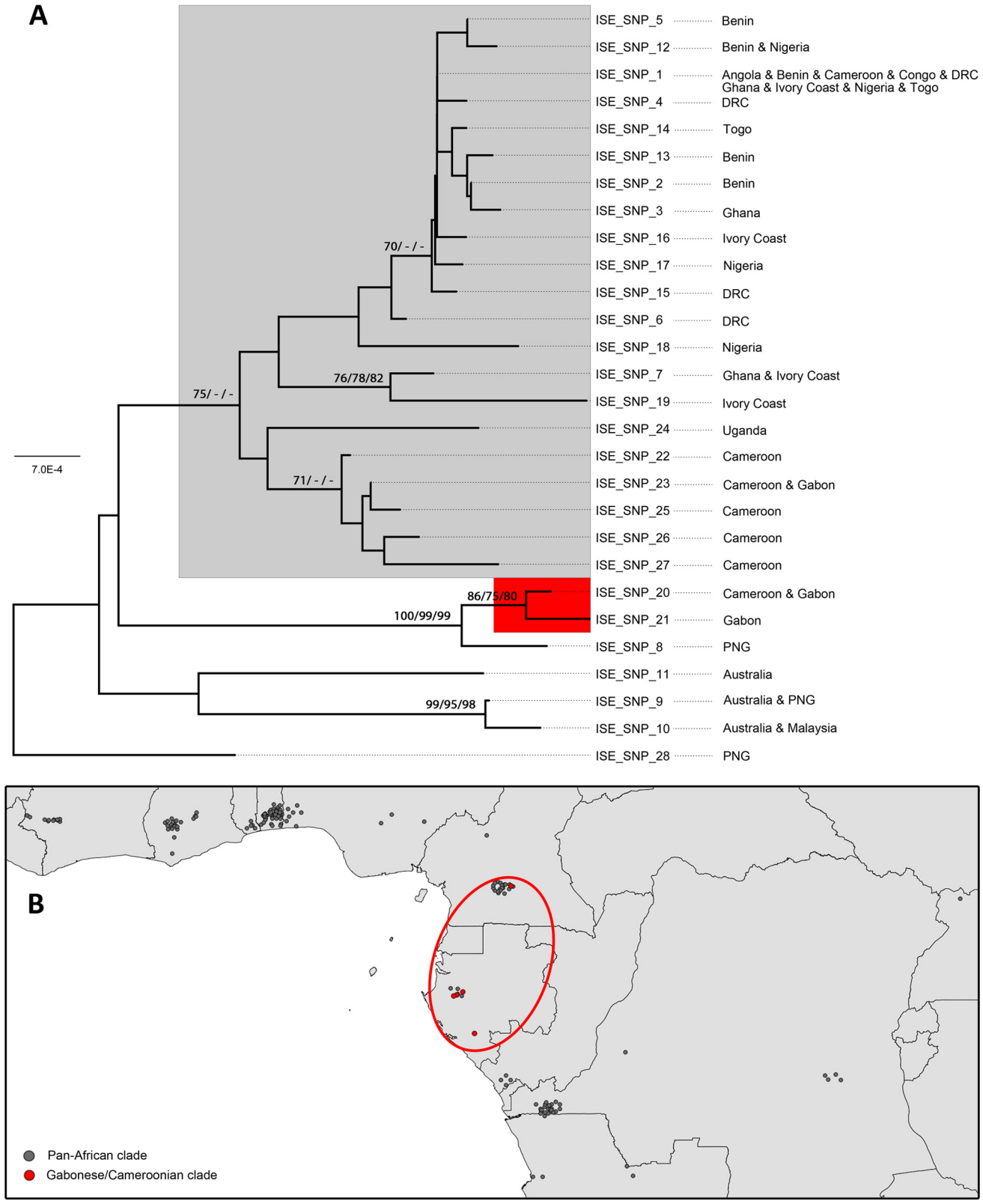

FIG 3 (A) Neighbor-joining tree showing the phylogenetic relationships between the 28 currently known ISE-SNP types of *M. ulcerans*, with haplotype ISE-SNP 28 from Papua New Guinea as an outgroup. Bootstrap values (if >70%) for the neighbor-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) analyses are indicated at the nodes as NJ/ML/MP. ISE-SNP types belonging to the pan-African clade and the Gabonese/Cameroonian clade are highlighted in gray and red, respectively. (B) Geographical distribution of the pan-African clade and the Gabonese/Cameroonian clade. The location of residence of the individual BU patients at the time of clinical visit was retrospectively correlated with the ISE-SNP typing results.

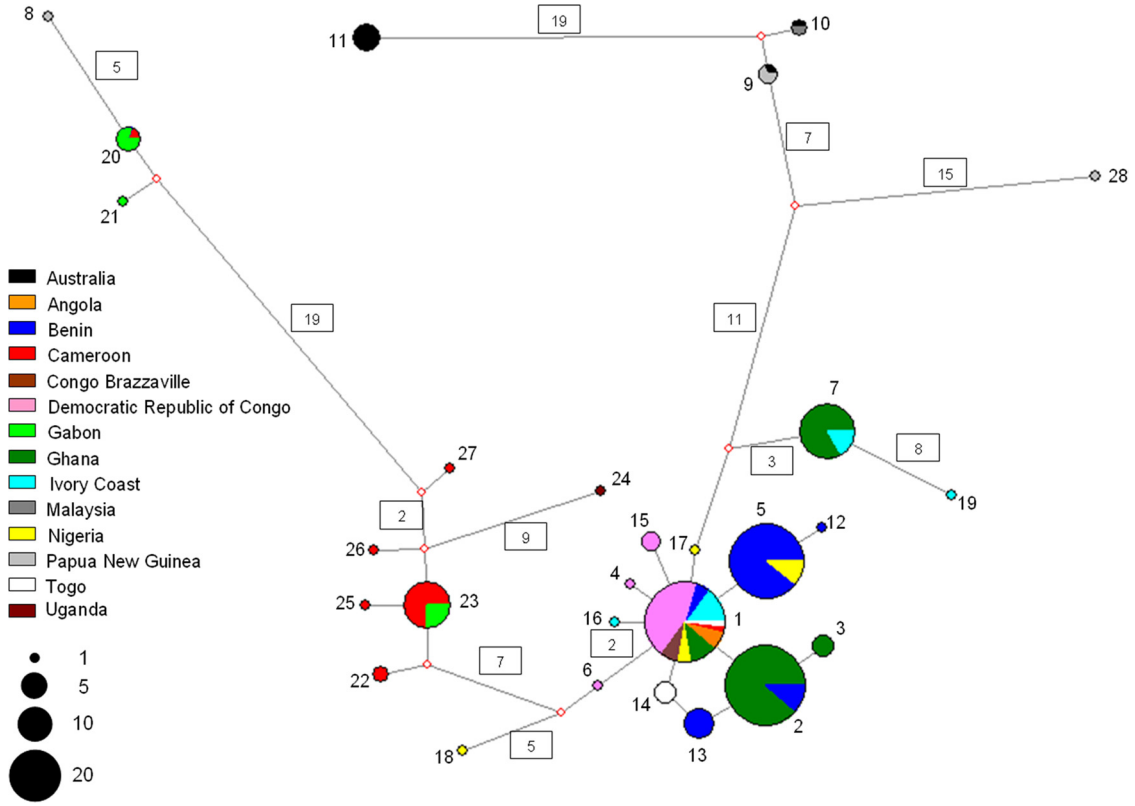

FIG 4 Phylogenetic network showing patterns of descent among the 28 currently know ISE-SNP types of *M. ulcerans* in relation to their geographic origin. The network was derived by using the median joining algorithm after processing the data with the reduced median method as implemented within Network v.4.6.1.0. Each circle represents a unique ISE-SNP type, and the size of the circle is proportional to the number of individuals sharing that type. Numbers in boxes represent the number of mutational steps (if not given, then there was a single mutational step). Positions at which mutations occurred are given in Fig. 1. Color codes represent the country of origin, as shown in the key.

that (i) an ancestral ISE-SNP type will be more geographically dispersed than a more recently derived type and (ii) that the geographical distribution of the ISE-SNP types is not explained by selective effects, then this common root node is represented here by ISE-SNP 1, the most common type distributed over the entire continent. The different ISE-SNP types thereby represent the initial stages of clonal diversification through *de novo* mutations from this possibly ancestral type, after its intracontinental spread.

The unevenly distributed ISE-SNP types circulating within small regions of West Africa are furthermore suggestive of the existence of independent transmission clusters. We found a strong association between the distribution of ISE-SNP types and the greater West African hydrological drainage basins. Genetic differences between clinical isolates originating from two neighboring drainage areas in Benin have previously been reported (10, 18). It appears that geographic barriers (e.g., elevated regions and salt water) bordering these hydrological basins separated an ancestral genotype to a certain extent into discontinuous parts by the formation of a physical barrier to bacterial gene flow. Our data suggest that this resulted in differentiation by the slow accumulation of point mutational changes of the original founder clone (ISE-SNP 1) into different closely related types distributed over the various basins (Fig. 2B and Table 3). New ISE-SNP types derived from the founder type did not easily spread but formed focal transmission clusters associated with the hydrological drainage areas. Hence, BU infections in these areas probably resulted from locally confined transmission of a single circulating clone, with only occasional transfer of clones between basins. Our findings confirm a study of Röltgen et al. (49), in which a number of *M. ulcerans* haplotypes within the Densu hydrological basin of Ghana (with SNP typing based on whole-genome data) were differentiated, revealing similar focal transmission clusters within the basin itself. Hence, our findings provide additional evidence that both transmission and fine-grained evolutionary events play roles at the local level and we consequently hypothesize that potential reservoirs have limited mobility. Such a scenario would correspondingly account for the presence of villages where BU is endemic and those where it is not endemic that are in close proximity to each other (<10 km) within the same drainage basin (45).

Our phylogenetic analyses did not result in a fully resolved phylogenetic tree, since most nodes had low bootstrap support. Nevertheless, there was support for a "pan-African clade" and a "Gabonese/Cameroonian" sister clade. The ISE-SNP types from the pan-African clade are widespread throughout Africa, while the ISE-SNP types of the Gabonese/Cameroonian clade are much rarer and are found in a more restricted area (Fig. 3), which suggests that the latter clade evolved more recently. Alternatively, this may also be the result of a sampling artifact; indeed, the Spearman's rank correlation indicated that the higher the sampling effort per country, the more ISE-SNP types found. However, the entirety of the Gabonese/Cameroonian region in itself was well sampled, with five isolates/clinical samples belonging to the Gab-

onese/Cameroonian clade and 25 isolates/clinical samples to the pan-African clade (Tables 1 and 2; Fig. 3). Furthermore, the fact that we did not encounter ISE-SNP types of the Gabonese/Cameroonian clade in neighboring countries like the Democratic Republic of the Congo (DRC), where sampling was higher, also suggests that the ISE-SNP types belonging to the Gabonese/Cameroonian clade are not only rare but also have a limited distribution. Interestingly, the only ISE-SNP 1 isolate from Cameroon (ITM_120140) came from a patient from Bankim, a district located along the Mapé River (Sanaga Basin), while other studied isolates all came from around the Nyong River Basin. Bankim has been recently identified as an additional area of BU endemicity in Cameroon. However, whether BU was emerging in Bankim or constitutes a newly recognized preexisting disease focus remains unclear (50, 51).

The Gabonese/Cameroonian clade was found to form a strongly supported monophyletic group with Papua New Guinean ISE-SNP type 8, which is distinctly unrelated to other ISE-SNP types found in Southeast Asia. With use of a different genotyping technique, the relatedness of a Papua New Guinean clinical isolate (not included in this study) to African rather than to Southeast Asian clinical isolates has been reported elsewhere (52). The process (historical events, restricted bacterial gene flow, etc.) that led to this intercontinental association of ISE-SNP haplotypes remains elusive.

In this report, we have analyzed a large collection of isolates representative of the African *M. ulcerans* population in order to characterize its population structure accurately and appropriately. The panel used in this study is, to our knowledge, the most comprehensive one studied so far. It covered disease foci from all 11 well-documented countries of BU endemicity, ranging from West, to Central, to East Africa. Six countries (Burkina Faso, Equatorial Guinea, Guinea, Kenya, Liberia, and South Sudan) that have reported a limited number of BU cases in the past (53) were not included in the study as we were unable to include specimens, or isolates, from them. Moreover, cases from the Central African Republic, Senegal, and Sierra Leone were never confirmed by laboratory tests (53). Although we tried to maximize spatial diversity within our panel, some countries are better represented than others, again due to the limited availability of clinical isolates. We might have missed some ISE-SNP types in these countries, because there was a significant relationship between the sampling effort per country and the amount of different ISE-SNP types identified per country. Because of all these limitations, we successfully optimized the genotyping PCR technique for application directly on clinical specimens, which allowed us to include clinical specimens from certain geographical regions of Gabon and Nigeria in which no *M. ulcerans* isolates were available (Table 2).

Nonetheless, the quality of these kinds of bacterial population studies largely depends on the quality of the patient information connected with the clinical isolates and specimens. Isolates ITM_070123 and ITM_070404 originated from the same patient, an Angolan refugee fleeing the civil war in his country (54), who was diagnosed before he was sheltered in humanitarian camps across the border in the DRC. We believe that ISE-SNP type 15, the haplotype to which his isolates belong, is North Angolan rather than Congolese, as another isolate, ITM_092479, also originated from a patient who likely is of Angolan origin. During the civil war, however, identity fraud was common in the camps in the DRC, causing misclassification of patient origins.

To our knowledge, ISE-SNP typing currently yields the greatest resolution within *M. ulcerans*, save for whole-genome sequencing. The method may be an easy, low-cost, powerful, reliable, and reproducible tool for reference laboratories to assist in the tracking of *M. ulcerans* ISE-SNP types for epidemiological studies on a continental scale (55).

Because African *M. ulcerans* shows such low genetic variation, further studies require a whole-genome approach to comprehensively evaluate the genetic diversity, the evolution, and the phylogenetic relatedness of African *M. ulcerans* and to delineate the exact origin and spread of the pathogen at the local and the continental levels. It is specifically the paucity of genetic diversity and the sequential order of the genetic changes that have occurred between individual isolates that render *M. ulcerans* such a promising model to reveal evolutionary bacterial mechanisms. Furthermore, given the comprehensive nature of full-genome data, sequences could also serve in large-scale microepidemiological studies that are focused on the elucidation of transmission pathways and relevant reservoirs of *M. ulcerans*. Indeed, different studies of mycobacterial genomics (18, 49, 56) have already shown that, at the whole-genome level, substantial genetic variation exists in African *M. ulcerans*, which can be exploited for phylogenetically robust strain classification. In order to capture as much diversity as possible and to minimize phylogenetic discovery bias (57) in such impending large sequencing endeavors, it will be desirable to select representative types from all the central and radial ISE-SNP types defined in this study.

ACKNOWLEDGMENTS

The present study pays tribute to the extensive collection of *M. ulcerans* isolates generated over decades by Françoise Portaels and her collaborators in countries where BU is endemic, who initiated and fueled research into the pathogenesis, diagnosis, and management of *M. ulcerans* disease.

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