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Phenotypic and Genotypic Characterization of *Mycobacterium africanum* Isolates from West Africa

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The *Mycobacterium tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. Most clinical isolates are *M. tuberculosis* or *M. bovis*. These species can be distinguished by phenotypes and genotypes. However, there is no simple definition of *M. africanum*, and some authors question the validity of this species. We analyzed 17 human isolates from Sierra Leone, identified as *M. africanum* by biochemical and growth characteristics. We sequenced polymorphic genes and intergenic regions. We amplified DNA from six loci with variable numbers of tandem repeats (VNTRs) and determined the exact number of repeats at each locus in each strain. All *M. africanum* isolates had the ancestral CTG Leu at *katG* codon 463. Drug-resistant *M. africanum* isolates had *katG* and *rpoB* mutations similar to those found in drug-resistant *M. bovis* and *M. tuberculosis*. Fourteen Sierra Leone *M. africanum* isolates (designated group A) had *katG* codon 203 ACC Thr, also found in *M. africanum*[T] (the T indicates type strain) from Senegal. Group A isolates clustered with *M. africanum*[T] by VNTR analysis. Three *M. africanum* isolates (group B) had *katG* codon 203 ACT Thr, found in *M. tuberculosis*[T], and clustered with *M. tuberculosis*[T] by VNTR analysis. Phenotypic identification of *M. africanum* yielded a heterogeneous collection of strains. Genotypic analyses identified a cluster (*M. africanum* group A) which included *M. africanum*[T] and was distinct from the rest of the *M. tuberculosis* complex. Future studies of *M. africanum* should include both phenotypic and genotypic analyses.

The *Mycobacterium tuberculosis* complex includes four recognized species (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*), which together cause human and animal tuberculosis (37). *M. tuberculosis* and *M. bovis* are the most common isolates in clinical laboratories and can be distinguished by biochemical tests, growth phenotypes, and several genetic markers. However, *M. africanum* isolates have substantial phenotypic heterogeneity with some strains resembling *M. bovis* and others resembling *M. tuberculosis* (4, 10, 12, 15, 36). This has led several authors to question the validity of the species designation *M. africanum* (33, 37, 39).

We conducted a detailed genotypic analysis of a panel of 17 recent *M. africanum* isolates. All were isolated from humans in Sierra Leone between 1991 and 1993 and were identified as *M. africanum* on the basis of biochemical tests and growth patterns. These strains are a subset of a collection described previously (12, 14, 29). We sequenced portions of several genes and intergenic regions which are known to have polymorphisms in *M. tuberculosis* and *M. bovis*. We also analyzed six loci with variable numbers of tandem repeats (VNTRs). Our goal was to determine whether *M. africanum* strains have distinct genotypes which set them apart from *M. tuberculosis* and *M. bovis*.

MATERIALS AND METHODS

Mycobacterial strains. Mycobacterial strains are listed in Tables 1 and 2. *M. africanum* isolates were obtained from humans in Sierra Leone with pulmonary tuberculosis (12, 29). These strains were part of a large collection of 96 strains originally collected for the purpose of drug resistance surveillance organized by the National Tuberculosis Control Program in Sierra Leone and supported by the German Leprosy Relief Association. Isolates were characterized as *M. africanum* based on biochemical and biophysical properties as described previously (12). Susceptibility testing was performed by the proportion method using the critical concentrations of 40 μg/ml for rifampin, 0.2 μg/ml for isoniazid (INH), 4 μg/ml for streptomycin, and 2 μg/ml for ethambutol. Pyrazinamidase production was determined as described by Wayne (35). The 17 *M. africanum* isolates in this study were selected from the larger collection to include isolates with a maximal divergence in antibiotic susceptibility phenotypes. *M. africanum* ATCC 25420[T] (the T indicates type strain), *M. bovis* ATCC 19210[T], *M. microti* ATCC 19422[T], *M. tuberculosis* ATCC 27294[T], and *M. bovis* ATCC 35734 (BCG Pasteur) were obtained from the American Type Culture Collection in Manassas, Va.

DNA preparation. Mycobacterial isolates were killed by immersion in 70% (vol/vol) ethanol (40). DNA was released from bacterial cells by snap freeze-thawing (41).

DNA sequence analysis. A portion of the *rpoB* gene was amplified by PCR and sequenced as described previously (41). Similarly, the first 500 codons of the *katG* gene, the upstream noncoding region of *inhA*, and the *oxyR-ahpC* intergenic region were amplified and sequenced as described previously (21, 26). Sequences were compared to wild-type sequences found in GenBank under accession no. L27989 (*rpoB*), Z97193 (*katG*), U02492 and Z79701 (*inhA*), and U16243 (*oxyR-ahpC*). We did not sequence the *gyrA* gene. Although polymorphisms at *gyrA* codon 95 are useful for separating isolates of *M. tuberculosis*, this codon is not variable in *M. africanum* (26).

VNTR analysis. Six loci containing variable numbers of tandem repeats (VNTRs) were amplified by PCR as previously described (9). The exact number of tandem repeats at each locus in each strain was determined by the length of the PCR products on agarose gels. One of these loci (MPTR-A) contains the major polymorphic tandem repeat (MPTR), and five loci (ETR-A through ETR-E) contained exact tandem repeats (ETRs). Primers, PCR conditions, map locations, and other details related to these VNTR loci were published previously (9).

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TABLE 1. Phenotypes of 17 *M. africanum* clinical isolates from Sierra Leone[a]

Group[b]	Strain no.	Nitrate reduction	Niacin accumulation	Catalase activity at 22°C (mm)	Niveau depth (mm)	Pyrazinamidase activity	Susceptibility to: Isoniazid	Susceptibility to: Rifampin	Susceptibility to: Streptomycin	Susceptibility to: Ethambutol
A	014	Weakly positive	Weakly positive	3	7	Negative	R	R	R	S
A	031	Weakly positive	Weakly positive	0	8	Weak	R	R	R	S
A	034	Weakly positive	Weakly positive	2	8	Positive	R	R	R	S
A	037	Weakly positive	Negative	10	13	ND	R	R	R	R
A	073	Weakly positive	Negative	1	12	Positive	R	R	R	R
A	213	Weakly positive	Weakly positive	1	5	Negative	R	R	R	R
A	279	Weakly positive	Weakly positive	1	8	Positive	S	S	ND	S
A	354	Weakly positive	Negative	1	8	Positive	S	S	ND	S
A	370	Weakly positive	Weakly positive	1	4	Positive	S	S	ND	S
A	481	Weakly positive	Weakly positive	1	6	Positive	R	S	S	S
A	556	Weakly positive	Weakly positive	2	5	Weak	R	S	ND	S
A	566	Weakly positive	Negative	0	9	Positive	R	S	ND	S
A	736	Weakly positive	ND	1	6	Positive	S	S	S	S
A	754	Weakly positive	Weakly positive	1	10	Positive	R	R	ND	B
B	053	Weakly positive	Weakly positive	2	10	Positive	R	R	ND	S
B	203	Positive	Weakly positive	1	ND	Positive	B	S	ND	S
B	246	Positive	Positive	16	8	Positive	S	S	ND	S

[a] All 17 strains had no catalase activity at 68°C, produced no color change on bromcresol medium, and displayed dysgonic growth on Löwenstein-Jensen medium. Abbreviations: ND, not done; R, resistant; B, borderline; S, susceptible.
[b] *M. africanum* group based on the sequence of *katG* codon 203.

TABLE 2. Genotypes of 17 *M. africanum* clinical isolates from Sierra Leone and five reference strains of the *M. tuberculosis* complex

Group[a]	Species and strain	Polymorphism not associated with drug resistance: *katG* codon 203 sequence	*katG* codon 463 sequence	VNTR allele profile[b]	Mixed-linker cluster[c]	Polymorphism associated with drug resistance: *katG* codon 315 sequence	*rpoB* genotype
A	*M. africanum* 014	ACT Thr	CTG Leu	663535	3	AGC Ser	Wild type
A	*M. africanum* 031	ACT Thr	CTG Leu	564545	1	AGC Ser	Codon 519 Asn deletion CAA
A	*M. africanum* 034	ACT Thr	CTG Leu	564544	2	ACC Thr	Codon 526 His→Arg (CAC→CGC)
A	*M. africanum* 037	ACT Thr	CTG Leu	564544	2	ACC Thr	Codon 526 His→Arg (CAC→CGC)
A	*M. africanum* 073	ACT Thr	CTG Leu	663535	3	ACC Thr	Codon 526 His→Asp (CAC→GAC)
A	*M. africanum* 213	ACT Thr	CTG Leu	664535	Unique	ATC Ile	Codon 526 His→Tyr (CAC→TAC)
A	*M. africanum* 279	ACT Thr	CTG Leu	564544	1	AGC Ser	Wild type
A	*M. africanum* 354	ACT Thr	CTG Leu	565544	1	AGC Ser	Wild type
A	*M. africanum* 370	ACT Thr	CTG Leu	664536	Unique	AGC Ser	Wild type
A	*M. africanum* 481	ACT Thr	CTG Leu	564536	1	AAC Asn	Wild type
A	*M. africanum* 556	ACT Thr	CTG Leu	663535	4	ACC Thr	Codon 533 Leu→Pro (CTG→CCG)
A	*M. africanum* 566	ACT Thr	CTG Leu	564544	Unique	AGC Ser	Wild type
A	*M. africanum* 736	ACT Thr	CTG Leu	564544	1	AGC Ser	Wild type
A	*M. africanum* 754	ACT Thr	CTG Leu	663535	4	ACC Thr	Codon 531 Ser→Leu (TCG→TTG)
B	*M. africanum* 053[d]	ACC Thr	CTG Leu	642432	Unique	AGC Ser	Codon 531 Ser→Leu (TCG→TTG)
B	*M. africanum* 203	ACC Thr	CTG Leu	642432	ND[e]	AGC Ser	Wild type
B	*M. africanum* 246	ACC Thr	CTG Leu	642432	Unique	AGC Ser	Wild type
	M. africanum[T]	ACT Thr	CTG Leu	564544	ND	AGC Ser	Wild type
	M. bovis BCG Pasteur	ACT Thr	CTG Leu	655623	ND	AGC Ser	Wild type
	M. bovis[T]	ACT Thr	CTG Leu	675542	ND	AGC Ser	Wild type
	M. microti[T]	ACT Thr	CTG Leu	653571	ND	AGC Ser	Wild type
	M. tuberculosis[T]	ACC Thr	CGG Arg	633433	ND	AGC Ser	Wild type

[a] *M. africanum* group based on the sequence of *katG* codon 203.
[b] The number of tandem DNA repeats at each of six genetic loci (MPTR-A, ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E) was determined in each strain (9). The results were combined to yield a six-digit allele profile. Each digit represents the number of tandem repeats at a particular locus (except locus MPTR-A, for which the number of copies is reduced by 10). For example, the *M. africanum* type strain has the VNTR allele 564544. This strain has 15 tandem repeat copies at locus MPTR-A, 6 copies at locus ETR-A, 4 copies at locus ETR-B, 5 copies at locus ETR-C, 4 copies at locus ETR-D, and 4 copies at locus ETR-E.
[c] Mixed-linker fingerprints were previously published (12). Eleven strains fell into one of four clusters (designated cluster 1, 2, 3, or 4), and five strains had unique fingerprints.
[d] This strain also had a nucleotide substitution at *katG* codon 253 and a frameshift mutation at *katG* codon 254. This genotype is predicted to be associated with INH resistance.
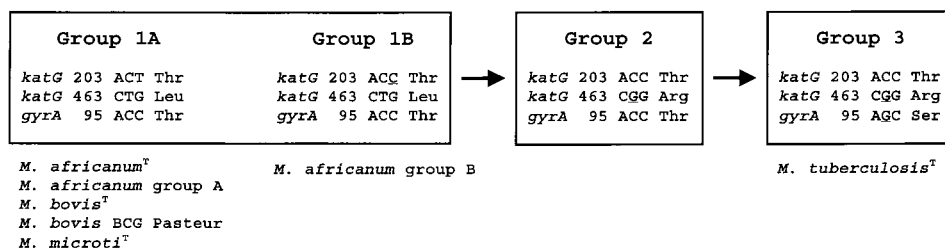[e] ND, not done.

FIG. 1. Broad evolutionary scenario for *M. tuberculosis* complex organisms. Sreevatsan et al. (26) defined groups 1, 2, and 3 based on the sequence of *katG* codon 463 and *gyrA* codon 95. We subdivided group 1 strains based on the sequence of *katG* codon 203. The distribution of the 22 strains described in this manuscript is shown.

Phylogenetic analysis. Phylogenetic trees were constructed based on the VNTR allele profiles by maximum-parsimony analysis using PAUP software (30). Maximum parsimony and other methods for phylogenetic analysis are described in recent reviews (22, 28). The data were resampled with 10,000 bootstrap replications (6), and a majority-rule consensus was computed from the most-parsimonious trees. The analysis was conducted twice, treating character states as either ordered or unordered. Treating the character states as ordered assumes single-step transitions in the number of tandem repeats at each locus. This assumption is consistent with the results of sequence analysis of the MPTR-A locus (7). Genetic distances between pairs of VNTR allele profiles were calculated based on the minimum number of single-step transitions.

Mixed-linker PCR. Genomic DNA was digested, ligated with mixed linkers, and amplified in a nested PCR as previously described (13). The PCR products were separated by agarose electrophoresis to generate a DNA fingerprint based on the mobile IS*6110* insertion element. Mixed-linker fingerprints for this collection were previously published (12).

Other genetic analyses. RD1 is a 9.5-kb region deleted in substrains of *M. bovis* BCG. Two primers which flank RD1 and one primer within RD1 were combined in a multiplex-PCR amplification as previously described (31). Strains containing the RD1 region yield a 150-bp product, while strains with deletion of RD1 (all BCG substrains) yield a 200-bp product. Substrains of *M. bovis* BCG also have a sequence polymorphism at codon 252 of a 1,551-bp open reading frame (*orf1*) in GenBank under accession no. M15467 (gene homologous to *Rv0422c*). A portion of this gene was amplified by PCR, and the sequence at this codon was determined by digestion with the restriction enzyme *Ban*I as previously described (7).

RESULTS

Tables 1 and 2 list phenotypic and genotypic results for 17 *M. africanum* isolates from Sierra Leone. These isolates were identified as *M. africanum* on the basis of biochemical and biophysical properties as listed in Table 1 (12, 36). *M. africanum* isolates grew as smooth, dysgonic, nonpigmented colonies. Growth was inhibited by thiophene-2-carboxylic hydrazide (TCH), with the exception of growth by strains resistant to INH. All strains had pyrazinamidase activity except two isolates (014 and 213) which were resistant to multiple drugs. Acquisition of pyrazinamide resistance is associated with the loss of pyrazinamidase activity. Most *M. africanum* isolates had catalase activity less than or equal to 10 mm (height of bubbles above agar) at 22°C, and all were negative for catalase activity at 68°C. All isolates except 370 grew at least 5 mm below the surface (niveau depth) on Lebek's semisolid medium, indicating microaerophilic growth. All isolates produced no color change when grown on bromcresol medium. The nitrate reduction and niacin accumulation phenotypes were variable, as described for other collections of *M. africanum* strains (4, 36).

***katG* sequence.** *katG* codes for catalase peroxidase. Loss of catalase peroxidase activity can result from deletions or point mutations in *katG* and is associated with INH resistance. We sequenced the entire *katG* gene in the 17 *M. africanum* strains and the 5 reference strains and compared the sequences to the wild-type sequence (GenBank accession no. Z97193). Results are listed in Table 2. We found mutations at five *katG* codons (203, 253, 254, 315, and 463). Eleven strains were INH resistant; of these strains, seven had polymorphisms at *katG* codon 315. One INH-resistant strain had both a point mutation at

codon 253 and a frameshift mutation at codon 254. Identical or similar *katG* mutations are associated with INH resistance in *M. tuberculosis* (21).

We found polymorphisms at *katG* codons 203 and 463 which were not associated with INH resistance. Sreevatsan et al. (26) used polymorphisms at *katG* codon 463 and *gyrA* codon 95 to define a broad evolutionary scenario for the *M. tuberculosis* complex, as shown in Fig. 1. *katG* codon 463 CTG Leu is the ancestral genotype; it was found in all strains of *M. bovis* (109 strains), *M. microti* (9 strains), and *M. intracellulare* (1 strain) (26). Some *M. tuberculosis* strains (group 1) also have CTG Leu at this locus, but the majority of *M. tuberculosis* strains (including *M. tuberculosis*[T]) have CGG Arg (groups 2 and 3) (26). All 17 *M. africanum* strains from Sierra Leone and *M. africanum*[T] from Senegal had the ancestral CTG Leu and thus belong to group 1 of the *M. tuberculosis* complex.

The only site which varied among the *M. africanum* strains and which was not associated with drug resistance was *katG* codon 203. This synonymous polymorphism was used to divide the *M. africanum* strains into two groups (Fig. 1). Fourteen *M. africanum* strains from Sierra Leone (designated group A) had *katG* codon 203 ACT Thr. This genotype appears to be the ancestral genotype; it is found in *M. africanum*[T], *M. bovis*[T], *M. microti*[T], *M. bovis* BCG, and clinical *M. bovis* isolates. Three of the *M. africanum* strains (designated group B) had *katG* codon 203 ACC Thr, which is also found in *M. tuberculosis*[T] and in all of several hundred clinical *M. tuberculosis* isolates sequenced to date.

***rpoB* sequence.** *rpoB* codes for the β subunit of the DNA-dependent RNA polymerase which is the target of rifampin activity. Mutations in *rpoB* codons 509 to 533 are strongly associated with rifampin resistance in pathogenic mycobacteria, including *M. tuberculosis*, *M. leprae*, and *M. avium* (17, 41). Eight of the *M. africanum* isolates from Sierra Leone, seven of which had *rpoB* mutations, were resistant to rifampin. Nine of the *M. africanum* isolates were rifampin susceptible, and eight of these had the wild-type *rpoB* sequence in this segment of the gene. Interestingly, *M. africanum* 556 was rifampin susceptible but had a codon 533 Leu-to-Pro (Leu→Pro) mutation previously associated with rifampin resistance in clinical *M. tuberculosis* isolates (41). *M. africanum* 556 is no longer viable, so we were unable to repeat these analyses. Overall, we observed six distinct *rpoB* mutations in these *M. africanum* strains, all of which have been described in rifampin-resistant *M. tuberculosis* strains.

VNTR analysis. We amplified DNA at six loci with variable numbers of tandem repeats (9). The number of tandem DNA repeats at each locus in each strain was determined by the length of the PCR product on an agarose gel. The results of the six loci (MPTR-A, ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E) were combined to yield a six-digit VNTR allele profile (9). Each digit represents the number of tandem repeats at a
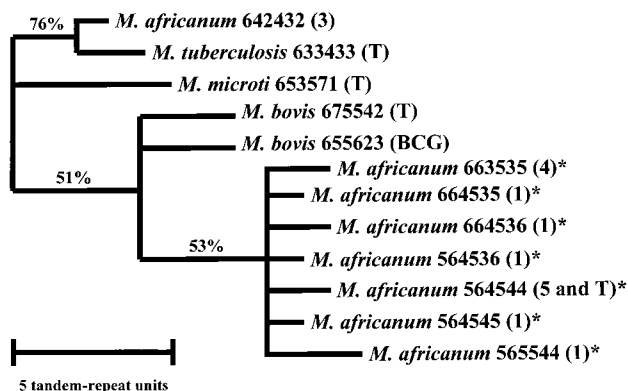
FIG. 2. Parsimony phylogenetic tree based on VNTR allele profiles. The tree includes 12 VNTR allele profiles generated from 22 strains. The number of clinical isolates is indicated in parentheses, as are the profiles associated with the type strains (T) and BCG Pasteur (BCG). VNTR allele profiles found in *M. africanum* group A are marked by an asterisk. Horizontal lengths represent genetic distances; vertical lengths are not meaningful. All clusters shown on this tree were found in 100% of the most-parsimonious trees. The data were resampled with 10,000 bootstrap replications (6, 30). The percentage of bootstrap replications which yielded each grouping is indicated. The tree was constructed with the assumption that the number of tandem repeats in each strain changes by a single copy at a time (ordered character states). When we conducted the same analysis with unordered character states, the *M. africanum* group A isolates remained clustered.

particular locus, with the exception of locus MPTR-A, where it represents the number of copies minus 10. For example, *M. africanum*[T] has the VNTR allele profile 564544. This strain has 15 tandem repeat copies at locus MPTR-A, 6 copies at locus ETR-A, 4 copies at locus ETR-B, 5 copies at locus ETR-C, 4 copies at locus ETR-D, and 4 copies at locus ETR-E. VNTR allele profiles are listed in Table 2. Figure 2 displays phylogenetic relationships among the VNTR allele profiles, and Table 3 shows genetic distances between pairs of VNTR allele profiles.

The 14 *M. africanum* group A isolates had seven VNTR allele profiles, indicated by asterisks in Fig. 2. One of the group A VNTR profiles was identical to that of *M. africanum*[T] (564544). The group A isolates, including *M. africanum*[T], clustered by VNTR analysis. They were more closely related to *M. bovis*[T] than to the rest of the *M. tuberculosis* complex (Fig. 2 and Table 3). In contrast, the three *M. africanum* group B

isolates had the VNTR allele profile 642432 and clustered with *M. tuberculosis*[T].

Mixed-linker PCR fingerprints. The mixed-linker PCR fingerprints for these strains were previously published (12). Eleven of the *M. africanum* isolates in group A were clustered by mixed-linker analysis, and three were unique. Two of the group B isolates were unique, and the third was not analyzed by mixed-linker PCR. Cluster 1 and cluster 2 have mixed-linker PCR fingerprints which differ by a single band and together accounted for 40% of all *M. africanum* isolates from Sierra Leone in a previous report (12).

The clustering of strains by mixed-linker analysis and VNTR analysis was consistent with the results of the other genotypic methods. Mixed-linker cluster 2 contained two strains with identical VNTR allele profiles (564544), identical *katG* codon 315 mutations, and identical *rpoB* mutations. The two strains in mixed-linker cluster 3 and the two strains in cluster 4 all shared the VNTR allele profile 663535, though their drug resistance mutations varied. The five strains in mixed-linker cluster 1 had four closely related VNTR allele profiles.

Other analyses. All 17 *M. africanum* isolates had wild-type sequences in the upstream noncoding region of *inhA* and the *oxyR-ahpC* intergenic region (26). All 17 *M. africanum* strains had the wild-type sequence AGC Ser at *orf1* codon 252 (7). All 17 *M. africanum* strains contained RD1, as previously reported (31).

DISCUSSION

The *M. tuberculosis* complex includes four species on the Approved List of Bacterial Names: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (37). A fifth group, characterized by the Canetti strain, has distinct features, but has not been proposed as a separate species (34). Strains from all four species show >85% DNA-DNA relatedness and have identical 16S rDNA and 16S-23S rDNA internal transcribed spacer sequences (8, 16, 18). Comparative sequence analysis of multiple structural genes revealed minimal polymorphism among strains of all four species (26). Although several authors have suggested that the four species should be reclassified as subspecies of *M. tuberculosis*, no formal proposal has been made (34, 37).

M. tuberculosis is the major cause of human tuberculosis worldwide. *M. bovis* causes tuberculosis in multiple mammalian species, including humans. The distinction between *M.*

TABLE 3. Genetic distances between pairs of VNTR allele profiles[a]

VNTR allele profile	M. africanum group B 642432	M. tuber-culosis type 633433	M. microti type 653571	M. bovis type 675542	M. bovis BCG 655623	M. africanum group A						
						663535	664535	664536	564536	564544	564545	565544
M. africanum group B 642432	0	3	8	8	8	7	8	9	10	9	10	10
M. tuberculosis type 633433	3	0	9	9	7	6	7	8	9	8	9	9
M. microti type 653571	8	9	0	8	10	9	10	11	12	9	10	10
M. bovis type 675542	8	9	8	0	6	7	6	7	8	5	6	4
M. bovis BCG 655623	8	7	10	6	0	7	6	7	8	7	8	6
M. africanum group A 663535	7	6	9	7	7	0	1	2	3	4	3	5
M. africanum group A 664535	8	7	10	6	6	1	0	1	2	3	2	4
M. africanum group A 664536	9	8	11	7	7	2	1	0	1	4	3	5
M. africanum group A 564536	10	9	12	8	8	3	2	1	0	3	2	4
M. africanum group A 564544	9	8	9	5	7	4	3	4	3	0	1	1
M. africanum group A 564545	10	9	10	6	8	3	2	3	2	1	0	2
M. africanum group A 565544	10	9	10	4	6	5	4	5	4	1	2	0

[a] The box shows distances within the group A cluster of *M. africanum* strains from Sierra Leone. All three strains in *M. africanum* group B had an identical VNTR allele profile.

tuberculosis and *M. bovis* has epidemiologic implications, since infected animals are often a source for human *M. bovis* infections, and therapeutic implications, since *M. bovis* strains are resistant to pyrazinamide. *M. bovis* may be less virulent than *M. tuberculosis* in humans, but it is clearly more virulent in some animal models. Strains of *M. tuberculosis* and *M. bovis* can be distinguished on the basis of epidemiology, biochemical tests, growth phenotypes, and several genetic markers. *M. tuberculosis* strains nearly always have a functional nicotinamidase, produce niacin, reduce nitrate, have enhanced growth in the presence of glycerol, and are resistant to TCH (36, 37). In contrast, *M. bovis* strains lack nicotinamidase function, do not produce niacin or reduce nitrate, are not stimulated by glycerol, and are sensitive to TCH (36, 37). *M. tuberculosis* and *M. bovis* strains can be distinguished by the sequence of *oxyR* nucleotide 285 (5, 25). Also, most, but not all, *M. bovis* strains have a guanine at *pncA* nucleotide 169 and lack the *mtp40* gene (5, 20, 24, 27, 38).

M. africanum was described by Castets and colleagues in 1968 (2). The type strain (ATCC 25420) was isolated from the sputum of a tuberculosis patient in Senegal. Strains of *M. africanum* display substantial phenotypic heterogeneity, and there is no simple phenotypic definition of the species (10, 12, 15, 36, 37). Difficulties in precisely defining or identifying *M. africanum* have complicated recent studies of this species (15). Both genotypes and phenotypes of *M. africanum* vary by geographic area (4, 10, 12, 14), so reports from different regions are not directly comparable. Collins et al. (4) used the nitrate reduction assay to divide *M. africanum* strains into two groups, African I (negative for nitrate reduction) and African II (positive for nitrate reduction). African I isolates are generally associated with West Africa, and African II isolates are generally associated with East Africa (4, 11, 12). However, a recent survey identified similar numbers of African I and African II isolates in the West African nation of Guinea-Bissau (15).

Several researchers have questioned whether *M. africanum* is actually distinct from the other members of the *M. tuberculosis* complex (33, 37, 39). However, the existing *M. africanum* literature suggests that it differs from *M. tuberculosis* and *M. bovis* in host range, source of infection, geographic distribution, virulence, and pathogenesis. *M. africanum* has been reported in monkeys as well as humans (32). It is a common cause of human tuberculosis in both East and West Africa (12, 19, 23). Strains resembling *M. africanum* are isolated at low rates from humans on other continents (4, 11, 42). These strains cause a different pattern of disease than *M. tuberculosis*. For example, they are rarely isolated from humans with genitourinary tuberculosis (11). In experimental models, *M. africanum* has reduced virulence compared to *M. tuberculosis* (1, 3).

We identified isolates from Sierra Leone as *M. africanum* on the basis of biochemical and biophysical properties. In general, these strains differed from *M. tuberculosis* and *M. microti* in having negative or weak niacin production and differed from *M. bovis* in their pyrazinamidase production and their susceptibility to TCH. However, these phenotypes were variable (Table 1), as has been reported by other investigators. Phenotypic analysis is complicated by some changes associated with drug resistance. Acquisition of INH resistance is associated with TCH resistance (12), and acquisition of pyrazinamide resistance is associated with a loss of pyrazinamidase (24, 27).

These 17 *M. africanum* strains shared a number of genotypes with the rest of the *M. tuberculosis* complex, including the wild-type sequences of *inhA*, the *oxyR-ahpC* intergenic region, *orf1* codon 252, and the presence of RD1. These *M. africanum* strains have mutations leading to INH and rifampin resistance similar to those seen in *M. tuberculosis* and *M. bovis*.

The 17 *M. africanum* strains fell into two distinct genotypic groups (Fig. 1). Fourteen group A strains had *katG* codon 203 ACT Thr and codon 463 CTG Leu and had closely related VNTR allele profiles. Their genotypes and phenotypes were similar to those of *M. africanum*[T] from Senegal. The geographic distribution of group A *M. africanum* strains is unknown. In addition to the isolates described here, we have identified *M. africanum* strains from New York and The Netherlands with VNTR allele profiles that cluster with group A (data not shown). In contrast, the three group B strains had *katG* codon 203 ACC Thr and VNTR allele profile 642432. In these respects, they cluster with *M. tuberculosis*[T].

VNTR analysis and mixed-linker PCR fingerprinting are based on different genetic targets and were independently informative for strain differentiation. The 14 group A *M. africanum* strains formed 7 clusters by VNTR analysis, 7 clusters by mixed-linker analysis, and 10 clusters when the results of both analyses were combined. VNTR analysis and the sequence at *katG* codon 203 yielded identical separation of the *M. africanum* strains into genotypic groups A and B. Each cluster identified by mixed-linker analysis was also restricted to a single genotypic group. VNTR analysis may be useful for evolutionary study when combined with other techniques. VNTR analysis samples multiple independent genomic loci. VNTR results are digital and are amenable to phylogenetic and genetic distance analyses as shown in Fig. 2 and Table 3. However, the number of alleles at each VNTR locus is limited, so genetic convergence may occur frequently. Convergence would make strains appear more closely related than they actually are.

All 14 *M. africanum* group A strains had typical *M. africanum* phenotypes. One group B strain had two features more typical of *M. tuberculosis*, namely, it was positive for niacin production and showed 16 mm of catalase activity. Under the classification system proposed by Collins et al. (4), all 14 group A strains and one group B strain are African I (negative or weakly positive for nitrate reduction) and two group B strains are African II (positive for nitrate reduction). However, there was no phenotype which clearly separated the genotypic groups A and B.

Strains with *M. africanum* phenotypes have substantial genotypic variability. *M. africanum* group A isolates had *M. africanum* phenotypes and clustered with *M. africanum*[T] in genotypic analyses. *M. africanum* group B strains also had *M. africanum* phenotypes but clustered with *M. tuberculosis* strains in genotypic analyses. Haas et al. (14) identified strains from Uganda with *M. africanum* phenotypes and *katG* codon 463 CGG Arg, a sequence previously found only in *M. tuberculosis* (26). These data suggest that multiple genetically distinct strains have converged toward an *M. africanum* phenotype. Our results support the retention of the designation *M. africanum* for the group A strains, since they cluster with *M. africanum*[T] in multiple analyses and are distinct from the rest of the *M. tuberculosis* complex. Future studies of *M. africanum* should include both phenotypic and genotypic analyses.

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