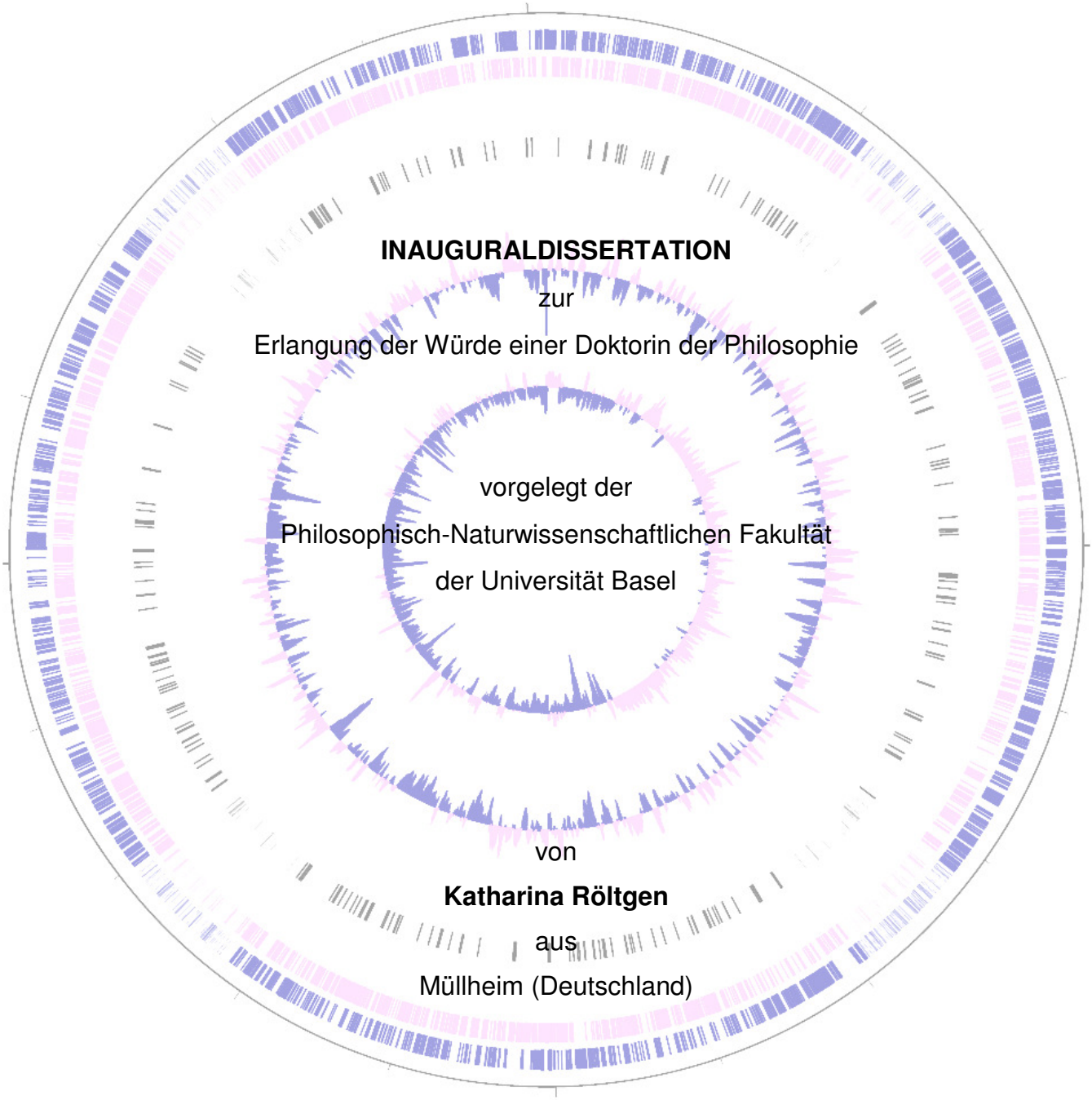
# Development and application of new approaches to study the epidemiology of *Mycobacterium ulcerans* disease (Buruli ulcer) in Ghana



**INAUGURALDISSERTATION**

zur

Erlangung der Würde einer Doktorin der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus

Müllheim (Deutschland)

Basel 2012

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von:

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Basel, den 16. Oktober 2012

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Dekan

*There are only two mistakes one can make along the road to truth; not going all the way, and not starting.*

*Buddha*

## ACKNOWLEDGEMENTS

My first and foremost gratitude goes to my PhD supervisor Professor Gerd Pluschke for guiding me throughout my PhD studies with exceptional scientific and moral support. Thank you for giving me the opportunity to work on different interesting aspects of Buruli ulcer and for worthwhile and enjoyable stays in Ghana.

The work presented here was carried out in cooperation with scientists at the Noguchi Memorial Institute in Ghana and would not have been possible without their commitment. I am especially thankful to Dr. Dorothy Yeboah-Manu for a fruitful collaboration, for her hospitality and help during my stays in Ghana as well as for agreeing to be part of my thesis committee as external expert. Thank you Kobina Ampah and Grace Kpeli for your friendship and memorable times in Ghana (and now in Basel!).

I am especially grateful to Professor Thomas Junghanss, who kindly agreed to be the co-referee of my thesis committee.

Furthermore, I would like to thank Dr. Weihong Qi and Professor Timothy Stinear for their bioinformatics help and contributions to the *M. ulcerans* genomics studies.

My special appreciation is addressed to Nicole Scherr for a great collaboration on different aspects of our work, scientific advices and for being a true friend. Many thanks go to Marie-Thérèse Ruf for her friendship and for always having an answer and solution to every question or problem. I truly acknowledge being part of a great Molecular Immunology research team and am particularly grateful to all current and former members of our group for creating such a nice and encouraging working atmosphere: Warm thanks go to Maja Jud, Sarah Kerber and Julia Hauser for so many happy and funny lab moments, huge thanks go to Angelika Silbereisen, Anna Frommherz, Jean-Pierre Dangy, Marco Tamborrini, Paola Favuzza, Martin Bratschi, Andrea Vettiger, Raphael Bieri, Miriam Bolz, and Anita Dreyer for entertaining coffee and lunch breaks as well as scientific advices.

I am very happy to have met and worked with Professor Paul Johnson during his 6-months sabbatical at our Institute in Basel.

My warmest thanks go to Jörg and my parents for all their love and support.

The work presented here was supported by the Stop Buruli Initiative funded by the UBS-Optimus Foundation. I was supported by a stipend of the Ghanaian-German Centre for Health Research funded by the DAAD-German Academic Exchange Service.

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## SUMMARY

*Mycobacterium ulcerans,* causing the devastating skin disease Buruli ulcer (BU), has evolved from the fish pathogen *M. marinum.* By the acquisition of a virulence plasmid (pMUM) a progenitor of *M. ulcerans* has gained the unique property of producing a polyketide-derived macrolide toxin termed mycolactone. The toxin is responsible for the characteristic chronic ulcerative pathology of BU. Adoption of pMUM was probably the key event in the evolution of *M. ulcerans*, constituting a population bottleneck that led to the development of a new species with a highly clonal population structure. Subsequent reductive evolution is indicative for the adaptation to more stable environmental niches and led to the emergence of three *M. ulcerans* lineages considered as different ecotypes. *M. ulcerans* lineage 3, responsible for BU infections in Africa and Australia, is characterized by extremely low levels of genetic diversity. This genetic monomorphism has long hindered molecular epidemiological studies aiming at the identification of transmission pathways and environmental sources of *M. ulcerans*.

Our whole genome sequence comparison of Ghanaian *M. ulcerans* strains representing three previously identified variable number of tandem repeat (VNTR) types in Ghana, enabled the identification of single nucleotide polymorphisms (SNPs). Real-time PCR-based SNP typing assays at the detected SNP loci facilitated medium-throughput analyses of a comprehensive collection of Ghanaian *M. ulcerans* isolates. Typing results of *M. ulcerans* strains isolated between 1999 and 2007 from patients residing in the BU endemic Densu River Basin and another BU endemic area in the Amansie West district of Ghana demonstrated the presence of two different clonal complexes in the two regions. Local clustering of certain haplotypes within the Densu River Basin revealed that *M. ulcerans* is very focally transmitted, which excludes some of the currently discussed modes of transmission. Our recently developed temperature-switch PCR-based typing strategy is used by a reference laboratory in Ghana to monitor the spatio-temporal distribution and further spread of the detected *M. ulcerans* variants in that region.

*M. ulcerans* infection foci are commonly connected with close proximity to human-disturbed aquatic environments. While BU in Ghana is highly associated with

residence along the lower part of the Densu River, where the construction of an impoundment has led to the formation of a lake and wetlands surrounding this area, no infections have so far been reported from villages located further upstream. However, results of our sero-epidemiology studies indicated that healthy inhabitants of communities both downstream (BU endemic) and upstream (BU non-endemic) of the Densu River are equally exposed to *M. ulcerans*. About one third of healthy individuals from both areas showed specific humoral responses against the *M. ulcerans* 18KDa small heat shock protein. Moreover, environmental samples collected in the BU endemic and non-endemic communities showed no significant difference in PCR positivity targeting *M. ulcerans* sequences.

Elusive transmission pathways and reservoirs of *M. ulcerans* combined with the lack of a vaccine against BU complicate the prevention of this disease. Since the current strategy to control BU relies on early case detection and rapid treatment, the identification of alternative drugs is of high importance. Therefore a compound screening platform for *M. ulcerans* was developed within the framework of this thesis and is currently used to screen compound libraries for new scaffolds which may eventually have the potential to replace rifampicin.

Taken together, the spatio-temporal patterns of *M. ulcerans* haplotypes in the Densu River Valley as well as the observed clustering of BU cases in certain communities along the Densu River indicate that the emergence of *M. ulcerans* infection foci cannot be solely explained by an interaction between *M. ulcerans* and the environment. We hypothesize that BU patients with ulcerative lesions containing vast loads of mycobacteria may play an active role in the dissemination of *M. ulcerans* in the environment. This would have major implications for strategies to better control the disease and remains to be investigated further.

## ZUSAMMENFASSUNG

*Mycobacterium ulcerans*, der Erreger der gravierenden Hauterkrankung Buruli-Ulkus (BU), hat sich aus dem Fischpathogen *M. marinum* entwickelt. Durch den Erwerb eines Virulenzplasmids (pMUM) hat ein *M. ulcerans*-Vorfahre die einzigartige Fähigkeit erlangt, ein Makrolid-Toxin namens Mycolakton zu produzieren, das für die BU-typische chronisch-ulzerative Pathologie verantwortlich ist. Die Aufnahme von pMUM war vermutlich das Schlüsselereignis für die Entstehung des Erregers und führte durch diesen genetischen „Flaschenhalseffekt“ zu der Entwicklung einer neuen Spezies mit hochklonaler Populationsstruktur. Die darauf folgende reduktive Evolution deutet auf die Anpassung an eine stabilere ökologische Nische hin und führte zu der Entwicklung von drei *M. ulcerans* Abstammungslinien, die verschiedene Ökotypen darstellen. Da die *M. ulcerans* Abstammungslinie 3, die BU Infektionen in Afrika und Australien hervorruft, nur minimale genetische Diversität aufweist, war die Erforschung der Herkunft und Übertragungswege von *M. ulcerans* durch Feintypisierung von Isolaten lange Zeit unmöglich.

Vergleiche der Genomsequenzen von Vertretern der drei in Ghana vorhandenen *M. ulcerans* VNTR (Variable Number of Tandem Repeat) Varianten ermöglichte die Identifizierung von Einzelnukleotid-Polymorphismen (single nucleotide polymorphisms, SNPs). Mit Hilfe der identifizierten SNP-Positionen wurden Real-Time PCR-basierende Assays entwickelt, mit denen *M. ulcerans* Isolate aus Ghana typisiert werden konnten. Die Typisierung von *M. ulcerans* Stämmen, die zwischen 1999 und 2007 von Patienten aus dem BU endemischen Densu Flussgebiet und einer anderen endemischen Region im Amansie West Bezirk isoliert wurden, hat gezeigt, dass in den beiden Regionen zwei unterschiedliche klonale *M. ulcerans* Komplexe vorherrschen. Da auch innerhalb des Densu Flussgebietes die Verbreitung der meisten *M. ulcerans* Varianten nur auf ein bestimmtes Teilgebiet begrenzt war, können einige der derzeit diskutierten Übertragungswege des offensichtlich sehr fokal übertragenen Erregers ausgeschlossen werden.

*M. ulcerans* Infektionsbrennpunkte sind im Allgemeinen mit aquatischen Umgebungen assoziiert, die durch den Menschen verändert worden sind. So tritt BU in Ghana vorwiegend entlang des unteren Densu Flussbereichs auf, in dem durch

den Bau eines Staudamms ein See mit umliegenden Feuchtgebieten entstanden ist, während aus Dörfern im oberen Flussbereich noch keine Krankheitsfälle gemeldet wurden. Unsere sero-epidemiologischen Studien haben gezeigt, dass dennoch sowohl Bewohner aus Gemeinden flussabwärts (BU endemisch) als auch weiter flussaufwärts (nicht BU endemisch) gleichermassen mit *M. ulcerans* in Kontakt kommen. Bei etwa einem Drittel der gesunden Studienteilnehmer aus beiden Flussgebieten konnten spezifische Antikörper gegen das so genannte „18KDa small heat shock protein“ von *M. ulcerans* nachgewiesen werden.

Da Herkunft und Übertragungswege von *M. ulcerans* unklar sind und bis heute kein Impfstoff verfügbar ist, wird BU weniger durch Prävention, als vielmehr mit der Behandlung von Patienten in möglichst frühen Krankheitsstadien bekämpft. Daher ist die Suche nach alternativen Chemotherapeutika essentiell. Wir haben im Rahmen dieser Arbeit eine Screening-Plattform für *M. ulcerans* entwickelt, die nunmehr dazu verwendet wird, ausgewählte Wirkstoffbibliotheken auf neue Substanzen zu durchsuchen, die im besten Fall das Potential haben Rifampicin zu ersetzen.

Zusammengefasst, weisen sowohl die räumlich-zeitliche Verbreitung von *M. ulcerans* Varianten im Densu Flussgebiet als auch das fokale Auftreten von BU in bestimmten Gemeinden entlang des Flusses darauf hin, dass die Entstehung von *M. ulcerans* Infektionsbrennpunkten nicht nur auf eine Wechselwirkung zwischen *M. ulcerans* und der Umwelt zurückzuführen ist. Basierend auf unseren Beobachtungen vermuten wir, dass BU Patienten mit bakteriell hoch belasteten ulzerierenden Läsionen eine wichtige Rolle bei der Verbreitung des Erregers in der Umwelt spielen. Dies könnte bedeutende Konsequenzen für die Vorgehensweise bei der Bekämpfung von BU haben und liefert einen wichtigen Ausgangspunkt für zukünftige Forschung.

# CHAPTER 1

# Introduction

*Mycobacterium ulcerans* (*M. ulcerans*) causes the chronic and destructive skin disease Buruli ulcer (BU). Until today, cases have been reported from more than 30 countries world-wide with the highest incidence rates in Western Africa, where BU constitutes a substantial public health problem. In spite of considerable advances during the past few years in understanding this enigmatic disease, important aspects such as environmental reservoirs and transmission pathways have yet to be explored. The following sections summarize the current understanding of BU and its causative agent as well as unresolved research issues in this field.

**History and geographical distribution of Buruli ulcer**

Chronic skin ulcers consistent with *M. ulcerans* infection were first described in 1897 by Sir Albert Cook, a British physician working at the Mengo Hospital in Kampala, Uganda. In 1935, cases of unusual skin ulcers were reported in patients from the Bairnsdale district in Australia [1]. However, the first detailed characterization of BU patients living in an area around Bairnsdale was provided in 1948 by MacCallum and his colleagues, who were the first to isolate the etiologic agent of the disease, a mycobacterium henceforth designated as *M. ulcerans* [2]. The first large numbers of cases were recorded in the Buruli County (Nakasongola District) in Uganda during the 1960s, giving rise to the official designation Buruli ulcer for this disease [3]. In the following decade *M. ulcerans* infection foci were predominantly reported in a number of West and Central African countries such as Congo [4], Ghana [5], Uganda [6] and Nigeria [7]. Since then new BU infection foci were discovered in Togo [8] and Angola [9] and strong increases in the incidence rates were recorded in several West African countries including Benin [10], Ivory Coast [11], Ghana [12] and Cameroon [13]. In 1998, the World Health Organization established the global Buruli ulcer initiative in order to raise awareness about the hitherto neglected disease and to coordinate global BU control and research efforts [14]. Health workers and researchers from various countries have subsequently shared experiences and information regarding transmission, pathogenesis, molecular biology and treatment [15].

Until today BU has been reported from 32 tropical and subtropical countries in Africa (Angola, Benin, Burkina Faso, Cameroon, Congo, Ivory Coast, Democratic Republic of the Congo (former Zaire), Equatorial Guinea, Gabon, Ghana, Guinea, Kenya,

Liberia, Malawi, Nigeria, Sierra Leone, Sudan, Togo Uganda), Latin America (Brazil, French Guiana, Mexico, Peru, Suriname), South-East Asia (China, Indonesia, Japan, Malaysia, Sri Lanka) and the Western Pacific (Australia, Kiribati, Papua New Guinea) [16].

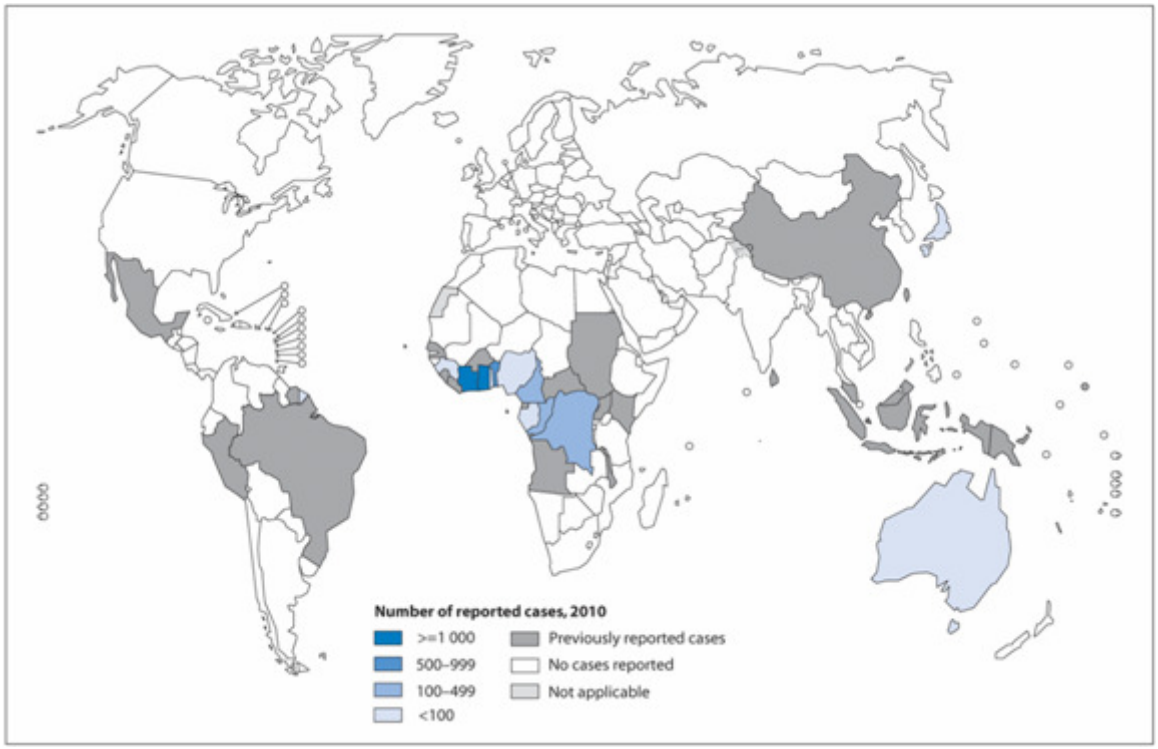


Figure 1: A global map representing countries that have reported cases of Buruli ulcer disease as of 2010. Data source: World Health Organization. Map production: Control of Neglected Tropical Diseases.

Accurate estimations of BU case numbers and burden of the disease is very difficult due to the characteristic focal distribution of BU within endemic countries affecting predominantly remote areas of Western Africa with limited access to the formal health sector, where comprehensive reporting systems are lacking. In 2010, the highest incidence rates were reported from Ivory Coast (2533 cases), Ghana (1048 cases), Benin (572 cases) and Cameroon (287 cases) (Figure 1) [17]. However, there is evidence of vast under-reporting [18].

**Clinical presentation of Buruli ulcer**

BU is a chronic, necrotizing infection of the subcutaneous adipose tissue. Most commonly it starts as a painless, mobile swelling in the subcutaneous tissue referred to as nodule (Figure 1A), which may eventually break down to form a slowly progressing ulcer with characteristic undermined edges. Other pre-ulcerative manifestations include papules (Figure 2B), which are predominantly observed in Australian BU patients as well as oedema (Figure 2C) and plaques (Figure 2D), often causing large ulcerations (Figure 2E) [19,20]. Untreated infection may lead to extensive tissue destruction followed by contractures and deformities (Figure 2F) due to uncontrolled self-healing processes.

WHO has defined lesions with a cross-sectional diameter of less than 5 cm as category I (small), 5-15 cm as category II (moderate), and more than 15 cm, lesions at crucial sites (eye, breast, and genitalia), or multiple lesions as category III (advanced) lesions. For various reasons, including the indolent nature of BU, lack of systemic symptoms, stigmatization, association with witchcraft and limited access to medical care, patients in rural regions of Africa tend to seek treatment at the formal health sector late and often present with large ulcers [20].

The percentage of BU patients with osteomyelitis was reported to be 13% in a study including 1611 BU patients from Benin [21]. Secondary infection of BU lesions occurs with *Staphylococcus aureus* and *Pseudomonas aeruginosa* being the most prominent causes (Yeboah-Manu et al., submitted).

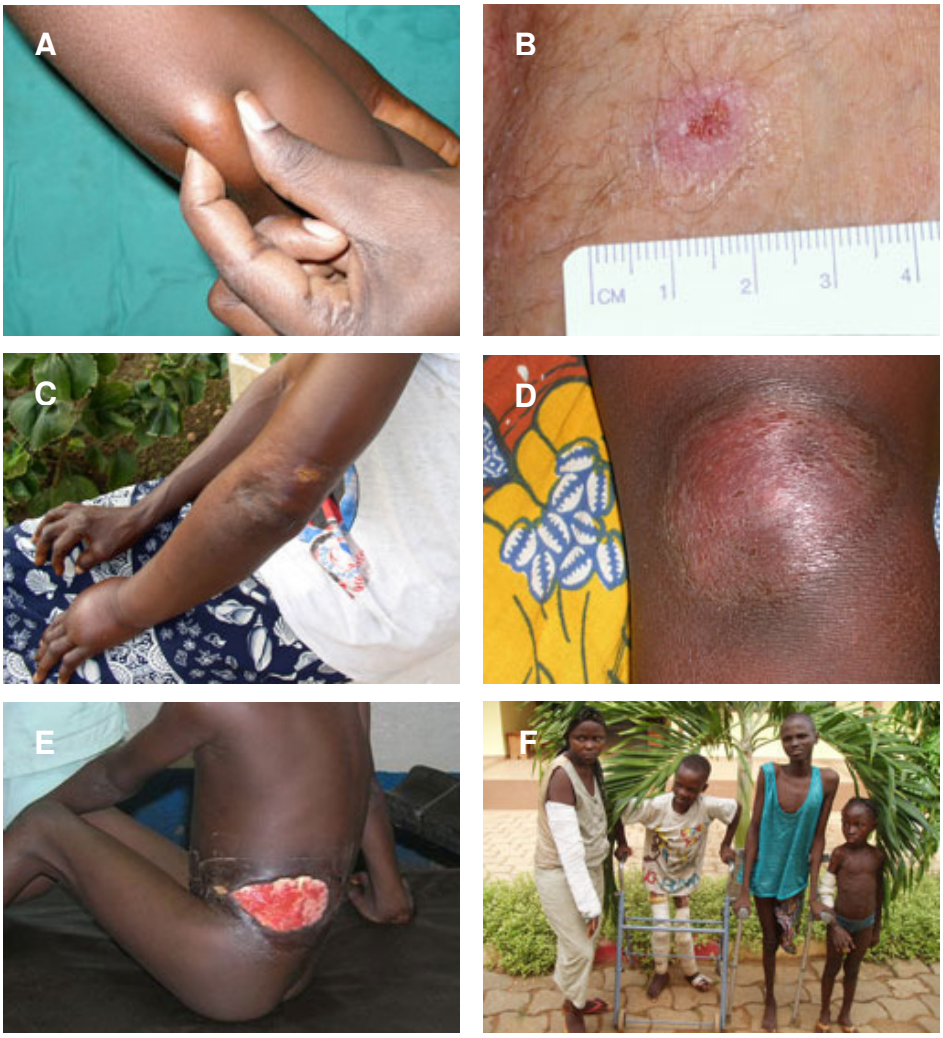


Figure 2: Non-ulcerative and ulcerative forms of Buruli ulcer, Complications: A: Nodule; B: Papule; C: Edema; D: Plaque; E: ulcer; F: Patients with disabilities. Source: WHO

Approximately 80% of the ulcers occur on the limbs, most commonly involving the lower extremities [22]. All age groups are affected by the disease with a nearly equal gender distribution, but children under the age of 15 make up at least 50% of all cases in Africa [23].

### Environmental reservoirs and transmission of *Mycobacterium ulcerans*

The epidemiology of BU is still poorly understood; however, *M. ulcerans* infection foci are typically associated with proximity to stagnant and slow-flowing water bodies (Figure 3) and often connected to human-disturbed aquatic environments. While it is known that other mycobacteria such as *M. leprae* and *M. tuberculosis* are transmitted

by person-to-person contact, it is commonly assumed that infection with *M. ulcerans* takes place through trauma of the skin [24] or insect bites via an environmental reservoir in the ecosystem [22].

Figure 3: Typical Buruli ulcer riverine endemic sites in the Densu River Basin of Ghana (2010).

Definite environmental reservoirs and transmission pathways have yet to be elucidated, since cultivation of the extremely slow growing *M. ulcerans* from possible environmental sources contaminated with faster growing mycobacterial species and other microorganisms is more than a challenge. Only one pure culture of *M. ulcerans* could ever be obtained from an environmental source, more precisely from an aquatic water bug collected in Benin [25]. Hence, environmental studies are largely based on the detection of *M. ulcerans* genomic DNA sequences. One of the DNA targets, the insertion sequence (IS) element *2404* has been detected by PCR in various environmental samples including water [26], aquatic insects [27] and plants [28], snails [29], and small fish [30]. However, the presence of this IS element in other environmental mycobacteria has been reported. Since 2007 confirmatory real-time PCR assays targeting not only IS*2404* but also another IS element (IS*2606*) and a sequence encoding the ketoreductase B domain of the mycolactone polyketide synthase genes are available to complement the specificity of the IS*2404* PCR. Analysis of the three targets facilitated the detection of *M. ulcerans* DNA in soil, sediment, and mosquito extracts from a BU endemic area in Australia [31]. However, since PCR is not able to prove for the presence of viable bacteria, results are difficult

to interpret. A recent study in South-Eastern Australia has implicated tree-dwelling native possums as a possible animal reservoir [32] and mosquitoes as potential vectors [33,34] of *M. ulcerans* in BU endemic settings of Victoria. A similar potential animal reservoir could not be identified in BU endemic regions of Africa so far. However, there are strong indications, that spread of *M. ulcerans* from chronic, ulcerated lesions to insect vectors or another currently unknown environmental reservoir and subsequent infection of individuals living in the same settlements should be considered.

### Evolution and diversity of *Mycobacterium ulcerans*

*M. ulcerans* is closely related to and has a common ancestor with the fish pathogen *Mycobacterium marinum* (*M. marinum*) [35]. Based on a universal molecular clock rate [36] it was estimated that *M. ulcerans* has diverged from *M. marinum* around a million years ago by the acquisition of a plasmid (pMUM001) encoding enzymes required for the production of the toxin mycolactone [35,37]. The exact species definition of *M. ulcerans* is currently complicated by the identification of other closely related mycolactone producing mycobacteria (MPM), which have so far not been associated with BU [38–41]. Though given distinct species names such as *M. shinshuense*, *M. marinum*, *M. pseudoshottsii* and *M. liflandii*, genetic analyses suggest that all MPM are derived from a common ancestor and are genetically coherent [42]. Hence, it has recently been proposed that all MPM should be renamed *M. ulcerans* [42,43].

After the acquisition of pMUM and insertion sequence (IS) elements *2404* and *2606*, *M. ulcerans* diverged into at least two principal lineages by reductive evolution including extensive gene loss predicted to be associated with adaptation to a new niche environment. *M. ulcerans* strains from Asia, South America and Mexico as well as fish and frog isolates belong to the ancestral lineage, whereas the classical lineage includes clinical isolates from Africa, Australia and South East Asia [44]. Whole genome comparison of 35 *M. ulcerans*, MPM and *M. marinum* strains suggests a further subdivision of ancestral strains into lineage 1 including human isolates from South America as well as fish and frog isolates with world-wide origin and lineage 2 consisting of clinical *M. ulcerans* isolates from Japan. In this study the

classical lineage - defined as lineage 3 - was largely represented by human and other animal isolates from Africa and Australia. It was suggested that "*each of the M. ulcerans lineages probably represents different ecotypes, reflecting adaptation to related but distinct niche environments*" and should be considered as *M. ulcerans* ecovars [42]. Deletion or inactivation of genes required for pigment biosynthesis, anaerobiosis, and intracellular growth suggests that the environment *M. ulcerans* is adapting to might be dark (especially lineage 3 isolates, which have lost UV-protecting pigment genes), aerobic and possibly extracellular [42,45].

*M. ulcerans* lineages are generally characterized by limited genetic diversity, which is typically found in bacterial populations that have recently gone through an evolutionary bottleneck such as the acquisition of a plasmid [46]. Further loss of gene function and chromosomal rearrangements among the closely related and highly clonal Australian and African isolates suggests that lineage 3 has passed through a second evolutionary bottleneck [42]. Extremely low levels of genetic diversity have been identified among West African *M. ulcerans* strains [47–50], indicating a relatively recent dispersal of one *M. ulcerans* clone throughout this region. Little is known about the evolution and geographical expansion of *M. ulcerans* in West Africa largely due to the lack of genetic typing methods with sufficient resolution to differentiate between closely related strains. In this PhD thesis the development and application of genetic fine-typing tools and the analysis of clinical *M. ulcerans* isolates from various African countries, in particular from Ghana, are described in chapters 2-4. The genome, evolution and diversity of *M. ulcerans* is reviewed in chapter 5 of this thesis.

**Pathology and the role of mycolactone**

The pathology of BU is determined by unique characteristics of *M. ulcerans*. While the low optimum growth temperature of 28-32 °C renders the skin a favorable site for infection and slowly progressing lesions can be attributed to the extremely long generation time of *M. ulcerans* (about 72 hours on routine mycobacteriological media), the production of mycolactone provokes extensive tissue necrosis [51]. Connor and Lunn have already suggested in 1965 that "*M. ulcerans elaborates a diffusible toxic substance that causes tissue necrosis*" after a histopathologic analysis

of a pre-ulcerative tissue specimen showing acid-fast bacilli in the center of a necrotic zone in the subcutaneous fat virtually devoid of inflammatory cells [52]. But it is only since 1999 that a polyketide-derived macrolide referred to as mycolactone can be definitely associated with the virulence of *M. ulcerans* infection [53]. Today, the classic histological features of BU lesions, namely the necrosis around clumps of extracellular acid-fast bacilli and the defective inflammatory response are explained by the cytotoxic and immunosuppressive properties of mycolactone [54]. While there is evidence for a transient intracellular stage of *M. ulcerans* during the establishment of infection, the release of mycolactone appears to be responsible for the killing of host cells and infiltrating leukocytes in advanced stages of the infection [55]. Mycolactone is present in cutaneous lesions but can also be detected in lymphoid tissues and serum [54,56]. It has been shown that *M. ulcerans* produces an extracellular matrix containing mycolactone but also other proteins and lipids that may play a role in the virulence of *M. ulcerans* [57].

Polyketides are produced by various organisms, but mycolactones are the first discovered macrolides in mycobacterial species and the first identified polyketide virulence determinants of a bacterial human pathogen [58]. Hence, the ability to produce mycolactone is restricted to *M. ulcerans* and other closely related MPM. So far, five structural variants (mycolactones A/B, C, D, E and F) have been identified among a worldwide collection of *M. ulcerans* and other MPM strains [59]. The genetic basis for mycolactone production is discussed in chapter 5 of this thesis.

**Diagnosis of Buruli ulcer**

Initial diagnosis of BU by experienced clinicians or health workers in endemic regions can be confirmed in the laboratory by four different methods including direct smear examination (Figure 4A), PCR (Figure 4B), histopathology (Figure 4C) and culture of *M. ulcerans* (Figure 4D) [20].

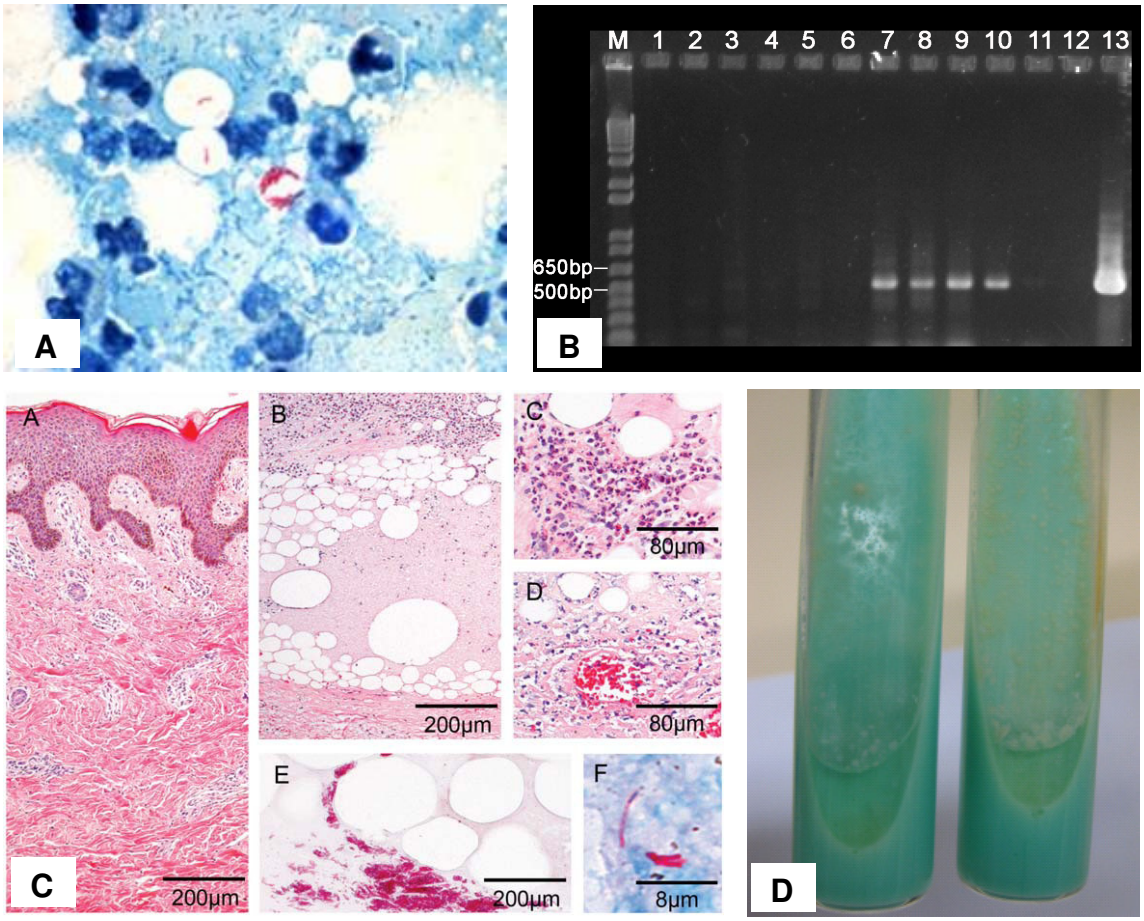


Figure 4: Laboratory techniques to confirm clinical BU diagnosis. A: direct smear examination (Yeboah-Manu, 2006); B: Agarose gel showing *M. ulcerans* insertion sequence *2404* PCR bands for positive samples (7-10 and 13); C: Histopathological characteristics associated with untreated BU lesions [60]; D: Culture of *M. ulcerans* on Löwenstein-Jensen medium.

While direct smear examination of swab specimens taken from undermined edges of the ulcers by Ziehl-Neelsen (ZN) stain is the simplest and most accessible diagnostic technique, the sensitivity of this method may be as low as 40%. The three remaining methods are largely confined to central reference laboratories. BU can be definitively diagnosed by *M. ulcerans* culture, but primary isolation may take 8 to 12 weeks. *M. ulcerans* recovery rates vary between laboratories but can be as high as 75% for tissue specimens [61], 41% for fine-needle-aspirates (FNA) and 43% for swab samples [62]. Culture, histopathology and PCR require good laboratory infrastructure and highly qualified personnel. PCR targeting IS*2404* is currently the gold standard for BU diagnosis in reference laboratories approaching 100% specificity. The

sensitivity is dependent on the PCR technique, but may approximate 100% (85% for FNA samples [63]) relative to the other diagnostic techniques [31,64]. Due to potential problems with amplicon contamination, strict quality control is required.

Diagnosis in many rural health facilities of endemic African regions is still mainly based on clinical findings. Since differential diagnosis of pre-ulcerative and ulcerative BU includes some important diseases like phagedenic ulcer and cutaneous tuberculosis, there is an urgent need for a specific, fast, cheap and simple point-of-care diagnostic test for BU, which can directly be applied at peripheral health centers and hospitals.

**Treatment of Buruli ulcer and prospects for a vaccine**

The only treatment option for BU has long been wide surgical excision of the necrotic tissue followed by skin grafting and, if available, rehabilitative physiotherapy [20]. In response to a pilot study evaluating the efficacy of rifampicin and streptomycin on early BU lesions [65], the WHO released provisional guidelines in 2004 advising a combination chemotherapy of oral rifampicin and intramuscular streptomycin administered daily for eight weeks [66]. Routine implementation of this regimen has greatly improved healing and reduced the frequency of relapses to less than 2% [67,68]. However, streptomycin is administered by daily injections and is potentially nephrotoxic and ototoxic [69]. Although rifampicin is currently the key drug for the treatment of BU, it has to be combined with another antibiotic to increase its effect and to avoid the development of resistance, which has been described after rifampicin monotherapy in a BU mouse model [70]. While pilot studies on fully oral chemotherapy using a combination of rifampicin and clarithromycin yielded promising results [71,72], identification of new compounds active against *M. ulcerans* that could replace rifampicin is crucial.

In chapter 7 of this PhD thesis the development of an Alamar Blue-based assay to analyze compounds for their activity against *M. ulcerans* growth is described. Screening of a set of azole compounds led to the identification of a low-molecular weight compound with good activity against *M. ulcerans*. The developed screening

platform is currently used in our laboratory to analyze further compounds for their activity against *M. ulcerans*.

Taking advantage of the temperature sensitivity of *M. ulcerans*, the application of heat in the form of phase change material as a treatment option has been tested in a proof-of-principle study for a limited number of BU patients [73]. A larger thermotherapy study is currently ongoing.

Patients in remote areas of Africa with limited or no access to adequate health facilities often seek traditional treatment including magico-religious rites as well as application of herbal preparations or other natural substances, which have no proven efficacy and bear a high risk of secondary infection of the wounds [15,74]. Although prompt and appropriate treatment of BU can minimize irreversible physical disabilities and treatment costs, patients in developing countries often seek adequate medical treatment only at late disease stages [74]. The obvious preference for traditional healing in remote African areas has a variety of reasons including, financial difficulties as well as problems associated with the duration of hospital admission (often more than 3 month per patient) and social stigma connected with the mysterious nature of the disease [74–76].

Until today, there is no specific vaccine against *M. ulcerans* and it remains controversial, whether BCG vaccination has a short-lasting protective effect [77]. However, it was reported that BCG vaccination at birth leads to protection against BU osteomyelitis in children and adults [21]. A major protective antigen of the BCG vaccine is the Ag85 complex, whose homologues are present in many mycobacteria. It was argued that a vaccine based on *M. ulcerans* antigens could offer a more specific and effective protection than BCG. Hence, the protective efficacies of two DNA vaccines encoding Ag85A from BCG and from *M. ulcerans* were compared in a *M. ulcerans* mouse model. Species-specificity was reflected in a higher protective efficacy of the *M. ulcerans* vaccine [78]. Hence, possible vaccine strategies proposed include adjuvanted subunit based protein vaccines or an attenuated, live vaccine based on *M. ulcerans* [18]. Even though mycolactone would represent a suitable target for the development of a toxoid-based vaccine, the polyketide itself is not

immunogenic and until now it was not possible to develop a protein conjugate that elicits high titers of potentially neutralizing mycolactone-specific antibodies [79].

**Immune response to *M. ulcerans***

Antigenic cross-reactivity between *M. ulcerans*, *M. tuberculosis*, *M. bovis* and various environmental mycobacteria complicates investigations on *M. ulcerans*-specific immune responses. One suitable serological marker for exposure of individuals living in BU endemic areas to *M. ulcerans* was identified as the immunodominant 18 kDa small heat shock protein (shsp), which has no homologues in *M. tuberculosis* and *M. bovis* [80]. In the largest sero-epidemiological study ever conducted for BU, we have determined anti-18 kDa shsp IgG titers in sera collected from healthy inhabitants of the BU endemic Densu River Valley of Ghana. The study, which is described in chapter 6 of this thesis, showed that a considerable proportion (about 32%) of exposed, but healthy individuals develop specific humoral responses against a *M. ulcerans* antigen, indicating that infection with *M. ulcerans* may lead only in a small proportion of exposed individuals to active BU. These results together with the observation that BU can heal spontaneously suggests the existence of protective immunity [81], even though the underlying protective mechanisms are largely unknown.

Apart from humoral immune responses, the control of *M. ulcerans* infection may be primarily dependent on adaptive cell-mediated immunity. Delayed-type hypersensitivity response to an extract of *M. ulcerans* (burulin) on skin testing was observed rarely in patients with early disease, but frequently in patients with healed or active disease [82], indicating a degree of T-cell sensitization. Despite evidence of Th1-mediated protective immunity it is not known to what extent BU patients can mount a *M. ulcerans*-specific Th1 response, since *M. ulcerans* infection has been widely associated with the suppression of local and systemic immune responses [81,83]. The production of interferon gamma (IFN-γ), which is preferentially secreted by Th1 cells, was shown to be reduced during active BU [83]. However, the suppression of systemic immune responses remains controversial. To give one example, Hong et al. have reported that mycolactone has immunosuppressive effects at the systemic level, since it is able to diffuse beyond the site of *M. ulcerans*

infection [54]. On the contrary, a recent study in a mouse model indicated that infection with *M. ulcerans* may lead to local and regional suppression of the immune response rather than induction of systemic immunosuppression [81]. In this vein, the lack of inflammatory responses in necrotic areas of BU lesions might be explained by mycolactone-induced apoptosis and necrosis of the inflammatory infiltrate [84].

After chemotherapy the local immunosuppression is reversed and leads to an active inflammatory process with leukocyte infiltrates cumulating in the development of ectopic lymphoid structures indicative for healing processes [60]. Some of the patients develop immune-mediated clinical deteriorations termed “paradoxical reactions” during the course of antibiotic treatment [85]. Recently, it has been reported that in some cases secondary *M. ulcerans* infection foci developing several months after successful treatment of the initial lesion may be resolved by the immune system [86].

**Objectives**

Efforts to identify transmission pathways and environmental sources of *M. ulcerans* have long been hampered by the lack of typing methods with sufficient resolution for micro-epidemiological studies. In this PhD thesis we anticipate the identification of useful SNP markers for the fine-typing of *M. ulcerans* strains from Ghana by the following strategy:

- Whole genome sequence comparison of *M. ulcerans* strains from Ghana in order to detect a comprehensive set of SNP markers.
- Development of genotyping assays based on real-time PCR as well as conventional PCR at the identified SNP positions.
- SNP typing of *M. ulcerans* strains from a BU endemic region in the Densu River Valley by the established SNP assays to be able to differentiate between *M. ulcerans* haplotypes and to monitor their circulation in this region.

A major mystery of BU is its focal distribution within endemic countries. In Ghana BU is highly endemic in communities along the lower part of the Densu River, while no cases have so far been reported from villages located further upstream. Until today it is not known, whether differences in BU prevalence can be attributed to a difference in the exposure to *M. ulcerans* or other currently unknown factors. It is unclear as well what proportion of a population in BU endemic and non-endemic communities is actually exposed to *M. ulcerans*. Our approach to gain better insight into the epidemiology of *M. ulcerans* in the Densu River Valley is:

- The determination of humoral responses against the *M. ulcerans* 18KDa small heat shock protein in blood sera collected from healthy inhabitants of BU endemic and non-endemic communities along the Densu River.

The identification of alternative drugs for BU, suitable for application at peripheral health centers in developing countries is one of the major research goals for BU. Therefore another aim of this PhD thesis is:

- The development of a drug screening platform to analyze compound libraries for new scaffolds with *M. ulcerans* growth inhibitory activity.

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CHAPTER 2

# Genomic Diversity and Evolution of *Mycobacterium ulcerans* Revealed by Next-Generation Sequencing

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This article has been published (September 11, 2009) in:
*Public Journal of Library Pathogens*

**ABSTRACT**

*Mycobacterium ulcerans* is the causative agent of Buruli ulcer, the third most common mycobacterial disease after tuberculosis and leprosy. It is an emerging infectious disease that afflicts mainly children and youths in West Africa. Little is known about the evolution and transmission mode of *M. ulcerans*, partially due to the lack of known genetic polymorphisms among isolates, limiting the application of genetic epidemiology. To systematically profile single nucleotide polymorphisms (SNPs), we sequenced the genomes of three *M. ulcerans* strains using 454 and Solexa technologies. Comparison with the reference genome of the Ghanaian classical lineage isolate Agy99 revealed 26,564 SNPs in a Japanese strain representing the ancestral lineage. Only 173 SNPs were found when comparing Agy99 with two other Ghanaian isolates, which belong to the two other types previously distinguished in Ghana by variable number tandem repeat typing. We further analyzed a collection of Ghanaian strains using the SNPs discovered. With 68 SNP loci, we were able to differentiate 54 strains into 13 distinct SNP haplotypes. The average SNP nucleotide diversity was low (average 0.06–0.09 across 68 SNP loci), and 96% of the SNP locus pairs were in complete linkage disequilibrium. We estimated that the divergence of the *M. ulcerans* Ghanaian clade from the Japanese strain occurred 394 to 529 thousand years ago. The Ghanaian subtypes diverged about 1000 to 3000 years ago, or even much more recently, because we found evidence that they evolved significantly faster than average. Our results offer significant insight into the evolution of *M. ulcerans* and provide a comprehensive report on genetic diversity within a highly clonal *M. ulcerans* population from a Buruli ulcer endemic region, which can facilitate further epidemiological studies of this pathogen through the development of high-resolution tools.

## AUTHOR SUMMARY

*Mycobacterium ulcerans* is the causative agent of Buruli ulcer (BU), a necrotizing skin disease and the third most common mycobacterial disease after tuberculosis and leprosy. It is an emerging infectious disease that afflicts mainly children and youths in West Africa. The disease is also found in tropical and subtropical regions of Asia, the Western Pacific, and Latin America. Limited knowledge of this neglected tropical disease is partially due to the lack of known genetic polymorphisms among isolates, which hinder the study of transmission, epidemiology, and evolution of *M. ulcerans*. Our aim is to systematically profile genetic diversity among *M. ulcerans* isolates by sequencing and comparing the genomes of selected strains. We identified single nucleotide polymorphisms (SNPs) within a highly clonal *M. ulcerans* population from a Buruli ulcer endemic region. Based on the SNPs discovered, we developed SNP typing assays and were able to differentiate a collection of *M. ulcerans* isolates from this Buruli ulcer endemic region into 13 SNP haplotypes. Our results lay the ground for developing a highly discriminatory and cost-effective tool to study *M. ulcerans* evolution and epidemiology at a population level.

## INTRODUCTION

*Mycobacterium ulcerans* causes Buruli ulcer (BU), a necrotizing skin disease and the third most common mycobacterial disease, after tuberculosis and leprosy [1]. In the past decade the incidence of BU has increased dramatically in West African countries, but the disease is also found in tropical and subtropical regions of Asia, the Western Pacific, and Latin America [2]. Due to the focal distribution of the disease and the fact that it affects mainly poor rural communities, BU belongs to the neglected tropical diseases. Limited knowledge about the disease is partially caused by the lack of molecular fine-typing methods, which hinder the study of transmission, epidemiology, and evolution of the clonal pathogen.

Genetic analyses suggested that *M. ulcerans* diverged from the fish pathogen *M. marinum* between 470,000 and 1,200,000 years ago by acquiring the virulence plasmid pMUM001 [3],[4]. Whole genome comparison of *M. marinum* strain M and *M. ulcerans* strain Agy99 revealed that the two strains share >98% nucleotide sequence identity, although extensive DNA insertions and deletions have been observed [4]. Our recent comparative genomic hybridization study found extensive large sequence polymorphisms (LSPs) among *M. ulcerans* clinical isolates of diverse geographic origins. Two distinct *M. ulcerans* lineages have been defined: the ancestral lineage of strains from Asia, South America and Mexico, which are genetically closer to the progenitor *M. marinum*, and the classical lineage of strains from Africa, Australia and South East Asia [5],[6].

Although continental types of *M. ulcerans* strains have been well established, differentiation between isolates within a geographic region, such as strains from African countries, has remained a challenge [7],[8]. Different genotyping methods have been applied to *M. ulcerans*, including IS*2426* polymerase chain reaction (PCR) [9],[10], amplified fragment length polymorphism (AFLP) [11], IS*2404* restriction fragment length polymorphism (RFLP) [12],[13], multi-locus sequence typing (MLST) [3],[14],[15], variable-number tandem repeat (VNTR) typing [7],[8],[16],[17] and IS*2404*-Mtb2 PCR [18]. Among these, AFLP [11] and recently established VNTR typing methods [7],[8] were the only techniques that have provided some resolution among clinical strains of *M. ulcerans* from Africa, confirming that genotypic diversity

attributable to insertions, deletions, and duplications of variable DNA fragments exists among African strains. However, protein coding genes in *M. ulcerans* African populations harbor extremely low levels of polymorphisms. No single nucleotide polymorphisms (SNPs) were detected in a multi locus sequence typing of a few chromosomal and plasmid genes [3],[14],[15].

To systematically and comprehensively study the genetic diversity and the evolution of *M. ulcerans* strains, a genome wide profiling is needed. The complete genome sequence of *M. ulcerans* Agy99 consists of a circular chromosome of 5632 Kb and a plasmid pMUM001 of 174 Kb [4], which can be used as the reference for comparative genome analysis. The ongoing revolution in massively parallel sequencing technology [19],[20], such as the availability of Roche 454 Life Sciences Genome Sequencer FLX [21], Applied Biosystems SOLiD System, and Illumina Solexa Genome Analyzer [22], has made it possible to sequence large numbers of bacterial strains within days [23]. Next generation sequencing platforms have been used for genome wide profiling of novel genetic variations in many different organisms, including viruses [24], bacteria [25],[26],[27], plants [28],[29], worms [30] and humans [31],[32]. Here we report the sequencing of the genomes of three selected *M. ulcerans* strains using pyrosequencing (Roche 454 Life Science) and Solexa (Illumina) sequencing by synthesis technologies. Based on these sequences we identified SNPs, which we used to estimate evolutionary times for the emergence of *M. ulcerans*. We also developed SNP typing assays as high-resolution genotyping methods for *M. ulcerans*. Genetic fingerprinting of bacterial isolates will be a valuable tool for distinguishing relapses from new infections, tracing infection chains, and identifying environmental reservoirs. Molecular epidemiological analyses based on SNP typing may finally contribute to better disease control by identifying preventable risks for infection.

## RESULTS

### Selection of *M. ulcerans* strains for genome sequencing

With the aim to comprehensively investigate genome diversity of *M. ulcerans* strains from an individual geographical region, we selected two Ghanaian patient isolates from different residential districts and sequenced their genomes with 454 and Solexa technologies, respectively (Table 1). These two strains were isolated from the same African country as the fully sequenced reference strain Agy99 [4]. Whereas Agy99 was isolated in 1999 from a BU patient, the two selected Ghanaian patient isolates NM20/02 and NM31/04 were isolated after an apparent process of replacement of VNTR types in Ghana [7] in the years 2002 and 2004, respectively. Agy99, NM31/04 and NM20/02 represent the three VNTR types (Table 1) previously identified in Ghana [7]. While the Ghanaian strains belong to the classical lineage, we also included a Japanese patient isolate, ITM Japan8756 (denoted as Jp8756 from here on in the paper), as a representative of the ancestral lineage in our analysis. Its genome was sequenced with the Solexa Genome Analyzer. Selected genomic regions of this strain were also sequenced with a NimbleGen comparative genome sequencing (CGS) microarray and the results were compared.

### Single nucleotide polymorphisms in *M. ulcerans* strains

We sequenced three *M. ulcerans* strains with single end reads generated by two different next–generation sequencing platforms. For NM20/02, we obtained 424,494 GS FLX reads (Roche 454) with an average length of 213 bases. For NM31/04 and Jp8756, we obtained 2.5 and 2.7 million 35-bp Solexa reads, respectively (Table 2). To identify SNPs, we mapped the reads to the reference genome, including both the Agy99 chromosome and the plasmid pMUM001.

We used the 454 software gsMapper for GS FLX reads and MAQ [33] for Solexa reads. The MAQ places Solexa reads mapped to multiple locations randomly, while gsMapper excludes reads mapped to repeated regions, such as insertion sequences (IS)*2404* and IS*2606*, which are present in high copy numbers in the *M. ulcerans* genome [4]. Therefore, the Agy99 chromosome was better covered by Solexa reads

than by GS FLX reads. 94% of the Agy99 chromosome was mapped with NM20/02 GS FLX reads, 99.99% with NM31/04 Solexa reads, 94.47% with Jp8756 Solexa reads. The average depth was 14 to 15 fold. We identified 135 chromosomal SNPs in NM20/02, 83 SNPs in NM31/04, and 26,564 SNPs in Jp8756 (Table S1).

The coverage for pMUM001 varied a lot from strain to strain. 33% of the pMUM001 was mapped with NM20/02 reads, 100% with NM31/04 reads, and 20% with Jp8756 reads. The average depth ranged from 63 for NM31/04 reads to 17 for Jp8756 reads (Table 2). Because the low coverage of the plasmid in NM20/02 could be an artifact from the gsMapper, which excluded reads mapped to non-unique regions, we mapped NM20/02 reads to pMUM001 using another software, MOSAIK (http://bioinformatics.bc.edu/marthlab/Mosaik), which allowed us to compare mapping results with non-uniquely mapped reads included or excluded. When all mapped reads were assembled regardless of their uniqueness, the full length of pMUM001 was well covered (Figure S1 A), suggesting the presence of a pMUM001-like plasmid in NM20/02. When only uniquely mapped reads were recorded, pMUM001 was partially covered (Figure S1 B). Reads mapped to regions such as those encoding Type I modular polyketide synthase genes, transposase genes, IS elements (IS*2606* and IS*2404*) were excluded due to their non-uniqueness. Although mapping analysis using MOSAIK confirmed that the lack of pMUM001 coverage in NM20/02 was a data analysis artifact, the lack of pMUM001 in Jp8756 was confirmed. MAQ didn't exclude non-uniquely mapped reads and was able to map the full length of pMUM001 with NM31/04 reads, which were analyzed exactly the same way as Jp8756 reads. Mapping using MOSAIK not only confirmed the lack of pMUM001 coverage by Jp8756 reads (Figure S1 E), but also revealed that the depth of pMUM001 regions covered by uniquely mapped Jp8756 reads were very low, which ranged from one read to four reads (Figure S1 F), while the depth of pMUM001 regions covered by uniquely mapped reads ranged from 10 to 50 in NM20/02 and NM31/04 (Figure S1 B and D). Most likely the under-representation of plasmid DNA in the total DNA sample is due to either complete or partial loss of plasmid sequences, which is frequently found in *M. ulcerans* strains that have been cultured over extended periods of time. Previous plasmid sequences analyses have found that plasmids in Japanese and African *M. ulcerans* strains are highly conserved in

size and sequence [15],[34]. However, a recent study found different strains of *M. ulcerans* were capable of producing structurally distinct mycolactones, which could be due to presence of sequence variations in pMUM001[35]. Our study suggests that the plasmids from all three African strains are highly similar. We only found one intergenic SNP and one synonymous SNP shared by NM31/04 and NM20/02 plasmids. The two non- synonymous SNPs found in the NM20/02 plasmid were within *IS*2606 genes. In Jp8756 we identified one intergenic SNPs in the plasmid regions with sufficient coverage (Table S1). To elucidate how the sequence variations affect mycolacton production, future experiments in the lab will be needed to enrich and analyze the plasmids in each test strain, together with characterization of mycolacton production.

Before next generation sequencing technologies became widely available, we have sequenced Jp8756 using comparative genome sequencing microarrays covering selected regions of the Agy99 chromosome and pMUM001. A total of 1,618 SNPs were identified in the selected 1.2 Mb chromosomal protein coding regions. 1,389 (86%) of these SNPs were confirmed by Solexa sequencing (Table S2). On both mutation mapping arrays and re-sequencing arrays, probes targeting pMUM001 showed very low signals (Figure S2), as compared to probes targeting the Agy99 chromosome, which also confirmed the lack of plasmid DNA in the Jp8756 DNA sample.

In total 26,669 SNPs were identified by comparing the Jp8756 to the Agy99 chromosome; 18,510 in 3,597 protein coding genes, and 8,159 in 1,768 different intergenic regions (Table S1). 99.35% of the SNPs were found only in the Japanese strain (Figure 1). In comparison to Agy99, the average number of SNPs ranged from 1 per 210 bp in Jp8756 to 1 per 68 Kb in NM31/04. While the Japanese strain Jp8756 and the Ghanaian strain Agy99 share 99.53% nucleotide sequence identity, the average percentage of polymorphic nucleotide sites between Ghanaian strains is only 0.0015% (NM31/04 vs. Agy99) and 0.0024 (NM20/02 vs. Agy99), respectively. These results are consistent with previous findings that strains of the ancestral lineage are genetically distant to the classical lineage strains [6]. There are 103 SNPs specific to the Ghanaian strains with an average of 0.0018% of polymorphic nucleotide site differences from the Agy99 genome (Figure 1).

We classified identified SNPs into five different categories: strain specific SNPs, transversions, synonymous SNPs (sSNPs), non-synonymous SNPs (non-sSNPs), and intergenic SNPs. Figure 2 shows the total numbers of SNPs and the percentage of different categories of SNPs found in each strain. Overall, only 34 SNPs were found parsimoniously informative (PI), common to at least two strains or "non-strain-specific." The numbers of "strain-specific" SNPs varied from 33 (40% of 83 SNPs) in NM31/04 to 26,496 (99.7% of 26564 SNPs) in Jp8756. In all three strains the percentage of intergenic SNPs was around 40%, indicating a similar distribution of point mutations in coding and non-coding regions. Interestingly, the majority of coding region SNPs (63%) found in Jp8756 was synonymous, while in the two Ghanaian strains the majority was non-synonymous. About 100 non-synonymous SNPs found in strain Jp8756, but none of the non-synonymous SNPs found in the two Ghanaian strains caused premature stop codons. While the accumulation of pseudogenes seems to play an important role in both the divergence of *M. ulcerans* from *M. marinum* and the emergence of *M. ulcerans* continental types [4], our observation suggests that there is no further formation of pseudogenes within the studied Ghanaian strains. The percentage of SNP transition ranged between 65% and 68%, suggesting a substitution bias in favor of nucleotide substitution within the purine or pyrimidine group.

We did not try to identify insertions or deletions (indels) of any size. First of all, single-base/small indels were difficult to identify based on 454 data due to higher indel error rate observed in pyrosequencing [36]. Secondly, identification of single-base/small indels using short reads, such as solexa or SOLiD data, requires paired-end data [33], which were not provided in our study. Without paired-end data, discovery of single-base/small indels using short reads requires gapped alignments of millions of short reads, which is computational challenged. Most currently available short read mapping tools supports only non-gapped alignments. Gapped alignment tools such as BLAST is not feasible for mapping short reads in a reasonable time scale and not guaranteed to give the right answer [37]. Last but not least, identification of large indels with high confidence also requires paired-end data. This is because missing regions can be explained by other factors, such as insufficient sequencing coverage,

differences in mapping algorithms when dealing with reads mapped to repeated regions, and so on.

**Genes potentially under selections**

Among the 94 protein coding genes containing SNPs found in the Ghanaian strains, most genes harbored only one SNP (Figure 3). Only three genes contained two SNPs: MUL_2118 (hypothetical protein), MUL_3524 (diphosphomevalonate decarboxylase) and MUL_3716 (nucleoside diphosphate kinase). When considering all strains, 3,597 genes harbored SNPs, most of which contained one to five SNPs (Figure 3), but much higher numbers of SNPs per gene were also observed. For example, the peptide synthetase Nrp gene MUL_2638 contained 59 SNPs, the Pks12 gene MUL_2266 harbored 55 SNPs, and the fatty acid synthase Fas gene MUL_3818 had 30 SNPs. However, all three genes were over 9 Kb in length, which may account for the high number of SNPs. We did find 70 genes with a SNP density higher than 1 per 80 bp, which was one standard deviation higher than the average SNP density (Table 3). More than 50% of these consisted of genes encoding hypothetical proteins. Others included genes associated with antigenic proteins (i.e. *esxE*, *esxF*, *mpt64*), lipoproteins (i.e. *dsbF*, *lppN*, *lpqV*), PE/PE-PGRS family proteins (MUL_4359, MUL_0355), membrane proteins (*mmr*) and transcription regulators (MUL_2645, MUL_0993). The high number of SNPs may represent evidence for selection pressure on these genes. We thus calculated values of synonymous differences per synonymous site ($p_S$) and nonsynonymous differences per nonsynonymous site ($p_N$) across all SNP harboring loci. The average $p_N–p_S$ value was –0.00276±0.0073 ($P<0.001$, $p_S$ = 0.0073±0.0063, $p_N$ = 0.0046±0.0033), suggesting that on average the frequency of synonymous mutation was significantly higher than the non-synonymous mutation frequency; i.e. there was no evidence for diversifying selection. Among the 70 genes found earlier with high SNP density, five genes (*esxE*, *mmr*, MUL_0355, as well as the hypothetical protein genes MUL_0161 and MUL_2106) showed significantly high $p_N–p_S$ values (above the mean + 3× standard deviations) and might be under diversifying selection, while six genes (*glbO*, *mcmA2b*, MUL_1435, MUL_2645, MUL_4312 and MUL_4846) showed significantly low $p_N–p_S$ values (less than the mean–3× standard deviations), which might be under

negative selection (Table 3). Meanwhile, we found 32 genes with average SNP density but significant low $p_N$–$p_S$ values, including genes related to transcription and translation (*whiB4*, *trxC*, *trpG*, MUL_0058, MUL_2937, MUL_4776) and another ESAT-6 family protein gene, *esxR* (Table 3).

There has been growing evidence suggesting that some of the ESAT-6 family proteins are involved in the interplay between host and pathogen via either antigenic variation or antigenic drift [38]. ESAT-6 protein encoding genes (*esxA esxB*) are deleted in *M. ulcerans* strains of the classical lineage [4],[39], which could contribute to antigen variation and enable the pathogen to escape the immune defense of the host. The Agy99 genome contains 12 genes encoding ESAT-6 like proteins [4]. This may represent a genomic basis for antigenic variation. The duplicated genes might encode antigenically different proteins with the same function. The differential expression of individual genes could enable them to substitute for each other functionally but escape from host immune recognition. The high number of SNPs and significantly high/low $p_N$–$p_S$ values we observed here on ESAT-6 family protein genes such as *exsE*, *esxF*, *esxR*, and another secreted antigenic protein gene *mpt64*, may be the result of selective pressure imposed by the immune system of the host. Mutations that lead to replacement of amino acids within the immunodominant epitopes have been proposed as a mechanism producing antigenic drift [40]. However, it remains to be investigated whether the esx genes are expressed, if their expression is controlled and coordinated, and if the mutations identified here lead to antigenic drift.

We further examined whether certain functional classes of genes were under positive selection by comparing $p_N$–$p_S$ values according to the Clusters of Orthologous Group (COG) classification. We found no significant overrepresentation of any functional class in gene group with positive $p_N$–$p_S$ values (under diversifying selection), except the “Function unknown” class (p value <0.05). Five functional classes showed overrepresentation of genes with negative $p_N$–$p_S$ values, indicating possible selections against amino acid changes, including functional classes J (translation, ribosomal structure and biogenesis), O (posttranslational modification, protein turnover, chaperones), F (nucleotide transport and metabolism), H (coenzyme transport and metabolism) and V (defense mechanisms).

**Phylogeny and estimation of the divergence time of *M. ulcerans* strains**

To evaluate the phylogenetic relatedness of the *M. ulcerans* strains sequenced in this study, we first analyzed the 34 PI SNP sites using the compatibility matrix program [41] to detect the effects of recombination on sequence divergence among the genes harboring these SNPs (Figure 4). In the square matrix, each white square corresponds to two compatible nucleotide sites, at which all nucleotide changes can be inferred to have occurred only once in a phylogeny. Black squares represent incompatible sites, where nucleotide changes are inferred to have occurred multiple times either due to recombination or repeated mutation. We found the 34 PI SNPs formed three groups: SNPs shared by the two Ghanaian test strains (at genomic locations of 1289632, 2366378, 2631719, 3621904, 3670882, 3692657), SNPs shared by NM31/04 and Jp8756 (at genomic locations of 119244, 3594811, 4144236, 4144237) and the rest of the SNPs shared by NM20/02 and Jp8756. The SNPs were compatible within each group but not across groups. The overall compatibility score of all 34 PI SNP sites was 0.5294, which measures the extent of the sites consistent with one phylogeny. The neighbor similarity score of the matrix was 0.6506, which was not significantly higher than scores of 1000 random matrices produced by shuffling the order of sites (mean score 0.6168, P = 0.30), suggesting that recombination among these regions has been rare.

We then used the split decomposition method to detect possible conflicting phylogenetic signals (Bandelt and Dress 1992). NeighborNet Network analysis of the 34 PI SNP sites revealed two parallel paths indicative of the presence of phylogenetic incompatibilities in the divergence of *M. ulcerans* strains, which could arise from recurrent mutation or recombination in the genomes. However, one parallel path showed much lower bootstrap support value (65.5%) than the other parallel path (100%). The paths with high bootstrap support values (over 99.5%) showed a tree-like network (Figure 5), suggesting that a bifurcating tree is an appropriate model for constructing strain phylogeny. Therefore, we constructed a minimum evolution tree of *M. ulcerans* strains rooted with *M. marinum* M to estimate the divergence time (Figure 6). We first compared the 3,597 SNP harboring *M. ulcerans* genes with the *M. marinum* M genome and identified 3,059 genes that have homologous genes in *M. marinum* M [42]. We then generated concatenated allelic

sequences of the 3,059 genes for the three test strains and two reference strains (a combined total of 1,032,790 codons in 3,113 kb of coding sequences per strain), based on which a minimum evolution tree of *M. ulcerans* strains was constructed. Estimated divergence time based on comparison of numbers of synonymous substitutions per nucleotide site ($d_S$) in the 1,032,790 allelic codons are shown in Table 4. The calculations were based on the estimated rate of synonymous substitution in bacteria of $5.8\times10^{-9}$ to $7.8\times10^{-9}$ substitution per site per year [43]. Our results suggest that Agy99 and *M. marinum* M diverged from a common ancestor about 1.13 to 1.52 million years ago, which confirms the recent divergence of *M. ulcerans* from its *M. marinum* progenitor, and seems more precise than the earlier estimation of between 1.2 and 4.7 million years [3]. The African classical lineage strain Agy99 and the Japanese ancestral lineage strain Jp8756 diverged about 394 to 529 thousand years ago (Table 4). Although the ancestral lineage was found genetically closer to the progenitor *M. marinum* in regions of difference (RD) composition [6], the SNP data suggested the Japanese strain was closer to the Ghanaian strains than to *M. marinum*. The discrepancy between the SNP phylogeny and the RD phylogeny could indicate that regions of *M. ulcerans* genomes harboring these genetic variations have diversified through different mechanisms at variable rates. The discrepancy could also be due to the small number of strains analyzed in our study. Among Ghanaian *M. ulcerans* strains the divergence times is less than 3,000 years. However, there are uncertainties in our phylogenetic estimates because of rate heterogeneity. The two-cluster test in LINTREE showed that all the interior nodes within the Ghanaian clade evolved at a uniform rate, while the Japanese strain evolved at a significantly different rate (CP = 99.96%). The branch-length test further indicated that the Japanese strain evolved significantly slower than average, and that all three Ghanaian strains evolved significantly faster than average. Thus, the divergence time of three Ghanaian strains is likely to be shorter than estimated here.

**Genetic diversity among Ghanaian clinical isolates**

We are developing hairpin primer SNP assays [44] based on the 173 SNP loci discovered through pairwise comparisons of the three *M. ulcerans* Ghanaian strains and are analyzing our collection of Ghanaian *M. ulcerans* isolates at these loci. At the

current stage it is possible to resolve the nucleotides at 68 SNP loci in 54 Ghanaian strains with 13 distinct SNP haplotypes identified (including the three reference strains). Previously only 3 haplotypes were found in this strain collection by VNTR typing, which was the best resolution achieved [7]. The nucleotide diversity ranged from 0.05 to 0.40 across the 23 sSNP and 31 intergenic SNP loci with an average diversity of 0.09 (Figure 7). Across the 14 non-sSNP, the average nucleotide diversity was 0.06. This level of nucleotide diversity means that two isolates selected at random from this collection will differ at a SNP locus in 6–9% of the cases. The alleles at the sSNP loci were highly nonrandom in their haplotype distribution. This statistical association can be seen in the distribution of the linkage disequilibrium coefficient (D) for 1,176 pairwise comparisons of alleles at 54 sSNP and intergenic SNP loci (Figure 8). A total of 751 (64%) of these comparisons were significant by a chi-squared test, and 600 (51%) were significant using a highly conservative Bonferroni correction for multiple tests [45]. The standardized coefficient of linkage disequilibrium (D′) was strongly U-shaped with 96% of the locus pairs in complete linkage disequilibrium. This observation indicates that there are at most three out of the four possible haplotypes for most locus pairs. It also suggests that recurrent mutation and recombination events have played only a minor role in generating haplotype diversity. Detailed epidemiological analysis of the typing results is out of the scope of this paper and will be summarized elsewhere (manuscript in preparation).

## DISCUSSION

In this study we have sequenced genomes of two Ghanaian and one Japanese *M. ulcerans* disease isolates by using two different massively parallel sequencing platforms. By comparison of genome sequences of these strains with the genome of the Ghanaian reference strain Agy99, we have identified over a hundred SNPs within Ghanaian strains and about 26,000 SNPs in the Japanese strain. The SNPs identified among Ghanaian strains for the first time allow to resolve the population structure and evolutionary relationship of an intra-continental population of *M. ulcerans*. The SNP data confirmed the recent divergence of *M. ulcerans* and *M. marinum* from a common ancestor and refined the estimated time of emergence to about 1.3 million years. We also estimated that the classical *M. ulcerans* lineage (represented by the Ghanaian strains) diverged from the ancestral lineage (represented by the Japanese strain) about 400,000 years ago, which is about the time when the modern human species, *Homo sapiens*, evolved (~250,000 to 400,000 years). However, we need to point out two limiting factors of our current estimates. First is the limited number of strains analyzed. Future studies should include many more strains from different countries and continents representing branches of the two lineages to improve the phylogenetic resolution and accuracy of the dating. Second is that our dating was based on the estimated rate of synonymous substitution in bacteria of $5.8\times10^{-9}$ to $7.8\times10^{-9}$ substitution per site per year, which was not the most appropriate clock rate for calculating the age of genetically monomorphic pathogens [46]. However, till now studies trying to establish the ideal molecular clock calibrated against a fossil record have not yielded much usable new information [46]. The clock rate we used has been used to date other monomorphic pathogens such as *M. ulcerans* [3], *M. tuberculosis* [43],[47] and *Escherichia coli* [48], and our estimated age of *M. ulcerans* is thus still informative and comparable to these previous estimations.

The SNP analysis suggested that the Japanese strain was closer to the Ghanaian strains than to *M. marinum*, which is contradictory to the previous finding based on RD composition, where the ancestral lineage was found genetically closer to the progenitor *M. marinum* [6]. The discrepancy might suggest that regions of *M.*

*ulcerans* genomes harboring these genetic variations have diversified through different mechanisms at variable rates. However, more strains within the ancestral lineage should be analyzed before firm conclusions can be drawn. Among Ghanaian strains the divergence time was less than 3000 years. Two-cluster and branch-length tests in this study revealed that the Ghanaian strains evolved significantly faster than average, thus the actual divergence time within Ghana could be much shorter than our current estimate. *M. ulcerans* was first isolated in 1948 [49], but large ulcers almost certainly caused by *M. ulcerans* have been already described by Sir Albert Cook in 1897. Since the late 1980s the number of reported cases has increased dramatically in West Africa [50],[51],[52],[53] and in Australia [54]. Increased disease incidence has been primarily attributed to factors such as environmental changes, increased exposure of the affected populations, and improved surveillance. VNTR typing results gave indications for the emergence and spreading of new genetic variants of *M. ulcerans* within Ghana [7]. More in-depth phylogenetic and functional analyses are needed to test if mechanisms such as virulence evolution and host adaptation of *M. ulcerans* play a role in the increasing incidence of BU.

Genome comparison of *M. ulcerans* Agy99 and *M. marinum* M revealed that *M. ulcerans* underwent reductive evolution with genomic signatures such as proliferation of ISE, accumulation of pseudogenes, chromosomal rearrangements, genome downsizing, and acquisition of foreign genes by acquisition of plasmids or bacteriophages [4]. Microarray based comparative genomic hybridization with a worldwide set of *M.* ulcerans isolates identified genomic regions of difference and demonstrated that the two major *M. ulcerans* lineages can be distinguished based on the location and size of genomic deletions [5],[6]. Due to the lack of paired-end sequencing data, we did not carry out systematic analysis on chromosomal deletions and rearrangements. But when we tried to close sequencing gaps using the Agy99 chromosome as the reference, there were more successful gap closure reactions in Ghanaian strains than in the Japanese strain. Failed gap closure attempts in Ghanaian strains were mostly around IS elements, while this was not the case for the Japanese strain (data not shown). These results suggest a high frequency of large chromosomal rearrangement events in the Japanese strain compared to the African classical lineage strains. In the two Ghanaian strain, NM31/04 and NM20/02, the

plasmid was fully covered by sequence reads and almost identical to pMUM001 in Agy99, while the plasmid in the Japanese strain was only partially covered with low depth. Altogether these observations suggest a more stabilized genome and a less important role of reductive evolution within *M. ulcerans* Ghanaian strains.

SNP analysis revealed no further pseudogene formation within the Ghanaian strains, and the Ghanaian strains were found to evolve significantly faster than average. While the majority of coding region SNPs found in Jp8756 was synonymous, the majority found in the two Ghanaian strains was non-synonymous. Further functional analysis of genes containing these non-sSNPs would help to elucidate if they could lead to “pathoadaptive” niche expansion, or provide a selective advantage in both sporadic infection and epidemic spread, which have been found in other bacterial pathogens [55],[56] and suggested for *M. ulcerans* [39],[57].

The low genetic diversity and high linkage disequilibrium within Ghanaian isolates supports the hypothesis that the *M. ulcerans* population spread over the African continent has gone through a severe bottleneck during adaptation to a possibly host-specific environment and has not yet accumulated much sequence diversity [15]. SNP typing of Ghanaian isolates was consistent with VNTR typing [7], but allowed to further differentiate between isolates coming from the same BU endemic focus. Closely related, but distinct clonal complexes including strains with minor variation seem to dominate in different BU endemic areas. Diversity of these local clonal complexes is indicative for ongoing microevolution.

Although there has been impressive recent progress in studying the transmission of BU, the precise environmental reservoirs and mode(s) of transmission are not fully understood [58],[59]. High-throughput genotyping platforms, such as Hairpin primer real time PCR assays [44], BeadArray [60] and OpenArray [61] will make genome wide SNP typing a highly discriminatory and cost-effective tool to study *M. ulcerans* evolution and epidemiology at a population scale. More in-depth phylogenetic and phenotypic analyses of a large number of disease isolates and environmental strains (once becoming available) is expected to shed more light into transmission and virulence evolution of *M. ulcerans* after its divergence from *M. marinum*. The study will also help to identify SNPs associated with host specificity and geographical

origins. However, the current set of SNP markers were obtained by comparing genomes of three very closely related Ghanaian strains and one very distant Japanese strain. Future genome sequencing of more representative strains from diverse locations around the world will be necessary to identify additional SNP markers to delineate the origin and spread of *M. ulcerans* at both local and global level.

## MATERIALS AND METHODS

### Bacterial strains and genomic DNA isolation

The three *M. ulcerans* strains sequenced with various sequencing platforms are listed in Table 1. Strain Jp8756 (ATCC 33728) from Japan was provided by Francoise Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Isolation and characterization of strains NM31/04 and NM20/02 from Ghana has been described elsewhere [7],[8],[62],[63]. We used the complete Agy99 genome sequence (chromosome, NC_008611, and the plasmid pMUM001, NC_005916) as the reference sequences. Genomic DNA was isolated as described [64].

### Pyrosequencing of *M. ulcerans* NM20/02

We sequenced the genome of *M. ulcerans* NM20/02 using the Roche 454 Life Sciences Genome Sequencer FLX following the manufacturer's instructions (Roche 454 Life Science, Branford, CT, USA). The shotgun library was prepared with 5 µg genomic DNA using the “Standard DNA Library Preparation Kit” (04852265001, Roche). Nebulized, purified, and adaptors attached single strand DNA fragments were clonally amplified using the “Emulsion PCR Kit I” (04852290001, Roche). Sequencing on the GS FLX was performed using the “Standard LR70 Sequencing Kit” (04932315001, Roche). Images were processed using the “Genome Sequencer FLX Data Processing Pipeline 1.1.02.15”. A total of 424,494 reads with average length of 213 bases were obtained.

We mapped 454 reads to the reference sequence using gsMapper (v1.1.02.15, Roche) to make SNP calls. To identify high confidence SNPs, we filtered substitutions reported by gsMapper using two rules: (1) supported by at least three non-duplicated reads, with at least one aligned in the forward direction and at least one aligned in the reverse direction. (2) Requirement for at least five conserved bases on both sides of the SNPs. We randomly selected 37 SNPs distributed across the genome for PCR and capillary sequencing. 29 SNPs were validated, three were miscalled due to sequencing errors in the Agy99 genome, and five failed due to unspecific PCR reactions (Table S3). After manual inspection, we found that each of

the five SNPs was supported by all the reads (7 to 16) uniquely mapped to the region, thus was likely to be a true SNP. Unspecific PCR reactions might be due to the presence of genes paralogous to genes harboring or flanking the SNPs.

**Solexa sequencing of *M. ulcerans* NM31/04 and Jp8756**

We sequenced the genomes of *M. ulcerans* NM31/04 and Jp8756 with the Illumina Genome Analyzer according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The DNA Colony/Cluster Template Library was prepared using the Illumina "Genomic DNA Sample Prep Kit" (Illumina). Briefly, 5 µg of genomic DNA was broken into fragments of approximately 100 bp by nebulization. After end repairing and adaptor ligation, the samples were gel-purified to recover fragments of 150–250 bp, which were PCR amplified for 15 cycles. For quality control, an aliquot of the library was cloned into a TOPO plasmid. Six clones from each bacterial strain were picked and subjected to capillary sequencing. The DNA Colony/Cluster Template Library was then used for flow-cell preparation using the "Standard Cluster Generation Kit" (Illumina). Sequencing on the Illumina Genome Analyzer was performed using "Genomic DNA sequencing primer V2" for 36 cycles. At the end of the run, images were processed using the "Solexa Data Analysis Pipeline 0.2.2.6". A total of 2.538 and 2.651 million reads of 35 bases in length were obtained for NM31/04 and Jp8756, respectively.

We mapped Solexa reads to the reference sequences using MAQ v0.6.3, which is particularly developed for building mapping assemblies from Illumina Solexa reads. For each read, un-gapped alignment against the reference was performed; all hits with up to 2 mismatches in the first 24 bp were found. Each read was placed to the position where the sum of quality values of the mismatched nucleotides is minimum [33]. For detection of high confidence SNPs, we first filtered the SNP calls reported by MAQ based on two rules: (1) supported by more than three non-duplicated reads; (2) covered by at least one read with a mapping quality higher than 40. We then randomly selected 84 SNPs called in NM31/04 or Jp8756 for PCR and capillary sequencing to determine the cutoff value of the consensus quality. We found that the consensus quality equal or higher than 40 excluded most false positive SNP calls and that the false positive rate was about 1% (Figure S3).

### NimbleGen comparative genome sequencing (CGS) of selective regions in *M. ulcerans* Jp8756

Before next-generation sequencing became widely available, NimbleGen CGS was the cost effective tool for the comparative analysis of microbial genomes to identify SNPs, insertions, or deletions with high speed and accuracy [65]. We first used a mutation mapping array tiling the reference genome to locate potential mutation harboring sites. Then a high density re-sequencing array tiling the putative sites was produced to identify the mutations [66]. The method has been applied successfully to survey the entire or partial genomes of several bacteria [48],[67],[68], including *M. tuberculosis* [69].

For CGS analysis of *M. ulcerans* Jp8756, we selected 1,265 of the 4,160 protein coding genes across the chromosome of Agy99 (1,210,734 bp out of 5,805,761 bp, 20%) and 51 of the 81 protein coding genes on the plasmid pMUM001 (135,612 bp out of 174,155 bp, 78%) (Table S2). These genes include drug resistance genes, known antigens, genes with housekeeping roles, and genes of hypothetical proteins. To avoid cross hybridization and ambiguous SNP calls, we excluded protein coding genes with paralogs in the *M. ulcerans* genome. Highly conserved genes between *M. ulcerans* and its ancestor *M. marinum* were also excluded. Mutation mapping and re-sequencing probes were designed for selected gene using the ArrayScribe software, synthesized using the Maskless Array Synthesis (MAS) technology [70],[71], and printed in a random layout. Genomic DNA samples from Jp8756 and the reference Agy99 were hybridized to the arrays separately following the NimbleGen protocol. Data were analyzed using NimbleScan software (NimbleGen). A list of 1,619 identified SNPs is provided in Table S2.

### SNP validation

We used Sanger sequencing of PCR products to validate a selected subset of SNPs. Primer sequences used for PCR and sequencing are provided in Table S3. PCR products were purified using the NucleoSpin Extract II Kit (Clontech Laboratories, Mountain View, CA).

**SNP typing**

We are developing hairpin primer (HP) assays [44] for SNPs discovered through pairwise comparisons of the three *M. ulcerans* Ghanaian strains (Agy99, NM20/02, and NM31/04) and analyzing our collection of clinical isolates from Ghana [7]. All HP assays were tested on the three reference strains to confirm the presence of each allele and to verify the performance of the SNP assays. Assays on the clinical DNA samples were considered reliable only if the cycle thresholds generated in the paired wells differed by three or more cycles. At the reported stage, it was possible to assign alleles to 68 SNP loci in 54 Ghanaian isolates (including the reference strains) using this approach.

**Phylogenetic analysis**

To identify putative regions of recombination or gene conversion, we used the Reticulate program and constructed a compatibility matrix [41]. We also used the SplitsTree program to detect conflicting phylogenetic information and determine if a bifurcating tree is an appropriate model to construct strain phylogeny [72]. A minimum evolution tree rooted with *M. marinum* was constructed by using the MEGA [73] software based on the numbers of synonymous substitutions per nucleotide site in concatenated SNP harboring protein coding genes in all strains. The numbers of synonymous substitutions per synonymous site were calculated from the concatenated nucleotide sequences using the modified Nei-Gojobori Jukes Cantor method. The Complete deletion method was used for handling alignment gaps. Because rate heterogeneity may have contributed to uncertainty in phylogeny estimates, we applied the two-cluster and branch-length tests in LINTREE [74] to identify significant rate heterogeneity in the phylogeny. The two cluster test was used to test the molecular clock hypothesis for the two lineages above each interior node of a tree and the branch-length test was used to examine the deviation of each root-to-tip branch length relative to the average length [74]. To generate the concatenated sequences, we first determined homologous genes in the *M. marinum* M genome [42] for each of the 3,597 protein coding gene harboring SNPs by standalone BLAST search [75]. Using the threshold of 90% nucleotide sequence identity over a minimum alignment length of 90% of both query and hit genes, 3,059 homologous

genes were identified. Protein sequences of the homologous genes were aligned using the CLUSTALW program [76]. Homologous genes were then aligned using the EMBOSS [77] Tranalign program so that the corresponding amino acid sequence alignment was imposed on the DNA sequence alignment. Allelic genes in *M. ulcerans* strains and homologous genes in *M. marinum* M with alignment gaps were then concatenated.

**Accession numbers**

The sequencing reads of the NM20/02, NM31/04 and Jp8756 genomes have been deposited in the NCBI Short Read Archive database under the accession number SRA008258.

**ACKNOWLEDGMENTS**

We thank the Michigan State University Research Technology Support Facility, Fasteris, and Roche NimbleGen for sequencing services, and the $[BC]^2$ Basel Computational Biology Center at the Biozentrum of the University of Basel for hardware and software support. We thank David Alland, Manzour Hernando Hazbón, and Alifiya S. Motiwala at the University of Medicine and Dentistry of New Jersey, as well as David W. Lacher at the US Food and Drug Administration for original protocols on hair-pin primer design and real time PCR experiments. We also thank Francoise Portaels at the Institute of Tropical Medicine, Antwerp, for providing us with strain ITM Japan8756, and Dieter Ebert of the Zoological Institute at the University of Basel for helpful discussions and a critical reviewing of the manuscript.

# TABLES

## Table 1. *Mycobacterium ulcerans* strains sequenced in this study.

| Strain | Year of isolation | Place of origin | MIRU 1 allele[2] | STI allele[2] |
|---|---|---|---|---|
| NM20/02 | 2002 | Ga District, Greater Accra region, Ghana | B | BD |
| NM31/04 | 2004 | Amansie West District, Ashanti region, Ghana | BAA | C |
| Jp8756 | 1980 | Japan | nd[3] | CF |
| Agy99[1] | 1999 | Ga district, Greater Accra region, Ghana | BAA | BD |

[1]Reference strain
[2]Hilty et al., 2006 [7]
[3]Not determined
doi:10.1371/journal.ppat.1000580.t001

## Table 2. Summary of next generation sequencing results.

| Strain | | NM20/02 | NM31/04 | Jp8756 |
|---|---|---|---|---|
| Sequencing method | | Roche 454 GS FLX | Illumina Solexa GA | Illumina Solexa GA |
| Total no. Reads | | 424,494 | 2,538,429 | 2,651,276 |
| Averaged read length (nt) | | 213 | 35 | 35 |
| Total sequences (nt) | | 90,299,836 | 88,845,015 | 92,794,660 |
| Map to Agy99 chromosome | Total no. reads mapped (%) | 382,116 (90.01) | 2,343,269 (92.31) | 2,279,741 (85.99) |
| | % genome mapped | 93.72 | 99.99 | 94.47 |
| | Average depth of mapped regions | 14.5 | 14.1 | 13.6 |
| Map to Agy99 plasmid pMUM001 | Total no. reads mapped (%) | 5,269 (1.24) | 326,541 (12.86) | 89,325 (3.37) |
| | % genome mapped | 32.56 | 100 | 20.35 |
| | Average depth of mapped regions | 19.8 | 63.4 | 17.2 |

doi:10.1371/journal.ppat.1000580.t002

## Table 3. Genes potentially under selection ordered by SNP density.

| Locus_tag | Locus | Product | COG | SNP density (bp per SNP) | Selection[1] |
|---|---|---|---|---|---|
| MUL_3769 | - | hypothetical protein | - | 39 | |
| MUL_4312 | - | hypothetical protein | - | 41 | – |
| MUL_4235 | - | hypothetical protein | - | 45 | |
| MUL_5054 | *esxE* | ESAT-6 like protein EsxE | COG1314U | 46 | + |
| MUL_3425 | *mmr* | multidrug-transport integral membrane protein Mmr | - | 46 | + |
| MUL_1135 | - | hypothetical protein | COG1902C | 46 | |
| MUL_5072 | *gid* | glucose-inhibited division protein B Gid | - | 47 | |
| MUL_4359 | - | PE family protein | COG0357M | 48 | |
| MUL_3746 | *glbO* | globin (oxygen-binding protein) GlbO | - | 48 | – |
| MUL_4906 | - | hypothetical protein | - | 50 | |
| MUL_0630 | - | hypothetical protein | COG0500QR | 51 | |
| MUL_5017 | - | hypothetical protein | - | 52 | |
| MUL_4764 | - | hypothetical protein | COG0500QR | 54 | |
| MUL_2201 | - | hypothetical protein | - | 55 | |
| MUL_0760 | - | hypothetical protein | - | 56 | |
| MUL_3596 | - | hypothetical protein | - | 56 | |
| MUL_1662 | *gloA* | lactoylglutathione lyase, GloA | - | 56 | |
| MUL_2106 | - | hypothetical protein | COG0315H | 57 | + |
| MUL_4509 | - | hypothetical protein | - | 57 | |
| MUL_4133 | *dsbF* | lipoprotein DsbF | - | 58 | |
| MUL_0355 | - | PE-PGRS family protein family protein | COG2346R | 60 | + |
| MUL_3885 | *echA4_2* | enoyl-CoA hydratase, EchA4_2 | COG1773C | 60 | |
| MUL_5108 | - | transposase | COG1119P | 60 | |
| MUL_0384 | - | hypothetical protein | - | 61 | |
| MUL_5010 | - | phosphoglycerate mutase | COG0406G | 62 | |
| MUL_0161 | - | hypothetical protein | COG2076P | 63 | + |
| MUL_4655 | *cysE_1* | serine acetyltransferase CysE_1 | COG1278K | 63 | |
| MUL_2839 | - | hypothetical protein | - | 64 | |
| MUL_2263 | - | hypothetical protein | COG0793M | 64 | |
| MUL_1479 | *trxB1* | thioredoxin TrxB1 | COG0526OC | 66 | |
| MUL_4386 | - | hypothetical protein | COG0620E,COG1309K | 67 | |
| MUL_5055 | *esxF* | ESAT-6 like protein EsxF | COG4842S | 67 | |
| MUL_0327 | - | oxidoreductase | COG1028IQR | 67 | |
| MUL_3206 | - | hypothetical protein | - | 67 | |
| MUL_3717 | - | hypothetical protein | - | 68 | |
| MUL_0010 | - | hypothetical protein | - | 70 | |
| MUL_3264 | *fdxA_1* | ferredoxin FdxA_1 | COG1146C | 70 | |
| MUL_1435 | - | exported protein | COG0704P | 70 | – |
| MUL_3277 | - | hypothetical protein | - | 71 | |
| MUL_3581 | - | phage-related integrase | COG0582L | 71 | |
| MUL_1216 | *tam* | trans-aconitate methyltransferase Tam | COG1522K | 72 | |
| MUL_2917 | - | hypothetical protein | - | 72 | |
| MUL_1771 | - | hypothetical protein | COG1670J | 72 | |
| MUL_0951 | - | hypothetical protein | - | 73 | |
| MUL_0889 | - | hypothetical protein | - | 73 | |
| MUL_0993 | - | transcriptional regulatory protein | COG0236IQ | 73 | |
| MUL_4846 | - | hypothetical protein | - | 73 | – |
| MUL_0366 | *mcmA2b* | methylmalonyl-CoA mutase alpha subunit, McmA2b | - | 73 | – |
| MUL_1003 | - | hypothetical protein | - | 74 | |
| MUL_4682 | - | hypothetical protein | COG3391S | 74 | |

| Locus_tag | Locus | Product | COG | SNP density (bp per SNP) | Selection[1] |
|---|---|---|---|---|---|
| MUL_4870 | - | short chain dehydrogenase | COG1028IQR | 74 | |
| MUL_0457 | - | hypothetical protein | COG2351R | 74 | |
| MUL_5109 | - | hypothetical protein | - | 75 | |
| MUL_0761 | - | hypothetical protein | - | 76 | |
| MUL_1274 | *lppN* | lipoprotein LppN | - | 76 | |
| MUL_2490 | - | hypothetical protein | - | 76 | |
| MUL_0820 | - | methyltransferase | COG0500QR,COG2226H | 76 | |
| MUL_2645 | - | AsnC family transcriptional regulator | COG0526OC | 76 | – |
| MUL_3440 | - | hypothetical protein | COG2185I | 77 | |
| MUL_3194 | - | hypothetical protein | COG2261S | 77 | |
| MUL_0424 | - | hypothetical protein | - | 77 | |
| MUL_5032 | *mpt64* | immunogenic protein Mpt64 | COG0425O | 77 | |
| MUL_4365 | - | hypothetical protein | COG0393S | 77 | |
| MUL_4394 | - | hypothetical protein | COG0526OC | 78 | |
| MUL_3305 | *ribD* | hypothetical protein | COG1985H | 78 | |
| MUL_3524 | - | diphosphomevalonate decarboxylase | COG3407I | 78 | |
| MUL_0217 | *lpqV* | lipoprotein LpqV | - | 78 | |
| MUL_0241 | *bioF2_1* | 8-amino-7-oxononanoate synthase BioF2_1 | COG0156H | 79 | |
| MUL_5058 | - | hypothetical protein | - | 79 | |
| MUL_4336 | - | PE family protein | - | 79 | |
| MUL_4899 | - | hypothetical protein | - | 80 | – |
| MUL_4670 | - | hypothetical protein | - | 82 | – |
| MUL_2937 | - | ArsR-type repressor | COG1846K | 82 | – |
| MUL_0017 | *trpG* | para-aminobenzoate synthase component II | COG1695K | 86 | – |
| MUL_5067 | *trxC* | thioredoxin TrxC | COG1522K | 89 | – |
| MUL_2060 | - | hypothetical protein | - | 92 | – |
| MUL_0670 | *rimL* | acetyltransferase, RimL | COG0664T | 92 | – |
| MUL_4330 | - | hypothetical protein | - | 96 | – |
| MUL_0430 | - | hypothetical protein | COG2608P | 99 | – |
| MUL_1434 | - | hypothetical protein | - | 99 | – |
| MUL_0058 | - | transcriptional regulatory protein | COG1670J | 101 | – |
| MUL_1897 | - | ABC transporter ATP-binding protein | - | 105 | – |
| MUL_5123 | - | hypothetical protein | COG3576R | 105 | – |
| MUL_4776 | - | hypothetical protein | COG1309K | 107 | – |
| MUL_2966 | - | hypothetical protein | - | 110 | – |
| MUL_5035 | - | hypothetical protein | COG0792L | 111 | – |
| MUL_0441 | *phoY2* | phosphate-transport system regulatory protein, PhoY2 | - | 112 | – |
| MUL_1835 | *secG* | preprotein translocase subunit SecG | - | 117 | – |
| MUL_0825 | - | hypothetical protein | COG1359S | 122 | – |
| MUL_4918 | *mce6B* | MCE-family protein Mce6B | COG1463Q | 129 | – |
| MUL_2369 | - | hypothetical protein | - | 132 | – |
| MUL_0065 | - | hypothetical protein | COG2353S | 136 | – |
| MUL_0035 | - | DNA-binding protein | COG1045E | 136 | – |
| MUL_3556 | - | integral membrane protein | COG0454KR | 140 | – |
| MUL_0015 | - | putative septation inhibitor protein | COG4842S | 141 | – |
| MUL_2243 | *esxR* | ESAT-6 family protein | - | 146 | – |
| MUL_4627 | - | hypothetical protein | - | 147 | – |
| MUL_2232 | - | molecular chaperone (small heat shock protein) | COG3585H | 150 | – |
| MUL_3337 | - | hypothetical protein | - | 152 | – |
| MUL_3714 | *rplU* | 50S ribosomal protein L21 | COG0261J | 156 | – |

| Locus_tag | Locus | Product | COG | SNP density (bp per SNP) | Selection[1] |
|---|---|---|---|---|---|
| MUL_3030 | *ureB* | urease beta subunit UreB | COG0832E | 156 | – |
| MUL_4051 | - | hypothetical protein | - | 165 | – |
| MUL_4256 | *whiB4* | transcriptional regulatory protein Whib-like WhiB4 | - | 183 | – |

[1]"+" represents potential diversifying selection indicated by $p_N - p_S$ values higher than mean + 3× standard deviation; "−" represents potential negative selection indicated by $p_N - p_S$ values lower than mean − 3× standard deviation.
doi:10.1371/journal.ppat.1000580.t003

**Table 4. Estimation of time of divergence between *M. ulcerans* Agy99 and other *M. ulcerans* and *M. marinum* strains.**

| Strain | $d_s$[1] | Est. divergence time[2] (yr) | Est. divergence time[3] (yr) |
|---|---|---|---|
| NM31/04 | 0.000017 ± 0.000004 | 1,466 ± 345 | 1,090 ± 256 |
| NM20/02 | 0.00003 ± 0.000006 | 2,586 ± 517 | 1,923 ± 385 |
| Jp8756 | 0.006141 ± 0.000081 | 529,397 ± 6,983 | 393,654 ± 5,192 |
| *M. marinum* M | 0.017642 ± 0.000138 | 1,520,862 ± 11,897 | 1,130,897 ± 8,846 |

[1]mean ± standard error
[2]Based on the rate of synonymous substitution of $5.8 \times 10^{-9}$ per site per year
[3]Based on the rate of synonymous substitution of $7.8 \times 10^{-9}$ per site per year
doi:10.1371/journal.ppat.1000580.t004

**FIGURES**

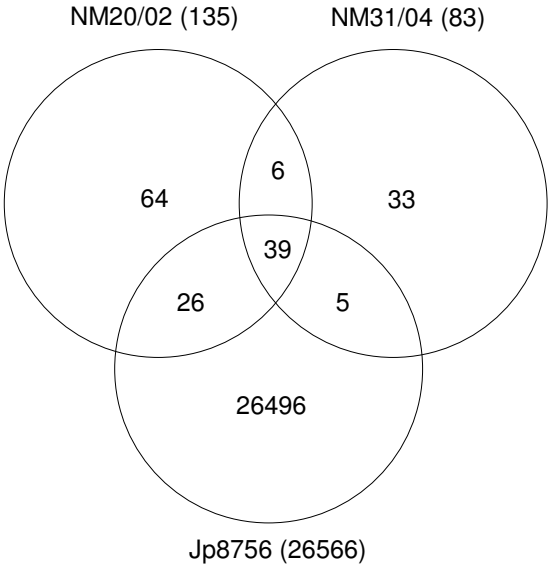


**Figure 1. Venn diagram of single nucleotide polymorphisms in *M. ulcerans* strains.**

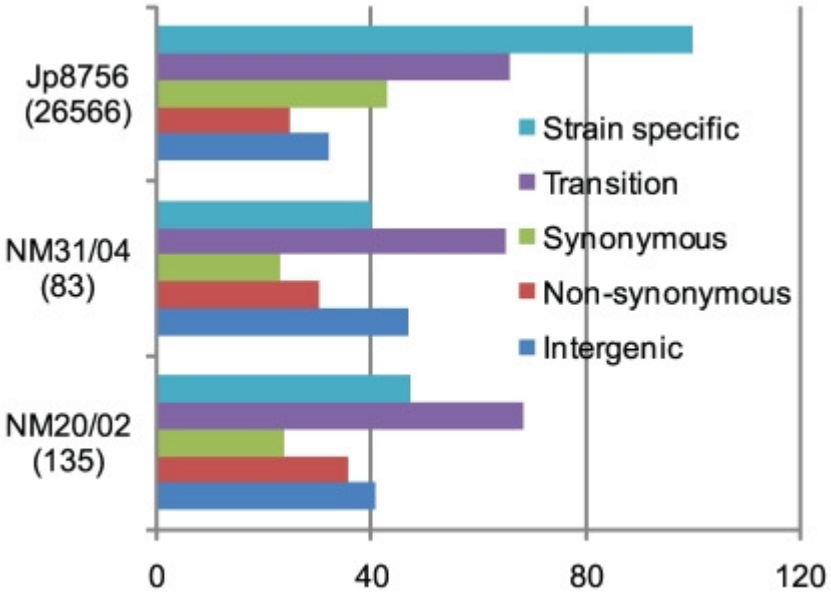


**Figure 2. Summary of single nucleotide polymorphisms in *M. ulcerans* strains.** The number in parentheses represents the total number of SNPs in each sequenced strain. The color bars show the distributions of different SNP categories in each strain.

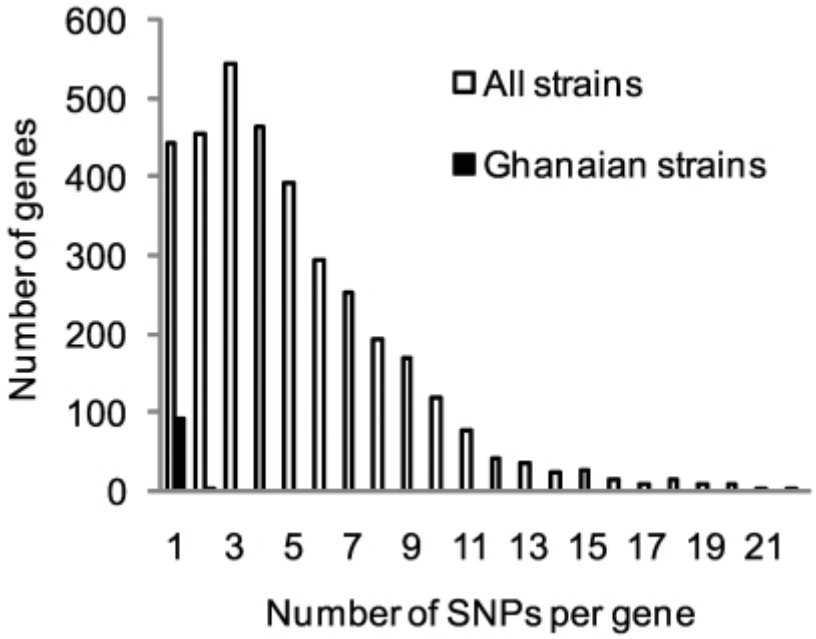


**Figure 3. Distribution of number of SNPs per gene.**

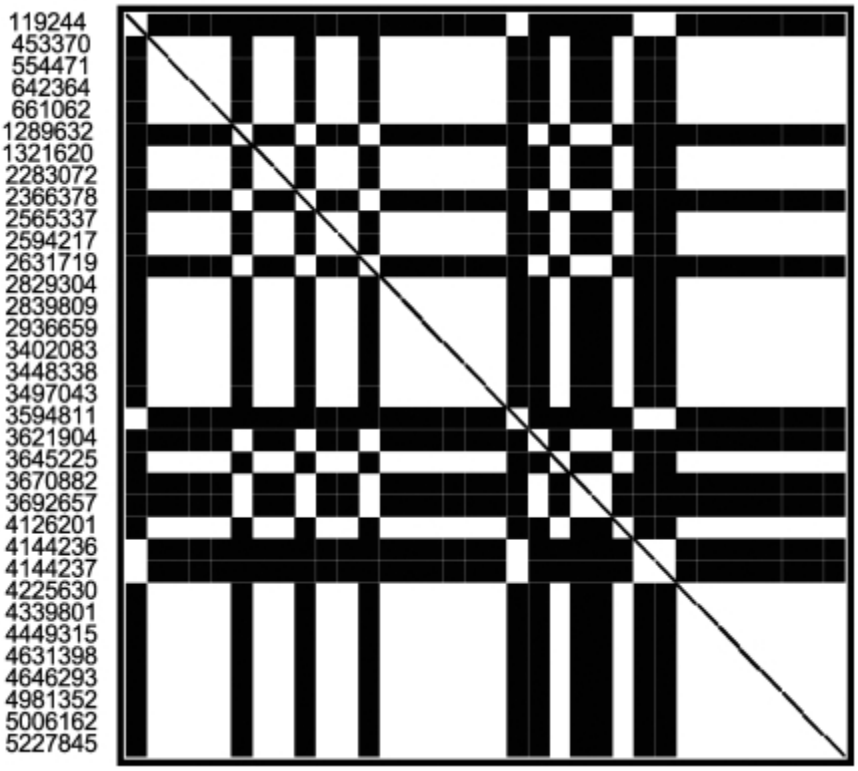


**Figure 4. Compatibility matrix of parsimony informative SNPs.** The genome positions are numbered to the left of the matrix. Black squares indicate incompatible sites, where nucleotide changes are inferred to have occurred multiple times either due to recombination or repeated mutation. White squares represent compatible sites, at which all nucleotide changes can be inferred to have occurred only once in a phylogeny.

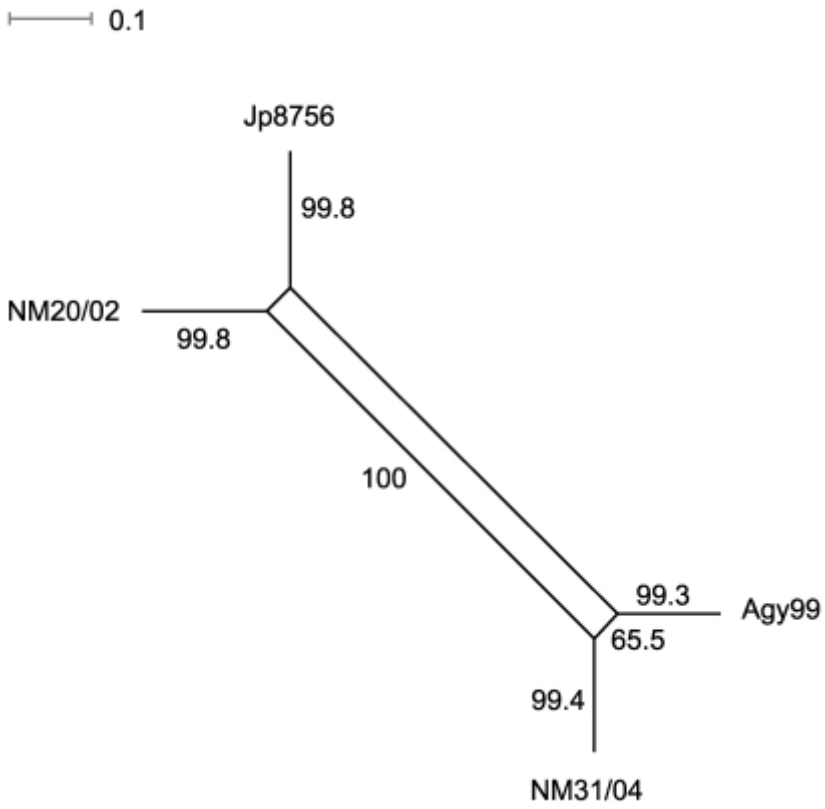


**Figure 5. NeighborNet network of the *M. ulcerans* strains based on the parsimony informative SNPs.** Bootstrap values shown close to branches are based on 1000 bootstrap replicates.

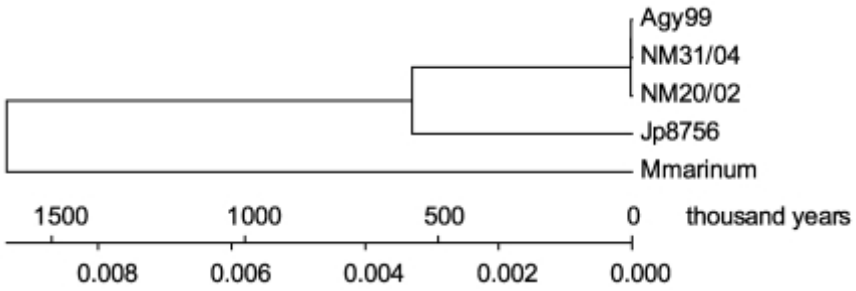


**Figure 6. Minimum evolution tree based on 1,032,790 allelic codons of the *M. ulcerans* and *M. marinum* strains.** The scale shows the divergence time frame and the number of synonymous substitutions per nucleotide site. The rate of synonymous substitution used for time calibration was $5.8\times10^{-9}$ substitution per site per year.

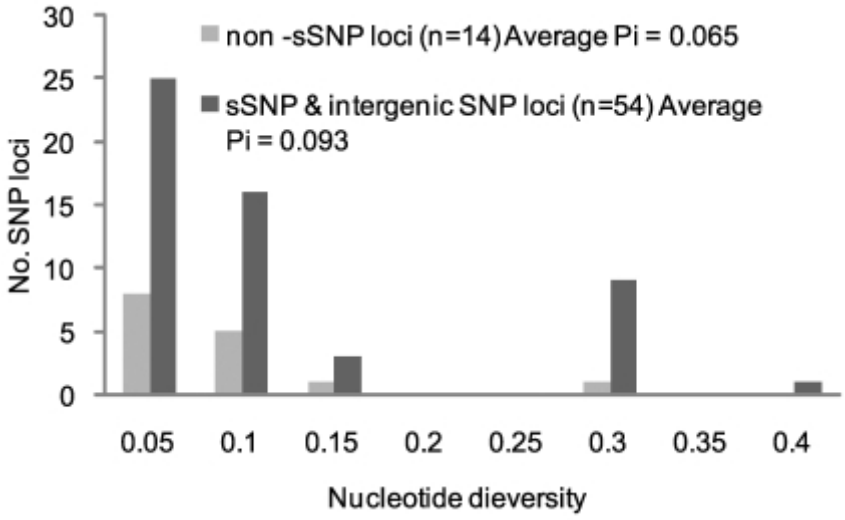


**Figure 7. Nucleotide diversity among SNPs identified through genome comparison of three Ghanaian strains, for which complete SNP data have been collected in 54 Ghanaian *M. ulcerans* strains.**

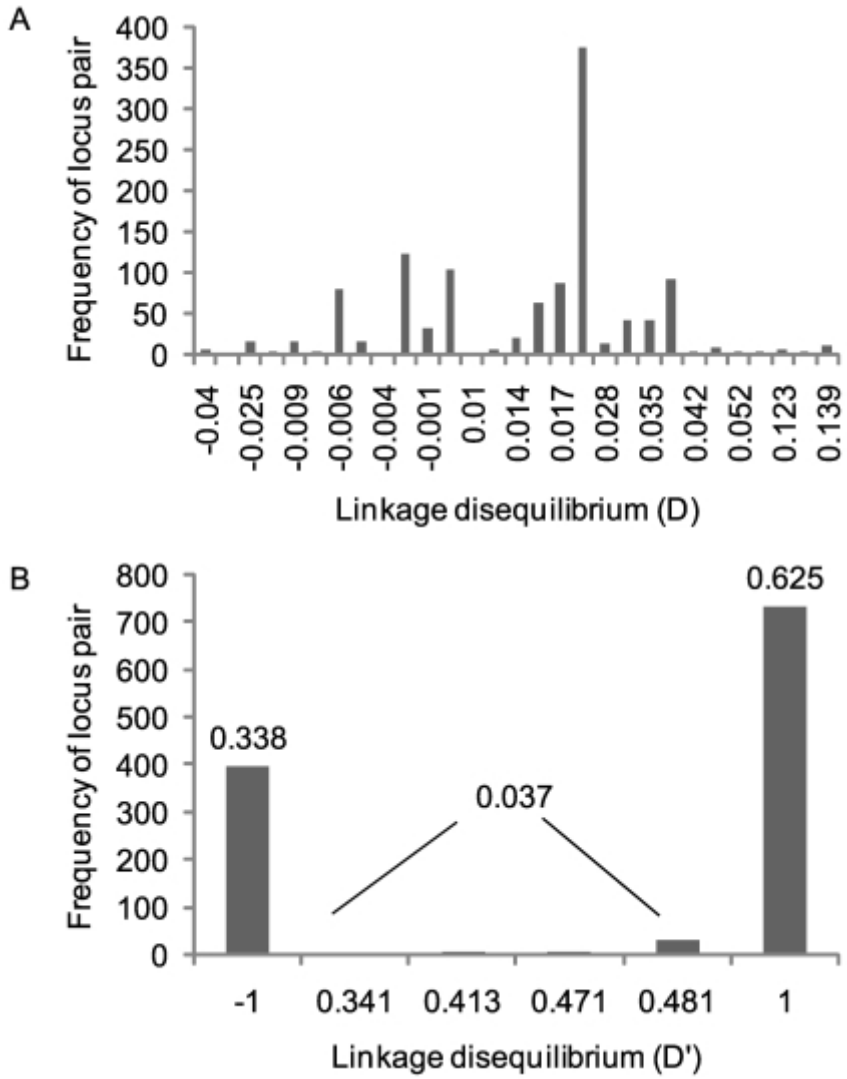


**Figure 8. Linkage disequilibrium among study loci.** A). The distribution of the linkage disequilibrium coefficient (D) for 1,176 pairwise comparisons of alleles at 54 sSNP and intergenic SNP loci. A total of 751 (64%) of these comparisons are significant by a chi-squared test, and600 (51%) remained significant using a Bonferroni correction for multiple tests. B). The distribution of the standardized coefficient of linkage disequilibrium (D'). Ninety-six percent of the locus pairs are in complete linkage disequilibrium.

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CHAPTER 3

# Single Nucleotide Polymorphism Typing of *Mycobacterium ulcerans* Reveals Focal Transmission of Buruli Ulcer in a Highly Endemic Region of Ghana

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This article has been published (July 20, 2010) in:
*Public Journal of Library Neglected Tropical Diseases*

**ABSTRACT**

Buruli ulcer (BU) is an emerging necrotizing disease of the skin and subcutaneous tissue caused by *Mycobacterium ulcerans*. While proximity to stagnant or slow flowing water bodies is a risk factor for acquiring BU, the epidemiology and mode of *M. ulcerans* transmission is poorly understood. Here we have used high-throughput DNA sequencing and comparisons of the genomes of seven *M. ulcerans* isolates that appeared monomorphic by existing typing methods. We identified a limited number of single nucleotide polymorphisms (SNPs) and developed a real-time PCR SNP typing method based on these differences. We then investigated clinical isolates of *M. ulcerans* on which we had detailed information concerning patient location and time of diagnosis. Within the Densu river basin of Ghana we observed dominance of one clonal complex and local clustering of some of the variants belonging to this complex. These results reveal focal transmission and demonstrate, that micro-epidemiological analyses by SNP typing has great potential to help us understand how *M. ulcerans* is transmitted.

## AUTHOR SUMMARY

*Mycobacterium ulcerans* causes a destructive skin disease known as Buruli ulcer (BU), which has been reported from more than 30 tropical or subtropical countries, with the highest prevalence in Western Africa. Due to the striking genetic monomorphism of African *M. ulcerans* populations, conventional genetic fingerprinting methods have largely failed to differentiate isolates coming from the same BU endemic area. Here we report a highly discriminatory fingerprinting method for *M. ulcerans* using a single nucleotide polymorphism-based genetic fine-typing technique. This method has enabled us for the first time to identify different *M. ulcerans* haplotypes within a BU endemic area. Linking the origins of *M. ulcerans* strains with the patients` residences unveiled the clustering of unique *M. ulcerans* haplotypes within the Densu river basin of Ghana. Results show, that haplotypes do not spread within a short time over the entire BU endemic region, but rather form independent focal transmission clusters.

## INTRODUCTION

Infection with *Mycobacterium ulcerans* causes a chronic, necrotizing disease of the skin and the subcutaneous adipose tissue commonly known as Buruli ulcer [1]. This serious infectious disease remains a major health problem in many parts of the world, but in particular, in Western and Central Africa [2]. In spite of considerable research efforts made during the past few years transmission and environmental reservoirs of *M. ulcerans* are still incompletely characterized [1]. Endemic foci are usually linked to wetlands and riverine areas, which has lead to the assumption that *M. ulcerans* is an environmental mycobacterium and that micro-traumata of the skin may initiate infection [3]. However, isolation of the slow growing *M. ulcerans* from an environmental source has been achieved only once so far, from an aquatic insect [4]. PCR screening of environmental samples for the presence of *IS2404* has implicated insects such as biting aquatic hemiptera and mosquitoes in the transmission of *M. ulcerans* [5-7], but their positivity for *M. ulcerans* DNA in polymerase chain reaction (PCR) tests may be only an indicator for the presence of *M. ulcerans* or other genetically closely related mycobacteria in the environment. Although BU is known to develop in all age groups with a nearly equal gender distribution, children 15 years of age or younger make up at least 50% of all cases in Africa [8]. Occasional clustering of cases within families may reflect a common source of infection or increased genetic susceptibility to infection rather than human-to-human transmission. Seroepidemiological studies have indicated that infection with *M. ulcerans* may lead to disease only in a minority of exposed individuals [9].

Many genetic fingerprinting methods have been applied for *M. ulcerans*, including *IS2404*, *IS2606* and *IS2426* PCR [10,11], amplified fragment length polymorphism analysis (AFLP) [12], *IS2404* restriction fragment length polymorphism analysis (RFLP) [13,14], multi-locus sequence typing (MLST) [15-17], variable-number tandem repeat analysis (VNTR) [18-21], *IS2404-Mtb2* PCR [22], and large sequence polymorphisms [23]. Among these, AFLP [12] and VNTR typing [19,21] were the only methods to reveal any genetic diversity among African strains.

MLST, which has now been developed for more than 50 microbial taxa [24], revealed extremely low levels of polymorphisms in several protein coding genes of African *M. ulcerans* strains [16,17]. Analyses of the population structure of bacterial pathogens such as *M. tuberculosis* [25], *Yersinia pestis* [26] or *Salmonella enterica Typhi* [27] have shown that single nucleotide polymorphism (SNP) typing is the most suitable fine-typing method for genetically monomorphic species [24]. This prompted us to develop a SNP typing method for *M. ulcerans* strains from a BU endemic area of Ghana. We selected two Ghanaian patient isolates representing two different Ghanaian VNTR types [19] for genome re-sequencing and compared obtained sequences with the published genome sequence of the reference strain Agy99 [28]. Whole genome comparison between these three strains detected 173 SNPs in total [29], which were used for the establishment of amplification refractory mutation system (ARMS) real-time PCRs using hairpin-shaped primers [30]. Typing of 74 strains isolated from patients living in the BU endemic Densu river basin at 65 SNP loci revealed the presence of five haplotypes in addition to the Agy99 reference haplotype. Sequencing of 4 additional strains chosen on the basis of detected haplotypes enabled further differentiation of isolates. Location of the homes of patients from whom the strains were isolated facilitated a phylogeographic analysis of haplotype distribution.

## MATERIALS AND METHODS

### Ethics statement

In the present study, *M. ulcerans* isolates were obtained from BU diagnostic samples. Data were analyzed anonymously and bacterial isolates delinked from the patients from whom they originated. Ethical approval to use the diagnostic specimens for immunological and microbiological research was obtained from the ethical review board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. Written informed consent was provided by all patients for standard surgical treatment and anaesthesia. In addition the ethical review board requested written informed consent for taking blood samples for immunological research on a special consent form, but not for potential later investigations of bacterial isolates generated during standard diagnostic procedures.

### Mycobacterial strains and genomic DNA extraction

A total of 74 *M. ulcerans* patient isolates from a BU endemic area located in the Ga West, Ga East and Akuapim South Districts were included in the SNP typing analysis. Patients were aged 2 - 75 years, while 71% of the patients were younger than 15 years. In addition, *M. ulcerans* patient isolates from the Ashanti Region (Amansie West District) of Ghana and from other West-African countries (Ivory Coast, Togo, Benin, Democratic Republic of Congo and Angola) were enclosed for further SNP typing analyses (Table 1). The complete Agy99 genome sequence published in 2007 [28] and re-sequenced genomes of isolates NM20/02, NM31/04 [29], NM14/01, NM43/02, NM49/02 and NM54/02 were used as reference sequences. The genomes of strains NM14/01, NM43/02, NM49/02 and NM54/02 were analyzed at Monash University using an Illumina GAIIx Genome Analyzer. A 100x coverage per genome was obtained on average. The Short Read Mapping Package (SHRiMP) software was used for aligning the genomic reads against the target Agy99 genome. For SNP identification the Nesoni software was used. Genomic DNA was isolated by cell wall disruption and phenol-chloroform extraction as described previously [31].

**SNP typing**

Real-time PCR hairpin primer (HP) assays [30] were used to detect SNPs in the 74 Ghanaian *M. ulcerans* strains. Real-time PCR was performed using Power SYBR green 1x PCR Master Mix (Applied Biosystems), 5 ng genomic DNA and 0.3 µM forward and reverse primers each in a total volume of 25 µl. Reactions were carried out in a Step One Plus Real-time PCR system (Applied Biosystems) with a 96-well block. Thermal conditions were as follows and as described previously [30]: stage 1, 95°C for 10 min, 70°C for 30 s; stage 2, 72°C for 30 s, 95°C for 20 s, 69°C for 30 s, lowering one degree in the last step for every cycle during 10 cycles; stage 3, 72°C for 30 s, 95°C for 20 s, and 60°C for 30 s, repeated 40 times; Melt curve stage, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Data were collected in the last step of stage 3 and after the Melt curve stage for analysis with the Step One Software version 2.0 (Applied Biosystems). SNPs were detected by ARMS assays and for each assay two PCRs with two sets of PCR primers were performed in parallel. Each PCR reaction contains SNP-specific primers, which are designed to be either fully complementary to the DNA template or mismatched at the 3`end nucleotide. As reactions with totally complementary primers have a more rapid developing fluorescence curve and an earlier cycle-threshold, differences between the two reactions allow the detection of SNPs.

The hairpin-shaped primers were designed as described previously [30]. In the first step linear primers were designed with Primer3 [32] to produce short amplicons (30 to 90 bp) and to anneal between 60 and 65°C. A tail was added to the 5' end of the SNP-detecting primer in order to produce a stem with the 3' end of the primer. The stem was designed with mfold software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) to have a melting temperature of 67 to 70°C with a free energy of between -0.5 and -2.0. Primers are provided in Table S1.

**Validation of SNP typing assays**

Assays were validated on the published genome Agy99 as well as the reference strains NM20/02, NM31/04, NM14/01, NM43/02, NM49/02 and NM54/02 to confirm

the presence of each allele and to verify the performance of SNP assays. Assays with genomic DNA samples from clinical isolates were considered reliable only if the cycle thresholds generated in the paired wells differed by three or more cycles and if the melting curves of paired wells were coherent. ARMS assays with low discriminatory power were not included into the standard set of 65 SNP typing assays. Sanger DNA sequencing of PCR products was used to validate a selected subset of SNPs. Primers used for PCR and sequencing were designed using Primer3 [32] software. PCR was performed using FirePol 10x buffer and 0.5 µl FirePolTaq-Polymerase (Solis BioDyne), 5 ng genomic DNA, 0.72 µM forward and reverse primers each, 2mM $MgCl_2$ and 0.4 mM dNTPs (Sigma) in a total volume of 25 µl. PCR reactions were carried out in a Gene Amp PCR System 9700 PCR machine (Applied Biosystems). Thermal conditions for PCR amplification of *M. ulcerans* genomic DNA were as follows: initial denaturation step, 94°C for 5 min; 32 cycles: 94°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 1 min (elongation); final extension step, 72°C for 10 min. PCR products were analyzed on 1% agarose gels. PCR products were purified using the Nucleo Spin Extract II Kit (Macherey-Nagel). Sequencing of purified PCR products was done by Macrogen (World Meridian Venture Center, Seoul/Korea). Sequencing was conducted by the Sanger method using BigDye™ terminator cycling conditions using the Automatic Sequencer 3730xl (Applied Biosystems).

Several random SNP loci were validated by Sanger sequencing in order to verify the ARMS approach for a differentiation of *M. ulcerans* isolates. Additionally, validation of pivotal SNPs loci revealing different haplotypes within the endemic area around the Ga District as well as differences between Amansie West District strains and isolates from other African countries was carried out. 100% of 36 randomly chosen SNP loci tested in reference strains Agy99, Nm20/02 and Nm31/04 were reconfirmed by Sanger sequencing. 100% of 70 significant SNP loci dividing Ga District strains into 5 haplotypes other than Agy99 and distinguishing Amansie West District as well as African strains were likewise verified by Sanger sequencing. In contrast, four real-time PCR typing results, which were validated because of unique allele occurrences in certain Ga District isolates diverged from reconfirmatory Sanger sequencing

analysis. Subsequent repetition of real-time PCRs in duplicates revised the initial real-time PCR analyses and confirmed Sanger sequencing results.

**Phylogenetic analysis**

MEGA software version 4.1 (beta) [33] was used to reconstruct the neighbor-joining tree based on SNP typing data (Phylogeny Test and options: Bootstrap 1000 replicates; Gaps/Missing Data: Complete Deletion; Codon Positions: 1st+2nd+3rd+Noncoding; Model: Nucleotide, Number of differences; Substitutions to include: Transitions + Transversions; Pattern among lineages: Same = Homogeneous; Rates among sites: Uniform rates).

We created a map of West Africa by using the map creator tool of Epi Info version 3.5.1 in order to illustrate detected SNP patterns in different African countries.

## RESULTS

### Development of ARMS SNP typing assays

Recently we have compared 454 and Illumina genome sequencing data of *M. ulcerans* patient isolates NM20/02 and NM31/04 originating from two different BU endemic areas of Ghana with the published genome sequence of the Ghanaian strain Agy99 [29]. Based on the identification of 173 SNPs we have developed medium-throughput ARMS-based real-time PCR SNP assays with hairpin-shaped (HP) primers. Initial ARMS assays were successful for 108 of 173 detected SNP loci at predefined optimal conditions. We were able to discriminate alleles under a single standard condition at 73/108 (67.6%) SNP loci. This is close to the success rate of 72.4% reported by Hazbon and Alland for their first round of HP-assay design [30]. Real-time PCR analysis revealed sequencing errors in the Agy99 reference sequence at eight of these loci, which were thus not suitable for further typing analyses. Redesign of the 100 initially failed assays and ongoing whole-genome sequencing of additional isolates will increase the pool of discriminatory SNP assays for future analyses of the population structure of African *M. ulcerans*. Developed SNP assays were used to type a collection of strains from two different BU endemic areas of Ghana and other African countries (Figure 1).

### Different clonal complexes of *M. ulcerans* dominate in the BU endemic regions of Africa

SNP typing of three strains from the Amansie West District of Ghana including the sequenced Amansie West reference strain NM31/04 revealed differences at 24 of the 65 SNP loci analyzed (37%) between these isolates (Figure 1B). In comparison to Agy99 differences at 29, 27 and 5 loci were found. A neighbor-joining tree analysis sub-grouped haplotypes from the two different BU endemic areas of Ghana into two clades (Figure 2). Clade 1 comprises strains isolated between 2001 and 2007 in the Densu river basin, which could be differentiated into 6 haplotypes (Figure 1A). All Amansie West District isolates are sub-grouped together with the 1999 isolate Agy99 into clade 2 (Figure 2).

Typing of patient isolates from other BU endemic African countries at the 65 SNP loci yielded 2 of 15 strains with patterns similar to haplotypes found in Ghana (Figure 1A and B). The only strain available from Togo had a haplotype which differed at only one locus from haplotype 6 found in the Densu river basin. One of the two analyzed strains from the Ivory Coast had a haplotype similar to the haplotypes of strains from the Amansie West District of Ghana. The other isolate from the Ivory Coast as well as all seven analyzed strains from Benin, three strains from the Democratic Republic of Congo (DRC) and two strains from Angola shared a distinct SNP pattern when compared to the Ghanaian isolates (Figure 1A and B). Thus, SNP typing results of clinical isolates from Ghana and isolates from other African countries revealed a neighbor-joining tree with 3 main branches. The strain from Togo is sub-grouped together with haplotypes 2-6 into clade 1, while strain 1 from the Ivory Coast is classed with Amansie West District isolates and Agy99 into clade 2. The other 13 West-African strains are sub-grouped into clade 3 (Figure 2).

**SNP typing of *M. ulcerans* patient isolates from a BU endemic area of Ghana identifies ten haplotypes belonging to a dominating clonal complex**

Using the 65 established ARMS assays we SNP-typed 74 *M. ulcerans* patient isolates collected between 2001 and 2007 from the BU endemic Densu river basin of Ghana, from which the sequenced strains Agy99 and NM20/02 originated. Within this group of 74 strains of common geographical origin, differences at 14 of the 65 SNP loci tested (22%) were observed (Figure 1A). Altogether five haplotypes (designated haplotypes 2-6) other than the Agy99 associated haplotype 1 could be distinguished. Haplotypes 2 - 6 differed at 41, 43, 47, 48 and 55 of the 65 analyzed SNP loci from haplotype 1 (strain Agy99), respectively.

Based on detected haplotypes we re-sequenced 4 representative strains NM14/01 (haplotype 5), NM43/02 (haplotype 3), NM49/02 (haplotype 6) and NM54/02 (haplotype 4) in order to further differentiate strains from the same haplotype. We detected new unique SNPs in NM43/02 (26 SNPs), NM54/02 (11 SNPs), NM14/01 (9 SNPs) and NM49/02 (4 SNPs) and established 24 new assays, which enabled a segregation of each haplotype into two haplotypes (Figure S1). A phylogenetic tree for haplotypes 1-10 is shown in Figure 3.

**Temporal and spatial distribution of haplotypes**

In contrast to the generally rarer haplotypes 2-4; 6-8 and 10, haplotypes 5 and 9 were found within each time interval (one year) from 06/2001 to 06/2006 (data not shown). Haplotypes 1 and 2 were not found again in the whole strain collection. Possible explanations include the actual absence of these haplotypes from the residual clonal *M. ulcerans* complex in the Densu river basin as well as phylogenetic or sampling bias.

For a phylogeographic analysis the homes of patients from whom the strains were isolated were marked in a map, depicting the distribution of haplotypes (Figure 4). Haplotypes 4, 6, 7, 9 and 10 appear to be unevenly distributed; i.e. they were found only in certain parts of the BU endemic area. Haplotype 10 was even found only within one small village. In contrast, the most prevalent haplotype (haplotype 5) co-localized with all other haplotypes. Interestingly, two *M. ulcerans* isolates of identical haplotype (haplotype 4) were isolated from two patients coming from the same household, suggesting a common source of infection.

## DISCUSSION

Previous investigations on the genetic diversity of *M. ulcerans* by comparative genomic hybridization analysis enabled differentiation of a world-wide collection of strains into two main lineages and six continental haplotypes [34]. However, phylogeographic and transmission pathway analysis requires high-resolution fine typing of strains from the same BU endemic region. This has been accomplished so far only for a few *M. ulcerans* populations and at low resolution. VNTR analysis of a Ghanaian strain collection based on the polymorphic loci ST1 and MIRU1 revealed the presence of three distinct allele combinations (BD/B, C/BAA and BD/BAA) in Ghana. All isolates from the Buruli ulcer endemic Densu river basin tested, except for Agy99, displayed combination BD/B [19]. Our recent genome re-sequencing analysis [29] compared the two Ghanaian VNTR type reference strains NM20/02 (BD/B) and NM31/04 (C/BAA) to Agy99 (BD/BAA) with the goal of detecting single nucleotide polymorphisms suitable for development of a fine-typing method. By selection of isolates with the three prevalent VNTR types in Ghana we expected to capture as much of the genetic variation present in the Ghanaian *M. ulcerans* population as possible. Our assumption was reinforced by re-sequencing four *M. ulcerans* strains initially grouped into haplotypes 3-6. Only 16 additional SNPs could be detected by comparison of the four strains to reference strain Agy99, while 50 unique SNPs could be identified by comparing the four strains among themselves. We anticipated that detected SNPs will provide the first useful genetic markers for phylogeographic and transmission pathway analyses at least in the Densu river basin and other BU endemic areas of Ghana.

We have identified 10 different haplotypes in a relatively small BU endemic area within the Densu river basin. Haplotypes 1-10 are descendants of a founder haplotype that has spread over the district; the most common haplotype 5 may represent this founder haplotype. Analysis of this spatial distribution of haplotypes indicates that emerging new haplotypes do not readily spread over the entire endemic area, but form focal transmission clusters.

Our data are comparable to studies of other genetically monomorphic organisms like *Salmonella typhi*, which report multiple strain types circulating within a specific

location [35-37]. Comparison of typing results in strains from two geographically separate BU endemic areas in the Densu river basin and the Amansie West Districts of Ghana uncovered a total of 61 differing alleles at 65 SNP loci. Within each of the two endemic areas SNP variation was significantly smaller (14 and 24 differences, respectively) than the overall variation between the two endemic areas. These results indicate the dominance of two different clonal complexes in the two separate Ghanaian BU endemic areas. SNP typing of two strains from Ghanaian neighboring countries showed similar SNP patterns when compared to AW District isolates (strain 1 from the Ivory Coast) or Ga District haplotypes (strain from Togo). Typing of 13 strains collected in additional African countries (Benin, DRC, Angola, strain 2 from the Ivory Coast) revealed a completely new SNP pattern compared to all other isolates. On the basis of typing with the Ghanaian set of SNPs these 13 strains could not be distinguished among each other and were thus clustered together into a clade. This clustering may however represent a phylogenetic discovery bias, i.e grouping of actually diverse strains leading to a so called “branch collapse”. Future addition of SNP loci identified by genome re-sequencing of a comprehensive pan-African selection of *M. ulcerans* isolates will lead to a further subdivision and differentiation of African strains. Phylogenetic discovery bias is implicit to SNP typing and will continue to exist as long as not every single sample will be sequenced. It will become smaller though with every additional re-sequenced genome. SNP typing based on a wider range of SNPs may therefore yield evidence for genetic divergence of strains from Benin, DRC, and strain two from the Ivory Coast. Hence, phylogeographic analyses in other African BU endemic areas will require whole genome comparison of strains from that area to develop a local set of informative SNPs. This is supported by our recent identification of seven insertion sequence element-related SNP types within Africa [38].

*M. ulcerans* has evolved from the aquatic environmental *M. marinum* and seems to have adapted to a more stable ecological niche [28]. Gain of the immunosuppressive toxin mycolactone is accompanied by loss of highly immunogenic proteins [39], suggesting an adaptation to survival in host environments that are screened by immunological defense mechanisms. Serological analyses have indicated that many individuals living in BU endemic areas are exposed to *M. ulcerans,* whereas relatively

few develop clinical disease [9]. There is no published evidence for direct person to person transmission of *M. ulcerans*. Based on numerous reports demonstrating an association of BU with slow flowing or stagnant water bodies, it is therefore commonly assumed that infection takes place through trauma of the skin or insect bites via an environmental reservoir in the aquatic ecosystems. Both biotic components, such as biofilms and aquatic invertebrate species are being considered as potential vectors and/or reservoirs [5,6,40-42]. Recent findings in south-eastern Australia have implicated mammals as environmental reservoir (Fyfe et al., submitted) and mosquitoes as vectors of *M. ulcerans* [43,44]. While large numbers of possums in a BU-endemic area of Australia are infected with *M. ulcerans*, a similar mammalian reservoir has not been identified in BU endemic regions of Africa. If such a reservoir plays a role in transmission, spread of *M. ulcerans* from chronic, ulcerated lesions to insect vectors or another currently unknown environmental reservoir should be considered. Subsequent infection of individuals living in the same settlements may be responsible for the focal transmission patterns, such as of haplotype 10, which was found only within one small village.

The Densu river basin represents one of the coastal drainage systems of Ghana. Its water is collected in the Weija Lake, which developed after construction of a dam at the river's mouth in the 1970s. If *M. ulcerans* bacteria would be carried freely by the flow of water of the Densu river and its tributaries, haplotypes present in upper parts of the river system should be represented in the lower parts. However, among patient isolates coming from the lower part of the basin close to the Weija Lake the rare haplotypes 4 and 7 dominated. *M. ulcerans* bacteria that are swept downstream in the river water thus do not seem to play a major role in transmission.

Results of this retrospective pilot study indicate that future longitudinal micro-epidemiological studies involving SNP typing of isolates may give deeper insight into transmission pathways and relevant reservoirs of *M. ulcerans.*

**ACKNOWLEDGMENTS**

We thank Prof. Dr. David Alland, Dr. Manzour Hernando Hazbón, Dr. Alifiya S. Motiwala at University of Medicine and Dentistry of New Jersey, as well as Dr. David W. Lacher at US Food and drug administration for original protocols on hair-pin primer design and real time PCR experiments. We also thank Francoise Portaels at the Institute of Tropical Medicine, Antwerp, for providing us with several *M. ulcerans* strains and Dr. Paul Johnson for critically reading our manuscript. We thank Kathrin Weise for her work on the Ga District map.

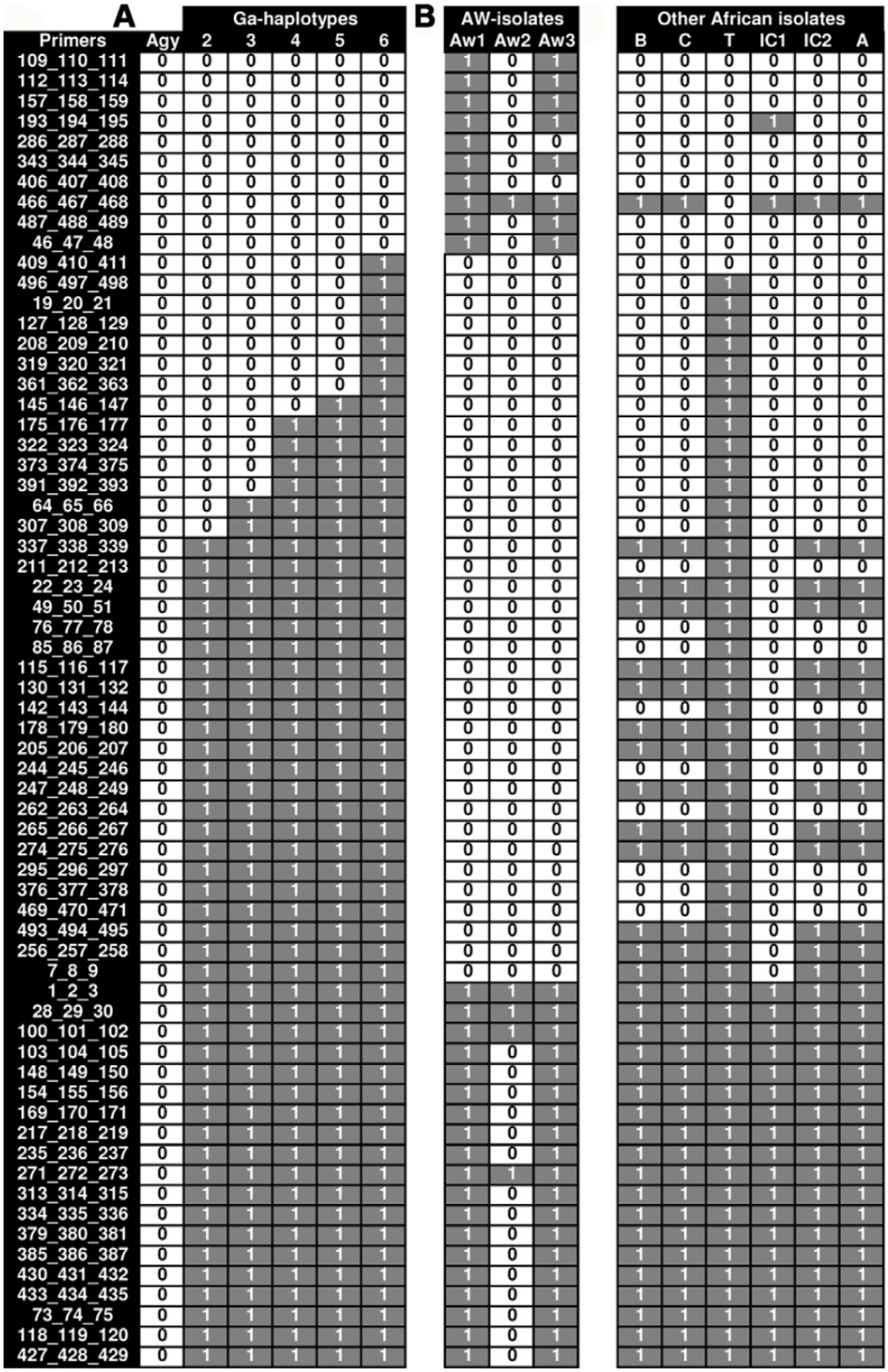
## TABLES

**Table 1. *M. ulcerans* strains included in the analysis.**

| Number of strains | Year of isolation | Place of origin | VNTR type[1] |
|---|---|---|---|
| 1 (Agy99) | 1999 | Ga District, Ghana | BD/BAA |
| 38 (NM20/02) | 2002 | Ga District, Ghana | BD/B |
| 21 | 2003 | Ga District, Ghana | BD/B |
| 5 | 2004 | Ga District, Ghana | n.t.[2] |
| 8 | 2005 | Ga District, Ghana | n.t. |
| 1 | 2006 | Ga District, Ghana | n.t. |
| 1 | 2007 | Ga District, Ghana | n.t. |
| 3 (NM31/04) | 2004 | AW District, Ghana | C/BAA |
| 7 | 1997–2001 | Benin | n.t. |
| 3 | n.p.[3] | DRC | n.t. |
| 1 | 1997 | Togo | n.t. |
| 2 | 1994 | Ivory Coast | n.t. |
| 2 | 1996 | Angola | n.t. |

[1]ST1/MIRU1 allele [19].
[2]not tested.
[3]not provided.
doi:10.1371/journal.pntd.0000751.t001

## FIGURES



| A | | Ga-haplotypes | | | | | B | AW-isolates | | | Other African isolates | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Primers | Agy | 2 | 3 | 4 | 5 | 6 | | Aw1 | Aw2 | Aw3 | B | C | T | IC1 | IC2 | A |
| 109_110_111 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 112_113_114 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 157_158_159 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 193_194_195 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 286_287_288 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 343_344_345 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 406_407_408 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 466_467_468 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 487_488_489 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46_47_48 | 0 | 0 | 0 | 0 | 0 | 0 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 409_410_411 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 496_497_498 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 19_20_21 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 127_128_129 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 208_209_210 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 319_320_321 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 361_362_363 | 0 | 0 | 0 | 0 | 0 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 145_146_147 | 0 | 0 | 0 | 0 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 175_176_177 | 0 | 0 | 0 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 322_323_324 | 0 | 0 | 0 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 373_374_375 | 0 | 0 | 0 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 391_392_393 | 0 | 0 | 0 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 64_65_66 | 0 | 0 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 307_308_309 | 0 | 0 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 337_338_339 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 211_212_213 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 22_23_24 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 49_50_51 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 76_77_78 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 85_86_87 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 115_116_117 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 130_131_132 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 142_143_144 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 178_179_180 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 205_206_207 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 244_245_246 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 247_248_249 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 262_263_264 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 265_266_267 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 274_275_276 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 295_296_297 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 376_377_378 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 469_470_471 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 493_494_495 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 256_257_258 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 7_8_9 | 0 | 1 | 1 | 1 | 1 | 1 | | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| 1_2_3 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 28_29_30 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 100_101_102 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 103_104_105 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 148_149_150 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 154_155_156 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 169_170_171 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 217_218_219 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 235_236_237 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 271_272_273 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 313_314_315 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 334_335_336 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 379_380_381 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 385_386_387 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 430_431_432 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 433_434_435 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 73_74_75 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 118_119_120 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 427_428_429 | 0 | 1 | 1 | 1 | 1 | 1 | | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

**Figure 1. SNP typing analysis of African *M. ulcerans* isolates. A** *M. ulcerans* isolates from the Densu river basin of Ghana were analyzed at 65 SNP loci (Primer IDs) by real-time PCRs. Base exchanges relative to the reference sequence of strain Agy99 were registered as 1 (grey). Allele matches with Agy99 were recorded as 0 (white). 5 haplotypes in addition to haplotype 1 (Agy99) could be distinguished on the basis of 14 SNP loci. **B** SNP typing results of strains from a second BU endemic area of Ghana as well as from additional African countries carried out with the set of SNP assays developed by whole genome sequencing of Ghanaian isolates. AW: Amansie West; Ga: strains from the Densu river basin; IC: Ivory Coast; T: Togo; B: Benin; C: Democratic Republic of Congo; A: Angola.

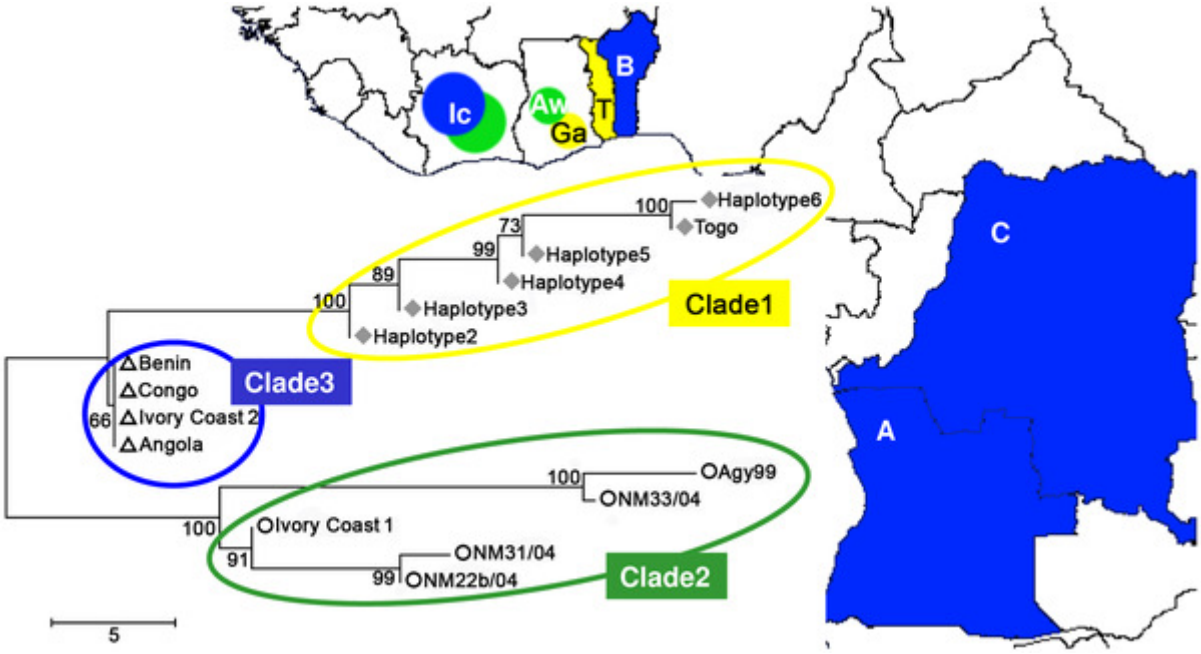



**Figure 2. Geographical distribution of African *M. ulcerans* clades.** Map of West-Africa, showing the distribution and SNP haplotypes of three African *M. ulcerans* clades. Clade 1: yellow; clade 2: green; clade 3: blue. AW: Amansie West; Ga: strains from the Densu river basin; IC: Ivory Coast; T: Togo; B: Benin; C: Democratic Republic of Congo; A: Angola. A neighbor-joining tree shows sub-grouping of detected haplotypes from the Densu river basin together with the only strain from Togo into clade 1, strains from AW together with strain Agy99 and strain 1 from the Ivory Coast into clade 2 and all other strains from additional African countries into clade 3 (scale: number of differences at the SNP loci tested).

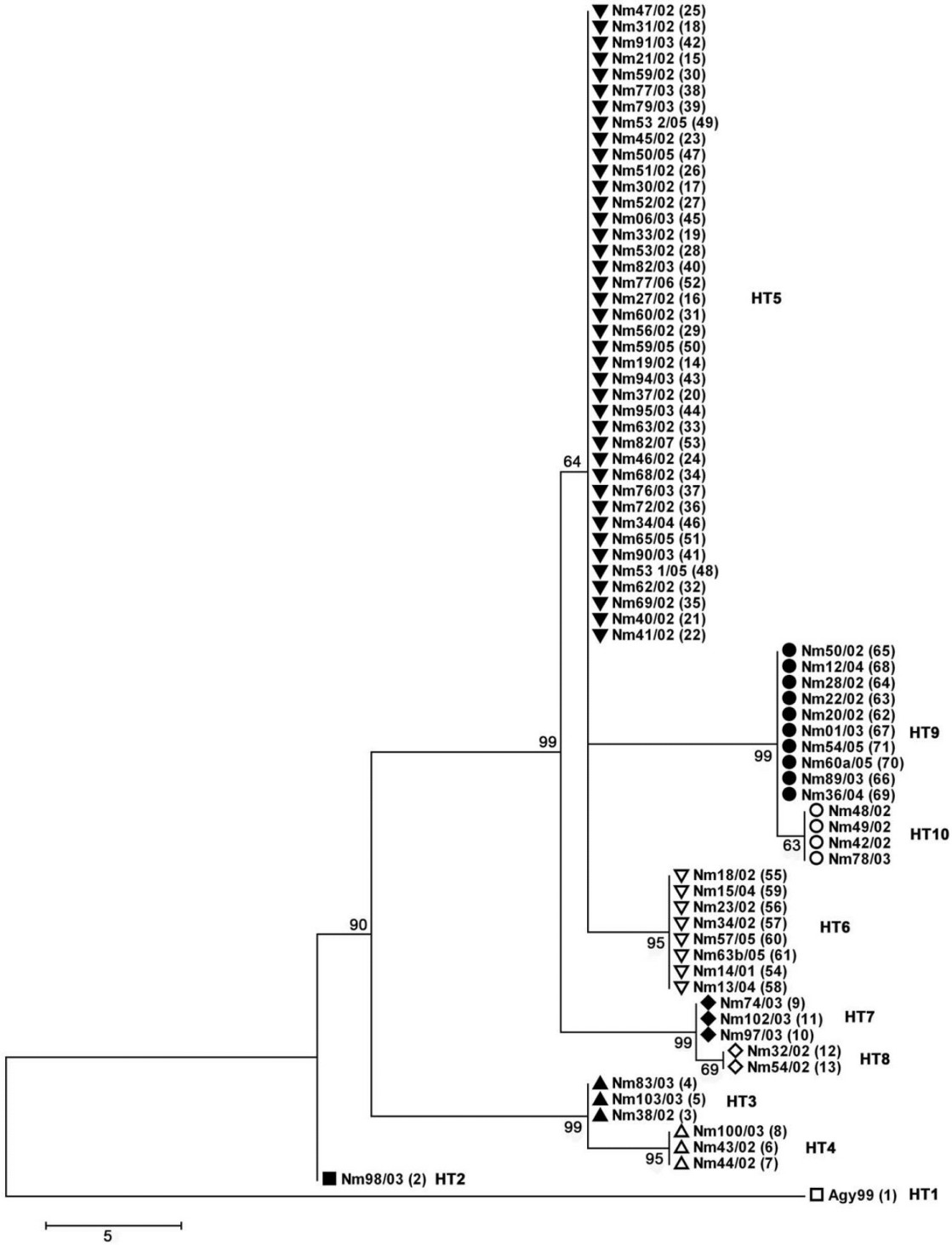



**Figure 3. Neighbor-joining tree of 75 Ghanaian *M. ulcerans* isolates.** 75 *M. ulcerans* isolates were aligned based on their SNP type (scale: number of differences at the SNP loci tested). HT = haplotype.

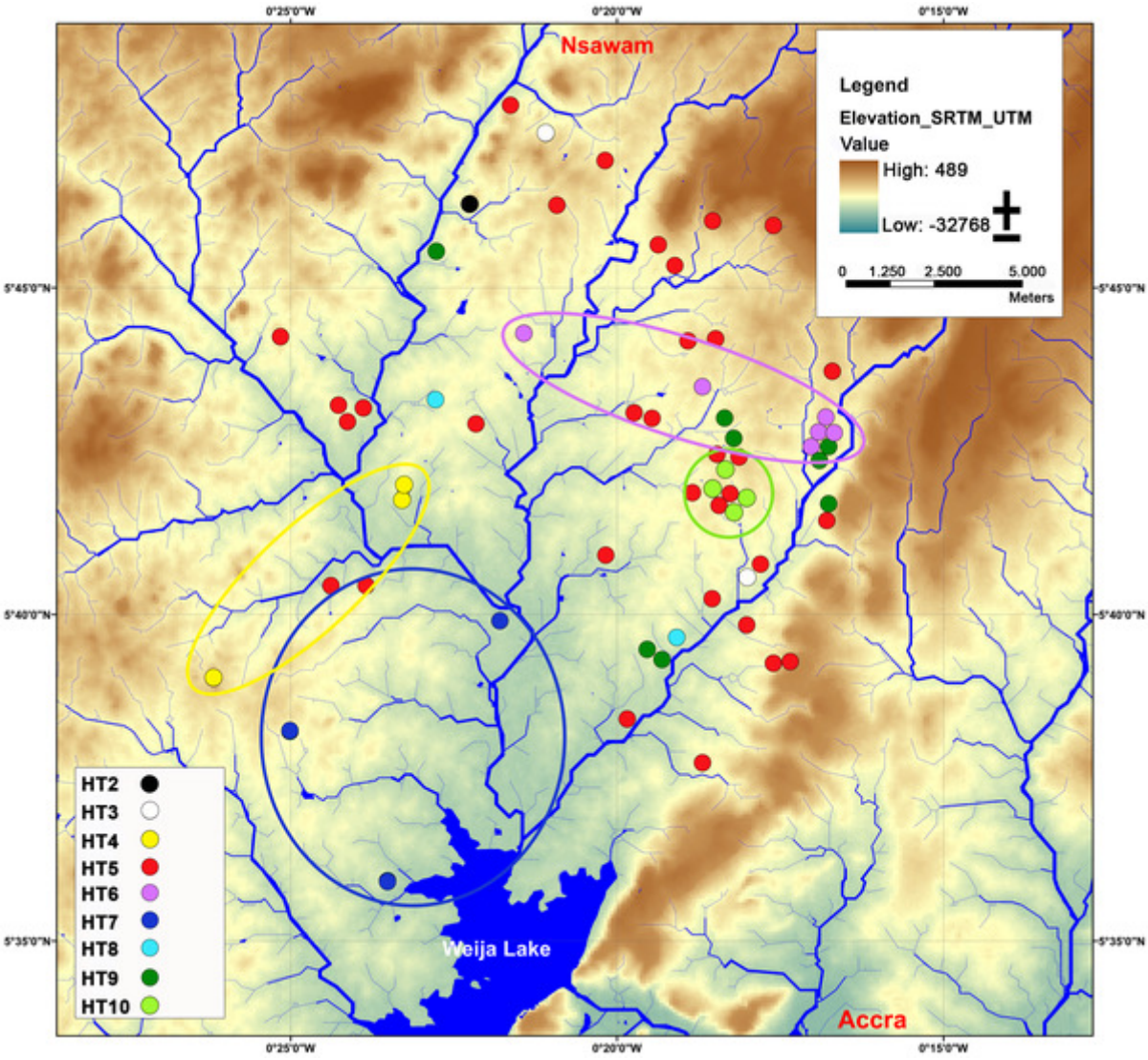



**Figure 4. Geographical distribution of *M. ulcerans* haplotypes.** Map of the Densu river basin, showing the homes of patients from whom the strains have been isolated between 2001 and 2006 (colored dots). Haplotypes 2 (black), 3 (white), 4 (yellow), 6 (purple), 7 (dark blue), 8 (light blue), 9 (dark green), 10 (light green) are unevenly distributed, whereas haplotype 5 (red) co-localizes with all other haplotypes. The background map was created using elevation data from the Shuttle Radar Topography Mission (SRTM). Water bodies were classified using optical data from Landsat ETM and radar data from TerraSAR-X.

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# CHAPTER 4

## Development of a Temperature-Switch PCR-Based SNP Typing Method for *Mycobacterium ulcerans*

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This article has been published (November 15, 2012) in:
*Public Journal of Library Neglected Tropical Diseases*

**ABSTRACT**

*Mycobacterium ulcerans* (*M. ulcerans*), the causative agent of the devastating skin disease Buruli ulcer (BU), is characterized by an extremely low level of genetic diversity. Recently, we have reported the first discrimination of closely related *M. ulcerans* variants in the BU endemic Densu River Valley of Ghana. In the study real-time PCR-based single nucleotide polymorphism (SNP) typing at 89 predefined loci revealed the presence of ten *M. ulcerans* haplotypes circulating in the BU endemic region. Here we describe the development of temperature-switch PCR (TSP) assays that allow distinguishing these haplotypes by conventional agarose gel-based analysis of the PCR products. After validation of the accuracy of typing results, the TSP assays were successfully established in a reference laboratory in Ghana. Development of the cost-effective and rapid TSP-based genetic fingerprinting method will thus allow investigating the spread of *M. ulcerans* clones by regular genetic monitoring in BU endemic countries.

## AUTHOR SUMMARY

As the third most common mycobacterial disease after tuberculosis and leprosy, Buruli ulcer constitutes a considerable health problem in many parts of the world, but in particular in West and Central Africa. Although this emerging skin disease is commonly associated with proximity to aquatic habitats, the mode of transmission remains obscure. While the clonal population structure of *Mycobacterium ulcerans* provides a great potential to trace transmission pathways and evolutionary relationships, micro-epidemiological studies have long been hampered by a striking lack of genetic diversity among African *M. ulcerans* populations. Whole genome comparison of *M. ulcerans* strains isolated from patients living in the BU endemic Densu River Valley of Ghana led to the identification of single nucleotide polymorphisms between these closely related strains. The subsequent development of SNP typing assays enabled a differentiation of ten *M. ulcerans* haplotypes in the BU endemic region. Here we selected canonical SNP markers for a spatio-temporal analysis of *M. ulcerans* variants in the Densu River Valley of Ghana using a temperature-switch PCR-based approach.

## INTRODUCTION

Infection with *M. ulcerans* causes a chronic and necrotizing skin condition known as Buruli ulcer. This emerging disease occurs focally in more than 30 predominantly tropical countries worldwide, but mainly affects impoverished populations of West and Central Africa with limited access to health care services [1]. Recent findings suggest that *M. ulcerans* has diverged about a million years ago from the fish pathogen *Mycobacterium marinum* by the acquisition of a plasmid encoding the enzymes required for the production of mycolactone [2–4]. Mycolactone is a cytotoxic macrolide toxin that plays a key role in the unique pathology of BU [5], characterized by the formation of progressive skin ulcers. While a potential transmission model implicating mammals as reservoirs and mosquitoes as vectors of *M. ulcerans* has been proposed for a local BU endemic region in south-eastern Australia [6,7], epidemiologic information for BU endemic African settings is sparse.

Remarkably little genetic diversity between *M. ulcerans* isolates from African BU patients has hindered molecular epidemiological studies tracing the spread of genetic variants of *M. ulcerans*. However, from a phylogenetic perspective the genetic monomorphism of this pathogen, which is associated with a clonal ancestry, holds a great potential to trace transmission pathways and evolutionary relationships. Several studies of other genetically highly homogeneous pathogens such as *Mycobacterium leprae* [8], *Bordetella pertussis* [9], *Yersinia pestis* [10] and *Bacillus anthracis* [11] have demonstrated the validity of genome-wide single nucleotide polymorphisms as markers for such phylogenetic analyses.

In our previous work we compared genome sequences of three Ghanaian *M. ulcerans* isolates, selected on the basis of the three earlier identified variable number of tandem repeat (VNTR) types among 57 *M. ulcerans* strains from Ghana [12], in order to detect a comprehensive set of SNP markers for genotyping studies. The subsequent development of 65 real-time PCR-based typing assays for the identified SNP loci allowed us to differentiate 75 *M. ulcerans* strains from a BU endemic area in the Densu River Valley of Ghana into six haplotypes. Genome re-sequencing of four haplotype representatives followed by further SNP detection and design of 24

additional real-time PCR assays led to the identification of ten haplotypes (HT1-10) among the 75 *M. ulcerans* isolates (Table 1).

Here we report the development of a simplified and cost-effective SNP typing method based on TSP and analysis of PCR products by conventional agarose gel electrophoresis. For this approach we have selected ten canonical SNP (canSNP) markers that facilitate a rapid differentiation of the ten described *M. ulcerans* haplotypes in the Densu River Valley of Ghana by the elimination of diagnostically redundant assays. This strategy is used to monitor the temporal as well as spatial distribution and spread of the *M. ulcerans* haplotypes in that region. Results of this ongoing study are expected to provide insights into the circulation of *M. ulcerans* variants in a BU endemic area.

## MATERIALS AND METHODS

### Ethics statement

*M. ulcerans* isolates analyzed in this study were cultivated for BU diagnosis. Ethical approval to use the isolates for immunological and microbiological research was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana (Federal-wide Assurance number FWA00001824). Written informed consent was provided by all patients involved in this study.

### Mycobacterial strains and genomic DNA extraction

We analyzed a total of 33 *M. ulcerans* isolates cultivated from wound specimen of BU patients living in the BU endemic Densu River Valley of Ghana. Ten of these isolates, used for the setup of SNP typing assays, had been typed previously by real-time PCR as SNP haplotypes 1-10 (Agy99 (HT1), NM98/03 (HT2), NM83/03 (HT3), NM100/03 (HT4), NM27/02 (HT5), NM18/02 (HT6), NM74/03 (HT7), NM32/02 (HT8), NM28/02 (HT9), and NM78/03 (HT10)) [13]. Genomic *M. ulcerans* DNA was isolated by cell wall disruption and phenol-chloroform extraction as described earlier [14]. DNA was quantified by using Qubit Fluorometer (dsDNA HS Assay Kit, Invitrogen).

### Selection of single nucleotide polymorphism loci and TSP assay design

In our previous work we have detected ten *M. ulcerans* haplotypes (HT1-10) in a relatively small BU endemic region within the Densu River Valley of Ghana by amplification refractory mutation system (ARMS) real-time PCR assays [13]. Here we constructed a phylogenetic tree of the ten identified haplotypes based on concatenated sequences of the 89 SNP loci (Figure 1A). For the TSP assay development, we selected ten representative SNP loci (TSP1, 3, 4, 6, 8, 9, 15, 16, 17 and 18), providing a discrimination of all described *M. ulcerans* variants in the Densu River Valley by haplotype-specific allele combinations (Figure 1B). TSP assay primers were designed on the basis of the strategy described by Hayden and Tabone [15,16]. For each SNP marker, locus specific (LS) primers amplifying the region

surrounding the SNP of interest as well as a nested allele-specific (NAS) primer fully complementary to the sequence of reference strain Agy99, but mismatched at the 3`end nucleotide for *M. ulcerans* strains harboring the SNP at this locus, were designed using Primer 3 Software [17] (Figure 2, Table S1). LS primers were designed to have an optimum melting temperature (Tm) of 63°C (range of 62-64°C) and to amplify a PCR product greater than 400 bp. The NAS primer was designed to have a core region with an optimum Tm of 46°C (range of 43-48°C) and a non-complementary 5`tail region that increased the overall optimum primer Tm to 53°C (range of 52-55°C). The forward (TSP assays 1, 3, 4, 6, 8, 9, 15, 16) or reverse (TSP assays 17, 18) NAS primer was positioned in at least 60 bp distance from the corresponding forward/reverse LS primer to ensure a clear distinction between the larger LS and the smaller NAS PCR product. Primer sequences and PCR product sizes are provided in Table S1.

**Differentiation of alleles by TSP and analysis of PCR product size**

The TSP method described by Hayden and Tabone [15,16] served as basis for the development of a TSP assay protocol for *M. ulcerans*. A number of technical details were modified, including standard PCR reagents, the addition of diluted DNA instead of DNA desiccation and the usage of a three-primer system with optimized primer concentrations. PCR assays were performed using 0.5 U FIREPol DNA Polymerase (Solis BioDyne), Buffer BD, 3 mM $MgCl_2$, 0.25 mM dNTPs (Sigma), 0.1 µM each of forward and reverse LS primer, 1 µM (0.25 µM, 0.5 µM, 0.75 µM) of forward/reverse NAS primer and 1-50 ng genomic DNA in a total reaction volume of 5 µl. In order to avoid evaporation of the relatively small reaction volume, PCRs were carried out in 0.2 ml eppendorf PCR tube strips, which could individually be closed after addition of the reagents. PCRs were performed in a T-Professional thermocycler (Biometra).

Thermal conditions for PCR amplification of *M. ulcerans* genomic DNA after an initial denaturation step (95°C for 5 min) were as follows: 15 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min in order to enrich the LS product at a relatively high annealing temperature (Figure 2A); 5 cycles of 95°C for 10 s and 45°C for 30 s to enable a possible incorporation of the NAS primer into the enriched LS PCR product at a low annealing temperature (Figure 2B); 15 cycles of 95°C for 10 s, 53°C for 30 s

and 72°C for 5 s to facilitate a competitive amplification of LS and NAS PCR products (Figure 2C); final extension step of 72°C for 10 min. 1 µl of the PCR products were analyzed on 2% agarose gels and stained for 1-2 hours in an ethidium bromide bath (1 µg/ml in 0.5xTBE). Whether the tested *M. ulcerans* strains harbored reference or SNP allele at the analyzed loci could then be determined by the presence of either the smaller or the larger PCR product, respectively.

**TSP validation by real-time PCR SNP typing**

All TSP SNP typing results were validated by the recently described amplification refractory mutation system real-time PCR SNP typing technique [13].

## RESULTS

### Optimization of TSP assays

TSP parameters described by Tabone et al. included the desiccation of genomic DNA by evaporation prior to PCR amplification [16]. In order to reduce the risk of contamination of the laboratory with DNA template, we eliminated this step. The addition of *M. ulcerans* DNA dissolved in water necessitated a new setup for all assay parameters.

While standard PCR reagents were used according to the manufacturer's (Solis Biodyne) recommendations, the most critical step for a specific detection of either the smaller NAS or the larger LS PCR product was to identify the optimal application of NAS and LS primers used for the PCR assays. Since initial PCR reactions using a four-primer system including both forward and reverse NAS and LS primers led to the amplification of additional non-specific PCR products (Figure 3A), we applied a three-primer system with only one NAS primer. In order to ensure an accurate differentiation of reference and SNP alleles we determined optimal primer concentrations by performing TSP assays for the ten haplotype representatives at NAS:LS primer ratios of 2.5:1, 5:1, 7.5:1 and 10:1. Results of all TSP assays provided a correct differentiation of reference and SNP alleles for the four NAS primer concentrations (Figure 3B-E). However, a clear-cut visualization of only the allele-specific PCR product was obtained by deploying a tenfold concentration of the NAS primer compared to the LS primers (Figure 3E).

The illustration of allele-specific PCR products could be further improved by loading only one-fifth of the PCR reaction on a 2% agarose gel. Gels initially overloaded by the whole PCR reaction volume showed traces of the non allele-specific PCR products.

The optimal amount of DNA for the TSP assay reactions was assessed by performing TSP assays for the ten haplotype representatives with a NAS:LS-primer ratio of 10:1 and different genomic DNA concentrations ranging from 1-50 ng. TSP assays provided accurate results for all DNA concentrations tested. However, subsequent standardized TSP strain typing was performed using 5 ng DNA for each

reaction. The performance of TSP assays was dependent on the purity of the extracted DNA. While samples with a high ratio of OD 260/280 (>1.7) facilitated a clear visualization of allele-specific PCR products on the agarose gels, traces of non-allele-specific PCR products were detected when using DNA extracts with poorer quality. TSP assays were evaluated on ten haplotype representatives in order to confirm accurate performance of all assays for both reference and SNP alleles (Figure S1).

**TSP typing of clinical *M. ulcerans* isolates**

We typed a total of 23 *M. ulcerans* strains isolated between 2009 and 2011 from BU patients living in the Densu River Valley of Ghana by the ten developed TSP assays using the optimized single standard condition (Figure S2). One additional strain served as a control for either the NAS or the LS PCR product amplification. Based on the resulting allele combinations at tested SNP loci, isolates could be differentiated into seven of the ten described *M. ulcerans* haplotypes (HT1 (1 strain), HT2 (7 strains), HT3 (1 strain), HT5 (3 strains), HT6 (5 strains), HT7 (1 strain) and HT9 (5 strains). ARMS real-time PCR SNP typing of the 23 isolates at the ten SNP loci confirmed the accuracy of TSP SNP typing results. In addition to clinical *M. ulcerans* strain typing, TSP assays were performed using DNAs directly extracted from BU lesion specimens. We selected four samples out of a panel of DNA extracts with the highest *M. ulcerans* DNA concentrations detected by IS*2404* real-time PCR. However, all attempts to SNP-type these samples by TSP failed, since additional, unspecific PCR products were amplified.

**Establishment of TSP assays in a BU endemic country**

Robustness of the assay protocol and suitability of the technique for technology transfer to laboratories in BU endemic countries was verified by analyzing TSP assays in a laboratory of the Noguchi Memorial Institute in Accra, Ghana. For this purpose we tested the ten haplotype representatives again using the same PCR materials and optimized assay parameters. Each assay provided the expected TSP genotyping products and endpoint detection by 2% agarose gels and ethidium bromide staining was comparable to the TSP setup results (Figure 4).

## DISCUSSION

A global overview of genetic diversity in *M. ulcerans* could be established by comparative genomic hybridization analysis detecting insertional-deletional variation in 35 clinical *M. ulcerans* isolates of world-wide origin [18,19]. However, efforts made to resolve the population structure of *M. ulcerans* strains from the same BU endemic countries were limited by the extensive genetic monomorphism of closely related isolates [12,20–25]. Genome sequencing of several clinical *M. ulcerans* isolates from a BU endemic region in the Densu River Valley of Ghana has facilitated for the first time the development of a SNP-based genotyping strategy with sufficient resolution for micro-epidemiological studies. This real-time PCR-based approach provided first insights into the actual diversity of such closely related *M. ulcerans* isolates as well as the distribution of *M. ulcerans* variants in the BU endemic area. Among 75 *M. ulcerans* strains isolated between 1999 and 2007 ten haplotypes showing different patterns of geographical distribution could be detected. The identification of geographically clustered emerging haplotypes provides a valuable opportunity to monitor the spatio-temporal spread of haplotypes in this region.

Cost is an important issue for all genetic fingerprinting analyses, and this particularly applies to neglected pathogens like *M. ulcerans*. While the established real-time PCR technique required relatively expensive equipment, the TSP-based assays described here, provide a simple and cheap alternative system, thus increasing the access of genetic typing assays for *M. ulcerans* to laboratories in tropical BU endemic areas. Low cost for the PCR assays, direct endpoint detection through PCR product staining on agarose gels and simple implementation of the method provided a practical means for rapid and cost-effective SNP typing.

The critical step for TSP assay development was to design primers with optimal melting temperatures for the envisaged reaction phase participations. Efficient primer design enabled the performance of all TSP assays under a single optimized standard condition. Genotyping accuracy and robustness of the TSP strategy was demonstrated by the successful application of the designed assays in two different research laboratories, yielding identical results.

SNPs represent highly stable phylogenetic markers in genetically homogenous pathogens as they occur at very low rates and convergent or reverse mutations are highly unlikely [26]. Numerous genotyping studies of monomorphic pathogens have demonstrated that a limited set of SNPs can be used to define phylogenetic relationships [11,27–30]. The idea of a canonical SNP, a SNP that can be used to define species [31], major genetic lineages of a species [27,28] or even specific strains [32,33] has recently been described in a review article focussing on the molecular epidemiology of *Bacillus anthracis* [26]. Here we present an extreme example of the canSNP concept where a small number of SNPs is used to distinguish between strains from a relatively small endemic region. For that purpose we replaced the 89 real-time PCR-based SNP typing assays by ten strategically placed canSNPs that enabled a differentiation of the ten described *M. ulcerans* haplotypes in our panel of strains.

The detected canSNPs provide useful genetic markers for the geographical and temporal analysis of *M. ulcerans* variants in the Densu River Valley of Ghana and the established TSP assays are now routinely used in the laboratories of the Noguchi Memorial Institute for *M. ulcerans* haplotype identification. However, a comprehensive overview of the *M. ulcerans* population structure in Africa can only be achieved by genome sequencing of *M. ulcerans* strains from other African BU endemic regions and subsequent identification of local sets of informative SNPs for further phylogenetic analyses.

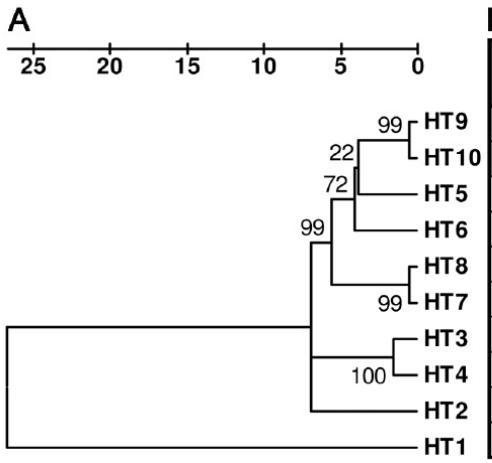
## TABLES

### Table 1. Summary of *M. ulcerans* genotyping in Ghana

| Genotyping method | No. of isolates | Origin (year of isolation) | No. of loci | No. of genotypes |
|---|---|---|---|---|
| [12] VNTR | 57 | Ghana (1997–2004) | 2 | 3 |
| [13] SNP[1] | 75 | Densu river (1999–2007) | 65 | 6 |
| [13] SNP[2] | 75 | Densu river (1999–2007) | **89** | **10** |
| canSNP | 24 | Densu river (2009–2011) | **10** | **10** |

[1]genotyping after comparison of 3 *M. ulcerans* genomes.
[2]genotyping after comparison of 7 *M. ulcerans* genomes.

## FIGURES




B

| TSP 1 | TSP 3 | TSP 4 | TSP 6 | TSP 8 | TSP 9 | TSP 15 | TSP 16 | TSP 17 | TSP 18 |
|---|---|---|---|---|---|---|---|---|---|
| 1 (C) | 1 (C) | 1 (C) | 0 (A) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 1 (C) | 1 (C) | 1 (C) | 0 (A) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 1 (A) |
| 0 (A) | 1 (C) | 1 (C) | 0 (A) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 1 (C) | 1 (C) | 0 (A) | 0 (T) | 0 (G) | 1 (C) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 0 (T) | 1 (C) | 1 (C) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 1 (G) | 0 (G) |
| 0 (A) | 0 (T) | 1 (C) | 1 (C) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 0 (T) | 0 (T) | 0 (A) | 1 (C) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 0 (T) | 0 (T) | 0 (A) | 1 (C) | 1 (A) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 0 (T) | 0 (T) | 0 (A) | 0 (T) | 0 (G) | 0 (T) | 1 (G) | 0 (A) | 0 (G) |
| 0 (A) | 0 (T) | 0 (T) | 0 (A) | 0 (T) | 0 (G) | 0 (T) | 0 (A) | 0 (A) | 0 (G) |

**Figure 1. Selection of TSP SNP typing assays. A** Linearized phylogenetic tree of the ten *M. ulcerans* haplotypes (HT1-10) detected in the Densu River Valley of Ghana (MEGA software version 4.1 (beta), scale: number of differences at the 89 SNP loci tested). **B** Schematic overview of reference (0) and SNP (1) alleles present in the sequence of the ten *M. ulcerans* haplotypes, which can be identified by TSP assays 1, 3, 4, 6, 8, 9, 15, 16, 17 and 18.

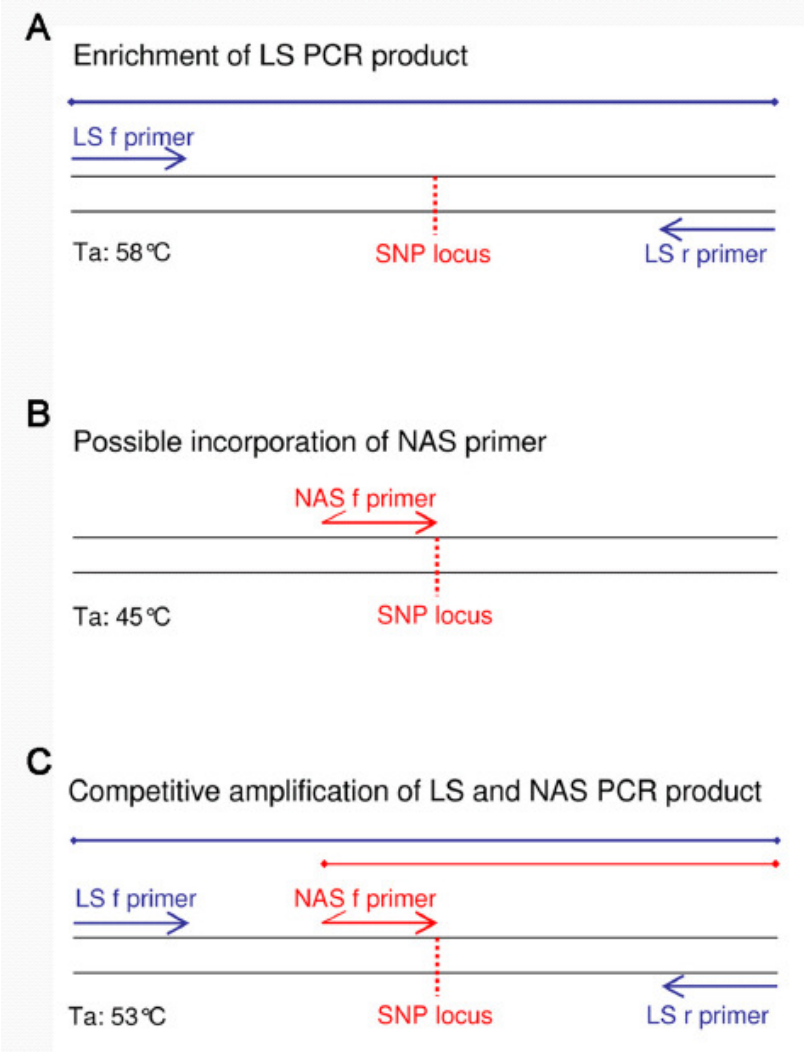



**Figure 2. Schematic illustration of TSP assay performance.** NAS and LS primer locations relative to the *M. ulcerans* DNA sequence surrounding a SNP locus are shown for the different PCR reaction phases. **A** Initial PCR conditions enable an amplification of the larger LS PCR product by applying an annealing temperature (Ta) of 58°C. **B** Reduction of Ta to 45°C facilitates a possible incorporation of the NAS primer into the enriched LS PCR product. **C** Competitive amplification of the larger LS and the smaller NAS PCR products are ensured by an increase of Ta to 53°C.

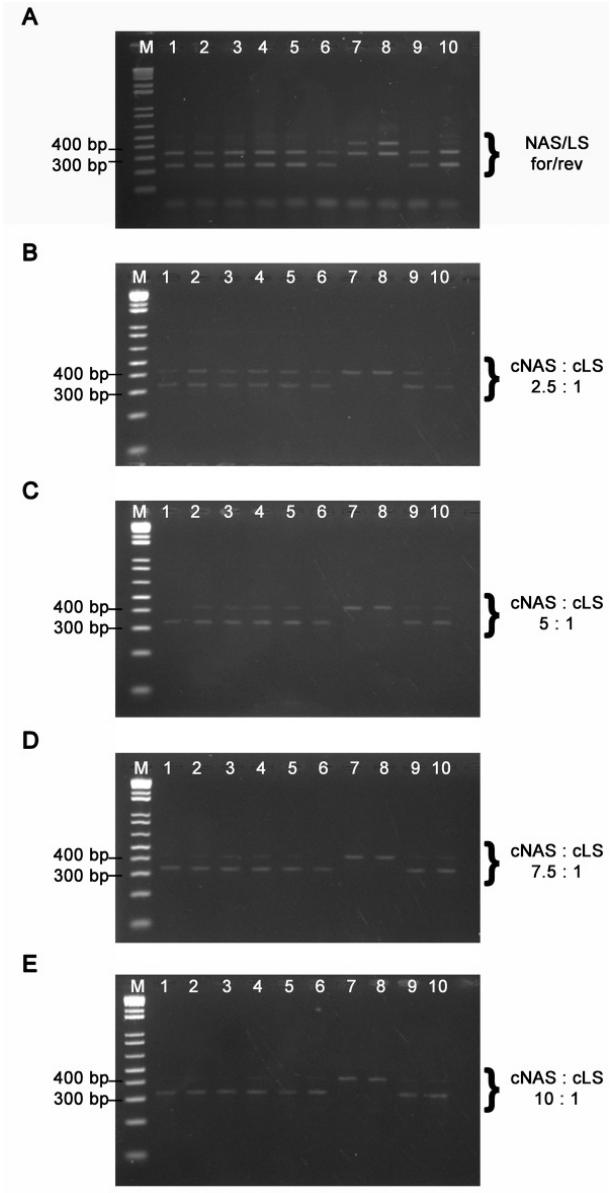



**Figure 3. Optimization of TSP SNP typing assays.** TSP endpoint detection by analysis of PCR product sizes on ethidium bromide-stained agarose gels for haplotypes 1-10 (lanes 1-10) at TSP assay locus 6. TSPs were performed using: **A** a four-primer system including both NAS and LS forward and reverse primers as well as a three-primer system with only one NAS primer and different NAS:LS primer ratios of **B** 2.5:1, **C** 5:1, **D** 7.5:1 and **E** 10:1.

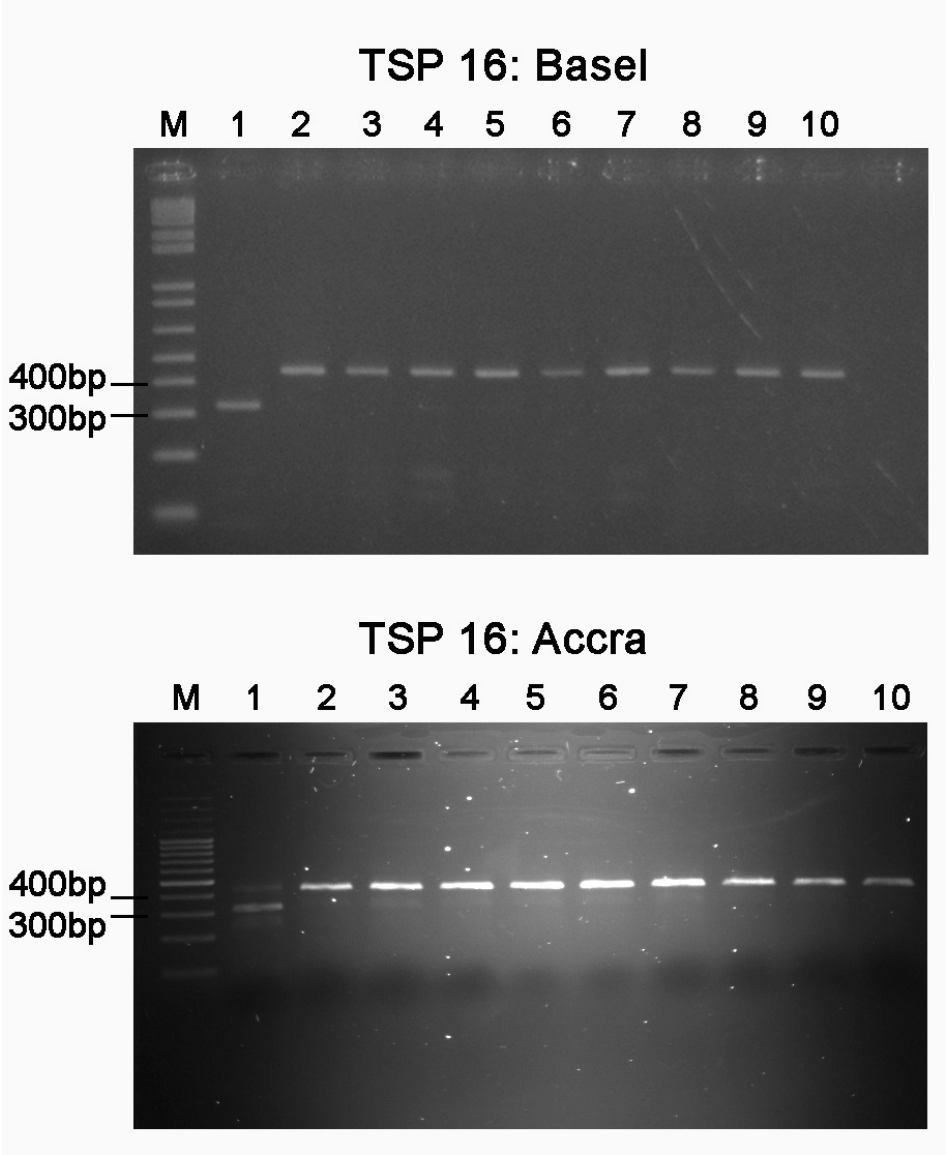



**Figure 4. Application of TSP assay 16 in two different research laboratories.** Comparison of TSP endpoint detection by analysis of PCR product sizes on ethidium bromide-stained agarose gels in two different laboratories in Basel, Switzerland and Accra, Ghana. PCR products are shown for haplotypes 1-10 (lanes 1-10).

**SUPPORTING INFORMATION**

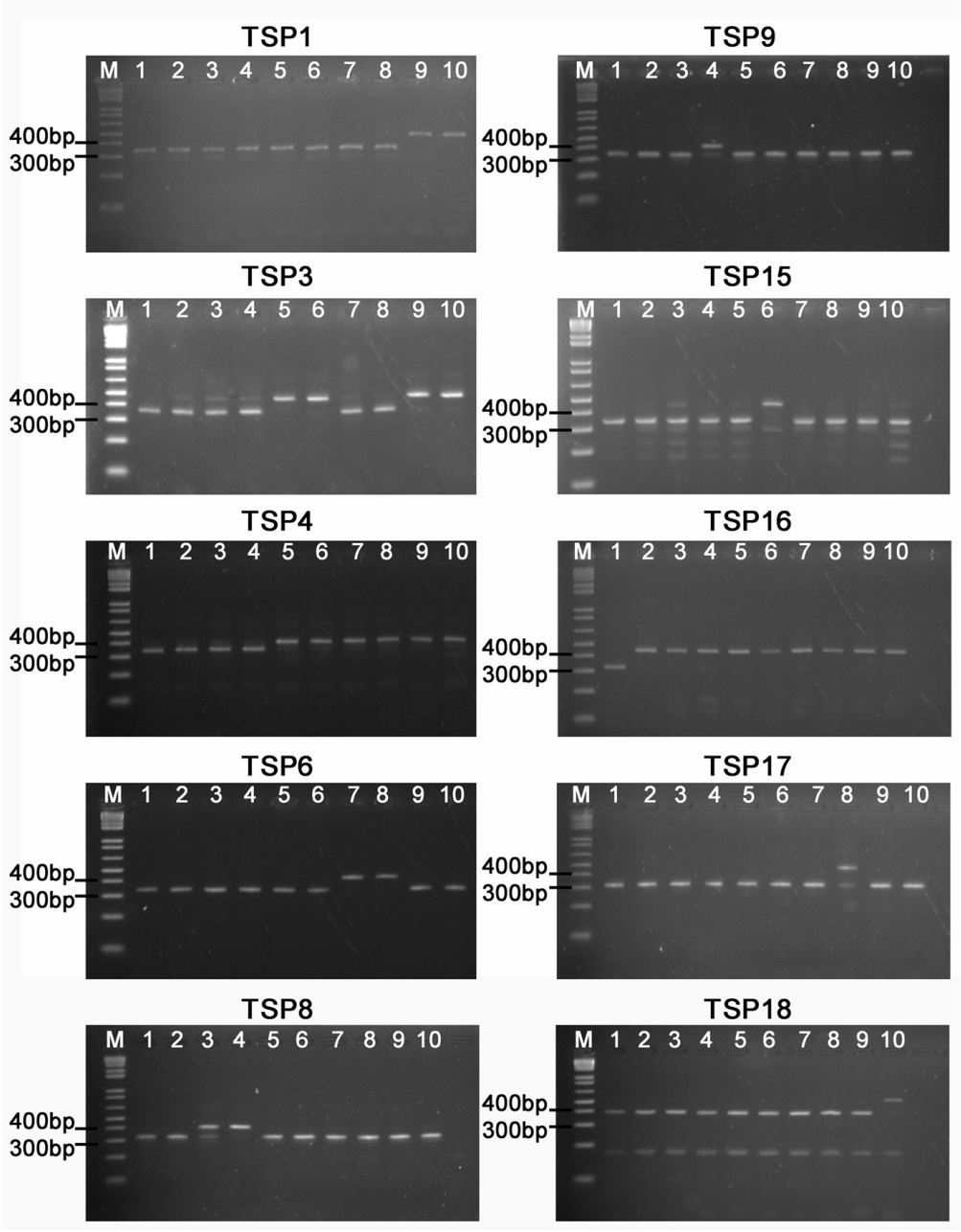



**Figure S1. Setup of TSP SNP typing assays.** TSP endpoint detection by analysis of PCR product sizes on ethidium bromide-stained agarose gels for all ten TSP SNP typing assays. PCR products are shown for haplotypes 1-10 (lanes 1-10).

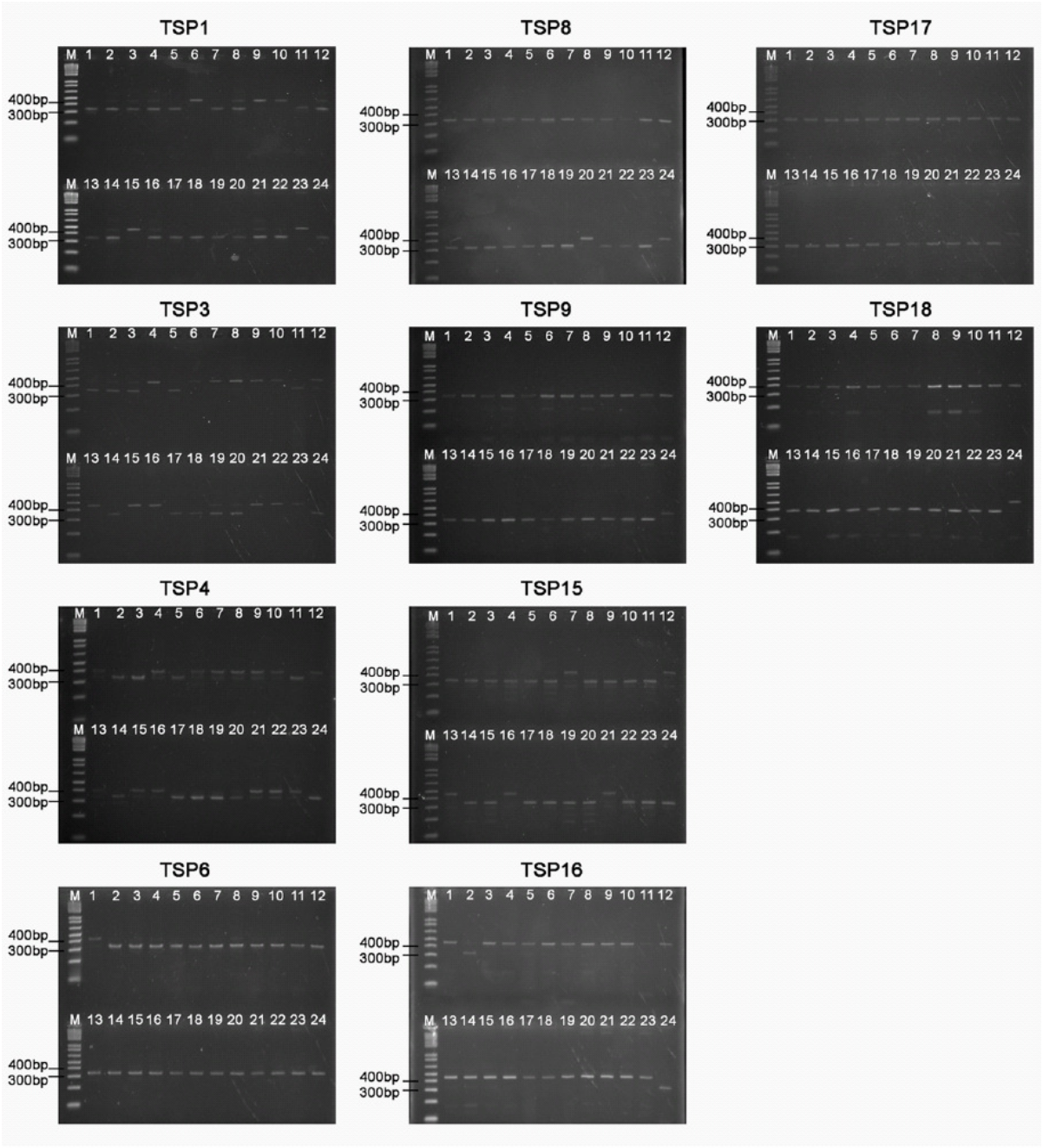



**Figure S2. TSP typing of clinical *M. ulcerans* isolates**

TSP endpoint detection by analysis of PCR product sizes on ethidium bromide-stained agarose gels for all ten TSP SNP typing assays. PCR products are shown for NM167, NM187, NM209, NM219, NM229, NM230, NM232, NM236, NM237, NM238(4), NM285, NM310, NM311, NM312, NM340, NM377, NM421C, NM465, NM491C, NM555, NM561, NM579, NM585, NAS or LS amplification control (lanes 1-24).

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CHAPTER 5

# The genome, evolution and diversity of *Mycobacterium ulcerans*

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This article has been published (April, 2012) in:
*Infection, Genetics and Evolution*

**ABSTRACT**

*Mycobacterium ulcerans* (*M. ulcerans*) causes a devastating infection of the skin and underlying tissue commonly known as Buruli ulcer (BU). Genetic analyses indicate that *M. ulcerans* has a common ancestor with *Mycobacterium marinum* (*M. marinum*) and has diverged from this fish and human pathogen perhaps around a million years ago. *M. ulcerans* is characterized by minimal genetic diversity and since it has a highly clonal population structure, genetic differences between individual isolates reflect changes that have occurred sequentially from their respective progenitors. This feature, which is shared by other bacterial pathogens with low sequence diversity, such as *Yersinia pestis* and *Bordetella pertussis* renders *M. ulcerans* a promising model to reveal evolutionary mechanisms. Until today transmission pathways and environmental reservoirs of *M. ulcerans* are not entirely explored. However, comparative genome analysis of closely related *M. ulcerans* isolates is anticipated to give deeper insights into the population structure of this enigmatic mycobacterium.

## 1. INTRODUCTION

The genus *Mycobacterium* comprises more than 120 species, many of which are important pathogens of animals and humans (Tortoli, 2006; Zakham et al., 2011). One species within this genus, *Mycobacterium ulcerans* (*M. ulcerans*), is the causative agent of Buruli ulcer (BU), a chronic, necrotizing infection of subcutaneous tissue. BU is reported from more than 30 countries worldwide, but predominantly affects impoverished populations living in remote areas of West Africa (Johnson et al., 2005). One hallmark of the evolution of *M. ulcerans* is the acquisition of a 174 kb virulence plasmid, referred to as pMUM001. This plasmid harbours genes required for the synthesis of the macrocyclic polyketide toxin mycolactone (George et al., 1999), which plays a key role in the pathogenesis of BU. While polyketides are produced by various bacteria, mycolactones are the first identified polyketide virulence determinants of a human bacterial pathogen (Hong et al., 2005a; Rohr, 2000). The cytotoxic and immunosuppressive activities of mycolactone are responsible for the formation of chronic ulcerative skin lesions with only limited inflammatory response in the centre of the lesions (Hong et al., 2008). Mycolactone production was initially assumed to be restricted to *M. ulcerans*, but recent studies revealed the existence of other closely related mycolactone-producing mycobacteria (MPM) (Ranger et al., 2006; Rhodes et al., 2005; Stragier et al., 2008; Trott et al., 2004). *M. ulcerans* and other MPM strains from around the world are characterized by limited genetic diversity, which is typically found in bacterial populations that have recently gone through an evolutionary bottleneck (Achtman, 2008). This feature may be related to the acquisition of the mycolactone plasmid and adaptation to a new lifestyle. Also other genomic signatures, like proliferation of the insertion sequence elements (ISEs) IS*2404* and IS*2606*, extensive gene loss through pseudogene formation and DNA deletion are suggestive of a bacterial population that has been transformed by DNA acquisition and is adapting to a new, perhaps protected environment, where genes once needed for survival under diverse conditions are no longer required (Stinear et al., 2007).

Although BU is commonly associated with proximity to aquatic habitats, an environmental reservoir of *M. ulcerans* is yet to be identified and so it is not clear to what conditions *M. ulcerans* is adapting. Consequently, the mode of BU transmission

remains an enigma. A recent study in South-Eastern Australia, which is the only non-tropical setting reporting significant numbers of BU cases, has implicated tree-dwelling native possums as a possible animal reservoir (Fyfe et al., 2010) and mosquitoes as potential vectors of *M. ulcerans* (Johnson, 2009; Lavender et al., 2011; Merritt et al., 2010). In African BU endemic areas numerous studies focusing on surveys of aquatic environments have suggested the potential of aquatic invertebrate and vertebrate species as well as aquatic vegetation as reservoirs and/or vectors of *M. ulcerans* but definitive evidence is lacking (Kotlowski et al., 2004; Marsollier et al., 2002, 2007; Merritt et al., 2005; Portaels et al., 1999, 2008; Williamson et al., 2008). Since routine methods for the cultivation of the extremely slow growing *M. ulcerans* from environmental sources are not available, these studies are largely based on the detection of *M. ulcerans* genomic DNA sequences by PCR. However, this approach is complicated by the presence of the target DNA sequences used in other MPM. Furthermore, only one *M. ulcerans* strain has ever been isolated from a non-vertebrate source (Portaels et al., 2008). Hence, the relevance of PCR positive environmental samples for the transmission of *M. ulcerans* to humans is not clear.

Prior to the application of high-throughput genome sequencing, high-resolution genetic fingerprinting methods for *M. ulcerans* suitable for micro-epidemiological studies in BU endemic areas could not be developed due to the distinctive genetic monomorphism of *M. ulcerans* strains within a BU endemic region. Comparative genome sequencing has now enabled a genome-wide search for single nucleotide polymorphisms (SNPs). High-resolution SNP typing allows for a differentiation of *M. ulcerans* disease isolates from BU endemic areas into sets of haplotypes. A first retrospective study using this approach has revealed a focal transmission pattern of certain haplotypes (Röltgen et al., 2010), and future longitudinal micro-epidemiological studies involving SNP typing are anticipated to provide deeper insight into *M. ulcerans* transmission pathways and relevant reservoirs.

## 2. EMERGENCE OF *M. ULCERANS* FROM *M. MARINUM*

Comparison of partial DNA sequences, commonly used for the identification and differentiation of mycobacteria, has revealed a close genetic relationship between *M. ulcerans* and *M. marinum* long before whole-genome sequences became available.

In the highly conserved 16S ribosomal RNA (rRNA) gene *M. ulcerans* and *M. marinum* isolates differ only at one dimorphic position (Portaels et al., 1996; Tønjum et al., 1998) and no difference was found in the 16S-23S rDNA internal transcribed spacer region (Roth et al., 1998). Analysis of eight housekeeping and structural genes revealed average nucleotide sequence identities of >98% between *M. ulcerans* and *M. marinum* (Stinear et al., 2000b). Genomic subtraction analyses have shown, that the major genetic acquisition by *M. ulcerans* was the polyketide synthase locus (Jenkin et al., 2003) harbored in the virulence plasmid pMUM001 (Stinear et al., 2004). Comparative analyses in geographically diverse strains of *M. ulcerans* have demonstrated a common evolutionary origin of their pMUM plasmids (Stinear et al., 2005a). Multi-locus sequence typing (MLST) of a panel of *M. ulcerans*, other MPM and *M. marinum* isolates has indicated that all MPM are genetically closely related. In the same study DNA-DNA hybridization analysis between MPM and *M. ulcerans* strains from Africa, Australia and China unveiled overall mean relative binding ratios of 98%, while mean ratios between MPM and non-MPM strains were only 40% (Yip et al., 2007). These data suggest that MPM have evolved from a common *M. marinum* ancestor (Yip et al., 2007) and comply with proposed criteria for species delineation (Vandamme et al., 1996). Therefore, it is currently debated whether this genetically coherent group of strains should be reclassified as *M. ulcerans* (Pidot et al., 2010a). MLST has shown that the ongoing process of evolution among MPM has generated at least two lineages, which occupy different ecological niches. One lineage includes strains, which typically cause disease in ectotherms such as frogs and fish, while strains of the other lineage cause BU in humans and can infect other endotherms such as possums (Yip et al., 2007). *M. ulcerans* isolates from BU patients can be distinguished from other MPM by the analysis of large sequence polymorphisms (LSPs) (Käser et al., 2009a) and by variable number of tandem repeat (VNTR) typing (Stragier et al., 2007).

Detailed analyses of LSPs between *M. ulcerans* isolates from BU patients of a world-wide origin revealed an intra-species evolutionary scenario with two distinct phylogenetic lineages (Käser et al., 2007; Rondini et al., 2007). *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 (Käser et al., 2007). While BU disease caused by strains belonging to the ancestral lineage is only sporadically reported (Faber et al., 2000; Guerra et al., 2008; Nakanaga et al., 2011), focal prevalence of BU in areas of Africa and Australia endemic for disease caused by the classical lineage is much higher. This may indicate that the ancestral lineage, which is genetically closer to *M. marinum* (Käser et al., 2007), is less virulent, but not necessarily less pathogenic, than the classical lineage (Käser et al., 2007; Käser and Pluschke, 2008; Mve-Obiang et al., 2003).

In order to estimate the divergence time of the two *M. ulcerans* lineages, a systematic analysis of synonymous SNPs between African classical lineage isolates and a Japanese isolate belonging to the ancestral lineage has been carried out. Based on a universal clock rate (Ochman et al., 1999), it was estimated that the classical lineage diverged about 400,000 years ago from the ancestral lineage (Qi et al., 2009). Data furthermore suggested that the African strains and *M. marinum* strain M diverged from a common ancestor about 1.1 to 1.5 million years ago (Figure 1). However there are many uncertainties associated with such estimates and the major underlying assumption here - that all strains have been evolving at equal rates – needs to be verified.

## 3. GENOME CHARACTERISTICS AND REDUCTIVE EVOLUTION

The first complete genome sequence of *M. ulcerans* was from a clinical isolate, called Agy99, isolated in Ghana in 1999 from a BU lesion. The *M. ulcerans* Agy99 genome is composed of a 5,631,606-bp chromosome with the 174,155-bp virulence plasmid pMUM001 (Stinear et al., 2007). The chromosome of *M. ulcerans* Agy99 was shown to harbor 4160 protein-coding genes as well as 771 pseudogenes (Stinear et al., 2007). It is rich in insertion sequences (IS), particularly IS*2404* (209 copies) and IS*2606* (83 copies) and contains two prophages, phiMU01 (18 kb, 18CDS) and phiMU02 (24 kb, 17CDS) (Stinear et al., 2007). The chromosome has a G+C content of about 65%, which is comparable to the G+C contents of *M. tuberculosis*, *M. bovis* and other mycobacteria (Marri et al., 2006). The virulence plasmid pMUM001 has a slightly lower G+C content of 62.5% and harbors 81 protein-coding DNA sequences (Stinear et al., 2007) including four copies of IS*2404* and eight copies of IS*2606* (Figure 2) (Stinear et al., 2004). By comparison of the *M. ulcerans* genome to 21

other available mycobacterial genomes, forty-seven chromosomally-encoded open reading frames (ORF) potentially unique to *M. ulcerans* have been identified (Pidot et al., 2010b). While these ORFs seem to have been acquired by horizontal gene transfer, comparative genomics of *M. ulcerans* and *M. marinum* strain M, which has 5426 protein-coding sequences and only 65 pseudogenes (Stinear et al., 2008), has shown that extensive genome downsizing and pseudogene formation is the dominating feature of genome adaptation of *M. ulcerans* (Stinear et al., 2007). Accumulation of pseudogenes has been reported from obligate intracellular organisms such as *M. leprae* (Cole et al., 2001), which harbours 1116 pseudogenes, and has been related to adaptation to a highly specialized ecological niche. Losses of genes, which are not essential for the survival in the new host, are tolerated due to a reduced pressure to maintain these genes (Moran and Plague, 2004). However, there are significant differences to *M. leprae*. While the majority of *M. ulcerans* bacteria have an extracellular location in advanced BU lesions, an intracellular stage may play a role only in an early stage of the infection (Schütte et al., 2009; Silva et al., 2009; Torrado et al., 2007). The unique cytotoxic and immunosuppressive toxin mycolactone is of crucial importance for both the pathogenesis of BU and for the chronic nature of the *M. ulcerans* infection. However, reductive genome evolution may be contributing further to immune evasion. Comparative genomic analyses of classical and ancestral *M. ulcerans* strains have indicated that the genes *esxA*, *esxB* and *hspX* encoding the highly immunogenic proteins ESAT-6, CFP-10 and HspX are hot-spots for genomic variation in the development of *M. ulcerans* and its adaptation to a new environment (Huber et al., 2008). While the genome of *M. marinum* M contains two copies of the *esxB-esxA* gene cassette, members of the *M. ulcerans* ancestral lineage have retained only one copy of this gene cluster. Both copies are deleted from the genome of *M. ulcerans* strains belonging to the classical lineage. Loss of these strong B- and T-cell immunogens may help *M. ulcerans* to evade host immune responses and may confer a survival advantage in host environments that are screened by immunological defense mechanisms.

In this vein, members of the PE and PPE multigene families are over-represented among the pseudogenes of *M. ulcerans* and *M. leprae*. While *M. marinum* has about 170 PE and 105 PPE genes, *M. ulcerans* has retained only 70 intact PE and 46 PPE

genes (Stinear et al., 2007). Members of these protein families, which contain highly conserved Proline-Glutamate (PE) or Proline-Proline-Glutamate (PPE) residues, are surface-associated cell wall proteins believed to provide a diverse antigenic profile and thus may play an additional role in immune evasion (Sampson, 2011; Voskuil et al., 2004). Considering that *M. leprae* has lost almost all genes belonging to this family, the decreased number of PE/PPE proteins further indicates an intermediate stage of reductive evolution and niche adaptation of *M. ulcerans* (Marri et al., 2006; Stinear et al., 2007).

Some of the genetic differences between *M. ulcerans* and *M. marinum* are reflected in their corresponding phenotype. While *M. marinum* grows relatively rapidly with a generation time of 4-6 hours at its optimal growth temperature (Stinear et al., 2008), *M. ulcerans* has a very long generation time of about 72 hours, which might be partly explained by a switch of essential protein coding sequences from the leading to the lagging strand and the associated deceleration of their transcription (Stinear et al., 2007). In contrast to *M. marinum*, which is capable of producing pigments in order to protect itself from light, *M. ulcerans* contains an inactive copy of the responsible *crtB* locus (Stinear et al., 2007), further suggestive of the adaptation of *M. ulcerans* to a new, possibly dark environment.

## 4. INSERTION SEQUENCE ELEMENTS (ISEs) AS DRIVERS OF GENOME REDUCTION

ISEs are mobile genetic entities encoding transposases that catalyze DNA copying or movement. ISEs can promote genome plasticity in the form of deletions, inversions and replicon fusions (Mahillon and Chandler, 1998; Mahillon et al., 1999). It has previously been shown that genetic diversity of mycobacteria is in part driven by ISEs such as IS*6110* of *M. tuberculosis* (Bifani et al., 1996; Sreevatsan et al., 1997), IS*1245* of *M. avium* (Motiwala et al., 2006; van Soolingen et al., 2001) and IS*900* of *M. paratuberculosis* (Green et al., 1989; Motiwala et al., 2006). *M. ulcerans* contains ten putative ISEs (Stinear et al., 2007) in addition to the well-characterized high-copy-number elements IS*2404* and IS*2606* (Stinear et al., 1999). IS*2404* is 1368 bp long, containing 41 bp perfect inverted repeats and producing 10 bp target-site duplications. IS*2606* is 1438 bp long, with 31 bp imperfect inverted repeats and

producing target-site duplications of 7 bp (Stinear et al., 2005b). The origin of IS*2404* and IS*2606* is not clear, but it has been shown, that both sequences are related to sequence elements found in the genus *Streptomyces* (Stinear et al., 2000b). Comparative genome analyses of *M. ulcerans* and *M. marinum* have shown that IS*2404* and IS*2606* are associated with many of the LSPs observed (Käser et al., 2007; Stinear et al., 2007). Furthermore, 111 of the predicted pseudogenes of *M. ulcerans* were created by insertion of one of the ISEs (Stinear et al., 2007).

## 5. PLASMID ASSOCIATED MYCOLACTONE PRODUCTION

While horizontal gene transfer among pathogenic mycobacteria is generally rare, *M. ulcerans* has not only acquired several ISEs, but also the 174 kb virulence plasmid pMUM001. Plasmid pMUM001 encodes a type I polyketide synthase locus, which occupies about 120 kb (Tobias et al., 2009). Bacterial type I polyketide synthases are characterized by a multi-modular structure comprising different enzymatic domains responsible for the stepwise biosynthesis of polyketides (Jenke-Kodama et al., 2005). The polyketide synthases of *M. ulcerans* are among the largest known cellular enzymes. These giant proteins referred to as MLSA1 (1.8 MDa), MLSA2 (0.26 MDa) and MLSB (1.2 MDa) are encoded by three large genes, *mlsA1* (51 kb), *mlsA2* (7 kb) and *mlsB* (42 kb), respectively. MLSA1 and MLSA2 synthesize the 12-membered conserved core structure and the upper side chain of mycolactone, while the highly unsaturated acyl side chain is generated by MLSB (Stinear et al., 2004). Both nucleotide and amino acid identities between domains with the same general function are remarkably high (>98%) (Tobias et al., 2009), indicative for the recent acquisition of a core gene set and subsequent gene duplication and recombination events (Stinear et al., 2004). In contrast to the assumption that high sequence identity among the domains would lead to genomic instability through homologous recombination (Stinear et al., 2004; Townsend, 2004), only five variants of mycolactone (mycolactones A/B, C, D, E and F) have been found so far (Figure 3), when analyzing a worldwide collection of *M. ulcerans* strains and other MPM (Mve-Obiang et al., 2003). Strong purifying selection is thought to ensure the maintenance of a mycolactone-producing form of the pMUM plasmid. In the absence of selection *in vitro*, deletion of plasmid sequences and a corresponding loss of mycolactone

production can readily arise (Stinear et al., 2005a). In addition to the *mls* genes, the polyketide synthase locus of pMUM001 harbors three putative accessory genes, *mup038*, *mup045* and *mup053* coding for polyketide-modifying enzymes. While the type II thioesterase encoded by *mup038* is thought to degrade abnormal polyketide extension products, the formation of an ester bond between acyl side chain and core structure of mycolactone is assumed to be catalyzed by the beta-ketoacyl synthase encoded by *mup045* (Stinear et al., 2004). The level of *mup053*-encoded cytochrome P450 hydroxylase activity seems to determine the oxidation state of the side chain C12` (Hong et al., 2003; Mve-Obiang et al., 2003). African *M. ulcerans* strains predominantly produce a mixture of *cis/trans* isomers of a mycolactone with oxidized C12`, designated mycolactone A/B. Australian *M. ulcerans* strains deficient in *mup053* produce a lower-mass metabolite with less of the hydroxylated C12` version, referred to as mycolactone C (Stinear et al., 2005a). A clinical isolate from China synthesized mycolactone D, which differs from mycolactone A/B by a methyl group at C2` of the side chain (Figure 3). The genetic basis for the production of mycolactone D was a recombination event within *mlsB* resulting in a different substrate specificity of one of its acyltransferase domains (Hong et al., 2005a).

Recent studies have identified additional structural variants of mycolactone produced by mycobacterial fish and frog pathogens closely related to *M. ulcerans*. Mycolactone E (Hong et al., 2005b; Mve-Obiang et al., 2005) was found in mycobacterial isolates causing a mycobacteriosis outbreak in a laboratory colony of *Xenopus tropicalis* (Trott et al., 2004). The designation *M. liflandii* has been proposed for these bacteria but this name has no standing in nomenclature (Mve-Obiang et al., 2005). Their close genetic relation to *M. ulcerans* is suggested by the presence of high copy numbers of IS*2404* in their genome (Trott et al., 2004). Furthermore, an unusual group of *M. marinum* strains from fish in the Red and Mediterranean Seas as well as an *M. ulcerans*-like *Mycobacterium* recovered from diseased striped bass and referred to as *M. pseudoshottsii* (Rhodes et al., 2005), also contain IS*2404* and produce another structural toxin variant, mycolactone F (Figure 3) (Ranger et al., 2006). These altered mycolactones are due to minor modifications in the acyl-side chains of mycolactones E and F caused by rearrangements between homologous modules within *mlsB* (Pidot et al., 2008).

## 6. DIVERSIFICATION OF LOCAL CLONAL COMPLEXES OF *M. ULCERANS*

*M. ulcerans* strains isolated in different West African countries are genetically homogeneous, indicative of the relatively recent dispersal of this clone throughout West Africa. Comparative genomic hybridization analyses have identified extensive insertional-deletional sequence differences between *M. ulcerans* isolates belonging to the ancestral and the classical lineage (Käser et al., 2007; Rondini et al., 2007). In contrast, no insertional-deletional genomic variation was detected within a panel of African disease isolates (Käser et al., 2009b). Furthermore, whole genome SNP analysis with a set of Ghanaian strains revealed no evidence for the formation of further pseudogenes (Qi et al., 2009). The genetic monomorphism of *M. ulcerans* has hampered development of a genetic fine-typing method for micro-epidemiological studies. Conventional methods, such as restriction fragment length polymorphism (RFLP) analysis (Jackson et al., 1995), 16S rDNA sequencing (Portaels et al., 1996), analysis of the regions between adjacent copies of IS*2404* and IS*2606* (2426 PCR) (Stinear et al., 2000a), RFLP analysis of IS*2404* (Chemlal et al., 2001a; Chemlal et al., 2001b), analysis of genomic regions between IS*2404* and a repeated GC-rich sequence (IS*2404*-Mtb2 PCR) (Ablordey et al., 2005) and mycobacterial interspersed repetitive units (MIRU) typing (Stragier et al., 2005) enabled only discrimination of continental lineages, but could not detect genotypic variation between strains coming from the same BU endemic region. Only variable number of tandem repeat (VNTR) typing based on newly identified polymorphic loci showed some limited potential to reveal genetic diversity within isolates from Africa (Ablordey et al., 2007; Hilty et al., 2006; Stragier et al., 2006). In one study VNTR typing with the VNTR loci ST1 and MIRU1 allowed for the differentiation of 57 strains from Ghana into three ST1/MIRU1 allele combinations. However, all 47 recent isolates from one BU endemic region in the Densu River valley of Ghana displayed the same ST1/ MIRU 1 haplotype (Hilty et al., 2006).

In a recent review, Achtman predicted that phylogenetic analyses of genetically monomorphic pathogens will focus on whole genome SNP analysis (Achtman, 2008). Phylogenetic analyses of monomorphic organisms like *M. tuberculosis* (Baker et al., 2004; Sreevatsan et al., 1997), *Bacillus anthracis* (Pearson et al., 2004; Van Ert et al., 2007), *Salmonella enterica* Typhi (Roumagnac et al., 2006) and *Yersinia pestis*

(Achtman et al., 2004) are now in fact based on high-resolution SNP typing. The first SNP typing study of *M. ulcerans* was based on a few SNPs identified in LSP-haplotye specific ISEs and led to the identification of seven SNP types in Africa (Käser et al., 2009c). In 2010 our research group reported the first SNP typing method for *M. ulcerans* with sufficient resolution for micro-epidemiological analyses within one BU endemic area, namely the BU endemic Densu river valley of Ghana (Röltgen et al., 2010). In this study SNPs were detected by whole-genome comparison of three Ghanaian *M. ulcerans* strains with different VNTR type (Hilty et al., 2006; Qi et al., 2009; Röltgen et al., 2010; Yip et al., 2007;). Developed SNP typing assays enabled the differentiation of six haplotypes. Subsequent genome re-sequencing of four haplotype representatives from the Densu River Valley of Ghana increased the resolution and allowed distinction of altogether ten local haplotypes (Röltgen et al., 2010). Analysis of the distribution of these haplotyes within the Densu River basin has shown that some haplotypes constitute focal transmission clusters. These haplotypes have probably emerged only recently. In contrast, the presumed founder haplotype was distributed over the entire BU endemic area. One possible explanation for these findings is the spread of *M. ulcerans* from ulcerated BU lesions to a currently unknown environmental reservoir and subsequent infection of individuals living in the same surrounding (Röltgen et al., 2010). Preliminary results obtained with isolates from other West African BU endemic areas speak for diversification of local *M. ulcerans* populations by acquisition of point mutations. High resolution SNP typing methods for individual BU endemic regions therefore have to be developed by whole genome sequencing of sets of local isolates. With the rapid development of next generation sequencing technology, micro-epidemiological studies may be performed in future by sequencing the genomes of all available isolates.

Minimal genetic diversity of bacterial populations is often accompanied by a clonal population structure with low levels of recombination and horizontal genetic exchange. Hence, genetic typing of such genetically monomorphic microbes holds a great potential to explore their evolutionary histories by monitoring sequentially accumulated genetic differences of isolates from their respective progenitors (Suerbaum and Achtman, 2001). Genome sequencing of large numbers of *M.*

*ulcerans* strains of diverse origin will thus provide deep insight into the evolutionary history of this emerging pathogen.

## 7. IMPACT OF ANTIBIOTIC USE ON *M. ULCERANS* GENOMIC DIVERSITY

Since 2004 the World Health Organization has recommended combined antibiotic treatment of BU with rifampicin and streptomycin for eight weeks (WHO, 2011), which has routinely been implemented in the BU endemic African countries. Given the history of antibiotic resistance in *M. tuberculosis* (Borrell and Gagneux, 2011), there have been concerns that inappropriate rifampicin and streptomycin use may drive similar patterns of resistance in *M. ulcerans.* However, no cases of rifampicin or streptomycin resistant clinical *M. ulcerans* strains have been reported, although the development of phenotypic and genotypic resistance of *M. ulcerans* under rifampicin monotherapy was observed in experimentally infected mice (Marsollier et al., 2003). Sequencing of *rpoB* and *rpsL* genes (associated with rifampicin and streptomycin resistance respectively) of clinical *M. ulcerans* strains from Ghana isolated between 2004 and 2007 led to the identification of one isolate with a point mutation in the *rpoB* gene (Beissner et al., 2010). Systematic surveillance for drug resistance among *M. ulcerans* is thus crucial, particularly as there is no alternative drug to replace rifampicin in the current BU treatment regimen. Drug resistance might also become more than a single-patient issue if the bacteria present in chronic BU wounds represent an active reservoir of *M. ulcerans*, required for transmission. Here, genomics will be a powerful tool as we study the movement and evolution of *M. ulcerans* within humans and the wider environment.

**ACKNOWLEDGMENTS**

This work was supported by the Stop Buruli Initiative funded by the UBS-Optimus Foundation. Katharina Röltgen was supported by a stipend of the Ghanaian-German Centre for Health Research funded by the DAAD-German Academic Exchange Service.

**FIGURES**

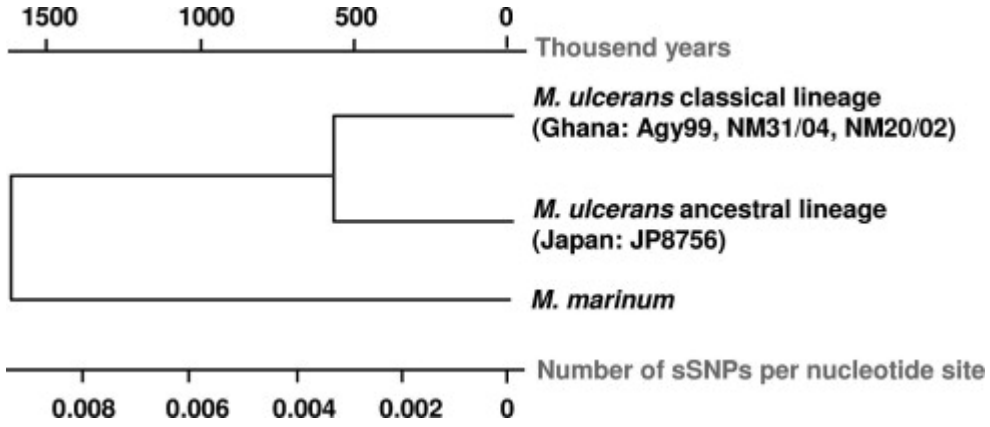


**Figure. 1. Minimum evolution tree of *M. marinum* (strain M) and *M. ulcerans* strains of the ancestral and classical lineages.** Top scale: divergence time frame per thousand years. Bottom scale: number of synonymous substitutions per nucleotide site. Rate used for the time calibration: $5.8 \times 10^{-9}$ substitutions per site per year.

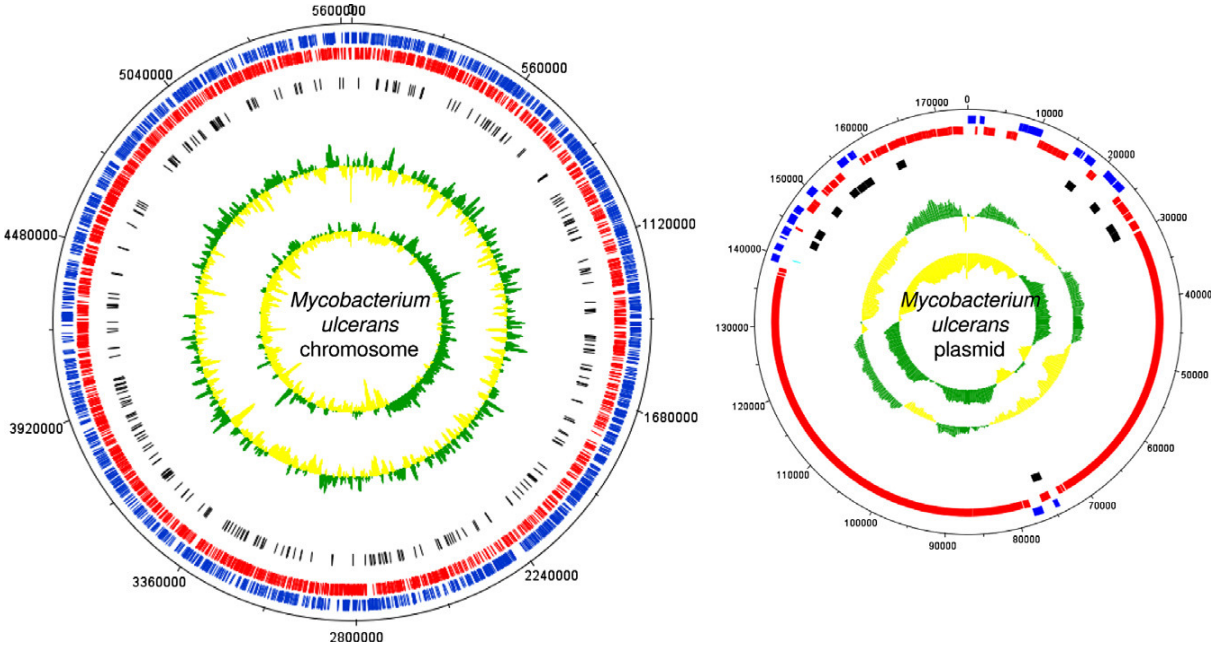


**Figure 2. Circular representation of the *M. ulcerans* Agy99 chromosome and plasmid.** The outer black circle shows the scale in bases. The next two inner circles illustrate the forward and reverse strand CDS in blue and red, respectively. IS element regions are displayed in the next inner circle in black. The innermost circles show GC plot and GC skew. The graph was designed using DNAPlotter Release 1.4 (Carver et al., 2009).

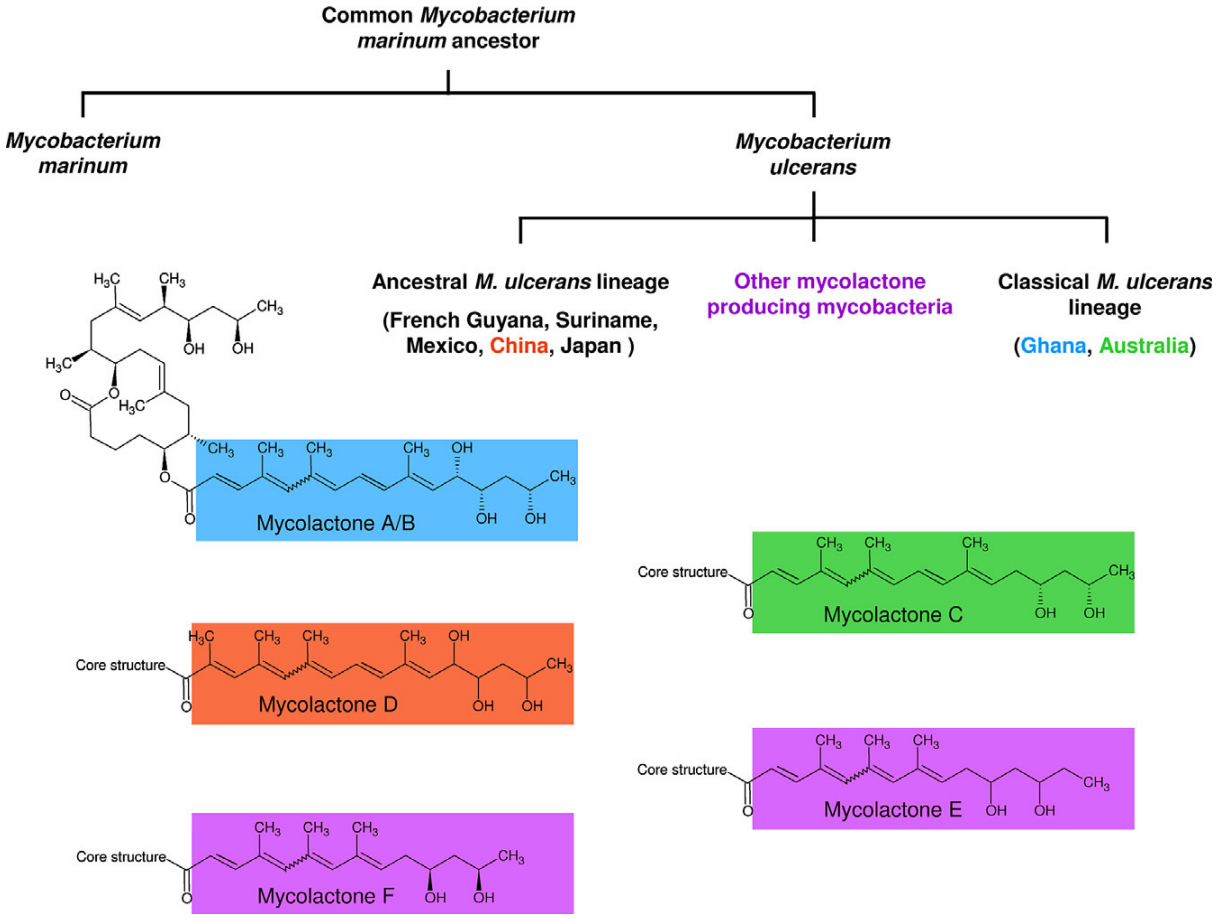


**Figure 3. Simplified family tree of *M. marinum*, *M. ulcerans* and other MPM strains.** Variants of mycolactone are shown with the corresponding color codes: mycolactone A/B produced by African *M. ulcerans* strains (blue); mycolactone C formed by *M. ulcerans* strains from Australia (green); mycolactone D produced by an *M. ulcerans* strain from China; mycolactones E and F formed by other MPM strains.

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# CHAPTER 6

## Sero-Epidemiology as a Tool to Screen Populations for Exposure to *Mycobacterium ulcerans*

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This article has been published (January 10, 2012) in:
*Public Journal of Library Neglected Tropical Diseases*

**ABSTRACT**

**Background:** Previous analyses of sera from a limited number of Ghanaian Buruli ulcer (BU) patients, their household contacts, individuals living in BU non-endemic regions as well as European controls have indicated that antibody responses to the *M. ulcerans* 18 kDa small heat shock protein (shsp) reflect exposure to this pathogen. Here, we have investigated to what extent inhabitants of regions in Ghana regarded as non-endemic for BU develop anti-18 kDa shsp antibody titers.

**Methodology/Principal Findings:** For this purpose we determined anti-18 kDa shsp IgG titers in sera collected from healthy inhabitants of the BU endemic Densu River Valley and the Volta Region, which was so far regarded as BU non-endemic. Significantly more sera from the Densu River Valley contained anti-18 kDa shsp IgG (32% versus 12%, respectively). However, some sera from the Volta Region also showed high titers. When interviewing these sero-responders, it was revealed that the person with the highest titer had a chronic wound, which was clinically diagnosed and laboratory reconfirmed as active BU. After identification of this BU index case, further BU cases were clinically diagnosed by the Volta Region local health authorities and laboratory reconfirmed. Interestingly, there was neither a difference in sero-prevalence nor in IS2404 PCR positivity of environmental samples between BU endemic and non-endemic communities located in the Densu River Valley.

**Conclusions:** These data indicate that the intensity of exposure to *M. ulcerans* in endemic and non-endemic communities along the Densu River is comparable and that currently unknown host and/or pathogen factors may determine how frequently exposure is leading to clinical disease. While even high serum titers of anti-18 kDa shsp IgG do not indicate active disease, sero-epidemiological studies can be used to identify new BU endemic areas.

## AUTHOR SUMMARY

Sero-epidemiological analyses revealed that a higher proportion of sera from individuals living in the Buruli ulcer (BU) endemic Densu River Valley of Ghana contain *Mycobacterium ulcerans* 18 kDa small heat shock protein (shsp)-specific IgG than sera from inhabitants of the Volta Region, which was regarded so far as BU non-endemic. However, follow-up studies in the Volta Region showed that the individual with the highest anti-18 kDa shsp-specific serum IgG titer of all participants from the Volta Region had a BU lesion. Identification of more BU patients in the Volta Region by subsequent active case search demonstrated that sero-epidemiology can help identify low endemicity areas. Endemic and non-endemic communities along the Densu River Valley differed neither in sero-prevalence nor in positivity of environmental samples in PCR targeting *M. ulcerans* genomic and plasmid DNA sequences. A lower risk of developing *M. ulcerans* disease in the non-endemic communities may either be related to host factors or a lower virulence of local *M. ulcerans* strains.

## INTRODUCTION

Buruli ulcer (BU), a severe necrotizing skin disease, is caused by the environmental pathogen *Mycobacterium ulcerans* (*M. ulcerans*). Globally, it is the third most prevalent mycobacterial disease that affects immunocompetent individuals after tuberculosis and leprosy [1]. Currently more than 30 countries, mainly in the Tropics and sub-Tropics, are known to report BU cases [2]. The main countries that are severely affected lie along the Gulf of Guinea and include Ivory-Coast, Ghana, Togo, Benin and Cameroon. In the highly endemic countries BU is second after tuberculosis as the most prevalent mycobacterial disease [2], [3]. However, the global burden of BU is not clear, because efficient and comprehensive reporting systems are lacking in many of the BU endemic countries. One characteristic of BU is its focal distribution within highly endemic countries. Most cases occur in remote villages with limited access to the formal health sector, prompting affected people to seek health at traditional healers [4]. Even today, not all affected communities may be known to the National BU Control Programs. Therefore reliable tools to detect and monitor the presence of BU in communities are urgently needed.

The disease presentation, which varies between individuals, starts either as a papule, nodule, plaque or edema and if these non-ulcerative early forms are not treated, extensive tissue destruction leads to the formation of large ulcerative lesions with characteristic undermined borders. Extensive tissue destruction frequently causes disfigurement and long lasting deformities such as loss of limbs and essential organs, like the eye [5], [6]. Many features of BU such as the mode of *M. ulcerans* transmission and risk factors for an infection with the pathogen are not clearly understood. However, BU is known to occur mainly in children less than 15 years of age and affects people in wetlands and disturbed environments [3], [7]. The pathology of BU is primarily associated with the secretion of the cytocidal and immunosuppressive polyketide toxin mycolactone [8].

Current methods for a laboratory confirmation of clinical BU diagnosis include microscopic detection of acid fast bacilli (AFB), culture of *M. ulcerans*, histopathology and detection of *M. ulcerans* DNA by PCR. Currently, PCR detection of the *M. ulcerans* specific insertion sequence IS*2404* is the gold standard for BU diagnosis [9].

Yet, PCR requires elaborate infrastructure and expertise and therefore make it out of reach for primary health care facilities in BU endemic low resource countries. Serology represents a more attractive approach for the development of a simple test format that can be applied to facilities treating BU in low resourced countries. Unfortunately, various studies have shown that serological tests targeting *M. ulcerans* antigens are not suitable to differentiate between patients and exposed but healthy individuals as both groups may exhibit serum IgG titers against these antigens [10], [11]. However, serology may be a useful tool for monitoring exposure of populations to *M. ulcerans*, although great antigenic cross reactivity between *M. ulcerans*, *M. tuberculosis*, BCG and other environmental mycobacteria complicates this approach. We previously profiled an immunodominant 18 kDa small heat shock protein (shsp) absent from *M. tuberculosis* and *M. bovis* as a suitable target antigen for sero-epidemiological studies. In spite of the presence of sequence homologues in *M. leprae* and *M. avium*, Western blot analyses, using a limited number of sera indicated that this protein can be used to distinguish between *M. ulcerans* exposed and non-exposed populations [10]. Here we have extended these studies with larger sets of sera. These sero-epidemiological studies identified a BU index case in a region of Ghana that was regarded, so far, as BU non-endemic.

## MATERIALS AND METHODS

### Ethics statement

Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). Written informed consent was obtained from all individuals involved in the study. Parents or guardians provided written consent on behalf of all child participants.

### Study area

One part of this study was conducted in five districts of the Eastern Region including East-Akim (EA), New-Juaben (NJ), Suhum-Kraboa-Coaltar (SHC), Akwapim South (AS) and Akwapim North (AN) as well as two districts of the Greater-Accra Region comprising Ga-West (GW) and Ga-South (GS). While EA and NJ report no BU cases and AN only occasionally, the remaining four districts have communities that report BU regularly to the NBUCP. GW reports the highest number of cases with an annual average number of 100 new cases, followed by AS, GS and the SKC. This study focused on selected communities within these districts, which are all located along the Densu River.

The other part of this study was carried out in three communities of the Volta Region, namely Torgorme, Gblornu and Kasa. These communities are situated along the banks of River Volta in the North Tongu district of the Volta Region, which was so far regarded as BU non-endemic. Torgorme, Gblornu and Kasa have an estimated population of about a 1700, 350 and 160, respectively.

### Confirmation of BU endemicity in communities along the Densu river

Initial community entry was done by first meeting community opinion leaders, which included the disease control officer responsible for the area, the assembly man and chiefs in order to explain the importance of the activity and to solicit their cooperation. A rough sketch and count of houses along the length and breadth of the community was carried out by walking through the community in order to estimate an

approximate number of houses to be surveyed. The area was then divided into two blocks, with one research team being responsible for one block. Each habitable structure within a block was then numbered serially. A house to house survey was carried out and interviews involving the head of a house were done. A data collection chart was used to collect information on the number of people in the house, healed and active BU cases and if active cases were found, samples were collected for confirmation of BU. Collected data of the BU patients included age and sex, when the disease was contracted and GPS coordinates of their houses.

**Participants and sampling for serological analysis**

Two milliliters of blood were collected into vacutainer tubes (BD) from participants of ten different villages within a 5 km radius along the Densu River; six and four of the communities were confirmed as BU endemic and non-endemic, respectively, using active search and mapping activities as described above. The endemic communities were: Kojo-Ashong and Otuaplem in the GW, Kwame Anum and Ayitey Kortor in the GS, Sakyikrom and Tetteh Kofi in the AS district, respectively. The non-endemic communities were Obuotumpan and Abotanso in the NJ and Abesim Yeboah and Ntabea in the EA district. The study participants aged between 5 and 90 years recruited from these communities were individuals with no history of BU. 188 participants were from the non-endemic villages (94 each females and males); age range 5–84 years, arithmetic mean of 28.6 years, median 19 years and mode of 15. 294 participants were from endemic villages (139 and 155 were male and females, respectively); age range 5–90 years, mean age of 26.8 years, median 20 and mode 12 years.

In addition, whole blood samples were also collected from 99 community members in three villages along the Volta River in the Volta Region which has so far been considered one of the non-endemic regions in Ghana. The three communities Torgorme, Gblornu and Kasa were selected as having never reported leprosy in the past five years according to data of the North Tongu District Directorate of Health Services.

Blood samples were transported immediately at ambient temperature to the laboratory for separation of serum by centrifugation at 2,000 g for 10 mins to remove the clot. Sera were stored at –80°C until analysis.

**Western blot analysis**

25 µg of recombinant *M. ulcerans* 18 kDa shsp protein was separated on NuPAGE® Novex 4–12% Bis-Tris ZOOM™ Gels, 1.0 mm IPG well (Invitrogen) using NuPAGE ® MES SDS Running Buffer (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS), 0.1%Tween 20 (PBS-T) and cut into strips. Protein strips were incubated with serum samples at a 1:500 dilution in PBS-T for 1.5 hrs. Strips were washed with 0.3 M PBS, 1% Tween 20 and incubated with alkaline phosphatase-conjugated AffiniPure $F(ab')_2$ fragment goat anti-human immunoglobulin G (IgG, Milian). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (BioRad) were used for color development.

**ELISA**

96-well Nunc-Immuno Maxisorp plates (Thermo Scientific) were coated with 0.5 µg recombinant 18 kDa shsp per well in 100 µl PBS. Plates were incubated at 4°C overnight. Plates were washed with $dH_2O$, 2.5% Tween 20 ($dH_2O$-T) and blocked for 1 h with 200 µl blocking buffer (5% skim milk in PBS) at 37°C. Serial 2-fold dilutions of serum from 1:100 to 1:12800 in 50 µl blocking buffer per well were incubated for 1.5 hrs at 37°C. The wells were washed with $dH_2O$-T. 50 µl of 1:6000 diluted goat anti-human IgG (γ-chain specific) coupled to horseradish Peroxidase (HRP, SouthernBiotech) was added to each well and incubated for 1 h at room temperature. After the last washing step with $dH_2O$-T, 100 µl TMB Microwell Peroxidase Substrate (KPL) was added. The reaction was stopped after 5 min. The absorbance was measured using an ELISA plate reader (Sunrise, Tecan) at 450 nm.

Each ELISA plate contained two-fold dilutions of a negative control comprising a pool of 5 negative sera from people living in BU non-endemic communities in Ghana and a positive control consisting of 5 medium positive sera from people living in BU

endemic areas. The cut-off value for positivity was considered to be the mean optical density (OD) of negative and positive control at a 1:100 serum pool dilution. Statistically, data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego California USA). The nonparametric Kruskal-Wallis test with Dunn's post-test was used to compare OD values for the different groups.

**PCR analysis of environmental samples**

Sampling was done from aquatic environments and from communities. Water, insects, fish, snails, dominant vegetation (both dead and living) and soil were collected randomly from the ground and edges of rivers at various locations in both endemic and non-endemic communities. Soil, vegetation and animal droppings were collected from various locations within both endemic and non-endemic communities. All collected samples were transported on the same day to the laboratory, stored at 4°C and analyzed within a week of collection.

DNA was extracted from about 200 mg portions of all the environmental samples using the FastDNA Spin kit for soil (MP Biomedical) according to the manufacturer's instruction. For insect samples additionally glass beads were added to the lysing matrix and the breaking step with the Fast Prep instrument was substituted by heating specimens at 95°C for twenty minutes followed by vortexing full speed for two minutes. The extracted DNA was stored at –20°C until analysis by real-time PCR.

TaqMan real-time PCR was performed using primers and procedures as previously described with some modifications in reaction conditions [12]. The primers and TaqMan MGB probes detecting IS*2404*, IS*2606* and the ketoreductase (KR) domain were obtained from Applied Biosystems (Foster City, CA, USA). IS*2404* real-time PCR mixtures contained 1X Qiagen master mix (containing HotstarTaq plus DNA polymerase, dNTP mix and PCR buffer) 1 µl of extracted template DNA, 0.5 µM concentrations of each primer and 0.2 µM probe, 1× TaqMan exogenous internal positive control (IPC) and probe reagents (Applied Biosystems), in a total volume of 20 µl. Amplification and detection were performed with the Rotor-Gene Q (Qiagen) using the following program: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 15 s. Each PCR run contained 2 non-template controls and an IS*2404*

positive control. Analysis for IS*2606* and KR was in a multiplex PCR using KR and IS*2606* probes with FAM and VIC fluorescence labels respectively and reaction conditions as above.

**Data analysis**

Initial BU survey results were entered in Microsoft Access and exported for integration using Quantum Geographic Information System (GIS) for analyses. Google Earth aerial images of communities were obtained, geo-referenced and linked to ground contours, features and other characteristics. The prevalence of BU was calculated by counting all individuals in the community with a classical BU scar, together with those with laboratory confirmed active disease, divided by the total number of persons examined within a community. The rate was expressed as a percentage.

## RESULTS

### *M. ulcerans* 18 kDa shsp specific serum IgG responses in individuals living in the Volta region of Ghana and identification of a BU index case in the region

We determined *M. ulcerans* 18 kDa shsp-specific serum IgG titers in 482 sera from people living in the BU endemic Densu River Valley in the Gar and Eastern Region, 99 sera from people living in the BU non-endemic Volta Region and 20 sera from European controls without travel history to Africa (figure 1A). Based on the defined ELISA OD cut-off values, a sero-positivity rate of 32% was observed for the sera from the Densu River Valley. The sero-positivity rate of people living in the Volta Region (12%), as well as the mean ELISA readouts obtained with their sera were significantly lower ($p<0.001$). None of the sera from European controls exhibited a significant titer (figure 1A). Sero-positive individuals from the Volta region were re-visited and interviewed. It was determined that all of them have lived entirely or at least for most of their life in their home communities in the Volta Region. One of the sero-positive participants from the village Torgorme reported at the interview to have a non-healing chronic wound on the leg (figure 1B). The wound was clinically diagnosed by an experienced physician as BU and clinical diagnosis was laboratory reconfirmed by positive IS*2404* PCR of swab specimens. The serum of this reconfirmed BU patient had the highest anti-18 kDa shsp-specific serum IgG titer of all participants from the Volta Region tested (figure 1A).

Following the identification of this index case, the health directorate of the Volta Region sent us specimens from eleven other individuals with suspected BU lesions. Six of these, were reconfirmed as IS*2404* PCR-positive BU by our laboratory at the Noguchi Memorial Institute for Medical Research, which is one of the BU reference laboratories in Ghana. While sera of two laboratory confirmed BU patients contained anti-18 kDa shsp IgG, four patients were sero-negative.

**Comparison of anti-18 kDa shsp IgG sero-positivity in BU endemic and non-endemic communities along the Densu River**

Active case search surveys were performed to determine the prevalence of BU along the Densu River (figure 2). The average prevalence of BU in endemic communities with 3 km buffer was 3.4%. While in some communities upstream no BU cases were found, the disease burden increases as the River runs downstream (figure 2A). Of the ten communities included in the sero-epidemiological study, four (Ntabea, Abesim-Yeboah, Obotanso and Obuotupan) were confirmed as non-endemic, as both the passive surveillance by the National BU control program and our active case search identified neither healed nor active cases. The total prevalence rate, including both healed and active cases, of the six endemic communities ranged from 1% to 19% with Tetteh Kofi, Otuaplem and Sode having the highest rates (4.8%, 14.9% and 19.1%, respectively). The prevalence of active cases ranged from <1% to 2.4%, with Sode also having the highest active case prevalence rate.

When 18 kDa shsp-specific serum IgG titers of 295 sera from BU endemic and of 187 sera from non-endemic communities were analyzed by ELISA (figure 1A), comparable sero-positivity rates (33% versus 31%, respectively) were found. ELISA results were reconfirmed by Western blot analysis with a randomly chosen subset of sera. There was good agreement between Western blot band intensities and ELISA titers with a few discrepancies related to a higher sensitivity of the ELISA method (data not shown). Sero-responders were found in all age groups (>5 years) tested, but sero-negative individuals dominated throughout (figure 3).

**PCR analysis of environmental samples**

211 environmental samples were collected randomly from both aquatic and dry land environs. The sampled BU endemic communities included Kojo-Ashong (KA), Sode (SD), Amasaman and surrounding hamlets (AS), and Kudeha and surrounding hamlets (KD) located in the GW and GS districts. Samples from non-endemic communities were collected in Abesim-Yeboah (AY), Obuotumpan (OB) and Ntabea (NB) located in the EA and NJ districts further up-stream of the Densu River (figure 2A).

*M. ulcerans* DNA in an environmental sample was confirmed by the presence of all three tested loci (IS*2404*, IS*2606* and KR) as revealed by positive results with all three PCR tests performed. In all, 19/211 (9.0%) of the samples tested were positive, including 5/19 aquatic snails, 5/28 sand samples collected from the communities, 4/30 samples from river water and river bed sand, 2/30 samples from aquatic vegetation, 1/6 sand samples collected from farms, 1/12 aquatic insects and 1/1 millipedes. As shown in Table 1 the average positivity rates for samples from endemic communities were 13.4% (7.7%, 13.3%, 14.3% and 18.5% in KA, AS, SD and KD, respectively) and 6.2% for samples from non-endemic communities (26.0%, 2% and 1.8% for NB, OB and AY, respectively).

## DISCUSSION

Broad antigenic cross-reactivity between mycobacterial species represents a major challenge for the development of a serological test that is specific and sensitive enough to monitor immune responses against *M. ulcerans* in populations where exposure to *M. tuberculosis* and BCG vaccination is common. In our earlier work, we have identified the *M. ulcerans* 18 kDa shsp as an immunodominant antigen, which has no homologues in *M. tuberculosis* and *M. bovis* [10]. However, interspecies cross-reactivity of this protein with an 18 kDa protein of *M. leprae* as well as a 20 kDa protein of *M. chelonae* was detected. In the same study we evaluated the use of measuring anti-18 kDa shsp IgG titers for assessing the exposure of a population to *M. ulcerans* on the basis of a limited number of BU patients, household contacts and people living in areas where BU is not endemic [10]. Since sera from inhabitants of BU non-endemic regions showed largely no reactivity with the 18 kDa protein of *M. ulcerans*, immune responses against environmental mycobacteria, such as *M. chelonae*, do not seem to compromise the developed serological test for *M. ulcerans* exposure. Here we have extended our previous analysis by comparing sera from areas of Ghana, which rarely report leprosy cases, but differ in their reported BU endemicity.

In Ghana, a national case search performed in 1999 yielded a crude national BU prevalence rate of 20.7/100,000 and hence demonstrated that BU is the second most common mycobacteriosis in the country after tuberculosis [13]. In this study diagnosis of both active and healed lesions was based solely on clinical grounds without any microbiological confirmation. Since the creation of the national control program, 32 of the 166 nation-wide districts continuously report BU. Through this passive surveillance system, over 11,000 cases have been reported between 1993 and 2006 (http://www.who.int/mediacentre/factsheets/fs199/en/) from mainly six of the ten regions of Ghana. No BU cases have been reported from the Volta, Northern, Upper East and West regions, giving the impression that those four regions do not harbor BU cases and therefore are non-endemic. However, in our analysis of sera from the Volta Region, a relatively small, but significant number of serum samples contained anti-18 kDa shsp IgG. Follow-up visits and interviews revealed that one of the sero-

positive individuals had a chronic wound which was subsequently laboratory confirmed as BU [14]. After identification of this index case, additional laboratory confirmed BU cases were found by active case search in the Volta Region. In our previous analyses [10], only part of the sera from laboratory reconfirmed BU patients were tested postitive for anti-18 kDa shsp IgG. In accordance with these findings, not all of the BU patient sera from the Volta region were sero-positive. These data clearly show that anti-18 kDa shsp IgG titers are no indication for active disease. A large epidemiological survey is now required to determine the prevalence of BU over the entire Volta Region. Until today no serological test allows for a distinction of BU patients and healthy individuals, which are exposed to *M. ulcerans*. However, our results demonstrate that sero-epidemiological studies can be used to complement active case search in regions, where data about the BU prevalence are lacking. Future longitudinal sero-epidemiological studies are planned in order to monitor the exposure of certain populations to *M. ulcerans* over a longer period of time. At this stage we cannot conclude how timing and frequency of exposure influences antibody titers against the pathogen.

The prevalence of 18 kDa shsp sero-positive individuals within populations along the Densu River was >30%. This confirms our earlier conclusion that a large proportion of healthy individuals living in endemic communities who have responded immunologically to *M. ulcerans* exposure do not develop overt disease. While the percentage of 18 kDa shsp sero-positive individuals was higher compared to that found using a Burulin skin test in healthy controls, it is comparable to that obtained for the serologic response to *M. ulcerans* culture filtrate [15],[16]. Diverse outcome of infection with the causative agents of the main mycobacterial diseases such as tuberculosis seems to be a common feature of their natural history. Not all exposed individuals show immunological evidence of infection and of those who get infected by *M. tuberculosis* estimations indicate that only 10% will ever develop overt disease [17], [18]. Manifestation of the disease ranges from self-limited pulmonary infection to localized extra-pulmonary infection and disseminated disease [19]. Factors accounting for the diversity in outcomes are not entirely known, but may relate to both host and pathogen factors. Even though clinical *M. ulcerans* isolates from Africa are clonally related and genetically largely monomorphic [20]–[27], differences in

virulence among African *M. ulcerans* strains cannot be ruled out completely. Hence, the percentage of *M. ulcerans* infected individuals who proceed to develop BU remains to be established. BU is known to develop in all age groups with a nearly equal gender distribution but most cases occur in children 15 years of age or younger [28]. In our study we found anti-18 kDa shsp sero-responders in all age groups (>5 years) analyzed. A future cohort study with infants could provide important insight, at which age these immune responses start to emerge.

Both in endemic and non-endemic villages of the Densu River Valley we found *M. ulcerans* PCR positive environmental samples. This is indicative for the presence of *M. ulcerans* or of closely related environmental bacteria all along the Densu River. Our findings are consistent with earlier findings of Williamson et al. [29] in the same region. Since the mode of *M. ulcerans* transmission and risk factors for the exposure to the pathogen are still not entirely elucidated, it is not clear, whether the types of environmental samples that were PCR positive have direct relevance for infection with *M. ulcerans*. Hence methods for the routine isolation and characterization of *M. ulcerans* from the environment need to be developed.

Hypotheses on risk factors and the mode of infection with *M. ulcerans* include contamination of wounds from an environmental reservoir, inhalation of vaporized contaminated water and inoculation by insects [30]–[32]. Our molecular epidemiological studies have recently demonstrated a focal transmission pattern for *M. ulcerans* [27]. This may help to explain one of the mysteries of BU transmission, the close proximity of endemic and non-endemic villages. As indicated in figure 2, while *M. ulcerans* is endemic in some villages within the Suhum-Kraboa-Coaltar district, through active case search we did not find any case (both healed and active) in neighboring districts located at the upper part of the river, such as East-Akim and the New-Juaben. In contrast, communities of the four districts, which are situated downstream (Akwapim South, Akwapim North, Ga-West and Ga-South) regularly report BU cases. BU endemic and non-endemic communities along the Densu river differ in terms of their vegetation. Upstream, within the wet semi-equatorial zone, the vegetation is predominantly moist semi-deciduous rain forest, which gradually changes downstream into a short stretch of Guinea Savannah around Nsawam and ends with coastal scrub and savannah grassland in the Ga districts. In addition, there

is a variation in the features of the Densu River, which takes its source from the Atewa Forest Range near Kibi and flows for 116 km into the Weija Water Reservoir before entering the Gulf of Guinea through the Densu Delta Ramsar site. While upstream the river flows fast, has clear water and the river bed consists of rocky stones, downstream the river flows sluggishly, has a muddy river bed, and the water is turbid. We did not find significant differences in anti-18 kDa shsp IgG seropositivity rate or titers between people living in communities in the Densu River Valley that were classified based on active case search as BU endemic or non-endemic. These findings could imply at least one of the following: 1) people in the non-endemic communities in the upper Densu River Valley may be exposed to *M. ulcerans* lineages with low virulence; 2) currently unknown host genetic, behavioral or socio-economic factors trigger the development of subclinical *M. ulcerans* infection to clinical disease; 3) in the non-endemic communities 18 kDa shsp binding antibodies are triggered by subclinical infections with environmental mycobacteria harboring antigens that are cross-reactive with the 18 kDa shsp [10].

**ACKNOWLEDGMENTS**

We are very grateful to the Disease Control and Technical officers in the districts that we worked with. We thank the chiefs, opinion leaders and inhabitants of the various communities for their participation and time. We thank Charles Atiogbe, John Fenteng, Zuliehatu Nakobu for blood sample collection and sera preparation and Eric Koka for community interviews.

## TABLES

**Table 1. IS*2404*, IS*2606*, KR PCR positivity of environmental samples collected in communities along the Densu River.**

| Village | BU endemicity | BU prevalence* | PCR positivity** |
|---|---|---|---|
| Obuotupan | Non-endemic | 0% | 1/50 (2%) |
| Abesim Yeboah | Non-endemic | 0% | 1/56 (1.78%) |
| Ntabea | Non-endemic | 0% | 6/23 (26%) |
| Amasaman | Endemic | 5.1% | 2/15 (13.3%) |
| Kudeha | Endemic | 3.2% | 5/27 (18.51%) |
| Kojo Ashong | Endemic | 14.3% | 2/26 (7.69%) |
| Sode | Endemic | 19.1% | 2/14 (14.28%) |
| **Total** | | | **19/211 (9%)** |

*Prevalence rate is given as the total prevalence comprising both active and healed lesions.
**Positivity indicates the presence of all three loci targeted by RT-PC.
doi:10.1371/journal.pntd.0001460.t001

**FIGURES**

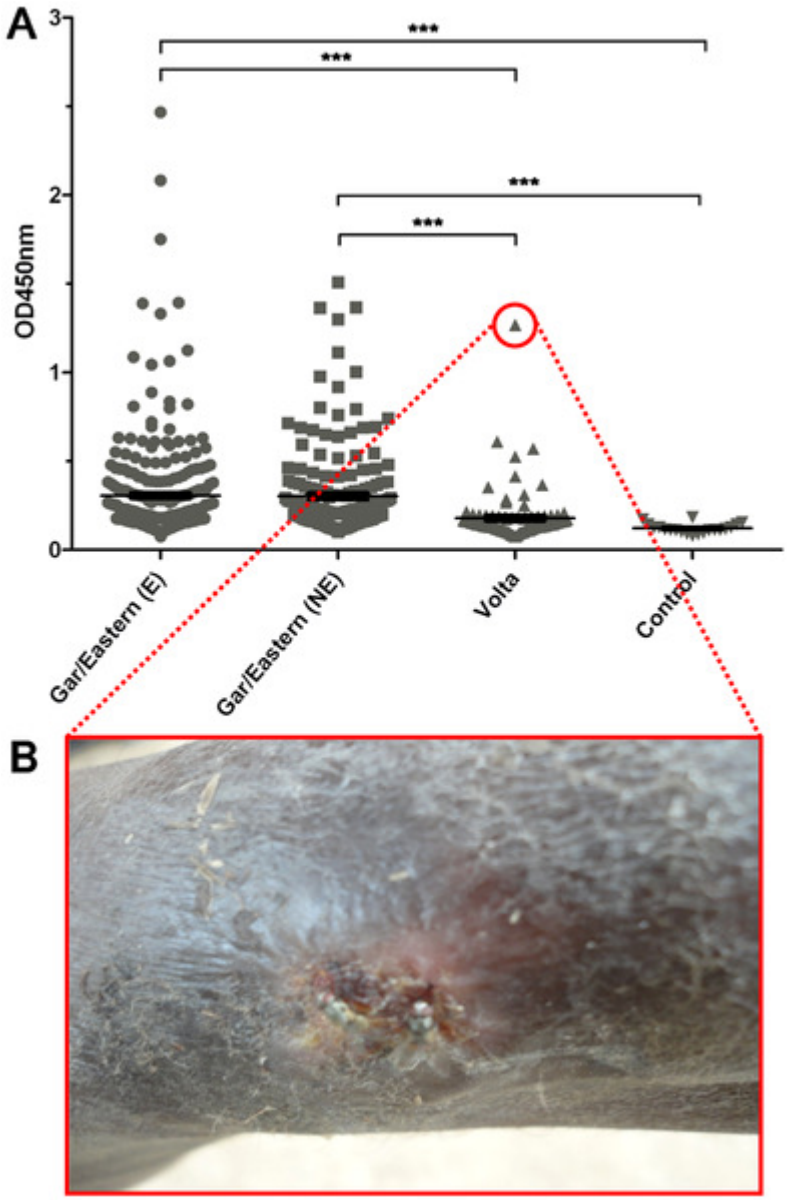
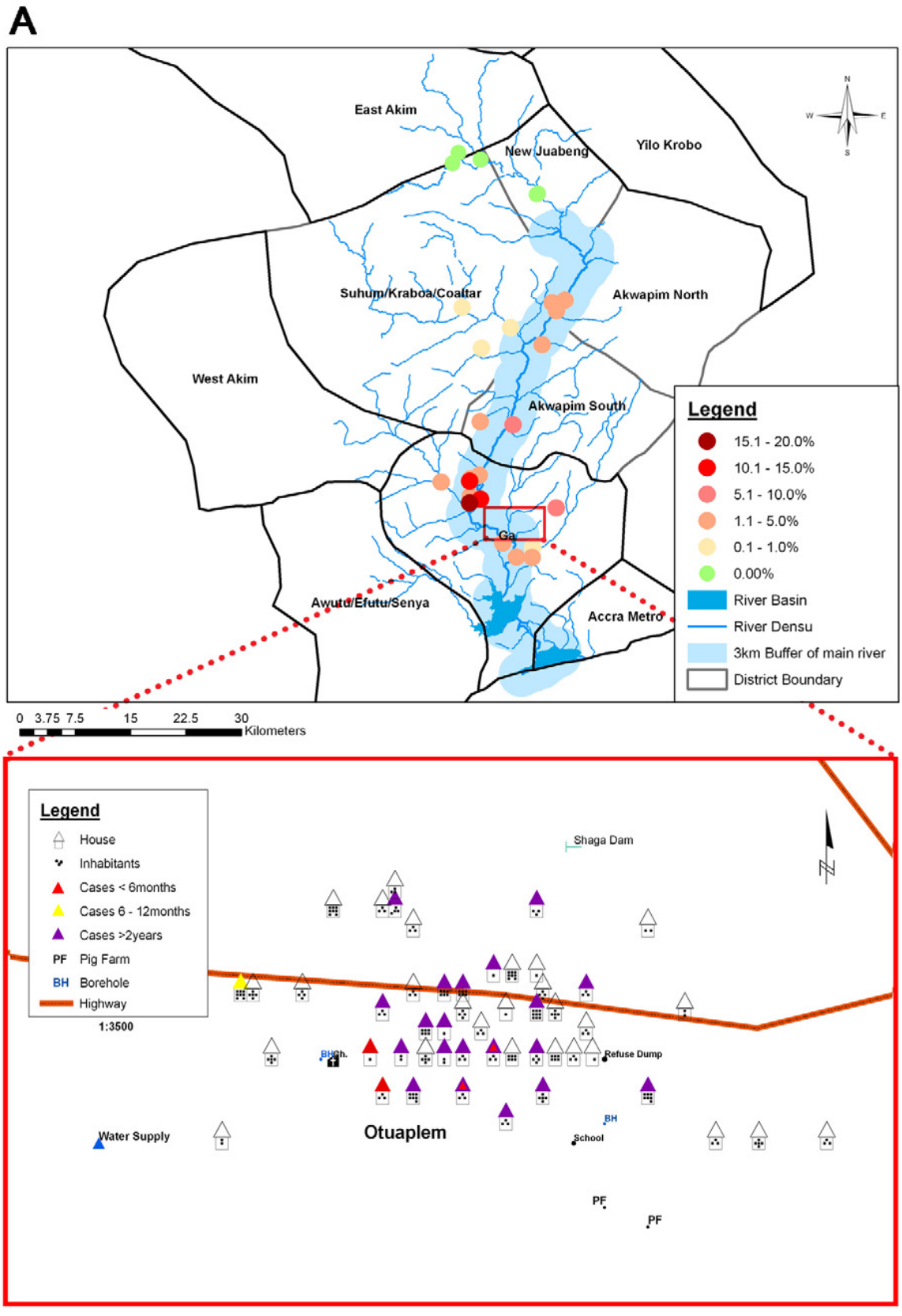



**Figure 1. Anti-*M. ulcerans* 18 kDa shsp IgG titers. A** OD values of sera from individuals living in BU endemic (E) and non-endemic (NE) communities along the Densu River (Gar/Eastern) or in the Volta Region (Volta) of Ghana obtained in an 18 kDa shsp ELISA for a 1 : 100 serum dilution are shown for individual sera. Sera from Europeans without travel history to Africa served as controls. Statistical differences between groups were calculated by the Kruskal-Wallis test with Dunn's post test (*** = $p<0.001$). **B** Laboratory confirmed BU lesion of a sero-positive participant from the Volta region.


A
East Akim
New Juabeng
Yilo Krobo
Suhum/Kraboa/Coaltar
Akwapim North
West Akim
Akwapim South
Ga
Awutu/Efutu/Senya
Accra Metro
N
W
E
S
Legend
15.1 - 20.0%
10.1 - 15.0%
5.1 - 10.0%
1.1 - 5.0%
0.1 - 1.0%
0.00%
River Basin
River Densu
3km Buffer of main river
District Boundary
0 3.75 7.5 15 22.5 30
Kilometers
Legend
House
Inhabitants
Cases < 6months
Cases 6 - 12months
Cases >2years
PF Pig Farm
BH Borehole
Highway
1:3500
Shaga Dam
Refuse Dump
Water Supply
Otuaplem
School
BH
PF
PF

B

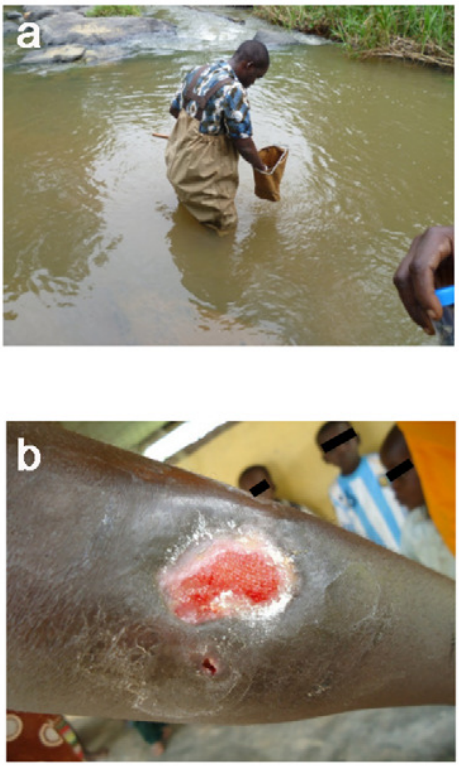



**Figure 2. Prevalence of BU and sample collection procedure. A** Map showing the prevalence of BU in selected communities along the Densu River. The prevalence rate was calculated by adding the number of individuals both with active or healed BU lesions divided by the total population number of the community. Exemplarily, the housing and population census is illustrated for the village Otuaplem. **B** Photographs illustrating environmental sampling (a), reconnaissance visit in the communities (b), an active BU lesion found in a community during active search (c) as well as a healed BU lesion also identified in a community (d).

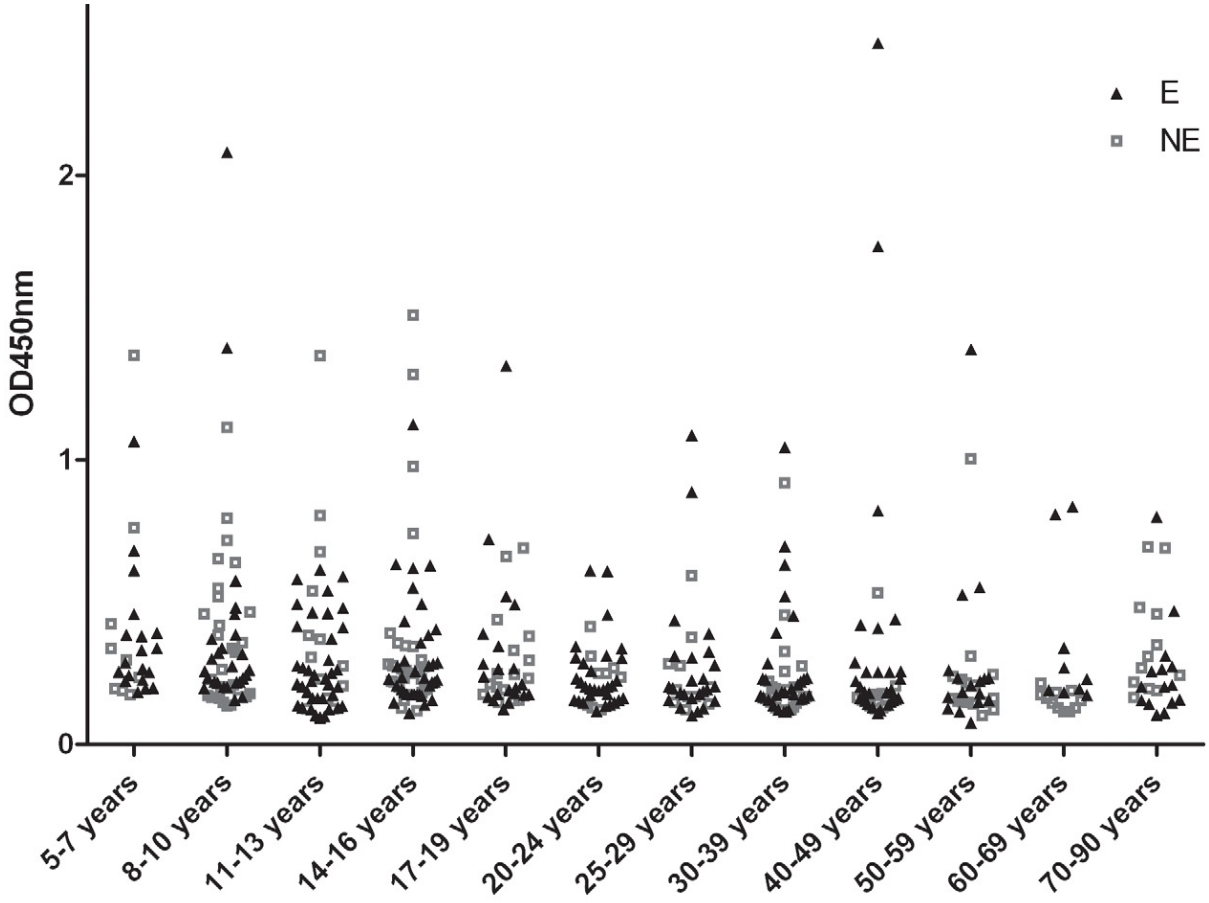



**Figure 3. Age distribution of anti-18 kDa shsp IgG titers.** OD values of sera from individuals living in BU endemic (E) and non-endemic (NE) communities along the Densu River obtained in an 18 kDa shsp ELISA for a 1 : 100 dilution are shown for individual sera.

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CHAPTER 7

# Screening of antifungal azole drugs and agrochemicals with an adapted Alamar Blue-based assay demonstrates antibacterial activity of Croconazole against *Mycobacterium ulcerans*

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This article has been published (September 24, 2012) in:
*Antimicrobial agents and chemotherapy*

**An Alamar Blue-based growth inhibition assay has been adapted for the thermosensitive and slow growing pathogen *Mycobacterium ulcerans* (*M. ulcerans*). The standardized test procedure enables medium-throughput screening of pre-selected compound libraries. Testing of a set of 48 azoles with known antifungal activity led to the identification of an imidazole antifungal displaying an inhibitory dose (ID) of 9 μM for *M. ulcerans*.**

*M. ulcerans* is the causative agent of Buruli ulcer, a disfiguring disease characterized by the formation of chronic, necrotizing skin ulcers. Buruli ulcer is a major public health problem primarily in West Africa, where it typically affects impoverished inhabitants of remote rural areas (25). Surgical excision of lesions was the only therapeutic option until the World Health Organization released provisional guidelines in 2004 advising an eight-week combination chemotherapy of oral rifampicin and intramuscular streptomycin (26). Implementation of this regimen has significantly reduced recurrence rates. However, there are some drawbacks associated with the administration of these antibiotics. Streptomycin requires daily injections and is potentially nephrotoxic and ototoxic (5). Monotherapy with rifampicin is not a solution because resistance can develop rapidly (14). While studies on fully oral regimens replacing streptomycin by clarithromycin yielded promising results (2, 6), identification of new drugs that could replace rifampicin would be most desirable.

Since lead compounds identified by target-based approaches are frequently inactive against the pathogen grown in culture (4, 13, 19), whole cell assays are highly valuable in the search for new antibiotics. The extremely slow growth rate of *M. ulcerans* complicates the identification of potentially effective new scaffolds. In this study, we describe the development of an Alamar Blue microplate assay, which enables screening of compounds for their activity against *M. ulcerans* within ten days. Here we report results of a pilot study with this medium-throughput screening procedure. Testing of a pre-selected group of azoles led to the detection of compounds with potency against *M. ulcerans*.

Alamar Blue-based assays have been shown to be powerful and sensitive tools for the identification of growth inhibitory compounds irrespective of the underlying mode

of action and chemical classification. They are widely used for screens both with mammalian cells and microorganisms, including mycobacterial species (4, 7, 9, 10, 13, 19). In order to develop a suitable test format for *M. ulcerans*, the specific growth features, a long generation time of about 72 hours and a low optimal growth temperature of 28-32°C, had to be taken into account. To reduce the problem that *M. ulcerans* tends to aggregate when grown in liquid medium, the inoculum was prepared in BacT/ALERT® culture bottles supplemented with enrichment medium according to the manufacturer's protocol (Biomerieux). NM20/02, a *M. ulcerans* strain isolated in 2002 from the Buruli ulcer lesion of a Ghanaian patient (22, 27) as well as additional strains from Togo (ITM 970680), Côte d'Ivoire (ITM 940511) and Australia (JS5147) were grown for 6-8 weeks at 30°C until exponential phase. The test inoculum was prepared by diluting the culture with BacT/ALERT® medium to a final $OD_{600}$ of about 0.02.

The Alamar Blue growth inhibition assay was performed in clear, flat-bottomed 96-well plates (BD Falcon). Stock solutions of the tested compounds were prepared in high-grade DMSO (Sigma) at a concentration of 10 mg/ml and diluted for the assay with DMSO to a concentration of 0.2 mg/ml. Then two-fold dilution series in medium starting from 0.0125 mg/ml to 0.0001953 mg/ml were prepared in a volume of 100 µl and set up in triplicates. As controls, streptomycin (Sigma) at a concentration of 0.0125 mg/ml to 0.0001953 mg/ml was used. In addition, medium and DMSO controls were included. To each well of a 96-well plate, 100 µl of the diluted *M. ulcerans* suspension was added. Plates were incubated for 8-10 days at 30°C, then 20 µl alamarBlue® (Invitrogen) was added and the plates were further incubated and monitored at 37°C as recommended for the reduction of the alamarBlue® component resazurin. During the short incubation period of 24 hours *M. ulcerans* viability was not affected by this elevated temperature (8). Since *M. ulcerans* is considered a biosafety level 3 pathogen in Switzerland, bacterial suspensions were afterwards inactivated for measurement of the fluorescence intensity outside of the BSL3 laboratory. 100 µl portions of each well were transferred to corresponding wells of a new 96-well plate containing 100 µl neutral buffered formalin solution (Sigma, final concentration 5% v/v). The plates were sealed with optical adhesive film (Applied Biosystems) and the

fluorescence intensity was measured using a SPECTRAmax Gemini XS device with Softmax Pro 5.2 software (λ = 540/588 nm). Procentual signal reduction was assessed by comparison with *M. ulcerans* control cultures containing compound-free DMSO and inhibitory dose (ID) values corresponding to 90% and 50% inhibition were determined.

For registered drugs, a complete toxicological profile is commonly available, but also registered agrochemicals have been broadly examined for their toxicological potential and for other features that have to be characterized prior to clinical phase I testing. Hence, compounds from both sources represent preferred candidates for medium-throughput testing for new indications. Here we have focused on azole-type fungicides, commercialized as drugs or agrochemicals. These compounds are cytochrome P450 inhibitors with partial specificity for the lanosterol 14-alpha-demethylase, cytochrome P450 51 (CYP51). The genomes of *Mycobacterium tuberculosis* (*M. tuberculosis*) and *M. ulcerans* (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList.woa/wa) encode at least 20 CYP enzymes. CYP inhibitors have been shown to inhibit *M. tuberculosis* growth (15) and among them, CYP51 seems to be highly relevant (21). Therefore, azole-type fungicides might have potential as drug candidates against mycobacterial infections. We have retrieved 48 commercialized azoles from the BASF compound repository (Table S1) and tested them in the Alamar Blue-based screening assay for *M. ulcerans* growth inhibitory activity. Of these 48 azoles, only the antifungal drug Croconazole, 1-[1-[2-[(3-chlorophenyl)methoxy]phenyl]ethenyl]-1*H*-imidazole (18), showed significant activity with an ID-90 of 2.9 µg/ml, equivalent to 9 µM, and an ID-50 of 0.8 µg/ml, equivalent to 2.6 µM (Figure 1A). In addition to the Ghanaian reference strain, we tested the activity of Croconazole on *M. ulcerans* isolates originating from Côte d'Ivoire, Togo and Australia and obtained similar ID-50 values of 0.7 µg/ml, 1.1 µg/ml and 0.6 µg/ml, respectively.

The characteristic feature of Croconazole, discriminating it from all other tested azoles, is a methylen-substitution at the imidazole-binding carbon. To further evaluate the structure-activity relationship of this compound class, eight additional analogues possessing this structural motif have been tested. Of these, only the 4-Cl-

isomer of Croconazole displayed a similar activity with an ID-90 of 2.8 µg/ml, equivalent to 8 µM, and an ID-50 of 0.95 µg/ml, equivalent to 2.7 µM (Figure 1B, Figure 2). While cytochrome binding via the imidazole is the essential interaction with the P450-enzyme, these data indicate that also the lipophilic side chain interaction is of critical importance, since already moderate side chain modifications lead to loss of activity. However, also metabolic deactivation cannot be ruled out. As all the other tested commercial azole drugs and agrochemicals, which show a very broad spectrum of substitution patterns and intrinsically high activity on a variety of CYPs, were inactive against *M. ulcerans*, the unique activity of Croconazole and its 4-Cl-analogue is rather surprising.

Croconazole is a potent imidazole antifungal that acts extracellularly on the cell membrane of fungi. It has been shown to inhibit predominantly CYP51, and thus interferes with the synthesis of ergosterol, the precursor of vitamine $D_2$ (11). The active sites of CYP51 are conserved in many mycobacterial species and virtually identical in *M. tuberculosis* and *M. ulcerans* while there are many differences to human CYP51 (21). It remains to be elucidated whether Croconazole exerts its inhibitory activity on *M. ulcerans* via inhibition of CYP51 or via another target.

Interestingly, apart from being employed as a broad spectrum fungicide (17, 24) developed by Shionogi Research Laboratories (Osaka, Japan), Croconazole is commercially also available as an active ingredient of a cream called Pilzcin® (Merz + Co., Frankfurt/Main, Germany (16)), indicated for topical treatment of dermatomycoses and candidiasis. Pilzcin® is in particular recommended for treatment of tinea pedis, an infection of the skin caused by fungi of the genus *Trichophyton* (3). Severe side-effects upon treatment with Croconazole containing drugs have not been described; however, a few cases of contact sensitization have been reported (23). Since Buruli ulcer primarily affects the skin and underlying tissue, topical treatment regimens have been considered and evaluated (1, 20). In active lesions clusters of toxin producing *M. ulcerans* are primarily found in deep layers of the subcutaneous fat tissue. Topical treatments thus may only be effective if the antimycobacterial agent is able to act in these deeper layers of the skin.

Since the development of new drugs is a very costly and time-consuming process, re-evaluation of already existing therapeutics for their potential effects against other diseases is a straightforward approach. A recent study focusing on the detection of new anti-tuberculosis drugs by re-evaluating a library of known human therapeutics led to the identification of promising new scaffolds (12). In a similar approach we identified Croconazole as a potent compound active against *M. ulcerans* by revisiting a panel of 48 well-characterized azole drugs and agrochemicals. In conclusion, screening of such pre-selected sets of compounds increases the chance to detect scaffolds on which future drug development efforts can be focused.

**ACKNOWLEDGEMENTS**

We would like to thank Marcel Kaiser for expert scientific advice.

**FIGURES**

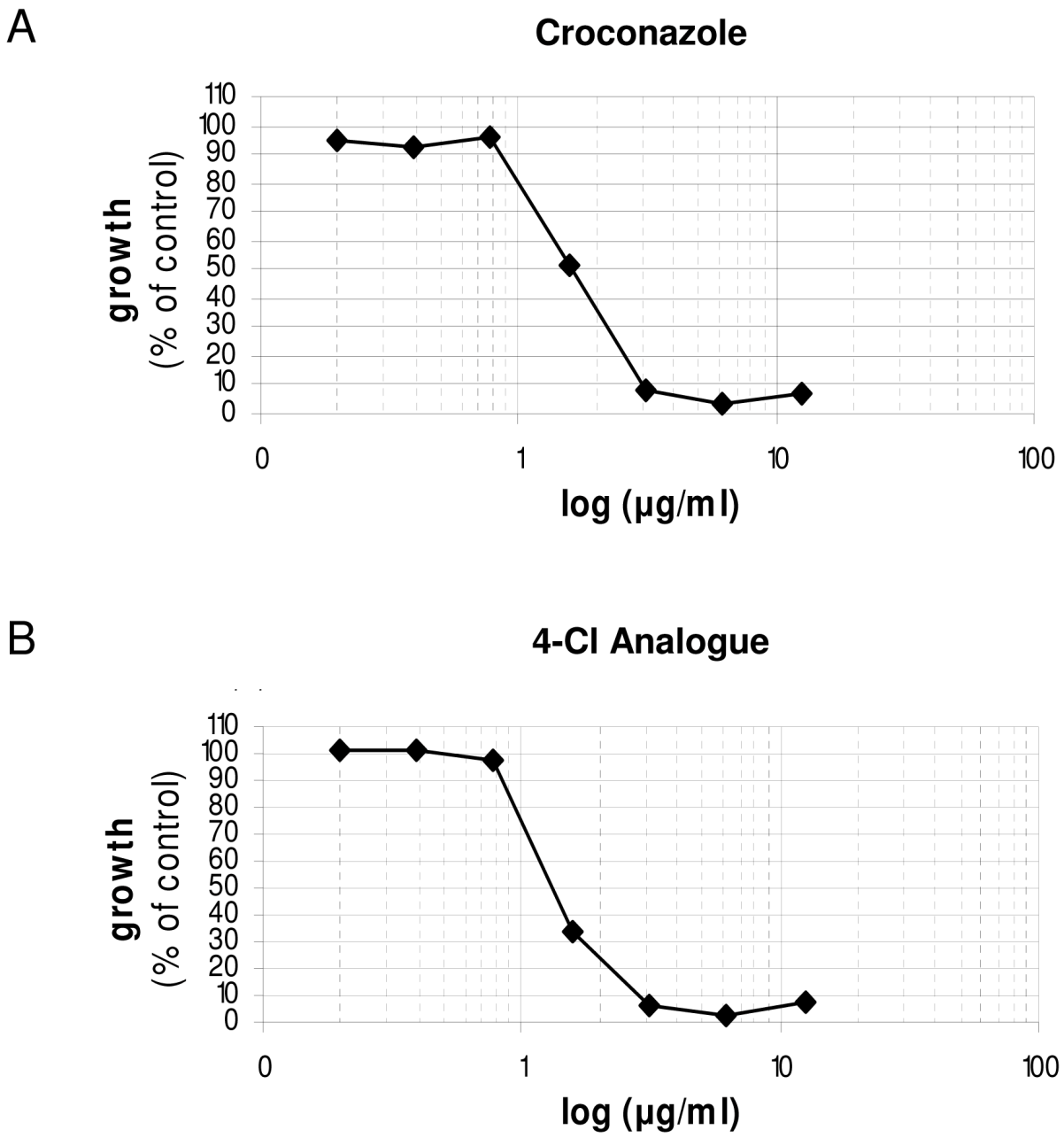


**Figure 1.** Inhibition of *M. ulcerans* growth upon treatment with the azole compound Croconazole (A) and its 4-Cl-analogue (B).

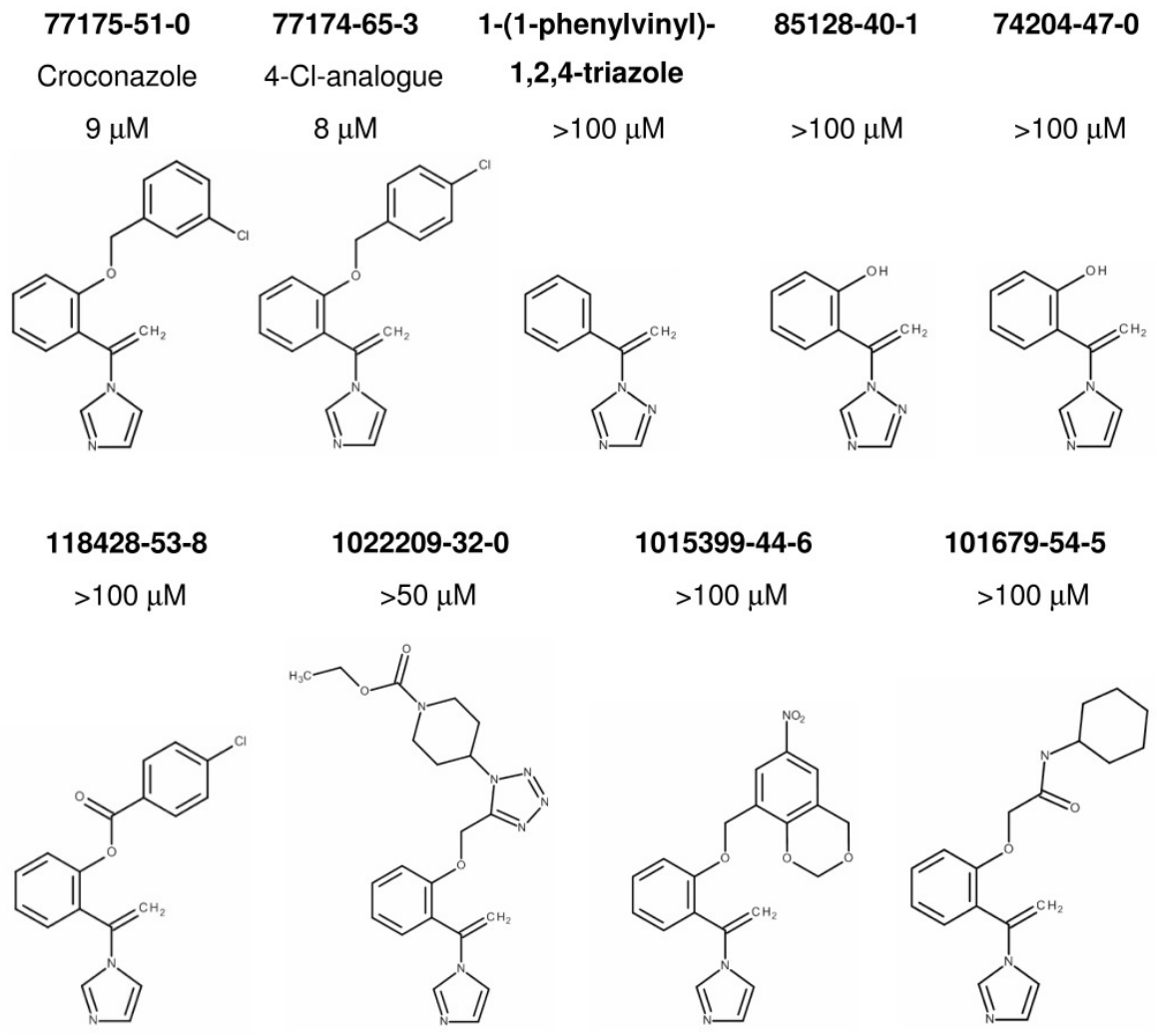


**Figure 2.** Overview of the Croconazole derivatives with corresponding CAS numbers and MIC values.

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# CHAPTER 8

# General discussion and conclusions

"*The war against infectious diseases has been won*", was proclaimed by U.S. Surgeon General William H. Stewart in the late 1960s, contemplating the benefits of antibiotics and vaccines [1]. However, the history of infectious diseases from that time until now has witnessed quite the opposite, namely the appearance and dissemination of Legionnaires` disease, Hantavirus pulmonary syndrome (HPS), Lyme disease and the acquired immune deficiency syndrome (AIDS), to mention but a few. Although the occurrence of these diseases often may seem mysterious, factors contributing to their emergence can be determined in virtually all cases studied [2–6]. While the continual evolution and adaptation of potentially pathogenic organisms provides an inexhaustible source for the emergence and re-emergence of infectious diseases, other determinants including ecological, environmental or demographic factors interact to precipitate disease emergence [2]. The recurring theme throughout all of these factors are human-induced changes [7], leading to an increased contact of individuals/populations to the pathogen, its vector or environmental reservoirs [2], which can be illustrated on the basis of the above and below mentioned disease examples. Outbreaks of Legionnaires` disease caused by *Legionella pneumophila* are largely associated with human-made aquatic environments containing water at elevated temperatures. Multiplication of the bacteria and subsequent inhalation of contaminated droplets through artificial water supply systems can lead to infection [6,8]. Human behavioral factors including farming, land development, deforestation and camping favor epidemics of HPS, which occur with increased exposure of people to faeces of specific rodent species, the natural reservoir of Hantaviruses [5,8]. The emergence of Lyme disease in the United States caused by *Borrelia burgdorferi* was probably the consequence of reforestation around settlements, which increased the population of deer and simultaneously the occurrence of the deer tick vector [3]. The unprecedented spread of HIV/AIDS was fostered by changes in human demographics and behavior including increased travel, movement of rural populations to large cities, urban poverty and a weakening of family structures promoting sexual practices that facilitate HIV transmission [8].

The resurgence of historically important diseases such as cholera, diphtheria and plague has been accelerated by wars or natural disasters such as earthquakes and

floods [8]. Recurrence or geographical dissemination of diseases transmitted by mosquitoes and other arthropod vectors have been mostly attributed to ecological changes resulting in the creation of standing water [9]. To give one example, dam-building and irrigation projects appear to favor the spread of schistosomiasis to new geographical areas, since they provide ideal habitats for both the schistosomes and their freshwater snail intermediate hosts [10].

Buruli ulcer, caused by infection with *Mycobacterium ulcerans*, is classified by the World Health Organization as one of currently 17 neglected tropical diseases [11]. Though not officially categorized by the former National Center for Infectious Diseases (CDC, currently reorganized into multiple national centers), as emerging or re-emerging, strong increases of BU case numbers in West Africa as well as the detection of new infection foci in the last decades [12–17], render BU an emerging infectious disease. While vast underreporting of BU cases in rural African regions results in an underestimation of the actual disease burden, it is believed that the incidence of BU in highly affected areas may exceed that of leprosy and in some regions even that of tuberculosis. Until today the emergence of BU can not entirely be explained and environmental reservoirs as well as transmission pathways remain to be established. However, several aspects mentioned above including evolution and niche adaptation of the pathogen *M. ulcerans* as well as ecological and environmental factors, which may contribute to the emergence of BU, will be discussed in the following sections and connected with the obtained results described in this PhD thesis.

**Buruli ulcer: emergence of a mysterious disease**

Morse suggested in 1991 that the emergence of infectious diseases can be thought of as a two-step process, in which the introduction of a pathogen into a new host population is followed by the establishment and further dissemination of the pathogen within the new host population [2,18]. But long before new host populations can be infected, a pathogen emerges itself by incessant evolution and adaptation processes.

Evolution and genetic diversity of *M. ulcerans*

The evolution of *Mycobacterium ulcerans* was coined by the acquisition of the virulence plasmid pMUM, probably enabling its divergence from the fish pathogen *Mycobacterium marinum* around a million years ago [19,20]. Although the genomes of these two species share more than 97% nucleotide identity, they cause different diseases in humans and exhibit widely different phenotypes [19,21,22]. The pathology of BU characterized by chronic and necrotic skin ulceration is largely mediated by the production of mycolactone, an immunosuppressive polyketide toxin encoded by pMUM and restricted to representatives of the species *M. ulcerans*, collectively referred to as mycolactone producing mycobacteria (MPM) [22,23]. *M. marinum* is a ubiquitous waterborne organism that usually infects fish and amphibians. Infection in humans occurs only occasionally and leads to the development of granulomatous skin lesions [24]. Thus, it seems likely that the ability to produce mycolactone was a first step in the emergence of a potentially infectious agent and that this evolutionary bottleneck has facilitated the development of a highly clonal species with increased virulence compared to that of its ancestor. A strong selective advantage is thought to preserve a mycolactone-proficient form of pMUM, since only seven structural variants of mycolactone (referred to as A/B, C, D, E, F, S1 and S2) could be identified so far and strains isolated from different African countries over a 40 year period were shown to produce the same mycolactone A/B [25,26]. Mycolactone production probably provided the ability for an early *M. ulcerans* population to persist in a new environment, where its generalist *M. marinum* progenitor could not be extant [22]. It is currently debated whether amoebae may act as a host for *M. ulcerans* in the environment [27]. However, until today it is not clear how *M. ulcerans* might benefit from mycolactone production in biotic surroundings. In the human host it has long been assumed that a potential anti-microbial activity of mycolactone might prevent secondary infections of the BU lesions and thus confer an advantage by circumventing competition with other microbes. As opposed to this, a recent study analyzing the bacterial flora of BU lesions has shown that a broad spectrum of bacterial species is present within the lesions (Yeboah-Manu, submitted). Presently it is assumed that the combination of cytotoxic as well as immunosuppressive properties of mycolactone confers a fitness advantage for *M.*

*ulcerans* by preventing a host immune system-mediated elimination of the bacteria. A second important event in the emergence of *M. ulcerans* was the acquisition and expansion of an insertion sequence (IS*2404*), which has resulted in the inactivation of genes and extensive loss of DNA [19]. While the genome of *M. marinum* strain M comprises a 6.6-Mb circular chromosome with 5424 coding sequences (CDS) and only 65 inactivated genes [21], the genome of *M. ulcerans* strain Agy99 consists of two circular replicons with a size of 5.8-Mb, 4241 CDS and 771 pseudogenes [19]. The described genomic signatures of *M. ulcerans* such as acquisition of foreign DNA, proliferation of IS elements, extensive gene loss through pseudogene formation and genome downsizing indicate that *M. ulcerans* is adapting to a new, apparently protected niche environment, where genes once needed for survival under diverse conditions are no longer required [19,22,28]. The current conception of a possible niche after analyses of the genome content of *M. ulcerans* strain Agy99 suggests that in contrast to the generalist *M. marinum*, *M. ulcerans* has become a specialist bacterium adapted to a dark, extracellular environment, where special characteristics such as the extremely slow growth rate, the reported depletion of highly immunogenic proteins [29] and the production of an immunosuppressive toxin provide a selective advantage [19,22]. Recent comparative genome sequencing data including mycolactone producing mycobacteria isolated from human, possum, fish, frog or insect sources of different geographical areas suggests a distinction of at least three *M. ulcerans* sublineages, which should be considered as *M. ulcerans* ecovars, in view of their adaptation to related, but distinct niche environments. The occupation of different ecological niches seems to be connected with the contact of MPM variants to different hosts, since one of these ecovars is responsible for BU cases in West Africa and Australia (sublineage 3), while representatives of the other two lineages are only occasionally reported to cause disease in humans (sublineage 2) or are exclusively associated with disease in ectotherms. *M. ulcerans* sublineage 3 is highly clonal and exhibits genome signatures indicating that this lineage has undergone another evolutionary bottleneck and further adaptation to its niche environment [22]. Comparative genome analysis of closely related lineage 3 isolates is expected to provide insights into possible sources and transmission routes of *M. ulcerans*.

In the past, the clonality and extremely limited genetic diversity of *M. ulcerans* lineage 3, most notably *M. ulcerans* strains from West Africa, combined with the lack of a typing method with sufficient resolution for micro-epidemiological studies have hampered attempts to identify evolutionary relationships between these closely related strains [30–33]. As a consequence, only fragmentary information is available regarding the routes of *M. ulcerans* dispersal both throughout West Africa and within BU endemic countries or even geographically confined *M. ulcerans* infection foci. Recent advances in genetic typing technologies and whole genome sequencing have provided the basis for investigations on the population structure of clonal bacterial populations characterized by limited genetic diversity such as *Yersinia pestis*, *Bacillus anthracis* or *Mycobacterium leprae*, leading to important insights into their spread, transmission routes and/or reservoirs [34–37]. To mention one example, a recent study investigated the phylogeography and molecular epidemiology of *Yersinia pestis* in Madagascar, one of the most plague-affected regions in the world. By typing 262 Malagasy isolates from 25 districts isolated between 1939 and 2005 on the basis of 56 single nucleotide polymorphisms (SNPs) and a 43-marker multiple locus variable number of tandem repeat (MLVA) system, island-wide geographic-genetic patterns and potential transmission routes could be identified. The results of this study, namely that significant geographic separation exists among the identified *Y. pestis* subclades, are consistent with the population genetics and ecology of the black rat, which is the main reservoir of *Y. pestis* in rural Madagascar and characterized by limited geographic ranges. The two primary flea vectors of *Y. pestis* may also play a role in maintaining genetically distinct subpopulations [37]. As highly stable phylogenetic markers that are unlikely to mutate again [38], SNPs represent ideal entities for detailed phylogenetic analyses of clonal pathogens with limited genetic diversity [39]. Once SNPs have been identified by comparative genome sequencing, various technologies are available for SNP typing analyses [40].

Our comparative genome sequencing of clinical *M. ulcerans* strains from Ghana has facilitated a genome-wide search for SNPs (chapter 2), enabling micro-epidemiological analyses within BU endemic areas of Ghana. In order to capture as much as possible of the genetic information present in the Ghanaian *M. ulcerans* population, one representative isolate of each of the three previously identified

variable number of tandem repeat (VNTR) types [31] was selected for genome comparison and SNP detection. Subsequent development of a real-time PCR method based on the identified SNPs facilitated for the first time a differentiation of 75 *M. ulcerans* strains isolated from BU patients of one geographically confined *M. ulcerans* infection focus in the Densu River Basin of Ghana between 1999 and 2007 into six haplotypes. Re-sequencing of four haplotype representatives led to the identification of additional SNPs enabling the establishment of even more SNP typing assays and consequently the differentiation of ten *M. ulcerans* haplotypes in the Densu River Basin of Ghana. Interestingly, several of the ten haplotypes showed a geographically clustered distribution within the small BU endemic region. Since the most prevalent haplotype was distributed over the entire area, it was hypothesized that this *M. ulcerans* variant was probably the founder haplotype in this area, from which the other haplotypes derived (chapter 3). However, whole genome sequencing of additional strains from the Densu River Valley is required to support this hypothesis. Typing results of clinical *M. ulcerans* isolates from another BU endemic region in the Amansie-West district of Ghana indicate the presence of two different clonal *M. ulcerans* complexes in the two geographically separate regions (chapter 3). The geographic separation of *M. ulcerans* haplotypes both between and within BU endemic areas is reminiscent of the distribution of *Y. pestis* subclades in Madagascar. While geographic-genetic patterns of *Y. pestis* could already be explained by the natural cycle between primary host, the black rat, and the flea vectors [37], reservoir(s) and vector(s) for *M. ulcerans* in West Africa have yet to be explored. Similar to *Y. pestis*, a transmission model has been established for *M. ulcerans* in Australian BU endemic settings pointing to mammals, most notably tree-dwelling native possums as possible reservoir [41] and mosquitoes as potential vectors for *M. ulcerans* [42]. In this concept, infection of humans is thought to take place via biting mosquitoes that fed on infected possums or had contact to vegetation contaminated by diseased possums [41]. However, this model is not easily translatable to African *M. ulcerans* infection foci. Firstly, because no *M. ulcerans* infections have been described in African mammals so far and, secondly, because higher BU case numbers would be expected, if mosquitoes were vectors of *M. ulcerans* in West African countries, where people are frequently bitten by mosquitoes. While BU has

consistently been linked to stagnant or slow-flowing water bodies in African BU endemic areas, it is controversially discussed whether the detection of *M. ulcerans* DNA in many biotic components of aquatic ecosystems, such as plants, snail, fish, insects or amoeboid protozoa is indicative for potential reservoirs or vectors of *M. ulcerans* or merely points towards the ubiquitous presence of *M. ulcerans* in these ecosystems [43–52,27].

The existence of different clonal *M. ulcerans* complexes in the two Ghanaian BU endemic areas under study indicates that *M. ulcerans* is maintained in geographically separate subpopulations. The observed focal clustering of certain *M. ulcerans* haplotypes in the Densu River Basin of Ghana shows that geographic separation of haplotypes even occurs within a relatively small BU endemic region. Based on these results we anticipate that if a reservoir in the aquatic ecosystem is involved in transmission, the spread of *M. ulcerans* from chronic BU lesions to such a currently unknown environmental reservoir and subsequent infection of individuals, in contact with the same reservoir, should be considered. Transmission could take place through trauma of the skin or vectors that still have to be identified (chapter 3).

While the evolution and adaptation of *M. ulcerans* to its new niche environment provided the basis for the emergence of a pathogenic organism for humans, ecological factors could be identified that probably have contributed to the emergence of BU in focal areas. Interestingly, literally all epidemiological investigations have associated BU outbreaks with close proximity to human-disturbed aquatic environments.

Introduction of *M. ulcerans* to the human host population

The emergence of infectious diseases is surprisingly often caused by human actions, with ecological changes being among the most frequently identified factors [2].

Emerging *M. ulcerans* infection foci have been related to various ecological and environmental disturbances, which can be considered as the introduction of *M. ulcerans* to the human host. Thus, increased BU incidence has been reported after alterations of water systems such as irrigation or damming of streams or rivers connected with the creation of wetlands and impoundments or severe flooding of

lakes and rivers exposing people to swampy terrain. Moreover, infection foci were associated with agricultural activities leading to flooding or the creation of irrigation systems as well as land development accompanied by resettlement near water bodies [53–55,13,56–59].

One of the major mysteries around BU is why residence near certain water bodies is associated with BU, whereas the disease seems to be absent along other aquatic environments of the same countries and why even along the same water body tremendous differences in case numbers can be recorded. To give one example, BU is highly associated with residence along the Densu River in Ghana, whereas only a few cases have so far been reported along the Volta River, which is the largest water system in Ghana. Furthermore, BU cases are found in the lower part of the Densu River, but not further upstream (chapter 6).

The Densu River system, one of the coastal drainage basins in Ghana, has been dramatically altered by a number of human activities such as farming, salt mining, sand winning, animal grazing and dam construction. These activities have significant implications on the fluvial processes of the river such as erosion, braiding and flooding [60]. An impoundment created downstream, at the southern part of the river (Weija Dam) led to the formation of the Weija Lake and wetlands surrounding this area [58]. Active BU case search surveys in 24 communities located in close proximity to and along the Densu River revealed prevalence rates of BU ranging from 0% to 20%. While in four communities upstream no BU cases were detected, increasing prevalence rates could be recorded as the river runs downstream approximating the Weija Lake (chapter 6). It is tempting to speculate that the creation of the Weija Dam and the associated environmental changes have contributed to the emergence of BU in the Densu River basin by increasing the contact of people to water bodies and consequently to *M. ulcerans*. However, so far, only a few BU cases have been reported from the Volta region [15], where the creation of the Akosombo dam and subsequent formation of the Lake Volta [61,62] resembles the situation in the Densu River basin and has led to the dissemination of other water borne diseases such as Schistosomiasis [61]. Moreover, our results of the largest sero-epidemiology study ever conducted for BU showed, that inhabitants of both six BU endemic communities mid- and downstream and four communities upstream the

Densu River seem to be equally exposed to *M. ulcerans*, since a considerable proportion (about 32%) developed specific humoral responses against the *M. ulcerans* 18KDa shsp antigen. In addition, environmental samples collected in BU endemic and non-endemic communities showed no significant difference in PCR positivity targeting *M. ulcerans* sequences (chapter 6). This indicates that not only increased contact to possible reservoirs of *M. ulcerans*, but also currently unknown host, pathogen or other environmental factors must contribute to the establishment and further dissemination of BU in certain focalized areas.

Establishment and further dissemination of BU in the human host population

The observation that more than 30% of healthy individuals living in BU endemic and non-endemic communities in the Densu River basin of Ghana develop a specific humoral immune response against an *M. ulcerans* antigen indicates that infection with *M. ulcerans* may lead only in a proportion of exposed individuals to active disease (chapter 6). Diverse outcome of infection with other mycobacteria such as *M. tuberculosis* or *M. leprae* has already been well described and associated with host genetic factors amongst others [63,64]. To date it is not clear, whether the development of BU may equally be determined by host genetic factors. Only one study reports that susceptibility to BU may be associated with a polymorphism in the natural resistance-associated macrophage protein gene, which had already been associated with tuberculosis and leprosy [65]. Various host behavioral factors that may lead to increased probability of acquiring BU have been reported for African and Australian BU endemic settings. In a recent review article summarizing results from currently available risk factor studies conducted in Australia and different African countries poor wound care, failure to wear protective clothing, and living or working near water bodies were identified as the most common risk factors [66]. A case-control study from south-eastern Australia reported reduced odds of having BU for those who frequently used insect repellent and protected their bodies from environmental exposure and increased odds for those who were bitten by mosquitoes on the lower extremities implicating mosquitoes in the transmission of *M. ulcerans* in this region [67].

In view of the limited genetic diversity of *M. ulcerans* strains from the same country, it is rather unlikely that pathogen factors such as differences in virulence account for the establishment of focalized transmission clusters within these countries. However, it cannot be ruled out that host-specific pathogen adaptation occurs as recently described for *M. tuberculosis* [68], considering that *M. ulcerans* is adapting to different niche environments and that one of these *M. ulcerans* ecovars (lineage 3) seems to be more virulent for humans than the others [22,69,70].

One of the most important environmental factors associated with the described creation of stagnant water bodies contributing to the establishment of BU, may be the promotion of optimal breeding sites for potential *M. ulcerans* vectors or animal/environmental reservoirs. However, since only certain water bodies seem to be connected with increased BU case numbers, the emergence of this disease cannot only be based on an interaction between *M. ulcerans* and the environment. Recently, it has been reported that the detection of *M. ulcerans* DNA in several environmental samples from the same villages appears to be a strong predictor of high BU case burden [71]. In this sense, the observed clustering of BU cases in certain endemic villages combined with the reported focalized *M. ulcerans* haplotype distribution in the Densu River basin encourages the assumption that humans and especially BU patients with ulcerative lesions may play an active role in the dissemination of *M. ulcerans* in the environment (chapters 3 and 6).

Outlook

Our ongoing research is focused on further genome comparison of closely related isolates from the Densu River Basin as well as different BU endemic areas in Ghana in order to trace transmission pathways and potential sources of *M. ulcerans* in this country. While spatial analysis of the *M. ulcerans* haplotype distribution in the Densu River Basin has already provided important insights into geographic-genetic patterns of this pathogen in a relatively small BU endemic area (chapter 3), future longitudinal analysis of the haplotype circulation is expected to give deeper insights into reservoirs and transmission routes of *M. ulcerans* in this area. First typing results of *M. ulcerans* strains isolated between 2009 and 2011 in the Densu River Basin at ten canonical SNP markers indicate that the geographic separation of *M. ulcerans*

haplotypes persists over several years (Chapter 4). Genome sequence comparison of additional isolates will show whether new *M. ulcerans* variants have emerged over time and how they are distributed within the BU endemic area. Genome comparison of *M. ulcerans* strains isolated from patients living in different BU endemic areas of Ghana as well as strains from other African countries is anticipated to help identifying sources and routes of dispersal of *M. ulcerans* on country and continental scale. In view of the ongoing enormous advances in next generation sequencing technologies facilitating whole-genome sequencing at ever-lower cost, future analyses might rather be performed by sequencing the genomes of all available isolates. Cultivation of *M. ulcerans* from potential reservoirs or vectors would prove that PCR positivity reflects at least in part presence of viable bacteria. Comparative genome analysis of strains obtained from potential reservoirs or vectors with human disease isolates could confirm or reject hypotheses on their involvement in transmission.

Understanding of transmission pathways and reservoirs of *M. ulcerans* may lead to the identification of preventable risks for infection. This could lead to the design of improved surveillance and control schemes for BU. Since no vaccine is currently available, the present control strategy of BU relies on early case detection and rapid treatment.

**Treatment of Buruli ulcer**

Until 2004 surgical excision of infected skin and underlying soft tissue, often accompanied by transplantation of healthy skin tissue, was the standard treatment for BU, since drug therapy has long been considered ineffective [72]. However, major drawbacks including the limited accessibility of surgery in remote areas, complications due to patients presenting at advanced disease stages as well as high recurrence rates [73,74], have rendered the identification of an effective drug treatment one of the major research priorities for BU. Following a pilot study demonstrating the efficacy of oral rifampicin and intramuscular streptomycin in a limited number of BU patients with early lesions [75], WHO released and promoted a new treatment protocol advising the daily administration of these antibiotics for eight weeks [76]. Routine implementation of this strategy in endemic countries such as Ghana and Benin has greatly improved healing and dramatically reduced

recurrences [77,78]. Even though effective, the daily injection of streptomycin represents one of the major obstacles to decentralize health care to facilities in remote areas in Africa where most of the patients live [79]. Furthermore streptomycin has several side effects including potential nephrotoxicity and ototoxicity [80]. Whether all-oral antibiotic treatment will be equally effective is currently under investigation. There is evidence that in combination with rifampicin, streptomycin administered for only four weeks followed and replaced by oral clarithromycin for another four weeks may be as effective as the standard protocol [81]. In a pilot study in Benin evaluating the efficacy of an all-oral regimen composed of rifampicin and clarithromycin for eight weeks all 30 BU patients were successfully healed [79]. In Australia, where surgery is widely accessible and the main treatment modality for BU, rifampicin is routinely combined with oral clarithromycin or moxifloxacin [82]. Taken together, rifampicin is currently the only key drug for the treatment of BU and has to be combined with other drugs to avoid the development of resistances. Hence the pipeline for drug combinations effective against BU is thin. Given the history of antibiotic resistance in other bacterial diseases such as tuberculosis [83,84], where resistance to most drugs has appeared soon after the introduction of the drug, the identification of alternative drugs or novel therapeutic approaches for the treatment of BU is of high importance.

While incubation times for drug candidate screening and susceptibility testing relying on the development of colonies or turbidity are exceedingly long for slow-growing bacterial species, Alamar Blue-based assays provide rapid and inexpensive tools and have widely been applied to analyze mycobacterial pathogens such as *M. tuberculosis*, *M. leprae* or *M. avium* [85–88]. The adaptation of a compound screening platform for *M. ulcerans* using Alamar Blue allows for the medium-throughput analysis of compounds with potential activity against this extremely slow-growing pathogen. In a pilot study analyzing a pre-selected set of azole compounds, one imidazole antifungal, referred to as Croconazole, could be identified as a potent compound active against *M. ulcerans* (chapter 7). Croconazole is commonly indicated for the treatment of dermatomycoses as topical application in the form of a cream termed Pilzin® [89]. Even though increased healing rates of BU lesions upon topical treatment with nitrogen oxide-releasing acidified nitrite creams have been

previously reported from a pilot randomized trial [90], their application has not been widely adopted. Since Pilzin® is commercially available and hence directly applicable, topical treatment of BU lesions with Pilzin® in combination with chemotherapy should be further investigated. The developed Alamar Blue-based screening procedure is currently used to identify further compounds inhibiting the growth of *M. ulcerans*.

Another alternative approach for the local treatment of BU lesions is the application of heat. Since *M. ulcerans* has a restricted, relatively low optimal growth temperature of 28-32°C, the maintenance of temperatures around 40°C in the ulcerated areas is expected to inhibit the growth of *M. ulcerans*. While successful treatment of individual patients or small case series by heat application has already been reported since 1950 [91–94], the recent employment of cheap and reusable phase change material (PCM) packs provided a major breakthrough for heat treatment of BU [94]. In a proof-of-principle trial six BU patients with ulcerative lesions could be successfully treated by the application of PCM devices. A larger trial evaluating the efficacy and applicability of treatment by PCM packs is currently ongoing.

## Future research priorities and required tools for the control and prevention of Buruli ulcer

In remote African BU endemic regions diagnosis and hence treatment of BU are still primarily based on clinical findings, since available diagnostic tools require expensive equipment and experienced personnel or are not sensitive enough. However, differential diagnosis includes other important diseases, which are prevalent in BU endemic areas and involve different treatment regimens. Thus, one of the major research priorities for BU is to develop a fast, low-tech, sensitive and specific point of care diagnostic test, which could directly be used at peripheral health centers. We are currently evaluating the use of an antigen capture assay based on ELISA and lateral flow techniques that will now be optimized to increase sensitivity.

Alternative treatment regimens for BU suitable for application at peripheral health centers are of urgent need. While trials replacing streptomycin by specific orally administered antibiotics provided promising results, no alternate drug is presently available to replace rifampicin. By using our Alamar Blue-based drug screening

approach, we have not only identified Croconazole, but also another compound with high activity against *M. ulcerans*. The potential application of this promising drug candidate will now be further evaluated with respect to its efficacy in animal models, safety and bioavailability. However, the development of a drug is an expensive and time-consuming endeavor. Hence, the pharmaceutical industry has no interest in the development of drugs for low-return neglected diseases. Therefore we are currently testing the potency of our drug candidate against *M. tuberculosis*, representing a potentially more profitable target.

While in recent years considerable progress has been made in the management of BU, the disease still imposes an enormous social and economic burden on endemic developing countries. The most desired solution to control BU in focal, highly BU endemic areas would be the vaccination of affected populations. Since no advanced vaccine candidate is presently available, research is focused on the development of new vaccine candidates including mycolactone-directed vaccines, attenuated live vaccines and subunit protein vaccines.

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