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Evolution of *Mycobacterium ulcerans* and Other Mycolactone-Producing Mycobacteria from a Common *Mycobacterium marinum* Progenitor▽†

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It had been assumed that production of the cytotoxic polyketide mycolactone was strictly associated with *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. However, a recent study has uncovered a broader distribution of mycolactone-producing mycobacteria (MPM) that includes mycobacteria cultured from diseased fish and frogs in the United States and from diseased fish in the Red and Mediterranean Seas. All of these mycobacteria contain versions of the *M. ulcerans* pMUM plasmid, produce mycolactones, and show a high degree of genetic relatedness to both *M. ulcerans* and *Mycobacterium marinum*. Here, we show by multiple genetic methods, including multilocus sequence analysis and DNA-DNA hybridization, that all MPM have evolved from a common *M. marinum* progenitor to form a genetically cohesive group among a more diverse assemblage of *M. marinum* strains. Like *M. ulcerans*, the fish and frog MPM show multiple copies of the insertion sequence IS*2404*. Comparisons of pMUM and chromosomal gene sequences demonstrate that plasmid acquisition and the subsequent ability to produce mycolactone were probably the key drivers of speciation. Ongoing evolution among MPM has since produced at least two genetically distinct ecotypes that can be broadly divided into those typically causing disease in ectotherms (but also having a high zoonotic potential) and those causing disease in endotherms, such as humans.

Mycobacterium ulcerans is a significant human pathogen and the causative agent of Buruli ulcer (BU), a disease with severe morbidity characterized by chronic skin ulceration and extensive necrosis of subcutaneous fat (40). Cases of BU have been reported in many parts of the world, with the greatest burden of disease occurring in West and Central Africa (16). However, other than travelers returning from countries where the disease is endemic, no cases of BU have ever been reported in the United States or Europe. *M. ulcerans* strains are characterized by the presence of a large circular virulence plasmid called pMUM (31, 33). This plasmid harbors three large genes (*mlsA1*, *mlsA2*, and *mlsB*) encoding polyketide synthases that are required for the synthesis of the lipid toxin mycolactone, which is the primary virulence factor for the pathogen (33). Comparisons of multiple plasmid and chromosomal genes among 10 *M. ulcerans* clinical isolates from diverse origins have suggested that plasmid acquisition was probably the key event that marked and permitted the recent emergence of *M. ulcerans* from a common *Mycobacterium marinum* progenitor (31). *M. marinum* is phenotypically distinct from *M. ulcerans*, producing photochromogenic pigments and generally growing more quickly. *M. marinum* causes granulomatous lesions in fish and other ectotherms and can also cause granulomatous skin lesions in humans. Comparisons between the 5.8-Mb genome of the *M. ulcerans* African epidemic strain Agy99 and the 6.6-Mb genome of *M. marinum* strain "M" confirmed this hypothesis and showed that *M. ulcerans* has recently passed through an evolutionary bottleneck, evolving from the generalist *M. marinum* to become a specialist bacterium, adapted to a more restricted (possibly host-specific) environment (32, 34; http://genolist.pasteur.fr/BuruList/). Like other niche-adapted pathogens, such as *Yersinia pestis* and *Bordetella pertussis*, *M. ulcerans* has undergone extensive gene loss due to DNA deletions, DNA rearrangements, and pseudogene formation. Many of these changes have been mediated by some of the 213 copies of IS*2404* and 91 copies of IS*2606* (34). Neither of these insertion sequence elements (ISE) is present in *M. marinum* (32).

It was assumed until recently that mycolactone production was restricted to *M. ulcerans.* However, in 2004, a previously unreported mycobacterium that contained both IS*2404* and IS*2606* was recovered from cases of an unusual, lethal edematous disease in laboratory-housed *Xenopus laevis* and *Xenopus tropicalis* frogs (37). Limited sequence comparisons of *hsp65*, the 16S rRNA gene, the 16S-23S rRNA gene internal transcribed sequence spacer, and *rpoB* gene fragments showed that this mycobacterium shared greater than 98% nucleotide identity with *M. ulcerans* and *M. marinum* (37). In a subsequent investigation, the frog mycobacterium was shown to contain a version of the *M. ulcerans* pMUM plasmid and to produce a new mycolactone, mycolactone E (13, 19). It was also given the unofficial epithet *Mycobacterium liflandii* (19). In 2005, another new mycobacterium, isolated during an epizootic of mycobac-

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TABLE 1. Mycobacteria used in this study

ST	Identity no.	Species designation	Yr isolated	Origin	Place of origin	Source[c]	Reference
1	99/84[a]	*M. marinum*	1999	Bilby (*Macrotis lagotis*)	Western Australia	PC	32
2	JKD2394[a]	*M. marinum*	1994	Human	Victoria, Australia	VIDRL	32
3	471[a]	*M. marinum*		Human	Norway	UTK	36
4	99/87[a]	*M. marinum*	1996	Human	Western Australia	PC	32
5	99/89[a]	*M. marinum*	1994	Human	Tasmania, Australia	PC	32
6	2000-372	*M. marinum*	2000	Human	France	IP	6
7	1726	*M. marinum*	1986	Armadillo (*Dasypus novemcinctus*)	Louisiana	ITM	35
8	1717	*M. marinum*	1986	Armadillo (*Dasypus novemcinctus*)	Louisiana	ITM	35
9	DL240490	*M. marinum*	1990	Fish (*Dicentrarchus labrax*)	Red Sea, Israel	NCM	38
10	CC240299	*M. marinum*	1999	Fish (*Cyprinus carpio*)	Freshwater, Israel	NCM	38
11	DL045	*M. marinum*	2002	Fish (*Dicentrarchus labrax*)	Mediterranean Sea, Greece	NCM	38
12	L15	*M. pseudoshottsii*	2002	Fish (*Morone saxatilis*)	Chesapeake Bay	VIMS	28
14	128FXT	*M. liflandii*	2005	Frog (*Xenopus* sp.)	California	UTK	37
15	5114[a]	*M. ulcerans*	1953	Human	Mexico	ITM	32
16	842[a]	*M. ulcerans*	1986	Human	Surinam	ITM	32
17	Agy99	*M. ulcerans*	1999	Human	Ga District, Ghana	VIDRL	32
18	13822/70[a]	*M. ulcerans*	1971	Human	North Queensland, Australia	QDRLMD	32
18	1615	*M. ulcerans*	1964	Human	Malaysia	UTK	22
19	98912[a]	*M. ulcerans*	1997	Human	People's Republic of China	ITM	9
19	753	*M. ulcerans*[b]	2004	Human	Japan	RIT	17
20	ATCC 19423[a]	*M. ulcerans*	1948	Human	Victoria, Australia	ATCC	32
22	M	*M. marinum*		Human	California	UW	25

[a] These strains used in a previous MLSA study (32).
[b] This isolate is also referred to as *Mycobacterium shinshuense*.
[c] PC, the Western Australian Centre for Pathology and Medical Research; VIDRL, Victorian Infectious Diseases Reference Laboratory, Victoria, Australia; UTK, Department of Microbiology, University of Tennessee, Knoxville, TN; IP, Institut Pasteur, Paris, France; ITM, Institute for Tropical Medicine, Antwerp, Belgium; NCM, National Centre for Mariculture, Eilat, Israel; VIMS, Virginia Institute of Marine Science, Gloucester Point, VA; QDRLMD,Queensland Diagnostic and Reference Laboratory for Mycobacterial Diseases, Australia; RIT, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan; UW, Department of Microbiology, University of Washington, Seattle.

teriosis from diseased striped bass (*Morone saxatilis*) in Chesapeake Bay, was also shown to contain IS*2404* and IS*2606* and to share >98% nucleotide sequence identity with *M. ulcerans*, *M. marinum*, and *Mycobacterium shottsii* (28). Based on some phenotypic traits that distinguished it from *M. ulcerans*, such as photochromogenicity, absence of growth at 37°C, and scant growth at 30°C on Middlebrook 7H10 agar, this mycobacterium was given a new species designation, *Mycobacterium pseudoshottsii*. Subsequent analysis of the strain showed that it too contained a pMUM-like plasmid with the mycolactone *mls* genes and that it made yet another mycolactone, called mycolactone F (26). In the same study, a cluster of *M. marinum* strains that had been isolated from diseased fish in the Red and Mediterranean Seas (38) were also positive for *mls* gene sequences, and they too produced mycolactone F (26). One of these strains (DL240490) shared identical *hsp65* gene sequences with *M. pseudoshottsii* and another fish pathogen, *Mycobacterium seriolae* (28).

Thus, the distribution of mycolactone-producing mycobacteria (MPM) appears to be much wider than first appreciated, and this raises several interesting questions regarding the mobility of pMUM and the evolution of *M. ulcerans* and *M. marinum*. In this study, we conducted a comprehensive genetic analysis of these strains by DNA-DNA hybridization (DDH), by sequence comparisons of eight chromosomal and four plasmid genes, and by comparing the distributions of pseudogenes and DNA deletions to better define the taxonomic status of all mycolactone-producing strains, including those that cause Buruli ulcer, and to gain insights into their evolution.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Among the isolates used in this study, 18 *M. ulcerans* strains and 22 *M. marinum* strains have been described in detail in a previous publication (32). Ten of these strains, each representing a unique multilocus sequence analysis (MLSA) sequence type (ST), are listed in Table 1, because they were used in additional analyses that included DDH, Southern hybridization, and pseudogene and deletion analyses. Table 1 also lists the details of five novel MPM and seven other mycobacteria that were examined in this study. The mycobacteria were cultivated in Middlebrook 7H9 medium or on egg yolk agar slopes at 30°C (32).

General DNA methods and oligonucleotides used in this study. Genomic DNA for DDH was extracted from mycobacteria harvested from egg yolk agar slopes. Briefly, 100 mg (wet weight) cells was resuspended in 2 ml of lysis buffer (15% [wt/vol] sucrose, 0.05 M Tris, pH 8.5, 0.05 M EDTA, 1 mg/ml lysozyme, and 2 mg/ml RNase) and incubated at 37°C for 1 hour. The volume was then increased to 5 ml by the addition of 2 ml of 10% (wt/vol) sodium dodecyl sulfate and 1 ml of 1× TE (10 mM Tris, pH 8.5, 1 mM EDTA) containing 5 mg of proteinase K. Following overnight incubation at 37°C, the DNA was separated by successive rounds of extraction with phenol-chloroform (1:1) and chloroform and then precipitated with a 2× volume of absolute ethanol in the presence of 4% NaCl at −20°C overnight. The resulting pellet was resuspended in 400 μl of 1× TE, and the extraction process was repeated. The final DNA pellet was washed twice in 70% (vol/vol) ethanol and resuspended in 100 μl of 1× TE.

Other methods for PCR, pulsed-field gel electrophoresis, and Southern hybridization were also performed as described previously (32). For the MLSA, sequencing of a 357-bp fragment of the *glcB* gene was achieved by PCR amplification using glcB-F (5′-GGACTTCACCATCACCACCT-3′) and glcB-R (5′-GACTCCAGGATCACGGTCCTT-3′) (Fig. 1). Other primer pairs used for MLSA have been described before (32). The oligonucleotides used for PCR amplification and sequencing of potential pseudogenes are listed in Table S1 in the supplemental material. Genome comparisons between *M. uclerans* Agy99 and *M. marinum* M identified 157 specific DNA differences between the species, referred to as *M. ulcerans* regions of difference (MURDs) (34). Most of the MURDs are deletions of DNA from *M. ulcerans*. To investigate the distribution of three deletions (MURD12, -54, and -152) among the strains in this study, we

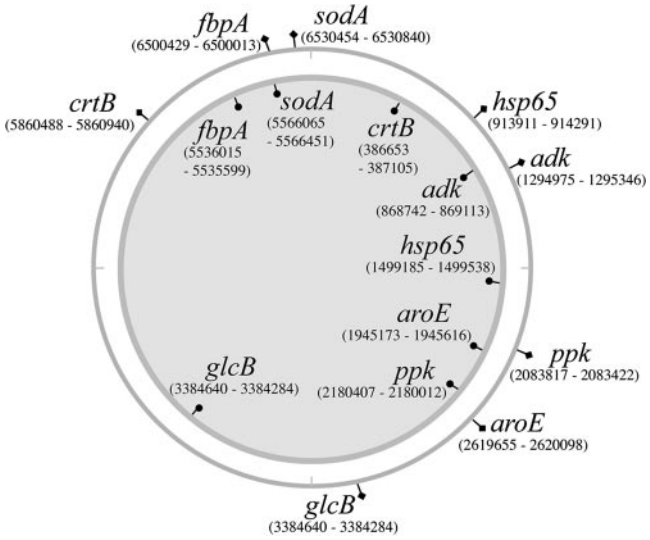

FIG. 1. Distributions and nucleotide positions of the eight loci on the 5.6-Mbp chromosome of *M. ulcerans* Agy99 (inner circle) and the 6.6-Mbp chromosome of *M. marinum* M (outer circle).

performed PCR with three oligonucleotides for each MURD. These primers were designed based on genome comparisons between *M. ulcerans* Agy99 and *M. marinum* M (34), where P1 hybridized to conserved sequences in both strains flanking the region deleted in *M. ulcerans*, P2 hybridized to sequence adjacent to P1 in *M. ulcerans*, and P3 hybridized to sequence adjacent to P1 in *M. marinum*. The oligonucleotide sets used for the detection of MURD12, -54, and -152 are listed in Table S2 in the supplemental material.

DNA-DNA hybridization. For each isolate, at least three biological repeats were performed, in which 100 ng of single-stranded genomic DNA was spotted in duplicate onto positively charged nylon membranes using a 96-well vacuum dot blotter (Schleicher & Schuell). DNA was cross-linked to the membranes with UV irradiation (1,200 mJ), washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and probed immediately. Genomic-DNA probes were prepared by incorporating digoxigenin dUTP by random prime labeling, following the manufacturer's instructions (Roche). Blots were hybridized and detected as described previously (15). Blot images were digitized and then analyzed with ImageJ (27). A standard spot area was used to measure the integrated pixel density. The relative binding ratio (RBR), defined as 100% for a blot hybridizing to a probe derived from autologous DNA, was calculated by dividing the mean integrated pixel density for each strain by the mean pixel density of the autologous reaction mixture and multiplying by 100. The mean was calculated from at least three independent hybridization reactions. Analysis of variance in conjunction with Dunnett's two-sided *t* test was used to determine if differences between RBRs were significant ($P < 0.05$). Where variance was not homogeneous (as determined by Levene's statistic), Dunnett's T3 post hoc test was employed. All calculations were performed using SPSS (version 14.0.1; LEAD Technologies, Inc.).

MLSA. The methods used for MLSA were as previously described (32), with the addition of an eighth locus, a 357-bp fragment of the *glcB* gene that encodes malate synthase, the enzyme involved in the second step of the glyoxylate bypass. The distribution and nucleotide positions of the eight protein-coding DNA sequences (CDS) on the 5.6-Mbp chromosome of *M. ulcerans* Agy99 and the 6.6-Mbp chromosome of *M. marinum* M are shown in Fig. 1. Double-stranded DNA sequences were determined from the PCR products of each locus using dye terminator sequencing as described previously (32). Sequence assembly was managed with Gap4 (3). An allelic profile was constructed for each strain, and the unique combination of alleles defined a sequence type (Table 1; see Table S3 in the supplemental material). The DNA sequences of the alleles are listed in Table S3 in the supplemental material. The allelic profiles for some isolates differed at more than three of the eight loci, so phylogeny was inferred by using a distance method rather than a pairwise comparison of the allelic profiles (29); the latter approach is referred to as multilocus sequence typing (10). For each ST, the nucleotide sequences of the alleles were concatenated in frame to generate an artificial CDS. Using the same approach, all MPM were subjected to further MLSA analysis by determining the partial DNA sequences of four pMUM plasmid CDS (*repA*, *parA*, MUP045, and the *mls* loading domain) as described previously (31).

Phylogenetic methods. Phylogenetic analysis was performed with MEGA software version 2.1.2 (18) and Splits Tree version 4 (14). "P" distances were used throughout, as the overall level of sequence divergence was small. Values for synonymous (dS) and nonsynonymous mutation frequencies were calculated by Nei and Gojobori's method (20), and standard errors (SE) for the means of these values were estimated by the method of Nei and Jin (21) using dSdNqw (7).

VNTR analysis. The primers and conditions used for PCR amplification of the nine variable-number tandem repeat (VNTR) loci 1, 4, 6, 8, 9, 14, 15, 18, and 19 were as described previously (2). Amplicon sizes were estimated following electrophoresis of the PCR products through a 2% agarose gel and comparison with a 100-bp DNA ladder (Promega).

Nucleotide sequence accession numbers. The DNA sequences of the alleles have been assigned GenBank accession numbers DQ985290 to DQ985355.

RESULTS

DNA-DNA hybridization. Whole-genome probes derived from the five novel MPM strains with MLSA ST9, -10, -11, -12, and -14 (see below); three *M. ulcerans* strains (ST17, -19, and -20); and a representative non-MPM *M. marinum* strain (ST22) were hybridized to each other in a reciprocal fashion. The five novel MPM strains were then tested against genomic DNAs from three additional *M. marinum* strains (ST1, -2, and -5). The results are summarized in Table 2, and an example is

TABLE 2. RBR for hybridization of mycobacteria using Digoxigenin-labeled genomic DNA probes

DNA source	Mean (range) RBR with DNA probe:								
	M. ulcerans			*M. liflandii* ST14	*M. pseudoshottsii* ST12	*M. marinum*			
	ST17	ST20	ST19			ST11	ST10	ST9	ST22
M. ulcerans ST17	100								
M. ulcerans ST20	97 (88–117)	100							
M. ulcerans ST19	94 (84–101)	104 (92–115)	100						
M. liflandii ST14	100 (89–109)	107 (95–105)	99 (94–106)	100					
M. pseudoshottsii ST12	98 (80–110)	102 (96–115)	101 (95–107)	102 (94–112)	100				
M. marinum ST11	94 (86–103)	100 (72–113)	88 (71–95)	98 (86–107)	102 (83–110)	100			
M. marinum ST10	103 (93–108)	100 (89–107)	95 (82–100)	100 (79–107)	100 (82–105)	96 (88–105)	100		
M. marinum ST9	91 (80–105)	101 (90–109)	88 (79–98)	96 (79–104)	99 (88–108)	100 (90–116)	98 (88–112)	100	
M. marinum ST22	52 (40–59)	60 (41–68)	29 (21–44)	33 (24–49)	41 (32–52)	32 (24–43)	39 (26–50)	37 (21–51)	100
M. marinum ST1	ND[a,b]	ND[b]	ND	19 (18–21)	15 (13–15)	27 (22–34)	21 (18–24)	23 (21–24)	ND
M. marinum ST2	ND[b]	ND[b]	ND	26 (24–27)	15 (15–17)	25 (20–34)	23 (21–25)	21 (18–25)	ND
M. marinum ST5	ND[b]	ND[b]	ND	32 (27–34)	21 (20–22)	30 (19–38)	33 (28–38)	23 (19–27)	ND

[a] ND, not done.
[b] Previous DDH experiments reported a mean RBR of 38 (range, 25 to 47) between these strains of *M. ulcerans* and *M. marinum* (36).

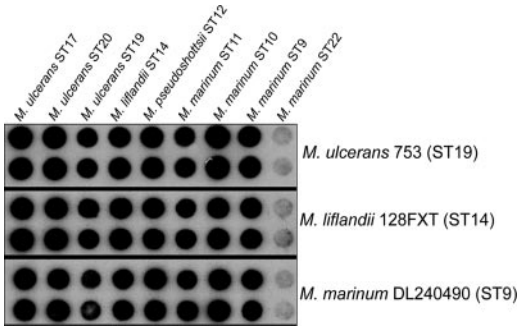

FIG. 2. Representative DNA-DNA hybridization result showing using whole-genome probes from three MPM against a panel of nine strains representing different MLSA sequence types.

given in Fig. 2, showing that by this technique the five new MPM are indistinguishable from strains of *M. ulcerans*, with an overall mean RBR of 98% (range, 79 to 117%). In contrast, the mean RBR between the MPM and non-MPM was significantly lower at 40% (range, 13 to 68%; $P < 0.05$).

MLSA of chromosomal genes. To improve chromosome coverage, we expanded our original MLSA method and added a 357-bp fragment of the *glcB* gene to the seven loci we had previously sequenced from 18 isolates of *M. ulcerans* and 22 isolates of *M. marinum* (Fig. 1) (32). A large difference in chromosomal location between the species was noted for some genes, and this was due to the extensive genome rearrangements that have occurred in *M. ulcerans* (34) (http://genolist.pasteur.fr/BuruList/). All eight loci were then sequenced for the 12 new isolates used in this study, which included five MPM recovered from diseased fish and frogs. Multiple alleles were obtained for each locus among the total of 52 mycobacteria tested. For example, 13 different *crtB* alleles, 7 different *adk* alleles, and 10 different *fbpA* alleles were obtained. A unique combination of alleles is referred to as an ST, and 20 STs were obtained (see Table S3 in the supplemental material). The eight alleles for each ST were concatenated in frame in the order *crtB*, *adk*, *fbpA*, *aroE*, *groEL*, *ppk*, *sodA*, and *glcB* to produce an artificial 3,210-bp CDS (Fig. 3A and B). DNA sequence alignments and comparisons of this CDS across all 20 STs revealed only 95 variable sites, representing a shared nucleotide identity of 97%, highlighting the very close genetic relationship between all the mycobacteria analyzed in this study (Fig. 3A and B). The MPM were represented by 11 STs, and they exhibited a very high level of nucleotide identity (>99%) (Fig. 3B). Significantly, all MPM contain the T-for-C transition within the *fbpA* gene, a polymorphic site suggested previously as a key discriminator between *M. ulcerans* and *M. marinum* (Fig. 3B) (32). A phylogeny inferred by split-decomposition analysis of the MLSA data shows a treelike structure among the MPM and divergence from other, non-MPM strains at a single node (Fig. 3C). Within the MPM, there is a second split into at least two distinct lineages that broadly correlate with those MPM causing disease in fish and frogs and those identified as *M. ulcerans* that cause Buruli ulcer in humans. Each of the novel MPM had related but distinct STs that clustered with a human *M. ulcerans* isolate from Surinam.

Among the nine non-MPM *M. marinum* sequence types, only ST2 contained isolates from a variety of sources, including humans, fish, and water. The *M. marinum* strain 2000-372 (ST6) has been proposed as a strain that represents an evolutionary "missing link" between *M. ulcerans* and *M. marinum*, because it does not make mycolactone but returns a positive result in a PCR test for IS*2404* (6). MLSA unambiguously grouped this strain within the STs most distant from *M. ulcerans*, and hence, it is very unlikely that this strain is an intermediate between the species (Fig. 3C). Sequence analysis of the IS*2404* PCR product from this strain showed that it contains an ISE that is distinct from IS*2404*, as it is only 86% identical at the nucleotide level.

MLSA of pMUM plasmid genes. Sequence comparisons and split-decomposition analysis of the 1,266-bp concatenated DNA sequences from four pMUM gene sequences among the MPM produced a tree topology very similar to that obtained from the eight chromosomal loci (Fig. 3D and E). There was clear separation between the fish and frog MPM and *M. ulcerans*. dS is a measure of the time a given coding sequence has been extant relative to another. Sequences with similar dS are likely to have been acquired at the same time. The parameter dS was not significantly different for plasmid (dS mean = 0.00639; SE = 0.00234) and chromosome (dS mean = 0.00515; SE = 0.00135) sequences, consistent with the idea that the plasmid has coevolved with all MPM from a common progenitor.

Comparison of plasmid sizes and distributions of IS*2404* and IS*2606*. Pulsed-field gel electrophoresis and Southern hybridization were used to compare the sizes of the pMUM plasmids among the novel MPM and to explore ISE copy numbers. *M. ulcerans* strains from Malaysia and Japan had plasmids of approximately 170 kb, as previously reported for strains from Africa and Australia (Fig. 4) (31, 33). The novel MPMs had plasmids of larger sizes. *M. liflandii* (ST14) and *M. marinum* strain CC240299 (ST10) harbor plasmids of approximately 180 kbp, while *M. pseudoshottsii* (ST12), *M. marinum* DL240490 (ST9), and *M. marinum* DL045 (ST11) all harbor plasmids of approximately 200 kbp (Fig. 4). The plasmid size correlated with restriction fragment polymorphism, as shown after digestion of DNAs from these strains with AseI and hybridization with probes derived from IS*2404* and IS*2606*. Of particular note are the patterns of bands, which are indistinguishable from each other for the strains with 200-kbp plasmids (ST9, -11, and -12). MLSA supported this finding with 99.9% shared nucleotide identity and only three variable nucleotides from the 3,210 bp sequenced among these strains (Fig. 3). Southern hybridization confirmed that all MPM contain many copies of IS*2404*, suggesting that this insertion sequence and possibly its expansion to a high copy number were characteristics of the common progenitor. The same is not true for IS*2606*. It was not detected in *M. marinum* CC240299, a finding confirmed by PCR (data not shown), and was present in low copy numbers in the *M. ulcerans* strain from Japan (ST19) and the novel MPM (Fig. 4), suggesting that it was acquired independently by the two different lineages of MPM.

Distribution of pseudogenes among novel MPM. Approximately 14% of the original coding potential of *M. ulcerans* Agy99 has been lost due to point mutations or insertion sequences that have disrupted CDS and led to the formation of pseudogenes. To explore the distribution of pseudogenes in the novel MPM, we used PCR to amplify and then sequence

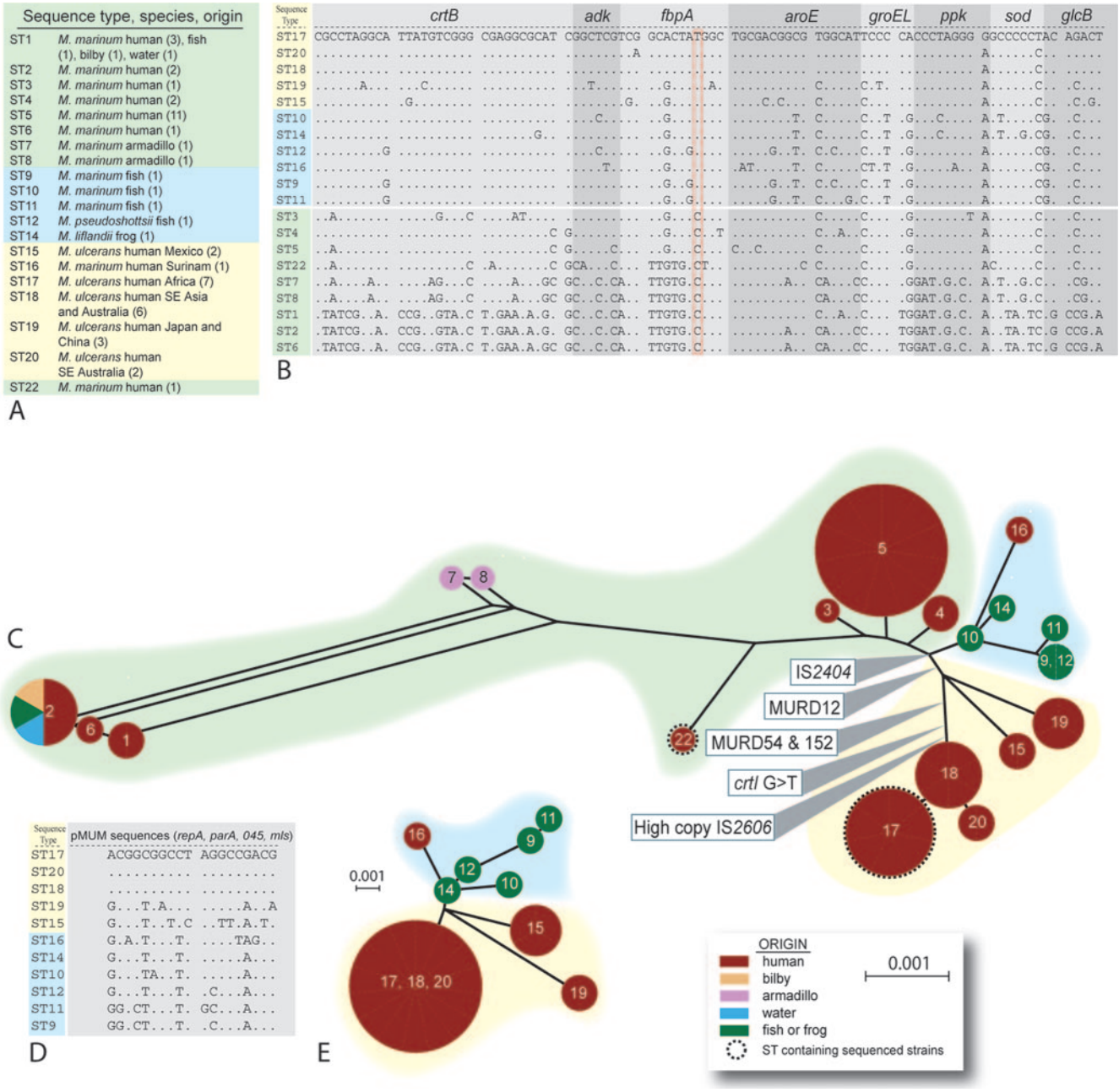

FIG. 3. Multilocus sequence analysis. (A) Species designations for the 20 sequence types, their origins, and (in parentheses) the numbers of isolates tested. (B) Alignment of the 3,210-bp sequences derived from the eight concatenated chromosomal CDS fragments among 20 different sequence types. Only variable nucleotides are shown. A period indicates identity with *M. ulcerans* ST17 (African type). (C) Split-decomposition representation of the phylogenetic relationships among *M. ulcerans*, other mycolactone-producing mycobacteria, and *M. marinum* strains. The circles at the vertices are labeled with the sequence types, and their sizes are proportional to the numbers of isolates within the groups, following a layout described previously (8). *M. marinum* ST1, -2, -3, -4, and -5 correspond to STI, -II, -III, -IV, and -V as previously reported (32). All edges had 100% bootstrap support (1,000 replicates). (D) Alignment of the 1,266-bp sequences derived from the four concatenated pMUM CDS fragments among 11 different sequence types. Only variable nucleotides are shown. A period indicates identity with *M. ulcerans* ST17 (African type). (E) Split-decomposition graph of the phylogenetic relationships among *M. ulcerans* and other mycolactone-producing mycobacteria, derived from the plasmid gene sequences. All edges had >60% bootstrap support (1,000 replicates).

the region spanning different mutations. These mutations were nucleotide changes that changed the reading frame or introduced a premature stop codon or disruptions by insertion of IS*2404*. Twenty of the 743 pseudogenes (including 21 mutations) identified in *M. ulcerans* Agy99 were selected for comparison with novel MPM of ST9, -10, and -11. These sequences were selected as a random sample of the total pseudogene complement with potential to explain phenotypic differences between strains. The results are summarized in Table S4 in the supplemental material and show that 15 of these pseudogenes

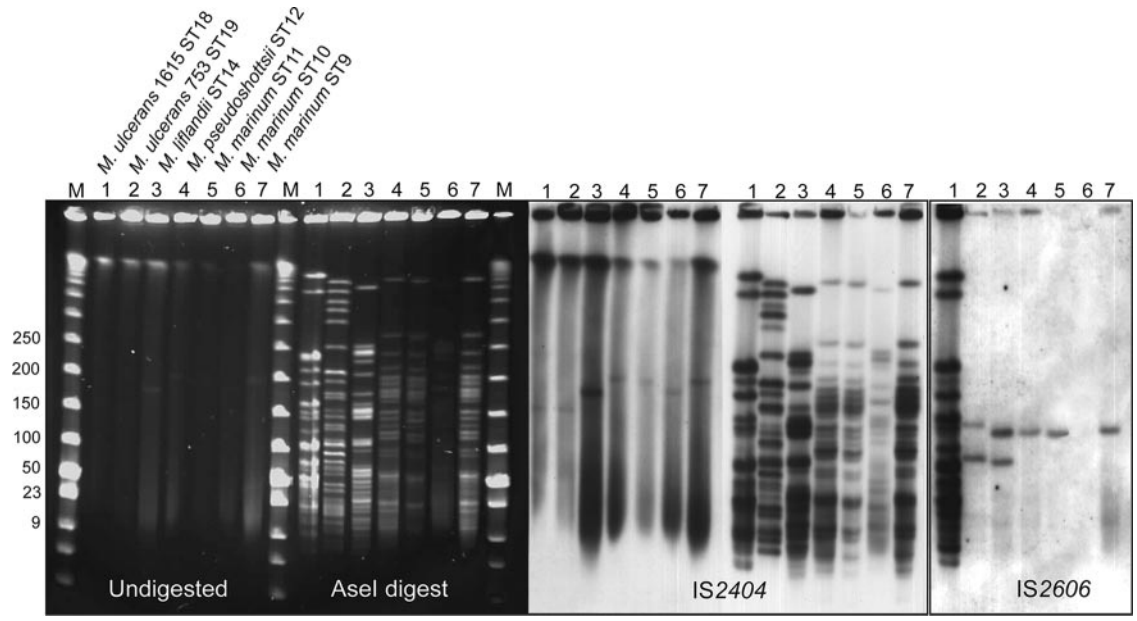

FIG. 4. Pulsed-field gel electrophoresis and Southern hybridization analysis of MPM showing the presence in all isolates of pMUM-like plasmids and the distributions of IS*2404* and IS*2606*.

are still intact in the novel MPM; of the five that were pseudogenes, only one (an insertion of 5 nucleotides in *arsC*) was caused by the same mutation as in *M. ulcerans* Agy99. The frequency of the G-to-T transition in *crtI*, encoding phytoene dehydrogenase, which is required for carotenoid pigment production, was more widely investigated by testing all 52 mycobacteria. This mutation was found to be restricted to *M. ulcerans* strains belonging to ST17, -18, and -20 (Fig. 3C).

Distribution of DNA deletions among MPM. Deletion of DNA fragments occurs frequently in mycobacteria, and their distribution among strains can be used to reconstruct evolutionary pathways (5). Comparative genomics revealed 157 between *M. ulcerans* strain Agy99 (ST17) and *M. marinum* M (ST22) (34). Three of these regions (MURD12, MURD54, and MURD152) that were shown to be deletions in *M. ulcerans* Agy99 were selected for further study. Using PCR, MPM and *M. marinum* were screened for the presence or absence of each MURD, using the approach developed for studying members of the *Mycobacterium tuberculosis* complex (5) (Fig. 5A). Analysis of strains representing each of the 20 STs revealed that MURD12 had been lost from all strains that constituted the endotherm lineage, represented by ST15, -17, -18, -19, and -20 (Fig. 3C and 5). *M. ulcerans* strains of the subcluster within the endotherm lineage (ST17, -18, and -20) have lost MURD54 and MURD152 (Fig. 3C and 5). *M. ulcerans* strain 5114 from Mexico (ST15) did not produce a product in the MURD152 assay, suggesting that this strain has undergone a different chromosomal modification in this region.

VNTR analysis distinguishes different MPM. Variable-number tandem repeat loci are widespread in mycobacterial genomes, and their varied distribution has been exploited for strain differentiation. A system of PCR and sequence-based VNTR typing has been described for high-resolution differentiation among *M. marinum* and *M. ulcerans* strains (1, 2, 12, 35), and we applied this technique to the MPM strains in this study. Comparisons of fragment sizes at VNTR loci 1, 4, 6, 8, 9, 14, 15, 18, and 19 permitted the discrimination of the novel MPM from other strains of *M. marinum* and *M. ulcerans*. However, *M. pseudoshottsii* L15 (ST12), *M. marinum* DL240490 (ST9), and *M. marinum* DL045 (ST11) shared the same VNTR profile (data not shown).

DISCUSSION

Initial MLSA analyses and subsequent whole-genome comparisons have shown that *M. ulcerans* has recently evolved from *M. marinum* by acquisition of the pMUM plasmid and reductive evolution (31–34). The recent discovery of MPM that are phenotypically distinct from *M. ulcerans* in diseased fish and frogs has highlighted the possibility that pMUM is being transferred among different mycobacterial species (19, 26). In this report, we show by a systematic genetic approach that all MPM are very closely related to each other and have evolved, not by multiple exchanges of pMUM, but from a common *M. marinum* progenitor that acquired the plasmid.

MLSA is widely used to understand the taxonomic relationships among bacterial populations (8, 10, 11), and it was the method we employed in an earlier study to suggest that *M. ulcerans* recently evolved from *M. marinum* (32). To improve genome coverage and increase resolution of the MLSA method for the present investigation, we added an eighth locus to create a 3,210-bp semantide (a large information-bearing molecule). We reanalyzed our original data set and then added sequences from 12 additional isolates that included 5 mycobacteria recently shown to contain pMUM and to produce mycolactones. There was significant sequence diversity among non-mycolactone-producing *M. marinum* isolates, and as shown by others (32, 39), the majority of isolates fell into two distinct clusters represented by ST1, -2, and -6 and ST 3, -4, and -5. The sample size in this study was too small to draw

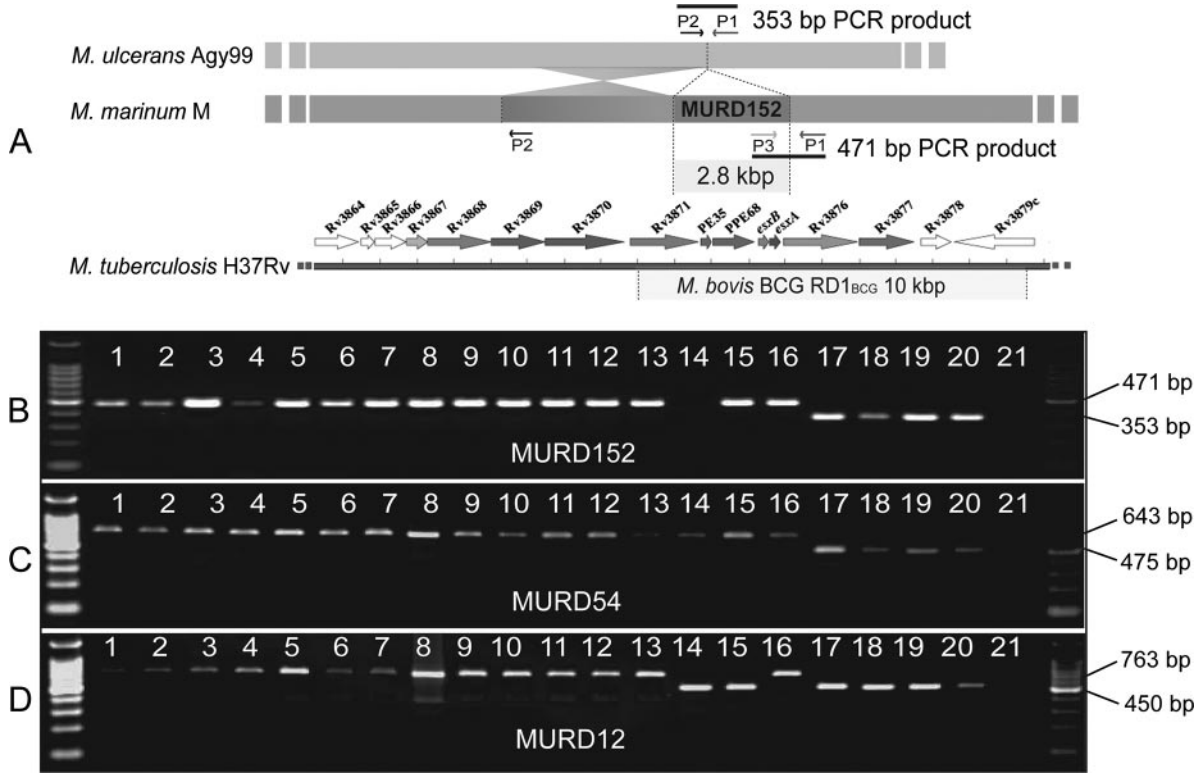

FIG. 5. PCR deletion analysis for MURD12, MURD55, and MURD152 of strains representing each MLSA sequence type. (A) Arrangement of the oligonucleotides used for PCR of MURD152, showing the 471-bp product predicted from the genome sequence of *M. marinum* strain M and the 353-bp product predicted from the genome sequence of *M. ulcerans* Agy99. Also shown for reference is the alignment of MURD152 with the RD1 region absent from *Mycobacterium bovis* BCG. (B, C, and D) Results of deletion PCR analysis for MURD152, MURD54, and MURD12, respectively. Lanes 1 to 11, *M. marinum* (1, 99/84; 2, JKD2394; 3, 471; 4, 99/87; 5, 99/89; 6, 2000-372; 7, 1717; 8, M; 9, DL240490; 10, CC240299; 11, DL045); lane 12, *M. pseudoshottsii* L15; lane 13, *M. liflandii* 128FXT; lane 14, *M. ulcerans* Mexico 5114; lane 15, *M. ulcerans* Japan 753; lane 16, *M. marinum* Surinam 842; lane 17, *M. ulcerans* Agy99; lane 18, *M. ulcerans* 1615; lane 19, *M. ulcerans* 13822/70; lane 20, *M. ulcerans* 19423; lane 21, no template control. On the left is a 100-bp DNA molecular size ladder (Promega).

conclusions linking specific *M. marinum* genotypes to virulence in humans, as has been proposed (39); however, such a correlation seems unlikely, given that *M. marinum* isolates of human origin spanned the spectrum of sequence diversity revealed by MLSA. Two *M. marinum* isolates recovered from armadillos showed intermediate sequence types (ST7 and -8), as did *M. marinum* strain "M" (ST22), whose genome has recently been sequenced (http://www.sanger.ac.uk/Projects/M_marinum).

MLSA unambiguously showed that, despite their varying phenotypes, all MPM have evolved from a single *M. marinum* clone that has since expanded into at least two distinct lineages, and this was supported by the congruent tree topology derived from the four pMUM plasmid loci (Fig. 3). Together with equivalent levels of synonymous nucleotide substitution frequency between chromosome and episome sequences, these data suggest that plasmid acquisition was probably the principal event that enabled an *M. marinum* progenitor to survive in a new environment.

The genetic homogeneity of MPM was also reflected in their high DDH values. DDH has been widely used for over 30 years in bacterial taxonomy to infer relatedness between genomes, and a DDH value greater than 70% is one criterion used to help define a bacterial species. The high DDH values among *M. ulcerans* and the other MPM were further evidence of their common origin and contrasted with the low (<55% RBR) values when the same MPM were tested against non-mycolactone-producing *M. marinum* strains. These data are consistent with an earlier investigation of *M. ulcerans* and *M. marinum* that showed intraspecies DDH values of >90% and interspecies DDH values of <50% (36). The presence of pMUM-like plasmids and the multiple copies of IS*2404* in all MPM may explain, at least in part, the striking DDH results. These data also indicate that IS*2404* acquisition (and possibly its expansion to high copy numbers) occurred before radiation of MPM around the world. In contrast, IS*2606* is present in high copy numbers only in the lineage of *M. ulcerans* strains that contain isolates from Africa, Australia, and Malaysia (ST17, -18, and -20) (30, 32). The varied distribution of this ISE among other MPM and its absence from the MPM *M. marinum* strain CC240299 (ST10) suggest it has been transferred independently to at least two different populations of MPM subsequent to IS*2404* and pMUM acquisition.

The *M. ulcerans* genome project and a recent microarray-based study have both revealed extensive DNA deletion polymorphism among *M. ulcerans* strains (34; M. Käser, S. Rondini, T. Stinear, M. Tessier, C. Mangold, G. Dernick, M. Naegeli, F. Portaels, U. Certa, and G. Pluschke, submitted for publication). In the current work, the pattern of DNA deletion

observed among MPM for three deletions was in good agreement with MLSA (Fig. 5). MURD12, a 10-kbp fragment containing CDS involved in secondary metabolism, was a marker for distinguishing between the ectotherm and endotherm lineages, as it was absent from *M. ulcerans* isolates from both Mexico and Japan (ST15 and -19) and the African, Malaysian, and Australian cluster (ST17, -18, and -20) (Fig. 3C and 5C). The MURD54 and MURD152 deletions differentiate the ST17, -18, and -20 subcluster from other MPM and are indicative of more advanced genome reduction in these strains. MURD152 is a 2.8-kb DNA fragment deleted from *M. ulcerans* strain Agy99 (ST17) that spans *esxA* and *esxB*, genes encoding key components of the ESX-1 secretion apparatus and virulence factor (4, 34). The MURD152 deletion PCR assay confirmed earlier findings that showed that *esxA* and *esxB* are absent from *M. ulcerans* strains from Africa, Australia, and Malaysia but present in other MPM (19, 26). Both the MURD54 and MURD152 assays may have diagnostic applications in countries where Buruli ulcer is endemic, such as Africa and Australia, where it will be useful, particularly when screening environmental samples, to distinguish between *M. ulcerans* strains that cause Buruli ulcer and other MPM.

The split of MPM into two distinct lineages, which include strains with different species names (e.g., *M. marinum*, *M. liflandii*, and *M. pseudoshottsii*) that typically cause disease in ectotherms but also have a high zoonotic potential and strains of *M. ulcerans* (ST17, -18, and -20) that cause Buruli ulcer in humans and target other endotherms, is an important finding. Some insights into the genetic basis for this separation have been gleaned from this study and, combined with previous research showing that these strains have different optimal growth temperatures and produce mycolactones with varying potencies (19, 26), they suggest MPM have evolved to occupy different ecological niches. *M. ulcerans* is not known to infect fish, while the diseases caused by *M. marinum* and *M. ulcerans* in humans differ greatly in their clinical, histopathologic, and epidemiologic aspects (32).

The large number of gene deletions and pseudogenes in the *M. ulcerans* Agy99 genome compared with *M. marinum* M is indicative of a bacterium adapting to a restricted and privileged environment, where mutations are tolerated in genes that are no longer required for survival. However, testing three MPM from the ectotherm lineage for 20 of these pseudogenes found only five inactivated CDS, indicating a much less advanced level of genome decay and metabolic streamlining, consistent with the hypothesis that they occupy different environments. Only one of the five mutations (an insertion in *arsC*) was conserved between the two MPM lineages (see Table S4 in the supplemental material), suggesting that a certain level of genome decay had begun before divergence. The other four mutations occurred at different positions in the same genes (*sigJ*, *echA13*, and *accD1*), and this may indicate that the products of these CDS are not only redundant but perhaps deleterious for survival of MPM and so have been subjected to independent, purifying selection.

The difference in pseudogene profiles may also help explain the phenotypic variation observed among MPM. For example, *crtI* encodes phytoene dehydrogenase, an enzyme essential for the production of carotenoid pigments in *M. marinum* (25). *M. ulcerans* Agy99 has the same *crt* locus as *M. marinum*, but it is nonpigmented, and this has been explained by a point mutation in *crtI* that introduces a premature stop codon and truncates the gene (see Table S4 in the supplemental material) (34). There was complete correlation between lack of pigment production and the disrupted *crtI* gene, as only MPM of the ST17, -18, and -20 cluster are nonpigmented (24, 26), and it was only this cluster that contained the mutation (Fig. 3C).

In this report, we have sought to clarify the genetic relationships among mycolactone- and non-mycolactone-producing mycobacteria, but this has in turn highlighted the recurring problem of assigning species status to highly related bacteria, as the question remains how much diversity is permissible in a genetically discrete cluster for it to be regarded as a distinct taxon. From a population genetics standpoint, the data presented here do not support the separation of MPM into different species. Employing a subspecies nomenclature might allow a more meaningful naming system that accurately reflects the common origin of MPM. A comprehensive polyphasic and multicenter study of MPM, as performed by Wayne et al. (41), would help decide their taxonomic positions.

Defining the host specificity and natural ecology of MPM is also a research priority. It may be that there are many different MPM but the only strains isolated are those producing mycolactones with sufficient potency to cause disease in humans, fish, frogs, possums, and koalas (23). A better understanding of MPM in the environment will be crucial to halting the spread of the diseases they cause, in particular Buruli ulcer.

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