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High Genetic Diversity among *Mycobacterium avium* subsp. *paratuberculosis* Strains from German Cattle Herds Shown by Combination of IS*900* Restriction Fragment Length Polymorphism Analysis and Mycobacterial Interspersed Repetitive Unit–Variable-Number Tandem-Repeat Typing[▽]

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***Mycobacterium avium* subsp. *paratuberculosis* is the etiologic agent of Johne's disease and is endemic to the national cattle herds of many countries. Because of the very low level of genetic heterogeneity of this organism, it is difficult to select a workable procedure for strain differentiation at a resolution sufficient to investigate epidemiological links between herds or different ruminant species and the suggested zoonotic potential of *M. avium* subsp. *paratuberculosis* for Crohn's disease. Analysis of restriction fragment length polymorphisms (RFLPs) based on the insertion element IS*900* (IS*900* RFLP) with four restriction enzymes and 10 markers of specific mycobacterial interspersed repetitive units (MIRUs) and variable-number tandem repeats (VNTRs) was applied to 71 bovine *M. avium* subsp. *paratuberculosis* isolates originating from 14 herds from different regions in Germany. Among these isolates, all of which belonged to the *M. avium* subsp. *paratuberculosis* type II group, 17 genotypes were detected by IS*900* RFLP and consisted of a combination of seven BstEII, eight PstI, nine PvuII, and four BamHI restriction patterns. Novel RFLP types were found. The diversity of the *M. avium* subsp. *paratuberculosis* isolates inside the herds was different depending on the frequency of animal purchase. The results of typing by IS*900* RFLP and MIRU-VNTR analyses were not associated. Fifteen MIRU-VNTR patterns were identified with a discriminatory index of 0.905. The most common BstEII-based IS*900* RFLP type, type C1 (72%), was subdivided into 14 types by MIRU-VNTR analysis. A combination of fingerprinting and PCR-based techniques resulted in 24 *M. avium* subsp. *paratuberculosis* genotypes and achieved a discriminatory index of 0.997. By using only BstEII and PstI digestion together with typing by MIRU-VNTR analysis, a discriminatory index of 0.993 was achieved. This is high enough to support epidemiological studies on a national as well as a global scale.**

Mycobacterium avium subsp. *paratuberculosis* is the etiologic agent of paratuberculosis (Johne's disease), a chronic infectious granulomatous enteritis principally affecting domestic and wild ruminants (20, 22). In Europe, cattle, sheep, goats, and farmed deer are predominantly affected (31, 34). Bovine paratuberculosis is endemic. The disease poses a significant economic problem primarily when it occurs in dairy herds (22) but also when it occurs in beef and sheep herds. The rapid spread of Johne's disease is caused by the trade in subclinically infected animals and infection in early calfhood (31). *M. avium* subsp. *paratuberculosis* is excreted in great quantities in the feces of the infected animals; it has a high tenacity, and it is therefore widely distributed in the environment. Furthermore, a role for *M. avium* subsp. *paratuberculosis* in the etiology of Crohn's disease in humans has long been discussed (7, 8, 16), but the zoonotic potential of the organism remains controversial.

So far, restriction fragment length polymorphism (RFLP) analysis based on the insertion element IS*900* (IS*900* RFLP) has been applied the most extensively to investigations of the genetic diversity among *M. avium* subsp. *paratuberculosis* strains for epidemiological studies of Johne's disease. Furthermore, macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) has been used. Based on the pattern groups obtained by these two techniques and on growth characteristics and pigmentation, *M. avium* subsp. *paratuberculosis* isolates are divided into three groups: the S (sheep) type, the C (cattle) type (3, 9), and the I (intermediate) type (13, 32). These three groups are also designated types I, II, and III, respectively (12, 37). These types show strong host preferences but no host specificity (3, 37). By using *M. avium* subsp. *paratuberculosis* isolates from different host species and different geographic regions of the world, a number of subtypes could be charac-

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terized, especially in the type II group of strains (9, 10, 25, 27, 32, 33, 46). Type II strains were mainly isolated from cattle; but they were also isolated from sheep, goats, different wildlife species, and humans.

Several other typing methods that target different structures in the genome have been developed and are faster and easier to perform and require smaller quantities of bacteria than IS*900* RFLP or PFGE. Among others, these include multiplex PCR of IS*900* loci (MPIL) (5) and multilocus short-sequence-repeat (MLSSR) sequencing (1). Some methods (e.g., MPIL) were shown to have an insufficient power for the discrimination of *M. avium* subsp. *paratuberculosis* isolates, and others (e.g., MLSSR sequencing analysis) were successfully applied by using a set of isolates from different host species and different geographic locations of the world. Moreover, recent studies have identified loci containing variable-number tandem repeats (VNTRs) of specific mycobacterial interspersed repetitive units (MIRUs) in *M. avium* subsp. *paratuberculosis* isolates (4, 6, 27, 35, 41). Those investigations were based on the screening of parts of or the whole *M. avium* subsp. *paratuberculosis* K10 genome and tested the polymorphisms of a diverse panel of strains. A typing system that included at least eight loci was proposed by Biet et al. (4) and Thibault et al. (41).

However, *M. avium* subsp. *paratuberculosis* isolates show a very low level of genetic heterogeneity in comparison to that of *Mycobacterium tuberculosis* and other bacteria. The use of a combination of several typing methods might lead to an increase in the overall discriminatory power of DNA typing, an approach which has already been established for mycobacteria of the *M. tuberculosis* complex (11, 23). For *M. avium* subsp. *paratuberculosis*, Stevenson et al. (37) and Thibault et al. (41) applied a combination of typing methods by using *M. avium* subsp. *paratuberculosis* isolates originating from different hosts and diverse geographic regions of the world.

The aim of this study was to verify the possibilities provided by the use of a combination of independent typing techniques for the differentiation of bovine *M. avium* subsp. *paratuberculosis* field isolates of different origins in Germany. The use of such a combination of independent typing techniques would permit epidemiological investigations on a national level within temporal and geographic limits. The genetic diversity of *M. avium* subsp. *paratuberculosis* isolates was analyzed by typing the isolates by IS*900* RFLP and MIRU-VNTR analysis of 10 genomic loci. For IS*900* RFLP, four restriction enzymes were used in order to facilitate a comparison of the resulting restriction patterns with those published previously by other authors and with strains from different regions of the world. The results obtained by the different techniques were compared by calculation of the discriminatory power of the individual or the combined typing methods.

MATERIALS AND METHODS

Collection and cultivation of bacteria. Field strains of *M. avium* subsp. *paratuberculosis* were isolated from the intestinal tissues (different lymph nodes, gut mucosa) or feces of dairy cattle between 2001 and 2004. The cattle originated from 14 herds (Table 1) from different geographic areas in Germany. Isolates originated from 1 to 34 single animals within the herds. The frequency of animal trade in these herds had differed in the past.

Primary isolates were cultivated on Herrold's egg yolk medium containing mycobactin (BD Biosciences, Heidelberg, Germany). The presence of acid-fast organisms was proved by Ziehl-Neelsen staining. After the identification of *M. avium* subsp. *paratuberculosis* on the basis of mycobactin-dependent growth, incubation period, colony morphology, and PCR, mass cultivation was performed on modified Loewenstein-Jensen medium containing mycobactin (Bioservice Waldenburg, Germany). The identities of the *M. avium* subsp. *paratuberculosis* isolates were again confirmed. Isolates were stored as glycerol stocks at −80°C. *M. avium* subsp. *paratuberculosis* type strain ATCC 19698 and reference strain DSMZ 44135, both from the German Collection of Microorganisms and Cell Cultures (DSMZ), served as controls for all techniques.

Identification of bacteria by PCR. DNA was isolated before PCR. Briefly, the cells were heat inactivated at 80°C for 20 min and ultrasonicated for 10 min. After heat treatment (100°C, 10 min) and centrifugation at 12,000 × *g* twice for 5 min each time, the DNA-containing supernatant was used for PCR.

The isolates were identified as *M. avium* subsp. *paratuberculosis* by PCR amplification of IS*900*, which was carried out with the primers and the PCR conditions described by Englund et al. (15), and the presence of the *M. avium* subsp. *paratuberculosis*-specific sequence f57 was determined as described by Vansnick et al. (43). To ensure the purity of the DNA and the absence of other *M. avium* subspecies, a lack of IS*901* and IS*1245* was proven by PCR by the methods of Bartos et al. (2) and Guerrero et al. (19), respectively. A total of 71 isolates identified as *M. avium* subsp. *paratuberculosis* (with an IS*900*-positive, IS*1245*-negative, IS*901*-negative, and f57-positive genotype) was included in the study.

DNA extraction. Genomic DNA for typing by IS*900* RFLP and MIRU-VNTR analysis was prepared by the cetyltrimethylammonium bromide method described by van Soolingen et al. (44). The concentration of DNA was measured with a spectrophotometer (Beckman Coulter).

Typing by IS*900* RFLP. (i) DNA digestion and Southern blotting. Two micrograms of genomic DNA was digested separately with BstEII, BamHI, and PvuII (Roche Diagnostics) and PstI (New England Biolabs) under the conditions specified by the manufacturers. DNA fragments were separated by horizontal electrophoresis on a 240-mm 1% (wt/vol) agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 65 V for 16 h. Bacteriophage lambda DNA cleaved with EcoRI and HindIII and labeled with digoxigenin-11-dUTP, with a size range of from 21.2 to 0.8 kbp (Roche Diagnostics), was run as an external molecular size marker in three lanes (both edges and the middle) of each gel. After incubation of the agarose gel with 0.25 N HCl for 15 min and washing with aqua bidest, the DNA was denaturized by incubation with 0.5 N NaOH for 30 min. The DNA fragments were vacuum transferred (model 785; Bio-Rad, United Kingdom) to a Hybond-N nylon membrane (Amersham Biosciences, United Kingdom) and fixed onto the membranes by treatment with heat for 2 h at 80°C.

(ii) DIG-labeled IS*900* probe. The probe for IS*900* RFLP was prepared by PCR amplification with the forward oligonucleotide 5′-TGG ACA ATG ACG GTT ACG GAG GTG G-3′ and the reverse oligonucleotide 5′-GAT CGG AAC GTC GGC TGG TCA GGA T-3′ (positions 209 to 233 bp and 662 to 638 bp, respectively, of IS*900*; GenBank/EMBL accession number X16293). These primers were previously described by Kunze et al. (24) and were recommended for use for probe amplification in the standardized protocol of Pavlik et al. (32). PCR was conducted with a digoxigenin (DIG)-labeled deoxynucleoside triphosphate (dNTP) mixture (DIG DNA labeling mix; Roche Diagnostics), according to the manufacturer's instructions. PCR mixtures of 20 μl contained 1× buffer, 1× DIG DNA labeling mixture, 0.5 μM (each) primer, 2.5 U *Taq* polymerase (Roche Diagnostics), and 50 ng of purified genomic DNA. The *M. avium* subsp. *paratuberculosis* reference strain (ATCC 19698) was used as the matrix for probe amplification. PCR was performed with the following cycling conditions: 94°C for 30 s for initial denaturation; 29 cycles consisting of 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min 30 s; and then 1 cycle consisting of 94°C for 30 s, 68°C for 2 min, and 72°C for 3 min. The amplification product was visualized by ethidium bromide staining after electrophoresis in a 1% (wt/vol) agarose gel and was purified by use of a QIAquick gel extraction kit (Qiagen). Sequence analysis confirmed the probe sequence (GenBank/EMBL accession number X16293).

(iii) Hybridization and detection. Hybridization was performed at 68°C overnight with the DIG-labeled IS*900*-specific probe. The membrane was subsequently washed under high-stringency conditions (twice with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate at room temperature for 5 min; twice with 0.1× SSC and 0.1% sodium dodecyl sulfate at 68°C for 15 min, and once with wash buffer at room temperature). The targets were submitted to immunological detection with anti-DIG antibody conjugated to alkaline phosphatase (DIG luminescent detection kit; Roche Diagnostics). The CSPD detection system was used according to the manufacturer's protocol to visualize the hybridized probe (Roche Diagnostics). The membrane was exposed to Hyperfilm ECL (GE Healthcare, Freiburg, Germany) and was developed for 1 to 30 min for optimal exposure.

TABLE 1. Origin of strains and results of typing by RFLP, MIRU, and VNTR analysis

Geographic origin[a]	Herd no.	No. of isolates	Type[b] by IS*900* RFLP after DNA digestion with:				Type[c] by MIRU analysis	TRs at loci 292-25-47-3-7-32	MIRU-VNTR pattern no.[d]
			BstEII	PstI	PvuII	BamHI			
Thuringia	1	2	C1	1	N_{14}	1	II	433228	3
	1	1	C1	1	1	1	I	333228	2
	1	1	C16	12	N_{15}	11	II	433218	1
	1	30	C17	9	3	11	II	433218	1
	2	2	C1	1	1	1	I	333228	2
	2	2	C1	1	1	1	I	343228	6
	2	2	C1	1	1	1	I	353228	7
	3	1	C_{new}	7	2C	15	I	333228	2
North Rhine-Westphalia	4	4	C1	3	1	11	IV	433218	4
	4	1	C1	3	1	11	IV	433228	8
	4	1	C1	3	1	1	III	352228	9
	4	2	C1	10	1	14	II	433218	1
	4	2	C1	10	N_{16}	14	II	433218	1
	4	4	C17	8	3	1	II	433228	3
	4	1	C22	11	N_{13}	11	IV	433218	4
Lower Saxony	5	6	C1	1	1	1	III	252226	5
Baden-Wurttemberg	6	1	C1	3	1	11	II	433218	1
	7	1	C1	7	1	1	I	433228	10
	8	1	C1	7	1	1	I	333218	11
	9	1	C1	7	1	1	V	333228	12
	10	1	C1	7	2	1	VII	333228	13
	11	1	C18	7	2	1	I	333228	2
Saxony-Anhalt	12	1	C5	1			VI	2221(1*)8[e]	14
Brandenburg	13	1	C1	3	N_{17}	11	II	433218	1
	14	1	C1	1	2	1	III	252228	15
Total no.[f]		71	7[f]	8[f]	9[f]	4[f]	7[f]	9[f]	15[f]

[a] Federal states of Germany.
[b] The nomenclature for the types obtained by IS*900* RFLP is described in the legend to Fig. 1.
[c] Types by MIRU analysis according to Bull et al. (6); for the designations, see Table 2.
[d] Types by MIRU-VNTR analysis with the combination of loci according to Bull et al. (6) and Thibault et al. (41); for the designations, see Table 4.
[e] (1*), some additional nucleotides were identified at one tandem repeat.
[f] Number of different profiles that resulted by the method.

(iv) Analysis of IS*900* RFLP results. The DNA fingerprints obtained by IS*900* RFLP were scanned and analyzed by using Gel Compare software (version 4.011; Applied Maths, Kortrijk, Belgium). All patterns were confirmed by retesting. The types obtained by IS*900* RFLP were analyzed by geographic location, by farm, and by individual animal.

(v) Nomenclature for IS*900* RFLP types. When the IS*900* RFLP profiles matched the patterns described by other authors, the designations of the other authors were used. Unknown new patterns were given a new numeric code. The IS*900* RFLP type designation after BstEII digestion was that described by Pavlik et al. (32); the IS*900* RFLP type designation after BamHI digestion was that described by Cousins et al. (10). Comparable RFLP patterns based on a specific RFLP probe on the left side of IS*900* have been described previously for patterns based on PstI digestion by Whipple et al. (46), Saunders et al. (36), and Palmer et al. (28) and for patterns based on PvuII digestion by Whipple et al. (46), Cousins et al. (10), Stevenson et al. (37), and de Juan et al. (12).

MIRU typing (MIRU PCR). (i) MIRU PCR. The amplification of specific MIRUs at four specific loci within the *M. avium* subsp. *paratuberculosis* genome, described as MIRU PCR by Bull et al. (6), was applied. The same DNA templates used for typing by IS*900* RFLP were used for the MIRU PCR. The primer sequences specific for loci 2 to 4 were those of Bull et al. (6). Reverse primer M1.rr (5′-CCG TTG TCC AGG TGG AGT-3′) was used for locus 1. *M. avium* subsp. *paratuberculosis*-specific DNA (approximately 50 ng) was amplified in a 50-μl reaction volume containing a 2 μM concentration of each primer, 1× reaction buffer containing 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide, 100 μM dNTPs, and 1 U of HotStarTaq DNA polymerase (Qiagen). The PCR started with an initial denaturation step at 94°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C (for the MIRU PCRs for loci 1 and 2) for 1 min, and 72°C for 30 s, with a final extension at 72°C for 1 min. The annealing temperature for the MIRU PCR for locus 3 was 52°C, and that for the MIRU PCR for locus 4 was 53°C. The MIRU PCR primers specific for locus 3 were used at a concentration of 6 μM. The PCR-amplified VNTRs were analyzed for their amplicon sizes on a 1.5% agarose gel. The results were proven by sequencing of the amplification products of isolates with different numbers of repetitive units at the four loci.

(ii) Nomenclature for MIRU types. The MIRU types were designated I to VII and represented the PCR results at the different MIRU loci (Table 2). Types I, II, and VII correspond to the MIRU patterns described by Bull et al. (6).

Typing by VNTR and MIRU analysis. Additionally, six other PCR-based typing markers that were identified by Thibault et al. (41) and that consisted of VNTRs at five specific genome loci (designated tandem repeats [TRs] 25, 47, 3, 7, and 32) and one TR with an MIRU structure (TR 292) were included in this study. The specific primer sequences and annealing temperatures described by Thibault et al. (41) were employed. DNA (approximately 50 ng) was amplified in a 20-μl reaction volume containing a 2 μM concentration of each primer, 1× reaction buffer containing 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide, 100 μM dNTPs, and 0.5 U HotStarTaq DNA polymerase (Qiagen). The PCR started with an initial denaturation step at 95°C for 15 min, followed by 35 cycles at 95°C for 1 min, 57°C (TR 32) for 1 min, and 72°C for 1 min 30 s, with a final extension at 72°C for 15 min. The annealing temperature for TR 25 and TR 292 was 58°C, that for loci 3 and 7 was 60°C, and that for TR 47 was 64°C. The PCR products were analyzed for their amplicon sizes on a 1.5% agarose gel to detect differences in repeat numbers. The results were proven by sequencing of the amplification products with different sizes at the different TR loci. The VNTR-MIRU profiles

TABLE 2. MIRU profiles and their frequencies

MIRU profile	No. of TR motifs[a]				No. of herds[b]	No. of strains[c]	No. of isolates[d]
	MIRU locus 1	MIRU locus 2	MIRU locus 3	MIRU locus 4			
I	3	7	5	1	6	7	11
II	3	9	5	1	4	8	43
III	3	5	5	1	3	3	8
IV	3	9	7	1	1	3	6
V	1	7	5	1	1	1	1
VI	3	7	9	1	1	1	1
VII	3	7	3	1	1	1	1

[a] The numbers indicate the total number of A and B motifs at each MIRU locus, corresponding to the consensus sequences published by Bull et al. (6) and calculated by the estimated sizes (in base pairs) of the amplicons.
[b] Epidemiologically unrelated herds.
[c] Genetically distinguishable isolates.
[d] Isolates not genetically characterized.

were designated by the number of complete TRs in the following order: TR 292-25-47-3-7-32.

Calculations. The discriminatory index of Hunter and Gaston (21) was calculated as a numerical index of the discriminatory powers of each typing technique and different combinations of these methods. The following formula was used to calculate the discriminatory index (DI):

$$\mathrm{DI} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} N_j(N_j - 1)$$

where N is the total number of epidemiologically unrelated strains in the respective typing scheme. Therefore, only isolates with different genotypes from a distinct herd detected by the method of interest were included in the particular calculation. S is the total number of distinct patterns or profiles discriminated by each typing method or by the combination of individual methods. N_j is the number of epidemiologically unrelated strains belonging to the jth pattern.

Calculation of the allelic diversity at the different MIRU loci was done as described by Supply et al. (38).

Definitions. Epidemiologically unrelated strains were defined as strains that were not derived from a common source; that is, they originated from different areas. A strain was defined as described by Tenover et al. (40) as an isolate or a group of isolates that can be distinguished from other isolates of the same genus and species by genotypic characteristics. In contrast, an isolate was defined as an isolate for which no information apart from its genus and species was available.

RESULTS

Characterization of strain types by growth characteristics and IS*900* RFLP. All 71 bovine *M. avium* subsp. *paratuberculosis* isolates from Germany analyzed had the following primary isolation characteristics: 6 to 12 weeks of growth for the appearance of cream-colored colonies, a rough colony morphology, and mycobactin dependence. During subcultivation they were moderately slowly growing. All isolates, including type strain ATCC 19698 and reference strain DSMZ 44135, showed the IS*900* cattle type after digestion with BstEII by IS*900* RFLP (Table 1). Therefore, they all belonged to the cattle group designated type II (12).

Typing by IS*900* RFLP with four different restriction enzymes. Hybridization of total DNA, digested separately with the enzymes BstEII, PstI, PvuII, and BamHI, resulted in at least 9 to 18 bands that ranged in size from less than 1 to 17 kb (Fig. 1 and 2). The use of DIG labeling for IS*900* RFLP resulted in band patterns with very high resolving powers. New IS*900* RFLP patterns could be identified. Depending on the restriction enzyme used, the number of distinct IS*900* RFLP profiles was different (Table 1). Seven BstEII-based IS*900* RFLP profiles, six of which (profiles C1, C17, C5, C16, C18, and C22) were described by Pavlik et al. (32) and one of which was a new profile (Fig. 1a), were detected at different frequencies (Table 1). Restriction analysis by PstI resulted in eight profiles; two of them (types 1 and 3) were previously described by Whipple et al. (46). The other six profiles (types 7 to 12) have not been published before (Fig. 2a). Nine IS*900* RFLP patterns were identified by PvuII restriction digestion (Fig. 2b). The clear differentiation of single bands, also within a range from 0.8 to 1.5 kbp, was remarkable. Three of these patterns

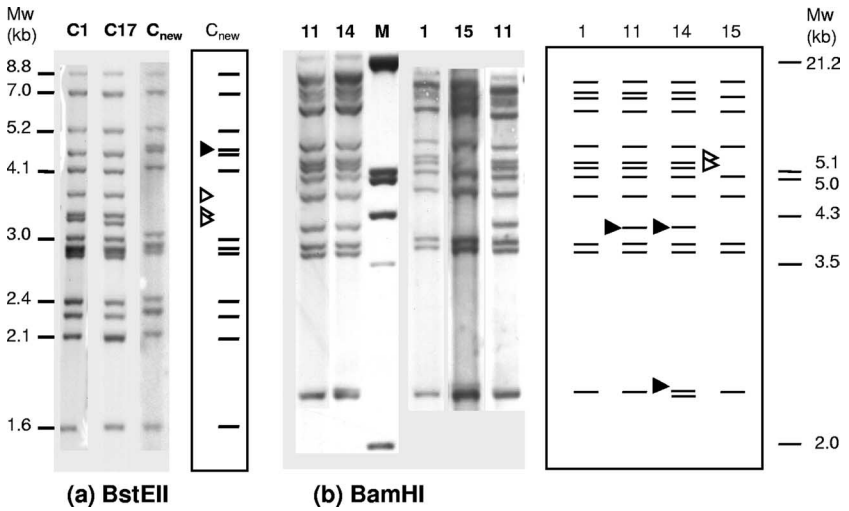

FIG. 1. IS*900* RFLP profiles of German *M. avium* subsp. *paratuberculosis* isolates from cattle. The numbers above the lanes are the IS*900* RFLP type designations. The numbers on the left or right side of the patterns indicate the sizes of the reference bands. The arrowheads in the schematic diagrams show the differences between type 1 and the other RFLP profiles. (a) IS*900* RFLP profiles after digestion with restriction endonuclease BstEII, designated according to the nomenclature of Pavlik et al. (32). One novel profile was detected. (b) IS*900* RFLP profiles after digestion with restriction endonuclease BamHI, designated according to the nomenclature of Cousins et al. (10). Types 14 and 15 have not been described previously. Lanes M, molecular weight (Mw) markers.

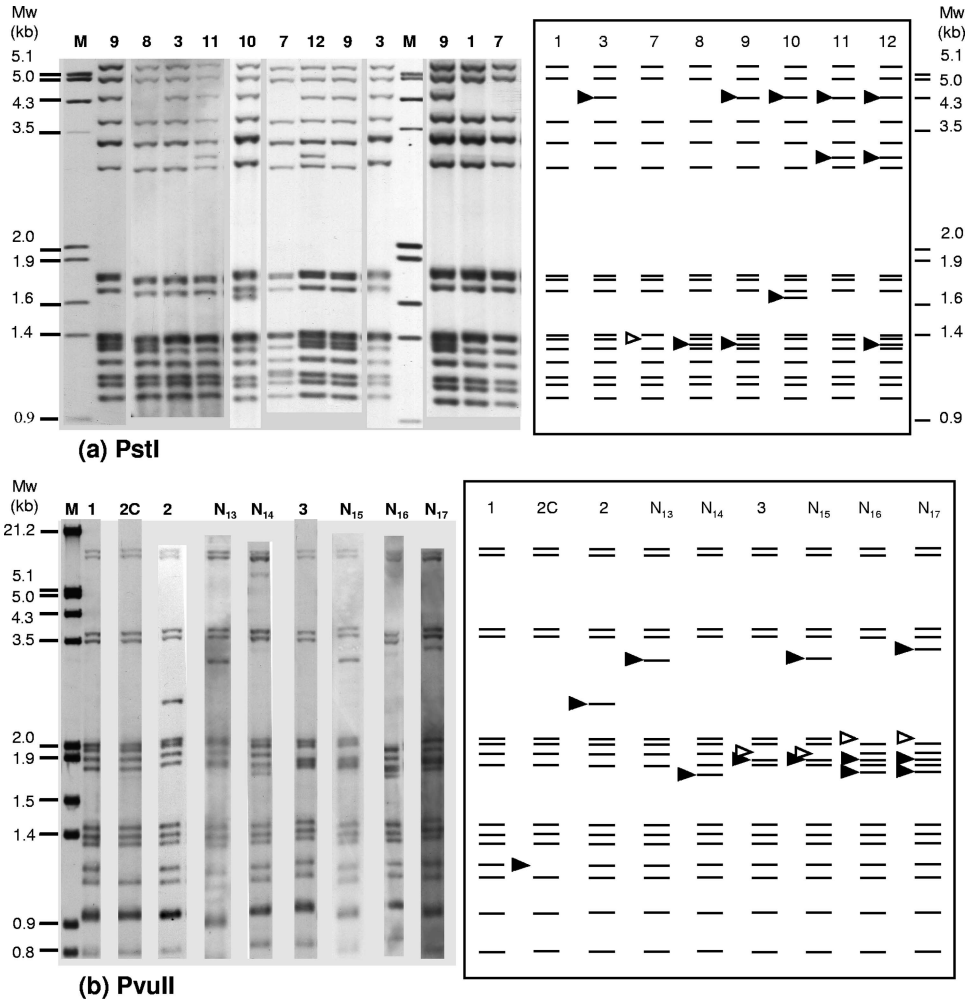

FIG. 2. IS*900* RFLP profiles of German *M. avium* subsp. *paratuberculosis* isolates from cattle. The numbers above the lanes are the IS*900* RFLP type designations. The numbers on the left or right side of the patterns indicate the sizes of the reference bands. The arrowheads in the schematic diagrams show the differences between type 1 and the other RFLP profiles. (a) RFLP profiles after digestion with PstI. Types 1 and 3 were designated according to Whipple et al. (46). Types 7 to 12 new profiles not previously published. (b) RFLP profiles after digestion with PvuII. Types 1 to 3 were designated according to the nomenclature of Whipple et al. (46), and type C2 is designated according to the nomenclature of Cousins et al. (10). Profiles N13 to N17 are novel profiles not described previously. Lanes M, molecular weight (Mw) markers.

(types 1, 2, and 3) were previously described by Whipple et al. (46), and type 1 and type 2C were also described by Cousins et al. (10). Five new IS*900* RFLP profiles were obtained. After BamHI digestion, four profiles were identified: two (types 1 and 11) were previously described by Cousins et al. (10), and two profiles were new (Fig. 1b).

As shown in Table 1, BstEII-based IS*900* RFLP type C1 was the most common type (identified in 11 herds [79%] and in 43% of the isolates) but could be subdivided into 10 different genotypes by use of the other three digestion enzymes. Likewise, BstEII-based IS*900* RFLP type C17, found in a total of 34 isolates (46%) from herds 1 and 4, was divided into two genotypes. Types C5, C16, C18, C22, and C_{new} were found in individual isolates.

Combination of the results obtained with all enzymes yielded 17 different restriction pattern profiles. Without BamHI digestion, 16 profiles could be found. By using the results of only BstEII and PstI digestion or only BstEII and PvuII digestion, 11 profiles were detected. Frequently observed IS*900* RFLP types (after BstEII, PstI, PvuII, and BamHI digestion) consisted of profiles C1-1-1-1 (herds 1, 2, and 5; 13 isolates), C1-7-1-1 (herds 7 to 9; 3 isolates), and C1-3-1-11 (herds 4 and 6; 6 isolates). Thirty isolates had the IS*900* RFLP pattern combination C17-9-3-11, but they originated from only one herd in Thuringia.

Typing by MIRU and VNTR analysis. As a result of MIRU PCR, three of the four MIRU loci studied showed polymorphisms in the number of repeat motifs (Table 2). Therefore, the results for MIRU locus 4, which did not contain polymorphisms, were not included in the subsequent analysis (Table 3 to 6). Altogether, seven different MIRU pattern profiles were identified (Table 1 and 2). MIRU profiles I to IV were each detected in several isolates, whereas MIRU profiles V to VII were found in individual isolates. *M. avium* subsp. *paratuberculosis* strains exhibiting MIRU profile I or II were designated *M. avium* subsp. *paratuberculosis* groups 1 and 2, respectively,

TABLE 3. TR copy numbers at the nine MIRU-VNTR loci

MIRU-VNTR pattern no.	TR copy no. at MIRU-VNTR locus:								
	1[a]	2[a]	3[a]	292[b]	25[b]	47[b]	3[b]	7[b]	32[b]
1	3	9	5	4	3	3	2	1	8
2	3	7	5	3	3	3	2	2	8
3	3	9	5	4	3	3	2	2	8
4	3	9	7	4	3	3	2	1	8
5	3	5	5	2	5	2	2	2	6
6	3	7	5	3	4	3	2	2	8
7	3	7	5	3	5	3	2	2	8
8	3	9	7	4	3	3	2	2	8
9	3	5	5	3	5	2	2	2	8
10	3	7	5	4	3	3	2	2	8
11	3	7	5	3	3	3	2	1	8
12	1	7	5	3	3	3	2	2	8
13	3	7	3	3	3	3	2	2	8
14	3	7	9	2	2	2	1	1[c]	8
15	3	5	5	2	5	2	2	2	8

[a] MIRU loci 1 to 3 according to Bull et al. (6).
[b] MIRU-VNTR loci according to Biet et al. (4) and Thibault et al. (41).
[c] Some additional nucleotides were identified at one tandem repeat.

by Bull et al. (6). Type strain ATCC 19698 and reference strain DSMZ 44135, which had MIRU profile I, belong to one of these large *M. avium* subsp. *paratuberculosis* groups. For one strain from Baden-Wurttemberg (herd 10), MIRU profile VII combined with BstEII-based IS*900* RFLP type C1 was detected. This pattern was recently shown for *M. avium* subsp. *paratuberculosis* vaccine strain 316F by Bull et al. (6).

Typing by VNTR-MIRU analysis with six of eight TR loci, as described by Thibault et al. (41), subdivided the *M. avium* subsp. *paratuberculosis* strains into strains with nine different profiles, as shown in Table 1. The most frequently identified profiles were 333228 (six herds), 433218 (four herds), and 433228 (three herds). By combining the results obtained for MIRU loci 1 to 3 and the six TR loci mentioned above, a total of 15 distinct MIRU-VNTR profiles (Tables 1, 3, and 4) were obtained. The allelic diversities of these nine TR loci calculated for the current strain panel were different (Table 5).

TABLE 4. Results of typing by MIRU-VNTR analysis in combination with IS*900* RFLP

MIRU-VNTR pattern no.	No. of strains[a]	BstEII- and PstI-based IS*900* RFLP pattern(s)[b]	No. of herds[c]	No. of isolates[d]
1	4	C1-3, C1-10, C16-12, C17-9	4	37
2	3	C1-1, C_{new}-7, C18-7	3	6
3	2	C1-1, C17-8	2	6
4	2	C1-3, C22-11	1	5
5	1	C1-1	1	6
6	1	C1-1	1	2
7	1	C1-1	1	2
8	1	C1-3	1	1
9	1	C1-3	1	1
10	1	C1-7	1	1
11	1	C1-7	1	1
12	1	C1-7	1	1
13	1	C1-7	1	1
14	1	C5-1	1	1
15	1	C1-1	1	1

[a] Genetically distinguished by typing by MIRU-VNTR analysis and IS*900* RFLP by using only the BstEII and PstI restriction enzymes.
[b] See Fig. 1 and Table 1 for the designations.
[c] Epidemiologically unrelated herds.
[d] The numbers include related and unrelated isolates.

TABLE 5. Allelic distribution among epidemiologically unrelated *M. avium* subsp. *paratuberculosis* isolates determined by MIRU-VNTR analysis[c]

Locus	No. of isolates with the following specific TR copy no.:									Allelic diversity[d]
	1	2	3	4	5	6	7	8	9	
1[a]	1		24							0.077
2[a]					3		11		11	**0.598**
3[a]			1		20		3		1	**0.342**
292[b]		3	10	12						**0.595**
25[b]		1	19	1	4					**0.394**
47[b]		4	21							**0.269**
3[b]	1	24								0.077
7[b]	10	15								**0.480**
32[b]						1		24		0.077

[a] MIRU loci 1 to 3 according to Bull et al. (6).
[b] MIRU-VNTR loci according to Biet et al. (4) and Thibault et al. (41).
[c] Boldface data indicate TR loci with detectable polymorphisms.
[d] The allelic diversity of specific TR loci obtained by MIRU-VNTR analysis was calculated as described by Supply et al. (38).

Comparison and combination of typing results obtained by IS*900* RFLP and MIRU-VNTR analysis. Altogether, IS*900* RFLP with the four digestion enzymes in combination with the results of typing by MIRU-VNTR analysis subdivided the 71 isolates originating from 14 farms from different geographic locations into 24 genetically distinguishable strains. The predominant BstEII-based IS*900* RFLP type, type C1, could be split into 14 different types by MIRU-VNTR analysis. The results of typing by IS*900* RFLP and MIRU-VNTR analysis were not associated (Tables 1 and 4). The discriminatory indices of the different typing techniques, calculated for each technique separately or in combination, are shown in Table 6. A combination of fingerprinting and PCR-based techniques resulted in a maximal discriminatory index (21) of 0.997. The individual methods had discriminatory indices if less than 0.9. For strain differentiation by IS*900* RFLP with BstEII digestion only together with typing by MIRU-VNTR analysis (nine loci), a discriminatory index of 0.986 was estimated.

Epidemiological results. The combination of results of all typing methods used enabled us to find isolates with different genotypes within distinct regions of origin and individual dairy cattle herds in Germany. Six to seven strains were found in three geographic regions. Up to seven different genotypes were found among the isolates from cattle on one farm (herd 4). The diversity of the *M. avium* subsp. *paratuberculosis* strains within herds was different. Among the isolates in herd 1 (n = 34) (Table 1), 91% of the isolates were genetically indistinguishable and the isolates thus corresponded to one strain. The other three profiles detected for the isolates in herd 1 appeared only once or twice. In contrast, the seven profile combinations detected among the isolates in herd 4 (n = 15) were more or less evenly distributed. In the past, cattle in this herd had frequently been purchased from other sources and had unknown infection states. The six isolates from the cattle in herd 5 showed the same genotype and thus corresponded to a single strain. The diversities of the isolates detected within these herds reflect the differences in the frequency of animal movements among these farms.

TABLE 6. Numerical index of discriminatory power calculated by separate and combined typing methods for the current strain panel

IS*900* RFLP with restriction endonuclease digestion	Locus no. or no. of loci			Discriminatory index[c]
	MIRU PCR[a]	VNTR-MIRU analysis[b]	MIRU PCR and VNTR-MIRU analysis	
BstEII				0.634
PstI				0.836
PvuII				0.813
BamHI				0.575
	Loci 1 to 3			0.838
		6 loci		0.874
	Loci 1 to 3	6 loci		0.905
BstEII + PstI				0.912
BstEII + PvuII				0.842
BstEII + PstI + PvuII				0.967
BstEII + PstI + PvuII + BamHI				0.970
BstEII + PstI + PvuII + BamHI			9 loci	0.997
BstEII + PstI			9 loci	0.993
BstEII + PvuII			9 loci	0.993
BstEII			9 loci	0.986
BstEII	Loci 2 and 3	6 loci		0.986

[a] MIRU PCR was performed as described by Bull et al. (6).
[b] VNTR-MIRU analysis was performed as described by Biet et al. (4) and Thibault et al. (41).
[c] The numerical index of discriminatory power was calculated as described by Hunter and Gaston (21).

Profile C1-1-1-1/2 (IS*900* RFLP/MIRU-VNTR analysis; Table 1) was detected in herds 1 and 2 from Thuringia. The isolates are likely epidemiologically linked. The trade of animals between these herds in the past could not be excluded.

DISCUSSION

This is the first report of a high number of genetically distinguishable *M. avium* subsp. *paratuberculosis* isolates in cattle on a national scale. A combination of independent typing techniques was applied to increase the discriminatory power of typing and to differentiate between *M. avium* subsp. *paratuberculosis* isolates.

IS*900* RFLP has been the technique used in most cases for the comparison of isolates in national and international studies. Standardization of this fingerprinting technique was proposed for the first time in 1999 (32). In preceding studies, a variety of restriction enzymes, labeling methods, and hybridization probes was used. In the present study, the IS*900* RFLP patterns resulting from restriction with four different enzymes were compared with the available reference patterns. However, by examination of the reference patterns obtained by IS*900* RFLP with PvuII or PstI digestion, the pattern types diverged from each other. The reason is that 1,451-bp insertion element IS*900* contains a restriction site for PstI as well as one for PvuII at position 891 bp and position 707 bp, respectively (based on the sequence with GenBank/EMBL accession number X16293). Therefore, the IS*900* RFLP profiles differ depending on whether the hybridization site of the probe is to the 5′ or 3′ site of the digestion site of the restriction enzyme or covers the digestion site. In the latter case, the probe recognizes IS*900* on both sides of the PstI site (25) and IS*900* RFLP results in a higher number of bands. Such patterns obtained by PvuII digestion have been published by Kunze et al. (24); and such patterns obtained by PstI digestion have been published by Thoresen and Olsaker (42), Pavlik et al. (29, 32), Moreira et al. (25), and Djonne et al. (14). The band pattern types obtained in the current study are in contrast to those results. The probe used here, originally recommended for use in the standardized protocol for IS*900* RFLP (32), was located on the left arm of IS*900* and did not cover a digestion site for PstI and PvuII. Comparable probes were applied by Whipple et al. (46), Francois et al. (17), Moreira et al. (25), Cousins et al. (10), Stevenson et al. (37), Saunders et al. (36), and de Juan et al. (12); and corresponding patterns were shown after digestion with one or both of these enzymes. In contrast, IS*900* contains no restriction site for BstEII or BamHI. Therefore, the BstEII- and BamHI-based IS*900* RFLP patterns are independent of the hybridization site of the probe. The numerous BstEII patterns published worldwide are quite comparable to those obtained in the current study.

As expected and in line with current knowledge, all isolates of the current study, which originated from cattle, belonged to the C type (type II). BstEII-based IS*900* RFLP type C1 was the predominant pattern found among the isolates in the panel of strains examined, in agreement with previous data for cattle herds from different countries in Europe (30, 31, 33). By using a second restriction enzyme, PstI, B-C1 was the most frequent of three IS*900* RFLP types (B-C1, E-C1, and L-C13) found among strains from cattle in Germany, as well as the most common type in Europe; this type has also been detected in the United States and Australia (33). The results obtained with the current panel of strains comprising 71 isolates from 14 German herds supplements these data with BstEII-based IS*900* RFLP types C17, C5, C16, C18, C22, and C_{new}, which are otherwise rare. Besides Europe, IS*900* RFLP type C1 is highly prevalent in the United States (33, 46) and New Zealand (9), whereas type C3 predominates in Australia (10, 36, 47) and type C17 predominates in specific regions of Argentina (25). As documented with our results for Germany, type C17 is rare in Europe and up to now has only occasionally been detected in isolates from Scotland (12, 18).

Using PvuII-based IS*900* RFLP, Bauerfeind et al. (3) detected only two different IS*900* RFLP patterns among nine German isolates, each of which was from an individual herd. The types were not comparable to the patterns obtained in the current study because a different hybridization probe was applied. However, the large panel of isolates in the present study yielded a higher diversity, as nine profiles were found only by PvuII digestion.

To increase the discriminatory power of *M. avium* subsp. *paratuberculosis* typing and to subdivide predominant BstEII-based IS*900* RFLP type C1, a combination of different methods was used by various authors. Whipple et al. (46) and Cousins et al. (10) applied four digestion enzymes for IS*900* RFLP to differentiate isolates from different regions in the United States and Australia. They detected 8 and 11 subtypes, respectively, among the cattle type II strains, whereas 17 IS*900* RFLP subtypes were detected in the present study. Depending on the distinct panel of strains used, different digestion enzymes showed the highest potential for differentiation. Whipple et al. (46) and Pavlik et al. (32) were able to subdivide isolates with BstEII-based IS*900* RFLP profile C1 into three subtypes by further digestion with another restriction enzyme. de Juan et al. (12) applied multiplex PFGE and found eight subtypes among 19 caprine isolates exhibiting IS*900* RFLP profile C1. Depending on the number of different isolates, multiplex PFGE subdivided BstEII-based profile C1 of cattle isolates into two types, BstEII-based profile C5 into three types, and BstEII-based profile C17 into six types (37).

By using PCR-based techniques but different markers and strain panels, IS*900* RFLP type C1 was divided into 4 (27), 11 (4), and 15 (41) different subtypes by MIRU-VNTR analysis. In the current study, 14 subtypes of the IS*900* RFLP C1 type were identified by typing by MIRU-VNTR analysis and 17 subtypes were identified by using a combination of IS*900* RFLP and this PCR-based technique. In contrast to the previous studies, however, our isolates originated from the cattle herds in only one country.

The overall hierarchy of the polymorphisms of the MIRU-VNTR loci used was similar to that observed in previous studies (6, 41). The six loci, loci 2, 3, 292, 7, 25, and 47 (Table 5), largely showed comparable variabilities in the current population. This suggests that the relative degrees of genetic information carried by the different loci are globally conserved among distinct epidemiological settings and bacterial populations, as described by Supply et al. (38) for some MIRU-VNTR loci of *M. tuberculosis.*

Motiwala et al. (26) compared the discriminatory powers of various *M. avium* subsp. *paratuberculosis* genotyping techniques by analysis of the data from former studies by various authors: IS*900* and IS*1311* RFLP with different digestion enzymes, MPIL, MIRU PCR, PFGE, and other methods. Except for the MLSSR sequencing approach (1), a discriminatory index of less than 0.9, which is comparable to that achieved in the current study, was achieved for all methods. This is not sufficient for epidemiological studies (21). It must be taken into account that only epidemiologically unrelated strains can be included in the calculation of discriminatory indices. Isolates from one herd with the same profile can be counted only once. However, although the current panel of strains exclusively originated from cattle herds in Germany, the discriminatory power was markedly increased by use of a combination of different typing methods. The discriminatory indices achieved (>0.98) were higher than the discriminatory index of 0.96 published by Amonsin et al. (1) for the typing of isolates originating from distinct geographic regions of the world and different host species by MLSSR sequencing analysis.

The reason for this high index is the lack of congruence of the typing results obtained by multiplex IS*900* RFLP and MIRU-VNTR analysis, as they are two fully independent genotyping methods. The two typing tools are apparently directed toward different targets of the *M. avium* subsp. *paratuberculosis* genome and provide complementary information (35). In contrast to typing by MIRU-VNTR analysis, IS*900* RFLP as well as PFGE provides information about alterations in the whole genomes of strains. Although RFLP is a cumbersome technique that requires the preparation of high-quality DNA, at present only a combination of such independent typing techniques allows the identification of sufficient heterogeneity among isolates in the *M. avium* subsp. *paratuberculosis* population. For phylogenetic studies, it should be considered that the modes of the evolutionary processes of the different independent target regions will differ and that the target regions will exhibit different evolutionary rates. According to Vergnaud and Pourcel (45), multiple-locus VNTR analysis does not provide a molecular clock. The mutation of TRs directly influences the phenotypes of the corresponding strains, so that these mutations are not neutral and probably contribute to the adaptation of the species to its environment in a reversible way. A simple replacement of RFLP by the use of additional TR markers to reduce the duration and the special technical demands is not recommended.

By applying discrimination criteria based on *M. tuberculosis* (39) to *M. avium* subsp. *paratuberculosis*, the high number of strains with IS*900* RFLP type C1 in frequent association with MIRU profiles I and II could characterize closely related, possibly clonally related strains. In contrast, typing by VNTR analysis resulted in diverse genotypes within these groups; therefore, their relations could not be confirmed. While the current multiplex IS*900* RFLP patterns suggest an association between the genotypes and the origins of many isolates, isolates with identical or similar MIRU-VNTR patterns were distributed rather randomly. As described in several studies that used microsatellites (2-bp to 8-bp repeat units) for multilocus VNTR analysis (45), highly polymorphic VNTR markers, which usually result from a higher rate of mutation events, usually have high homoplasy levels. To which of the currently used *M. avium* subsp. *paratuberculosis* markers this can be applied should be studied in further investigations. Evolutionary processes are certainly influenced by the extremely long generation time of *M. avium* subsp. *paratuberculosis* and the incubation time of paratuberculosis as a chronic disease. However, MIRU-VNTR typing must be combined with complementary methods not only for evolutionary studies but also for epidemiological studies.

In conclusion, despite the high level of homogeneity of the *M. avium* subsp. *paratuberculosis* isolates, typing by use of the combination of IS*900* RFLP and VNTR-MIRU analysis described here has shown a sensitivity sufficient to distinguish strains of type II, even within the bovine population, on a national scale. It offers the possibility of tracing the transmis-

sion of *M. avium* subsp. *paratuberculosis* among individual animals in one herd, between herds, and between different host species. For IS*900* RFLP we propose the selection of BstEII because it is the restriction enzyme most commonly used worldwide, and the resulting fingerprint patterns are quite comparable to those of previous epidemiological studies. When PstI or PvuII is also applied, a specific probe for hybridization situated on the left site of the IS*900* element without a restriction site for these enzymes should be used in further IS*900* RFLP studies. The standardized protocol should be changed accordingly. Use of the restriction enzyme PstI would be preferred and is recommended. Additional *M. avium* subsp. *paratuberculosis*-specific markers can further be applied and verified for use in a still more complex typing technique. Combined analysis of the types determined on the basis of independent genomic characteristics constitutes an approach to studying the population structure, the global epidemiology, and the evolution of *M. avium* subsp. *paratuberculosis.*

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