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Characterization of *Mycobacterium caprae* Isolates from Europe by Mycobacterial Interspersed Repetitive Unit Genotyping‡

Wolfgang M. Prodinger,[1]* Anita Brandstätter,[2] Ludmila Naumann,[3] Maria Pacciarini,[4] Tanja Kubica,[5] Maria Laura Boschiroli,[6] Alicia Aranaz,[7] György Nagy,[8] Zeljko Cvetnic,[9] Matjaz Ocepek,[10] Artem Skrypnyk,[11] Wilfried Erler,[11] Stefan Niemann,[5] Ivo Pavlik,[12]† and Irmgard Moser[11]†

Department of Hygiene, Microbiology, and Social Medicine[1] and Institute of Legal Medicine,[2] Innsbruck Medical University, Innsbruck, Austria; Bavarian Health and Food Safety Authority, Oberschleissheim, Germany[3]; Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy[4]; Forschungszentrum Borstel, National Reference Center for Mycobacteria, Borstel, Germany[5]; Agence Française pour la Sécurité Sanitaire des Aliments, Maisons Alfort, France[6]; Departamento de Sanidad Animal, Universidad Complutense, Madrid, Spain[7]; Central Veterinary Institute, Budapest, Hungary[8]; Croatian Veterinary Institute, Zagreb, Croatia[9]; Veterinary Faculty, University of Ljubljana, Slovenia[10]; German National Veterinary Reference Laboratory for Tuberculosis, Friedrich-Loeffler-Institut, Jena, Germany[11]; and Veterinary Research Institute, Brno, Czech Republic[12]

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Mycobacterium caprae*, a recently defined member of the *Mycobacterium tuberculosis* complex, causes tuberculosis among animals and, to a limited extent, in humans in several European countries. To characterize *M. caprae* in comparison with other *Mycobacterium tuberculosis* complex members and to evaluate genotyping methods for this species, we analyzed 232 *M. caprae* isolates by mycobacterial interspersed repetitive unit (MIRU) genotyping and by spoligotyping. The isolates originated from 128 distinct epidemiological settings in 10 countries, spanning a period of 25 years. We found 78 different MIRU patterns (53 unique types and 25 clusters with group sizes from 2 to 9) but only 17 spoligotypes, giving Hunter-Gaston discriminatory indices of 0.941 (MIRU typing) and 0.665 (spoligotyping). For a subset of 103 *M. caprae* isolates derived from outbreaks or endemic foci, MIRU genotyping and IS*6110* restriction fragment length polymorphism were compared and shown to provide similar results. MIRU loci 4, 26, and 31 were most discriminant in *M. caprae*, followed by loci 10 and 16, a combination which is different than those reported to discriminate *M. bovis* best. *M. caprae* MIRU patterns together with published data were used for phylogenetic inference analysis employing the neighbor-joining method. *M. caprae* isolates were grouped together, closely related to the branches of classical *M. bovis*, *M. pinnipedii*, *M. microti*, and ancestral *M. tuberculosis*, but apart from modern *M. tuberculosis*. The analysis did not reflect geographic patterns indicative of origin or spread of *M. caprae*. Altogether, our data confirm *M. caprae* as a distinct phylogenetic lineage within the *Mycobacterium tuberculosis* complex.

Several members of the *Mycobacterium tuberculosis* complex (MTC) that encompasses the causative agents of tuberculosis (TB) can be distinguished. Recently, *M. pinnipedii* (11) and *M. caprae* (1) have been added to *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. canettii*. *M. caprae*, with former names *M. tuberculosis* subspecies *caprae* (2) and *M. bovis* subspecies *caprae* (30), has originally been described as preferring goats to cattle as host within the same epidemiological settings in Spain (4, 19). *M. caprae* has been found to affect predominantly cattle among several other host species in Austria (34), France (20), Germany (15, 16), Hungary (16), Italy (M. B. Boniotti, L. Alborali, E. Tisato, and M. L. Pacciarini, Abstr. 25th Annu. Meet. Eur. Soc. Mycobacteriol., abstr. 61, 2004), Slovenia (16), and the Czech Republic (33). In addition, wild living species such as red deer (34) or wild boar (16, 27) infected with *M. caprae* may constitute a reservoir for resurgent TB in domestic animals. Human infections with *M. caprae* appear to be rare on a worldwide or a Europe-wide scale nowadays, due to the eradication campaigns and preventive measures taken against transmission of bovine TB in the last century. They primarily manifest in older individuals (31). However, in central European regions where *M. caprae* is the major cause of TB in cattle, it is also the predominant agent of "bovine TB" in humans (25, 34). Interestingly, genomic deletion analyses using large panels of MTC isolates (6, 29) suggested that both new MTC members have phylogenetically preceded *M. bovis*.

Genotyping techniques developed for MTC members have extended our understanding of the natural history of TB and have become an essential tool in TB epidemiology (47). This applies likewise to analyses of TB transmission among livestock (32) or between livestock and reservoir species such as badgers, possums, or deer (7). IS*6110* restriction fragment

* Corresponding author. Mailing address: Department of Hygiene, Microbiology, and Social Medicine, Innsbruck Medical University, Fritz Pregl-Straβe 3, A-6020 Innsbruck, Austria. Phone: 43 512 507 3401. Fax: 43 512 507 2870. E-mail: wolfgang.prodinger@uibk.ac.at.
† These authors contributed equally to this work.
‡ Supplemental material for this article may be found at http://jcm.asm.org/.

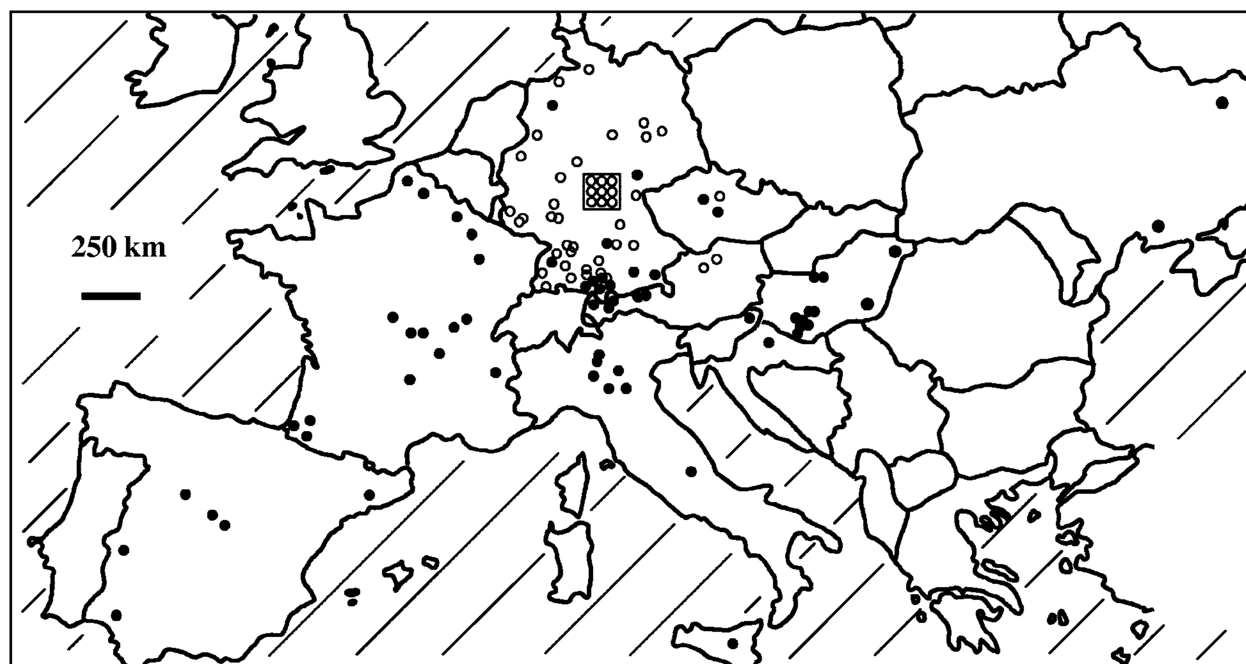

FIG. 1. Approximate locations in Europe of *M. caprae* isolates from animals (filled circles) or humans (open circles) included in the study. The exact provenance was unavailable for nine human isolates from Germany (shown boxed).

length polymorphism (RFLP) has been the standard technique for differentiation of human isolates with at least five IS*6110* copies (46) and can also discriminate *M. bovis* isolates, although such isolates most often possess only a single IS*6110* element (9, 40, 48). However, PCR-based methods have challenged this costly method, as they promise a faster throughput at equal quality, e.g., methods aimed at tandemly arranged repetitive sequences in the genomes of MTC members (13). These variable-number tandem repeats (VNTRs) are found at multiple loci, and some loci show substantial variation in the number of repeat units.

Different VNTR typing methods have been established. Initially, Frothingham and Meeker (18) used six exact tandem repeat (ETR) loci. Mazars et al. (28) described the analysis of 12 loci of mycobacterial interspersed repetitive units (MIRUs) by a single-PCR method which was later developed into a multiplex PCR with automated sequencer analysis (45). MIRU genotyping has proven highly discriminant for worldwide MTC isolate collections (43, 45) and analyses of human TB outbreaks (21, 26, 28), for population-derived samples of isolates (5, 44) and for population-based studies (12). MIRU patterns show sufficient stability and appear to evolve at a slower pace than IS*6110* patterns, as shown for serial isolates from patients (28, 38), and in some instances they discriminated IS*6110*-defined clusters (isolates with >6 bands) (12). MIRU typing has been studied for the discrimination of *M. bovis* isolates but has been found to be less discriminant than strategies aimed at other VNTRs (36). Skuce et al. (41) and Roring et al. (35) have described such VNTRs (termed QUBs) as particularly useful for *M. bovis* outbreaks and as more discriminant than ETR or MIRU typing with a standardized *M. bovis* panel (36).

M. caprae isolates have not been studied with VNTR analyses so far, and other techniques have their drawbacks with this MTC member. Spoligotyping, although instrumental in identifying *M. caprae* isolates (3), is little help in discriminating them: in central European countries, more than 50% of the isolates show the same spoligotype, lacking spacers 1, 3 to 16, 28, and 39 to 43 (16). IS*6110* RFLP is highly discriminant, as *M. caprae* isolates generally have two to eight IS*6110* copies (16), but is costly and time-consuming.

This study is the first to assess the potential of MIRU genotyping to differentiate *M. caprae* isolates. We have analyzed 232 *M. caprae* isolates from across Europe, representative of nearly all countries that have ever reported *M. caprae* isolates (Fig. 1), by MIRU typing and spoligotyping. We have analyzed the potential of the method to (i) discriminate between outbreaks among livestock, (ii) differentiate serial isolates obtained from natural TB foci over years, (iii) compare human isolates with each other and with animal isolates, and (iv) show the position of *M. caprae* among the MTC members on an evolutionary scale. The data obtained suggest that MIRU is a valuable tool in all of these aspects.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction. MTC isolates from TB patients (48 isolates) or from wild or domestic animals (camel, 2 isolates; deer, 7; cattle, 156; goat, 11; wild boar, 8) were determined to be *M. caprae* by their specific spoligotype and by allele-specific PCR for the typical *M. caprae* nucleotide sequence polymorphisms in *oxyR* and *pncA* (17). The isolates originated from 10 European countries (Austria, 59 isolates; Croatia, 11; Czech Republic, 4; France, 16; Germany, 83; Hungary, 17; Italy, 27; Slovenia, 1; Spain, 11; Ukraine, 3), including several *M. caprae* isolates reported in the literature (16, 20, 34). A detailed list of all 232 MIRU-typed *M. caprae* isolates is given in the supplemental material, and the approximate geographic origins of the isolates are shown in Fig. 1. Genomic DNA for PCR was obtained by standard isolation techniques (49) or by boiling a loop full of bacteria in 200 μl of 1× Tris-EDTA buffer for 15 min and using the supernatant.

MIRU genotyping. MIRU typing was performed for 205 isolates in Innsbruck, Austria, and for the remaining 28 samples in Borstel, Germany. MIRU typing in Innsbruck was performed by 12 single PCRs as described previously (28) with the primer sequences and final $MgCl_2$ concentrations as described previously (45) and the following modification: instead of a HotStar*Taq* DNA polymerase kit (QIAGEN, Hilden Germany), we used Dynazyme II DNA polymerase together with the appropriate 10× PCR buffer (Finnzymes, Espoo, Finland) and 10% dimethyl sulfoxide (molecular biology grade; Sigma, St. Louis, MO) in a 25-μl PCR. This protocol had been shown to yield identical MIRU results on a panel of RFLP-typed isolates and reference strains (data not shown). To minimize mislabeling and confounding errors, single strips of PCR tubes prefilled with 12 PCR mixes to amplify each of the 12 MIRU loci were used for each isolate. The PCR products were separated by electrophoresis on a 2% agarose (Seakem LE; Cambrex Biosciences, Rockland, Maine) gel in 1× Tris-acetate-EDTA containing 1 μg of ethidium bromide/ml in a Sub-Cell model 192 apparatus (Bio-Rad, Hercules, Calif.) and a 25- by 25-cm gel with three rows of 51 slots (slot width, 0.75 mm). Multichannel devices fitting both PCR strips and slot rows were used for loading the gel. A size marker (DNA molecular weight marker XIV; Roche, Mannheim, Germany) was loaded to every fifth lane. The fragments for each isolate were separated for 3 h at 150 V and photographed together. The MIRU copy number corresponding to the respective band size was calculated according to information provided by Philip Supply (www.ibl.fr/mirus/mirus.html) and entered into a spreadsheet file. At Borstel, MIRU codes were determined by T.K. and S.N. by multiplex-PCR MIRU typing according to a previously described method (45).

Spoligotyping and IS*6110* RFLP analysis. Spoligotyping was carried out as described previously (23) by using membranes (Isogen Bioscience BV, Maarsen, The Netherlands). The octal codes for spoligotypes were determined as proposed previously (14). IS*6110* RFLP was performed on selected series of isolates as described previously (49); for others, the results were retrieved from the literature (15, 16). The patterns were analyzed with Gelcompar version 4.2 (Applied Math, Sint-Martens-Latem, Belgium) by the unweighted-pair group method using arithmetic averages and the Dice coefficient for similarity with 1% band position tolerance.

MIRU-VNTR phylogenetic inference and evolutionary analysis of spoligotypes. For phylogenetic inference using the MIRU-VNTR data, we applied the neighbor-joining method (37) by the computer program PAUP* 4.0b10 (phylogenetic analysis using parsimony and other methods; D. Swofford, Sinauer Associates, Sunderland, Mass.). MIRU-VNTR data were treated as ordered character states. To bring the 76 different MIRU patterns for *M. caprae* into a phylogenetic context with those from other MTC members, we combined the data from this study with that from 90 individuals belonging to *M. tuberculosis* (n = 70), *M. africanum* (n = 2), *M. bovis* (n = 12), *M. pinnipedii* (n = 3; originally described as *M. bovis* [27]), *M. microti* (n = 2), and *M. canettii* (n = 1) (45), retrieved from http://www.ibl.fr/mirus/mirus.html. Identical haplotypes were identified and represented in the analyses by one sequence only. This led to a final data set comprising 167 different types, including *M. canettii* as an out-group taxon.

Statistical analyses. The Hunter-Gaston discriminatory index (HGDI) was calculated as described previously (22). Allelic diversity (h) of MIRU alleles was assessed according to Selander et al. (39).

RESULTS

MIRU-VNTR and spoligotyping analyses. A complete MIRU code was obtained for 229 of the 232 *M. caprae* samples. For three isolates, the copy number of certain loci could not be established as no amplification product was obtained (for two isolates at locus 16 and for the third isolate at loci 4, 16, and 23). This failure was probably caused by a limiting amount or quality of target DNA. As the three isolates were nevertheless unequivocally different from all other isolates, they were also included in the analysis. Identical isolates from one definite epidemiological setting (same farm within the same year) or from one person are counted as one representative isolate (RI), making a total of 128 RIs for this study. The cluster analyses by spoligotyping and MIRU genotyping, respectively, are summarized in Table 1 (fingerprint patterns and epidemiological data are given in the supplemental material).

TABLE 1. Clustering of *M. caprae* isolates by MIRU-VNTR typing or spoligotyping[a]

Spoligotyping cluster designation	No. of isolates in spoligotyping cluster		MIRU-VNTR cluster designation	No. of isolates in MIRU-VNTR cluster	
	RI (n = 128)	All (n = 232)		RI (n = 128)	All (n = 232)
S1	71	166	M1[b]	8	59
			M3[c]	2	2
			M4	2	13
			M5[d]	7	9
			M6	2	2
			M7	2	2
			M10	2	15
			M11[e]	1	1
			M12	3	3
			M13	2	2
			M16	2	7
			M17	3	4
			M18	2	2
			M21	2	3
			M22	4	4
			Unique	27	38
S2	4	5	M2	2	3
			Unique	2	2
S3	3	6	M8	3	6
S4	14	19	M5[d]	1	1
			M9	2	4
			M19	6	9
			M20	2	2
			Unique	3	3
S5	7	7	M11[e]	1	1
			M14	2	2
			Unique	4	4
S6	2	2	Unique	2	2
S7	9	9	M15	2	2
			M23[f]	1	1
			Unique	6	6
S8	2	2	Unique	2	2
S9	3	3	Unique	3	3
S10	2	2	M1[b]	1	1
			Unique	1	1
S11	2	2	M23[f]	2	2
S12	2	2	M11[e]	1	1
			Unique	1	1
S13	2	2	M24	2	2
S14	2	2	M25	2	2
Unique	3	3	M3[c]	1	1
			Unique	2	2

[a] A total of 17 spoligotyping patterns and 78 MIRU-VNTR patterns were observed.
[b] The M1 cluster was shared by spoligotypes S1 and S10.
[c] The M3 cluster was shared by spoligotype S1 and a unique spoligtyping cluster.
[d] The M5 cluster was shared by spoligotypes S1 and S4.
[e] The M11 cluster was shared by spoligotypes S1, S5, and S12.
[f] The M23 cluster was shared by spoligotypes S7 and S11.

Spoligotyping revealed 17 different patterns, and 71 of 128 RIs (55%) belonged to the "classical" spoligotype of *M. caprae*, S1. A further 54 RIs were grouped into spoligotypes S2 to S14 (group sizes, 2 to 14 RIs), and only three isolates had unique spoligotypes. In contrast, MIRU genotyping showed 25 clusters (group sizes, 2 to 9 RIs) and 53 unique types, making 78 different patterns altogether (Table 1). In particular, the prevailing S1 spoligotype isolates were split up into 15 MIRU clusters and 27 unique patterns. The HGDI for MIRU typing was 0.941 for the 128 RIs (0.896 for all 232 isolates), whereas

TABLE 2. List of isolates that display different spoligotypes, but identical MIRU patterns, in comparison to the typically observed combinations of MIRU and spoligotyping patterns

Isolate no. (RI)[a]	MIRU cluster[a]	MIRU code[b]	Spoligotyping cluster[a]	Spoligotype[c]
21	M5	2**3**6424253522	S4	.o..............ooooooooooo.o....ooooo.....
Prototype M5	M5	2**3**6424253522	S1	.o..............ooooooooooo.oooooooooo.....
Prototype M9	M9	2**6**6424253522	S4	.o..............ooooooooooo.o....ooooo.....
28	M23		S7	.o..............oooooooo...................
Prototype M23	M23		S11	.o..............oooooooo..........oooo.....
3	M1		S10	.o..............oo.oooooooo.oooooooooo.....
Prototype M1	M1		S1	.o..............ooooooooooo.oooooooooo.....
107	M3		Unique	........................ooo.oooooooooo.....
Prototype M3	M3		S1	.o..............ooooooooooo.oooooooooo.....
47	M11		S1	.o..............ooooooooooo.oooooooooo.....
8	M11		S5	.o..............ooooooooooo.ooo.oooooo.....
82	M11		S12	.o..............ooooooooooo.oooooooo.o.....

[a] Number and cluster designations are according to the table in the supplemental material.
[b] Lists the copy numbers in loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 (in this sequence). Copy numbers that are not the same for each code are shown in bold.
[c] The presence and absence of spacers 1 to 43 are denoted by "○"(present) and "."(absent).

spoligotyping had HGDI values of 0.673 ($n = 128$) and 0.480 ($n = 232$), respectively.

Five isolates within MIRU clusters showed spoligotypes that differed from the one typically associated with the respective MIRU cluster (Table 2). In these cases, spoligotyping was more discriminant than MIRU typing, as the spoligotypes showed additional deletions of a single spacer or of a block of contingent direct repeat (DR) spacers. The M5/S4 type combination of isolate 21 appears as an intermediate between the very closely related and actually observed combinations M5/S1 and M9/S4.

Of a total of 25 MIRU clusters, 15 comprised only animal isolates, 3 had only human isolates, and 7 had both. Thirteen of the 15 animal clusters were found within the same country, often in the same or bordering regions. The two exceptions are cluster M1, which is found in parts of the northern and southern Alps (separated by 300 kilometers), and cluster M5, found at very distant locations in France, Germany, the Czech Republic, and Ukraine. In contrast, only 2 of 14 shared spoligotypes comprised only animal isolates, but 9 of 14 were mixed of human and animal isolates. The S1 type was found distributed in all countries except Spain, with a higher proportion among isolates from Eastern European countries. In contrast, S4 and S7, the other types shared between several countries, were both restricted to Germany (human isolates only), France, Italy, and Spain.

Performance of MIRU genotyping versus that of IS*6110* RFLP. Since MIRU typing is being considered as the high-throughput alternative for IS*6110* RFLP typing for *M. tuberculosis*, we compared the discrimination of both methods for a subset of *M. caprae* isolates with available RFLP results. Three larger outbreaks or series of outbreaks among cattle (i.e., affecting one or two farms in the same or neighboring communities), with a total of 80 parallel outbreak isolates, were analyzed. The respective outbreak strains are represented by RIs 2, 10, and 36 (see the supplemental material). Two outbreaks showed microheterogeneity (Table 3, outbreaks A and B). In the first outbreak (RI 10), 2 of 11 parallel isolates differed by one IS*6110* band each, whereas all were MIRU identical (Table 3, outbreak A). In the second outbreak (RIs 35, 36, and 40), all 16 parallel isolates were RFLP identical, but one had a single MIRU copy number change (Table 3, outbreak B). Finally, in a border-crossing series of four outbreaks in the northern Alps with 53 parallel isolates (RI 2 of cluster M1), all isolates had an identical MIRU code, spoligotype, and RFLP pattern (Table 3, outbreak C). Interestingly, this MIRU type was also retrieved from distant sites in the southern Alps, but RFLP data are not available yet. In summary, among the 80 parallel isolates, two RFLP patterns differed from the respective outbreak RFLP pattern, and one MIRU type differed from the respective outbreak MIRU type.

Outbreaks among cattle or TB cases among wild boars that occurred separately over years, but in the same region within a range of 50 to 200 kilometers, showed greater heterogeneity among the isolates. With 23 isolates tested, the extent of copy number changes in MIRU typing agreed well with the number of band changes in RFLP typing in each setting (Table 3, outbreaks D to F).

***M. caprae* isolates from humans.** Forty-seven of the 232 *M. caprae* isolates were derived from 46 human TB cases, the large majority being reactivation cases in aged individuals (data not shown; see reference 25). Altogether, 38 different MIRU patterns and 14 different spoligotypes were observed (see the supplemental material). Twenty-eight isolates (61%) were unique by MIRU typing but only two had unique spoligotypes. MIRU typing thus discriminated 13 isolates of spoligotype S1. Nine human isolates clustered with isolates from cattle or free-living animals. Six of these seven clusters are unlikely to represent a direct epidemiological connection, as animals and humans were from different countries. In cluster M23, the two human isolates differed by a unique spoligotype from the cattle isolate showing spoligotype S1. In one instance, the isolates from a farmer and his cow shared the same type (corresponding to cluster M25).

TABLE 3. Discrimination of *M. caprae* genotypes by MIRU typing versus IS*6110* RFLP among series of isolates from outbreaks or endemic foci

Outbreak setting[a]	RI no.(s)[b]	RFLP pattern		MIRU pattern		No. of isolates
		No. of bands	No. of bands changed[c]	No. of MIRU loci changed[c]	No. of copies changed[c]	
A	10*	6	NA	NA	NA	9
	10	7	1	0	0	1
	10	7	1	0	0	1
B	36*	4	NA	NA	NA	11
	35	4	0	0	0	4
	40	4	0	1	1	1
C	30*	2	NA	NA	NA	1
	31	2	0	0	0	1
	3	2	0	0	0	1
	2	2	0	0	0	50
D	39*	5	NA	NA	NA	1
	38	5	0	1	2	1
	41	3	4	2	2	1
	30, 31	2	3	5	5	2
E	91, 92 (wild boar, cattle)*	5	NA	NA	NA	7
	87, 88 (wild boar)	4	1	1	1	2
	93, 94, 95 (wild boar, cattle)	4	5	3	4	4
F	4*	8	NA	NA	NA	2
	5	8	1	0	0	1
	6	7	3	1	2	1
	7	8	0	1	2	1
Total						103

[a] A, single outbreak (one cattle farm); B, single outbreak (two cattle farms); C, four outbreaks in neighboring communities (cattle); D, four outbreaks in the same region (cattle, range within 200 km); E, seven endemic foci (within 150-km range); and F, endemic focus in red deer (within 50-km range).
[b] RI numbers refer to the isolates listed in the supplemental material. The predominant genotype in each outbreak setting (A to F) is marked with an asterisk.
[c] As compared to the predominant genotype (marked with an asterisk) in each outbreak setting. NA, not applicable.

Nine further human isolates clustered with other human isolates only. Human-to-human transmission could not be ruled out or might even be suspected from the incomplete patient data. In the only case of childhood infection (isolate 56), a source (either animal or human) was not evident from this study. Cluster M11 comprised three individuals from the same region, aged 66, 70, and 80 years. However, their spoligotypes diverged by single DVR deletions. The other clusters did not show spoligotype diversity.

Allelic diversity in MIRU-VNTR loci. The distribution of MIRU allele numbers in our sample of 128 RIs is shown in Table 4. Two copies were found invariably at locus 39. On the other hand, a few isolates with more than two copies at locus 20 were detected, which has not been reported for other MTC isolates so far. Ten out of 122 isolates have copy numbers other than two at locus 24, with two having one copy. In 3 of all 232 isolates (1.3% [see the supplemental material]), two bands corresponding to different copy numbers were reproducibly found for one locus, indicating the emergence of new subclones. For 128 *M. caprae* isolates, allelic diversity (h) was highest for MIRU loci 26, 31, and 4, followed by loci 10 and 16. The other loci had low or no discriminatory potential.

MIRU-VNTR phylogenetic inference. As *M. caprae* isolates have not yet been included in any larger MIRU genotyping analysis of MTC isolates, we investigated how this method would group *M. caprae* within the MTC. Seventy-seven different MIRU patterns were observed for *M. caprae* in this study. Seventy-six (i.e., all except RI 126, which had three MIRU loci undetermined) were analyzed together with MIRU data for 90 well-studied MTC isolates published by Supply et al. (45). The resulting neighbor-joining tree for the 166 specimens, including *M. canettii* as the out-group individual, is shown in Fig. 2, and the positions of *M. caprae* isolates are indicated. All except one *M. caprae* isolate are separated from modern *M. tuberculosis* and found in one large group which has distinct branches for ancestral *M. tuberculosis*, classical *M. bovis*, *M. pinnipedii*, and *M. microti.* The only two *M. africanum* isolates are found between *M. pinnipedii* and *M. caprae* and between modern *M. tuberculosis* and *M. caprae*, respectively. Three *M. bovis* isolates (Fig. 2), i.e., one from *Oryx gazella* (isolate SAU 24) and two from humans (isolates NL 6 and NL 85), respectively, are positioned within *M. caprae*. The isolates of the dominant S1 spoligotype are evenly distributed among *M. caprae*, with the exception of the branch between RIs 26 and 19 that encompasses all S7 and most S4 type isolates.

DISCUSSION

Molecular epidemiology and epizootiology of TB have contributed significantly to a better understanding of the dynamics

TABLE 4. Number of occurrences of MIRU alleles and allelic diversity (*h*) at each MIRU locus for a representative sample of *M. caprae* (*n* = 128).

Parameter	Result at MIRU locus[a]											
	2	4	10	16	20	23	24	26	27	31	39	40
Allele no.												
0												1
1	2	1	1	0	12	1	2	0	6	0	0	3
2	125	21	0	22	114	2	118	7	8	5	128	120
3	1	75	7	13	3	9	7	2	114	13	0	5
4	0	22	13	85	0	115	1	30	1	21	0	0
5	0	6	13	4	0	0	0	73	0	86	0	0
6	0	2	88	1	0	0	0	15	0	3	0	0
7	0	0	2	0	0	0	0	1	0	0	0	0
8	0	0	3	0	0	0	0	0	0	0	0	0
9	0	0	1	0	0	0	0	0	0	0	0	0
Total tested	128	127	128	125	129	127	128	128	129	128	128	129
h	0.02	0.58	0.49	0.49	0.20	0.16	0.14	0.60	0.21	0.51	0.00	0.13
HGDI	0.05	0.60	0.51	0.50	0.21	0.18	0.15	0.61	0.21	0.51	0.00	0.13

[a] Results shown in rows 0 to 9 are number of occurrences of MIRU alleles at each locus. The copy number remained undetermined at locus 4 and 23 in one isolate and at locus 16 in three isolates. On the other hand, both copy numbers are included for three isolates with two alleles at one locus (loci 20, 27, and 40, respectively).

of transmission in human populations or between wild and/or domestic animals. In this study, we investigated the discriminatory potential of MIRU-VNTR genotyping for a comprehensive panel of isolates of *M. caprae*, a pathogen that contributes significantly to animal TB in continental Europe and to the evanescent number of "bovine TB" cases among humans in these countries. MIRU typing, as performed by electrophoresis of single-locus PCRs as readout (28), was found fully reproducible. Ninety-three percent of the isolates were fully typeable at the first run. The method also proved highly discriminant for this panel of *M. caprae* isolates, giving HGDI values of 0.941 and 0.896 for the representative set (*n* = 128) and the set including all outbreak isolates (*n* = 232), respectively. For MIRU genotyping, Sola et al. have reported HGDI values of 0.988 for a worldwide collection of 119 MTC isolates (43), and Sun et al. obtained values of 0.975 for 291 consecutive human *M. tuberculosis* isolates from Singapore (44).

Roring et al. calculated allelic diversity (*h*) for a defined set of 42 different *M. bovis* isolates and obtained a value of 0.74 for MIRU genotyping (36), in contrast to the *h* value of 0.933 for this *M. caprae* set (*n* = 128). These two panels cannot be compared directly, though, as the former was obtained selectively from Irish cattle and badgers, whereas the latter comprised human and animal isolates from across Europe. However, the most discriminating MIRU alleles were also found to differ between the two panels: MIRU 24 was moderately discriminating among the *M. bovis* set (36), as among *M. tuberculosis* (43, 45) isolates, but only poorly discriminating among *M. caprae* isolates. A similar finding was reported for a collection of 123 *M. bovis* isolates obtained from Belgian cattle over a period of 8 years (C. Allix, K. Walravens, V. Vandenpoorte, P. Supply, J. Godfroid, and M. Fauville-Dufaux, Abstr. 25th Annu. Meet. Eur. Soc. Mycobacteriol., abstr. 135, 2004). On the other hand, MIRU loci 10, 16, and 31 proved moderately discriminant among *M. caprae* samples but poorly resolving among the Irish *M. bovis* panel. Compared to large MTC panels analyzed by MIRU typing (and not including *M. caprae*) (43, 45), *M. caprae* isolates were found to be significantly less diverse at locus 40 and notably monomorphic at locus 39. Thus, an optimally reduced set of MIRUs for resolving *M. caprae* would consist of MIRUs 4, 10, 16, 26, and 31, which is different from other reduced MIRU sets postulated for *M. bovis* (36). Nevertheless, it will be interesting to test *M. caprae* within MIRU clusters with additional VNTRs, such as QUBs (41), reported to have a superior resolution in a well-defined *M. bovis* panel (36).

Direct comparison to IS*6110* RFLP is regarded as a challenge for MTC subtyping methods (3, 48), provided that the isolates do not harbor more than three copies of IS*6110*. *M. caprae* isolates generally have more than one IS*6110* copy and average around five (range, two to eight) copies (4, 16, 19, 34). Testing a subset of 103 *M. caprae* isolates from outbreaks or endemic foci, we found the same discrimination between isolates from endemic sources by MIRU typing and by IS*6110* RFLP and very little variation among outbreak isolates (Table 3). These data suggest that MIRU typing is an alternative that could substitute for RFLP typing. This experience supports the conclusions from an early evaluation of a two-step approach adopted in the United States for genotyping *M. tuberculosis* from humans, i.e., a combination of automated spoligotyping and MIRU typing that reduces the need for RFLP typing (12).

The geographical distribution of MIRU types among the 128 epidemiological settings suggests that MIRU clusters are also specific enough on a large scale. Of 15 clusters that comprise animal isolates only, 13 were found in a single country and thus provided clues for local and regional transmission or persistence of TB. In contrast, the locations found with mixed clusters (i.e., comprising animal and human isolates) do not suggest epidemiological connections, although the observed transmission between cow and farmer (RIs 43 and 85 [see the al material]) is an exception. This may be explained by the fact that human study isolates originated predominantly from el-

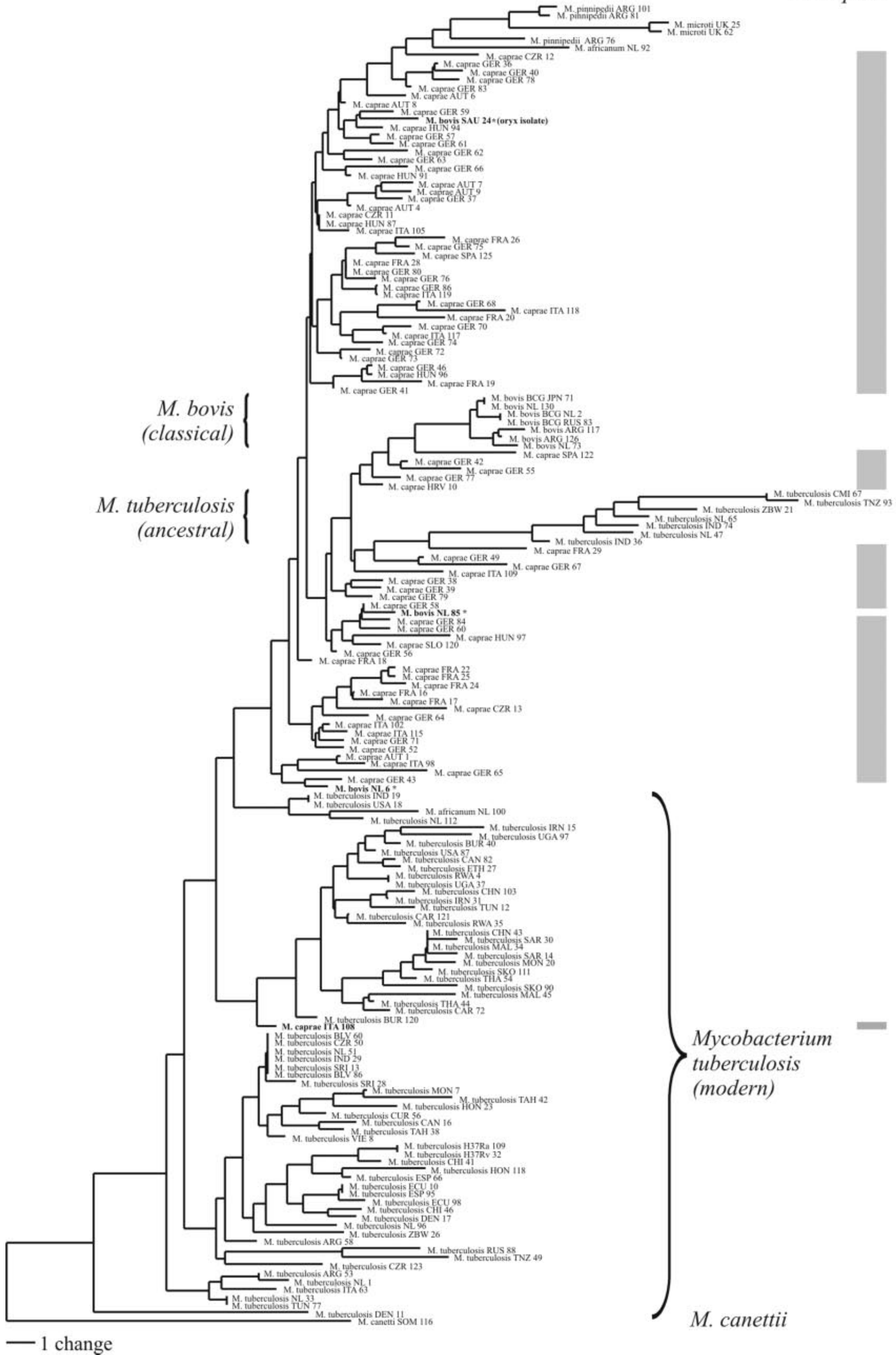

FIG. 2. Neighbor-joining tree based on 12 MIRU-VNTR loci and derived from 166 MTC isolates (76 *M. caprae* isolates from this study and 90 non-*M. caprae* MTC isolates taken from Supply et al. [45]). The out-group taxon for tree reconstruction was *M. canettii*. The linkage distance scale is indicated at the bottom. *M. caprae* isolates are indicated by the gray bar at the right and designated by country code of origin and RI number (see list in supplemental material). Other MTC isolates are designated by country code followed by the isolate number, as in reference 24. The three isolates printed in bold and marked with an asterisk are mentioned in the text.

derly patients and thus have been acquired several decades ago, whereas most animal isolates represent very recent infections. This hypothesis is supported by the high proportion of MIRU unique isolates from humans. In addition, a reduction of *M. caprae* strain diversity is very likely to have occurred after the eradication measures against "bovine tuberculosis" in the past.

A glimpse of ongoing strain differentiation could be caught in 3 of the 232 isolates, which showed two alleles for a single given MIRU locus. One would expect to "catch" the evolution of a subclone in rare instances, and this is actually an advantage of all VNTR-based methods. On the other hand, a potential problem associated with VNTR typing is homoplasia, i.e., that copy number changes in previously different isolates could lead to identical profiles, e.g., MIRU types. The instances in this study where different spoligotypes were found with identical MIRU patterns are best explained by additional deletion events in the DR gene locus (Table 2). Altogether, this data set does not infer that homoplasia is a major obstacle for the interpretation of MIRU genotyping results.

The underlying concept of MTC phylogeny has been demonstrated to be reflected by MIRU pattern analysis (45), except for *M. caprae*. These data were reanalyzed after addition of a comprehensive *M. caprae* sample (Fig. 2). *M. caprae* isolates (all but one) were distributed in one large group together with the distinct branches for ancestral *M. tuberculosis*, classical *M. bovis*, *M. pinnipedii*, and *M. microti*, respectively. All are set off from modern *M. tuberculosis*. Three "*M. bovis*" isolates (SAU 24, NL 6, and NL 85) were placed among *M. caprae* isolates at different sites. Those three have been shown to differ from any of the above MTC members of the *M. bovis* lineage by deletion analysis and single nucleotide polymorphisms (6). In accordance with these findings, 118 out of 128 *M. caprae* isolates had two copies at locus 24, a locus with a very slow evolution rate (45), as is the case for most isolates in the *M. bovis* lineage and for "ancestral" *M. tuberculosis*.

M. caprae has recently been defined as a separate member of the MTC (1). This development accommodates the findings from recent genomic deletion analyses (6, 29) that have likewise set apart "classical" *M. bovis* from *M. pinnipedii* or *M. microti*, i.e., pathogens that cause TB primarily in selected animal species. However, *M. caprae* causes TB among the same animal species as "classical" *M. bovis* does. In very practical terms, this implies that the lawful notification process should encompass *M. caprae* infections as TB and will have to be adapted wherever *M. caprae* is prevalent.

One interesting question remains. MIRU typing of this representative panel does yield a geographical pattern that would clearly suggest routes of spread of *M. caprae* in the past. On the one hand, the dominant S1 type is increasingly prevalent from the west part to the east part of Europe. As MTC strains are thought lose DR spacers and not to regain them, the S1 type can also be hypothesized to be ancestral to S4 and S7. On the other hand, a separate analysis of the human versus animal isolates in the same country shows a significantly higher proportion of the S1 type among cattle isolates (86%) than among human isolates (56%). This could reflect recent clonal expansions after an already achieved reduction of *M. caprae* prevalence by eradication measures. An analysis of 11,500 (classical) *M. bovis* isolates from the United Kingdom and Ireland by spoligotyping, followed by ETR-VNTR subtyping in part, has suggested clonal expansions leading to the current population of *M. bovis* in Great Britain (42). Historical animal trading across and from Europe has influenced the different repertoires of *M. bovis* spoligotypes, e.g., the ones in Great Britain and its trading-partner countries in contrast to those in continental European countries (8, 10, 16, 20, 41). *M. caprae* has been found almost exclusively in continental Europe and as far east as the eastern Ukraine. One human isolate has been reported from an inhabitant of the European part of Istanbul, Turkey (D. Satana, M. Uzun, Z. Erturan, Y. Yegenoglu, Abstr. 26th Annu. Meet. Eur. Soc. Mycobacteriol., abstr. 11, 2005), but information about the prevalence in Middle Eastern, Asian, or African countries is missing. The close relation of European *M. caprae* to an isolate from a gazelle found in Africa and Arabia is therefore very interesting and stimulating to further define its origin.

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