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Genetic Structure of *Mycobacterium avium* subsp. *paratuberculosis* Population in Cattle Herds in Quebec as Revealed by Using a Combination of Multilocus Genomic Analyses

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***Mycobacterium avium* subsp. *paratuberculosis* is the etiological agent of paratuberculosis, a granulomatous enteritis affecting a wide range of domestic and wild ruminants worldwide. A variety of molecular typing tools are used to distinguish *M. avium* subsp. *paratuberculosis* strains, contributing to a better understanding of *M. avium* subsp. *paratuberculosis* epidemiology. In the present study, PCR-based typing methods, including mycobacterial interspersed repetitive units/variable-number tandem repeats (MIRU-VNTR) and small sequence repeats (SSR) in addition to IS1311 PCR-restriction enzyme analysis (PCR-REA), were used to investigate the genetic heterogeneity of 200 *M. avium* subsp. *paratuberculosis* strains from dairy herds located in the province of Quebec, Canada. The majority of strains were of the "cattle type," or type II, although 3 strains were of the "bison type." A total of 38 genotypes, including a novel one, were identified using a combination of 17 genetic markers, which generated a Simpson's index of genetic diversity of 0.876. Additional analyses revealed no differences in genetic diversity between environmental and individual strains. Of note, a spatial and spatiotemporal cluster was evidenced regarding the distribution of one of the most common genotypes. The population had an overall homogeneous genetic structure, although a few strains stemmed out of the consensus cluster, including the bison-type strains. The genetic structure of *M. avium* subsp. *paratuberculosis* populations within most herds suggested intraherd dissemination and microevolution, although evidence of interherd contamination was also revealed. The level of genetic diversity obtained by combining MIRU-VNTR and SSR markers shows a promising avenue for molecular epidemiology investigations of *M. avium* subsp. *paratuberculosis* transmission patterns.**

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of Johne's disease, or paratuberculosis, a chronic granulomatous enteritis of domestic and wild ruminants, first described in 1895 (1). Paratuberculosis is a spectral disease characterized by an extended subclinical phase of several months or years, followed by an inevitable terminal clinical stage (2). Bovine paratuberculosis is endemic to most parts of the world, including Canada (3–5), and is considered one of the most costly infectious diseases of dairy cattle (6, 7). In Canada, paratuberculosis has also been detected in sheep (8), goats (9), and wild ruminant species (10–12). *M. avium* subsp. *paratuberculosis* is most likely introduced in ruminant herds through trading of subclinically infected animals, although wildlife reservoirs are also thought to play a role in spreading the bacteria to livestock (13). *M. avium* subsp. *paratuberculosis* is widespread in farm environments due to its excretion in high quantities in the feces of infected animals and to its environmental resilience. In addition, while *M. avium* subsp. *paratuberculosis* has been associated with human Crohn's disease, its role in causality remains to be demonstrated (14, 15). Nonetheless, *M. avium* subsp. *paratuberculosis* remains a public health concern.

Typing methods can be applied in molecular epidemiology studies to improve the understanding of transmission patterns of *M. avium* subsp. *paratuberculosis* and to investigate the role of wildlife reservoirs by tracking strains (16, 17). The knowledge thereby generated may improve the design of efficient control measures. Based on historical reasons, on the patterns obtained by pulsed-field gel electrophoresis (PFGE) analyses, and to a lesser extent, on growth characteristics and pigmentation, *M. avium* subsp. *paratuberculosis* strains have been divided into three major groups: the S (sheep) type (further segregated into subtypes I and III), the C (cattle) type (type II), and the B (bison) type (18, 19). However, the association of *M. avium* subsp. *paratuberculosis* types with either cattle, sheep, or bison is not absolute since strains from all lineages can cause diseases in these ruminants (19). IS*900* restriction fragment length polymorphism (RFLP) has been traditionally used to investigate the genetic diversity and epidemiology of *M. avium* subsp. *paratuberculosis* strains. However, RFLP is technically demanding and generates limited discriminatory power. Typing techniques such as multilocus short sequence repeat typing (MLSSR) and variable-number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) have gained interest mainly because of their ease of use and good discriminatory powers (17, 20). However, *M. avium* subsp. *paratuberculosis* is a highly monomorphic species, and using a combination of multilocus typing methods is necessary to increase their overall discriminatory power, as previously shown for the *M. tuberculosis* complex (21, 22) and *M. avium* subsp. *paratuberculosis*

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strains from different host species and different geographic locations (17, 23–26). A typing scheme based on 8 MIRU-VNTR loci (called INMV typing) has been previously proposed for the *M. avium* complex (26).

The goals of this study were (i) to investigate the genetic structure of bovine *M. avium* subsp. *paratuberculosis* populations originating from a total of 83 dairy herds, (ii) to describe the genetic diversity obtained by individual animal sampling versus environmental sampling, and (iii) to investigate the distribution of *M. avium* subsp. *paratuberculosis* genotypes in space and time. IS*1311* PCR-restriction enzyme analysis (PCR-REA) was used as a primary typing tool in combination with 17 distinct genomic loci (MLSSR and MIRU-VNTR) to study the genetic diversity of *M. avium* subsp. *paratuberculosis*.

MATERIALS AND METHODS

Source of strains and primary isolation. The present investigation was based on a collection of strains from 83 dairy herds located across the province of Quebec, Canada. These herds were sampled between one and five times between September 2007 and June 2011 in the context of different research projects, most of them including both environmental and individual sampling. There were no strict inclusion criteria applicable to all herds. Individual fecal samples from cows 3 years and older (maximum, 30 cows per herd), a single intestinal tissue sample, and pooled environmental manure samples were submitted to the Laboratoire d'épidémiosurveillance animale du Québec. Primary isolation of *M. avium* subsp. *paratuberculosis* strains was performed using 2 g of feces or pooled manure using the Bactec MGIT 960 mycobacterial detection system from Becton, Dickinson and Company. The protocol suggested by the manufacturer was strictly followed.

***M. avium* subsp. *paratuberculosis* subculture for molecular typing.** A total of 500 μl of growth medium from positive MGIT vials (231 samples in total) was diluted 1:5 in sterile phosphate-buffered saline (PBS). Aliquots of 100 μl of each dilution were then inoculated on 5 replicate slants of modified MiddleBrook 7H10 medium with mycobactin (27) and Herrold's egg yolk medium with mycobactin (28). Slants were incubated at 37°C and observed every 2 weeks for the appearance of colonies for up to 20 weeks.

Primary typing of *M. avium* subsp. *paratuberculosis* colonies (IS*1311* PCR_REA). Primary typing was performed using IS*1311* PCR followed by PCR-REA directly from individual *M. avium* subsp. *paratuberculosis* colonies growing on slants. IS*1311* PCRs were performed as described by Sevilla et al. (18) and Singh et al. (29) with slight modifications to adapt for colony PCR. Briefly, a single visible colony was harvested with a sterile toothpick and diluted in 10 μl of PCR grade water and heated at 95°C for 10 min. The mixture was then transferred to 40 μl of a PCR mixture consisting of 5 μl of 10× PCR buffer, 200 μM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, 0.6 μM primers M-56 and M-119, and 2 U of *Taq* polymerase. Amplification was performed under the following conditions: one cycle of 3 min at 94°C and 37 cycles of 30 s at 94°C, 15 s at 62°C, and 1 min at 72°C. Amplification reactions were analyzed on a Qiaxcel capillary electrophoresis instrument (Qiagen), which has greater resolution (3 to 5 bp) over that of traditional gel electrophoresis. The amplicon size was 608 bp. REA reactions were performed as described previously (18, 29). Briefly, restriction reactions were carried out in a volume of 30 μl, containing 20 μl of positive IS*1311* PCR, 3 μl of reaction 10× buffer, and 2 U of endonucleases HinfI and MseI. Reaction mixtures were incubated at 37°C for 2.5 h. Restriction reactions were analyzed using a Qiaxcel capillary electrophoresis instrument. Band patterns were interpreted following the recommendations of Sevilla et al. (18).

Genomic DNA extraction. *M. avium* subsp. *paratuberculosis* colonies were harvested from slants and stored in mycobacterial storage medium (20% glycerol solution containing 3.0% [wt/vol] tryptone soya broth). A total of 200 μl of the storage medium containing *M. avium* subsp. *paratuberculosis* cells was used to isolate genomic DNA using the QIAamp DNA minikit according to the recommended protocol, with a slight modification that included an initial enzymatic lysis step using 200 μl of 20 mg/ml lysozyme, and incubated for 3 h at 37°C before proceeding with the manufacturer's protocol. DNA was stored at −20°C until needed.

Molecular typing. MIRU-VNTR typing was carried out by PCR amplification on a total of 13 distinct loci: VNTR X3 (also known as MIRU-3), VNTR 3, VNTR 7, VNTR 10, VNTR 25, VNTR 32, VNTR 47, VNTR 292 (also known as MIRU-2), VNTR 259 (23), VNTR 1067 and VNTR 3527 (30), and MIRU-1 and MIRU-4 (31). For each marker, PCR conditions as described by the authors were strictly followed. Following PCR amplification, the reaction mixtures were analyzed on a Qiaxcel capillary electrophoresis instrument to determine the sizes of the amplicons at each locus. The number of repeats at each locus was determined according to the sizes of the amplicons using an allele-calling table as described by the authors of the above-cited works (23, 30, 31). The INRA (Institut National de Recherche Agronomique, France) MIRU-VNTR (INMV) nomenclature was used to analyze the results for MIRU-VNTR loci 292, X3, 25, 47, 3, 7, 10, and 32 as previously defined (26). For the remaining loci (MIRU 1, MIRU 4, VNTR 259, VNTR 1067, and VNTR 3527), a new nomenclature (MV typing) is proposed.

Multilocus short sequence repeat typing analysis was carried out by PCR amplification and sequencing of 4 short sequence repeat loci 1, 2, 8, and 9 (32). The primers and PCR conditions used were as described previously (32). All PCR amplicons were sequenced using BigDye v3.1 chemistry from Applied Biosystems on a 3730xl sequencer (Applied Biosystems). MLSSR types were expressed as the combination of number of repeats found in the amplified SSR loci. If the number of G repeats (stretches of guanosine residues) at loci 1 and 2 were equal to or greater than 11, G repeats for these loci were denoted as ≥11g. The allelic diversity at the different loci was calculated as previously described (33). This allelic diversity represents the probability that two alleles randomly selected from the population are different from each other.

Genetic diversity. The genetic diversity was estimated using Simpson's index of diversity with a 95% confidence interval (CI) for the individual typing methods and their combinations (34). The following analyses were based on a combination of methods offering the largest diversity. Descriptive statistics were first used to present the number of different genotypes found among strains from all samples collected in the same herd at a given sampling point. Individual rarefaction curves with 95% confidence intervals were used to compare the genetic diversity according to the sampling method used (i.e., fecal samples from individual cases versus environmental manure samples), performed in Past software version 2.17c (35). In brief, rarefaction methods can be used to compare genetic diversity in samples of different sizes (36). Spatial, temporal, and spatiotemporal scan tests were performed to detect clusters in the distribution of the more common genotypes, i.e., those detected in at least 5 herds. Herds were geocoded at the centroid of the area covered by the 6-digit postal code of the farm, which corresponds approximately to the municipality in rural areas of Quebec. The analysis was limited to one sampling point in time per herd, with a random selection of the sampling point for herds sampled more than once. This was done to avoid the detection of a temporal cluster of strains due to potential repeated sampling of the same animals. A Bernoulli model was used with cases defined as herds with the specific complete genotypes and controls defined as all other herds. The maximal size of a cluster was set as including a maximum of 50% of herds. The statistical significance of clusters was determined using the Monte Carlo simulation with 999 permutations performed in SaTScan version 9.1.1 (37).

Genetic relationship analysis. The genetic relationship between strains was investigated by creating minimum spanning trees (MST) using the BioNumerics software (Applied Maths, Austin, TX, USA) and a total of 17 combined VNTR and SSR markers. The MST is an undirected network that uses pairwise distances to describe the degree of dissimilarity between

TABLE 1 Distribution of *M. avium* subsp. *paratuberculosis* genotypes in 200 isolates from 69 dairy cattle herds of Quebec, Canada

Farm ID	Yr-mo of collection	No. of isolates	Source of isolates[a]	Main group[b]	Genotype profile			
					INMV	MV	MLSSR	Combined
1	2011-04	1	FC	C	INMV 2	MV 1	S7	MapGnt 10
2	2009-03	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
	2009-07	2	FC	C	INMV 2	MV 1	S1	MapGnt 5
	2009-10	2	FC	C	INMV 2	MV 1	S1	MapGnt 5
		1	FC	C	INMV 3	MV 1	S1	MapGnt 22
	2010-03	2	FC	C	INMV 2	MV 1	S1	MapGnt 5
	2011-06	1	FC	C	INMV 2	MV 1	S1	MapGnt 5
3	2007-09	1	FC	C	—[c]	MV 1	S13	—
		1	FC	C	INMV 8	MV 3	S6	MapGnt 31
4	2007-10	1	FC	C	INMV 3	MV 1	—	—
		2	FC	C	INMV 3	MV 1	S3	MapGnt 24
	2007-11	1	EV	C	INMV 3	MV 1	S3	MapGnt 24
5	2007-10	3	FC	C	INMV 2	MV 1	S1	MapGnt 5
6	2007-10	1	FC	B	INMV X	MV 2	S14	MapGnt 35
		1	FC	C	INMV 1	MV 1	S11	MapGnt 1
7	2007-11	1	FC	C	INMV 2	MV 1	S13	MapGnt 14
8	2008-01	1	FC	B	INMV X	MV 2	—	—
		1	FC	B	INMV X	MV 2	S20	MapGnt 36
9	2008-04	1	FC	C	INMV 1	MV 1	S12	MapGnt 2
10	2008-04	1	FC	C	—	MV 1	S18	—
11	2008-06	1	FC	C	INMV 2	MV 1	S1	MapGnt 5
12	2008-09	1	FC	C	INMV 3	MV 1	S6	MapGnt 26
13	2009-03	1	FC	C	INMV 2	MV 1	S6	MapGnt 9
14	2009-04	1	CO	C	INMV 3	MV 1	S15	MapGnt 28
15	2009-04	1	EV	C	INMV 2	MV 1	S2	MapGnt 6
17	2009-04	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
18	2009-04	1	EV	C	INMV 3	MV 1	S5	MapGnt 25
19	2009-05	1	EV	C	INMV 2	MV 1	S21	MapGnt 18
	2010-04	3	EV	C	INMV 2	MV 1	S21	MapGnt 18
		1	EV	C	INMV 2	MV 1	—	—
		2	FC	C	INMV 2	MV 1	S21	MapGnt 18
20	2009-05	2	EV	C	INMV 2	MV 1	S2	MapGnt 6
	2010-04	3	EV	C	INMV 2	MV 1	S2	MapGnt 6
		6	FC	C	INMV 2	MV 1	S1	MapGnt 5
		4	FC	C	INMV 2	MV 1	S2	MapGnt 6
		1	FC	C	INMV 2	MV 1	S8	MapGnt 11
	2010-05	1	FC	C	INMV 2	MV 1	S2	MapGnt 6
23	2009-09	1	FC	C	INMV 2	MV 1	S2	MapGnt 6
24	2008-04	1	EV	C	INMV 2	MV 1	S13	MapGnt 14
		1	FC	C	INMV 2	MV 3	S13	MapGnt 20
	2009-09	1	EV	C	INMV 2	MV 1	S18	MapGnt 16
		1	EV	C	INMV 3	MV 1	S18	MapGnt 29
	2010-06	1	EV	C	INMV 2	MV 1	S13	MapGnt 14
		2	FC	C	INMV 2	MV 1	S13	MapGnt 14
		3	FC	C	INMV 2	MV 1	S18	MapGnt 16
25	2009-10	1	EV	C	INMV 2	MV 1	S18	MapGnt 16
26	2009-11	1	EV	C	INMV 2	MV 1	S17	MapGnt 15
		1	EV	C	INMV 2	MV 1	S21	MapGnt 18
	2010-03	2	FC	C	INMV 2	MV 1	S21	MapGnt 18
27	2009-11	1	FC	C	INMV 1	MV 1	S14	MapGnt 3
28	2009-11	1	FC	C	INMV 3	MV 1	S3	MapGnt 24
29	2009-12	1	EV	C	INMV 2	MV 1	S2	MapGnt 6
	2010-03	5	FC	C	INMV 2	MV 1	S2	MapGnt 6
		1	FC	C	INMV 2	MV 1	S18	MapGnt 16
31	2010-03	1	EV	C	INMV 2	MV 1	S18	MapGnt 16
33	2010-03	1	FC	C	—	MV 3	S2	—
34	2010-03	1	FC	C	INMV 3	MV 1	S1	MapGnt 22
	2010-08	1	FC	C	INMV 3	MV 1	—	—
35	2010-04	1	FC	C	INMV 13	MV 1	S10	MapGnt 33
36	2010-04	1	FC	C	INMV 2	MV 1	S2	MapGnt 6

(Continued on following page)

TABLE 1 (Continued)

Farm ID	Yr-mo of collection	No. of isolates	Source of isolates[a]	Main group[b]	Genotype profile			
					INMV	MV	MLSSR	Combined
37	2010-04	2	EV	C	INMV 2	MV 1	S18	MapGnt 16
38	2010-04	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
		1	EV	C	INMV 2	MV 1	S2	MapGnt 6
	2010-07	1	FC	C	INMV 2	MV 1	S2	MapGnt 6
39	2010-04	2	EV	C	INMV 2	MV 1	S13	MapGnt 14
40	2010-04	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
		2	EV	C	INMV 2	MV 1	S2	MapGnt 6
		1	EV	C	—	MV 1	—	—
		2	FC	C	INMV 1	MV 1	S14	MapGnt 3
		1	FC	C	INMV 1	MV 1	S16	MapGnt 4
		7	FC	C	INMV 2	MV 1	S1	MapGnt 5
41	2010-05	1	EV	C	INMV 2	MV 1	S21	MapGnt 18
42	2010-05	1	FC	C	INMV 3	MV 1	S2	MapGnt 23
43	2010-08	1	FC	C	INMV 2	—	S18	—
44	2010-09	1	FC	C	INMV 3	MV 1	S2	MapGnt 23
47	2010-09	1	EV	C	INMV 3	MV 1	S2	MapGnt 23
		1	EV	C	INMV 3	MV 1	S18	MapGnt 29
48	2010-10	1	EV	C	INMV 2	MV 1	S9	MapGnt 12
49	2010-10	2	EV	C	INMV 2	MV 1	S17	MapGnt 15
		1	EV	C	INMV 2	MV 1	—	—
50	2010-10	2	EV	C	INMV 2	MV 1	S2	MapGnt 6
		1	EV	C	INMV 2	MV 1	—	—
52	2010-11	1	EV	C	INMV 3	MV 1	S1	MapGnt 22
		1	EV	C	INMV 3	MV 1	—	—
53	2010-11	1	FC	C	INMV 2	MV 1	S6	MapGnt 9
		2	FC	C	INMV 3	MV 1	S1	MapGnt 22
		2	FC	C	INMV 3	MV 1	S2	MapGnt 23
		1	FC	C	INMV 3	MV 1	—	—
54	2010-11	3	EV	C	INMV 3	MV 1	S8	MapGnt 27
56	2011-03	1	FC	C	INMV 2	MV 1	S3	MapGnt 7
57	2011-03	1	FC	C	INMV 3	MV 1	S1	MapGnt 22
58	2011-04	1	FC	C	INMV 2	MV 1	S1	MapGnt 5
60	2011-04	1	FC	C	INMV 2	MV 1	—	—
62	2011-05	1	FC	C	INMV 2	MV 1	S1	MapGnt 5
C	2007-10	1	FC	C	INMV 3	MV 1	S6	MapGnt 26
D	2007-11	1	FC	C	INMV 13	MV 1	S1	MapGnt 32
E	2007-11	1	EV	C	INMV 13	MV 1	S10	MapGnt 33
		1	FC	C	INMV 13	MV 1	S10	MapGnt 33
G	2007-11	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
		2	EV	C	INMV 2	MV 1	S2	MapGnt 6
		1	EV	C	INMV 2	MV 1	S4	MapGnt 8
		2	FC	C	INMV 2	MV 1	S2	MapGnt 6
H	2007-11	1	EV	C	INMV 2	MV 1	S19	MapGnt 17
		3	FC	C	INMV 2	MV 1	S19	MapGnt 17
I	2007-11	2	EV	C	INMV 2	MV 1	S2	MapGnt 6
		5	FC	C	INMV 2	MV 1	S2	MapGnt 6
J	2007-11	1	EV	C	INMV 1	MV 1	S16	MapGnt 4
		1	FC	C	INMV 2	MV 1	S2	MapGnt 6
		1	FC	C	INMV 1	MV 1	S16	MapGnt 4
L	2008-01	2	EV	C	—	MV 1	S1	—
		2	FC	C	—	MV 1	S1	—
M	2008-01	4	EV	C	INMV 2	MV 1	S1	MapGnt 5
		1	FC	C	INMV 2	MV 1	S1	MapGnt 5
		1	FC	C	INMV 2	MV 1	S6	MapGnt 9
N	2008-02	1	EV	C	INMV 2	MV 1	S2	MapGnt 6
		1	EV	C	INMV 2	MV 1	S3	MapGnt 7
O	2008-02	1	EV	C	INMV 2	MV 3	S1	MapGnt 19
		1	FC	C	INMV 2	MV 3	S1	MapGnt 19
P	2008-03	1	FC	C	INMV 2	MV 1	S13	MapGnt 14

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TABLE 1 (Continued)

Farm ID	Yr-mo of collection	No. of isolates	Source of isolates[a]	Main group[b]	Genotype profile			
					INMV	MV	MLSSR	Combined
R	2008-04	1	EV	C	INMV 2	MV 1	S1	MapGnt 5
		2	EV	C	INMV 2	MV 1	S3	MapGnt 7
		1	FC	C	INMV 2	MV 1	—	—
S	2008-04	2	FC	C	INMV 3	MV 1	S2	MapGnt 23
U	2008-04	1	FC	C	INMV 2	MV 1	S12	MapGnt 13
V	2008-05	1	FC	C	INMV 2	MV 1	S1	MapGnt 5
		7	FC	C	INMV 2	MV 1	S2	MapGnt 6
W	2008-05	1	FC	C	INMV 2	MV 1	S2	MapGnt 6
X	2008-06	2	EV	C	INMV 2	MV 1	S13	MapGnt 14
		1	EV	C	INMV 2	MV 1	S18	MapGnt 16
		1	FC	C	INMV 2	MV 1	S1	MapGnt 5
		3	FC	C	INMV 2	MV 1	S13	MapGnt 14
Total no.		200			6[d]	3[d]	21[d]	33[d]

[a] EV, environmental pooled sample; FC, individual fecal culture; CO, colon culture.
[b] C, cattle; B, bison.
[c] —, 8 isolates were not genotyped by INMV, 1 isolate was not genotyped by MV, and 11 isolates were not genotyped by MLSSR.
[d] Number of different genotype profiles for the method.

strains. The MST represents nodes (strains) linked together by the shortest possible distance.

RESULTS

Recovery of *M. avium* subsp. *paratuberculosis* strains in subculture. A total of 231 samples originating from 83 herds (1 to 14 samples per herd) were cultured in the Bactec MGIT 960 system and confirmed by PCR [TaqMan MAP (Johne's) Reagents; Life Technologies]. From this total, 200 samples originating from 69 herds gave visible colonies after 6 to 20 weeks on solid medium and are referred to as strains henceforth (Table 1). Colonies were rough, whitish, and generally small, although some samples yielded larger colonies.

Group typing (IS*1311* PCR-REA). Group typing of *M. avium* subsp. *paratuberculosis* strains revealed that the cattle type, or type II, was by far the most common group (Table 1). Of the 200 strains typed by IS*1311* PCR-REA, 197 (98.5%) were of the cattle type and only 3 (1.5%) were of the bison type (Table 1). In herd 6, both cattle-type and bison-type strains were present. In herd 8, the bison type was the only type detected. All bison-type strains were recovered from individual samples. No sheep-type strain was identified in this population.

Typing results (MIRU-VNTR and MLSSR). (i) MIRU-VNTR. Results from MIRU-VNTR typing are reported separately for INMV profiles (MIRU-VNTR loci 292, X3, 25, 47, 3, 7, 10, and 32) and MV profiles (MIRU-VNTR loci 1, 4, 259, 1067, and 3527) (Table 1).

A total of 6 distinct INMV profiles were observed, among which 1 had never been reported before and was therefore considered novel. The novel profile was given a tentative name (INMV X, profile 22532228). The new INMV X profile was specific to the bison-type strains. Table 1 shows the different INMV profiles found in this study: INMV 1 ($n = 8$), INMV 2 ($n = 146$), INMV 3 ($n = 30$), INMV 8 ($n = 1$), INMV 13 ($n = 4$), and INMV X ($n = 3$). INMV 2 (73% of strains and 64% of herds) and INMV 3 (15% of strains and 25% of herds) were the most frequent INMV profiles detected.

A total of three different MV profiles (MV 1, MV 2, and MV 3) were identified (Table 1). MV 1 was the most frequent profile observed (96% of strains in 94% of herds). The 3 strains with the MV 2 profile belonged to the bison-type group, whereas all strains with the MV 1 or MV 3 profile were from the cattle-type group.

(ii) MLSSR profiles. MLSSR profiling was applicable to only 189 strains from a total of 68 herds. Amplification failure occurred for at least one of the SSR loci in the remaining 11 strains. MLSSR segregated the 189 strains into 21 distinct profiles (Table 1). Types S1 (28% of strains in 22% of herds) and S2 (29% of strains in 22% of herds) were the most frequent profiles observed (Table 1).

Allelic diversity. The relative frequencies and diversity of the various alleles are shown in Table 2. INMV loci 3 and 32 and MV loci MIRU 1, MIRU 4, 259, and 3527 were all monomorphic. The highest allelic diversity (0.560) was observed for MLSSR locus 2.

Genetic diversity. Simpson's index of diversity was calculated for a total of 181 strains having a combined MLSSR, MV, and INMV profile (Table 1). The genetic diversities revealed by MLSSR, MV, and INMV typing methods were statistically different from each other based on the comparison of confidence intervals. The MLSSR method used individually or in combination with others was associated with high indices of diversity ranging from 0.83 to 0.88. Although the inclusion of MV and/or INMV in addition to MLSSR was not associated with a large increase in genetic diversity, it allowed an important increase in the number of different genotypes recovered, from 21 to a maximum of 33 (Table 3). All further analyses were performed based on the genotypes identified by the combination of the three typing methods (MV, INMV, and MLSSR).

A total of 33 distinct clusters (genotypes) were defined for 181 strains originating from 64 herds by combining the typing results from the MV, INMV, and MLSSR loci (Table 1). A total of 19 strains could not be typed using either MV, INMV, or MLSSR and had therefore no genotype assigned. The cattle-type group of strains was divided into a total of 31 distinct genotypes. Two strains of the bison-type group were segregated into 2 distinct genotypes; one bison type strain failed to give an MLSSR profile and could not be assigned a genotype (Table 1). *M. avium* subsp. *paratuberculosis* genotype 5 (MapGnt 5) and MapGnt 6 were the most frequent genotypes observed among all strains (20% and

TABLE 2 Relative frequency of isolates according to the specific allele copy number and allelic diversity for each locus

Locus	No. of isolates	Relative frequency (%) of isolates according to specific allele copy no.:											Allelic diversity
		1	2	3	4	5	6	7	8	9	10	≥11	
INMV													
292	192		3.6	92.2	4.2								0.147
X3	192		100										0.000
25	192			98.4		1.6							0.031
47	192			100									0.000
3	192		100										0.000
7	192		99.5	0.5									0.010
10	192	15.6	84.4										0.264
32	192								100				0.000
MV													
MIRU 1	199			100									0.000
MIRU 4	199	100											0.000
259	199		100										0.000
1067	199	2.5	96	1.5									0.078
3527	199		100										0.000
MLSSR													
1	189							29.1	1.6	2.1	5.8	61.4	0.535
2	189									6.9	43.4	49.7	0.560
8	189				5.8	87.3	6.9						0.230
9	189				10.6	89.4							0.189

23% of strains, respectively) and herds (20% of herds each). These two genotypes differed only in their MLSSR profile (Table 1).

The number of strains with a complete genotype available for each sampling point (i.e., one herd sampled at one time) ranged from 1 to 14, with a maximum of 4 different genotypes found per sampling point (Table 4). Among the 181 strains with a complete genotype, 66 were isolated from pooled environmental samples, 111 were from individual fecal samples, and 1 was isolated from the colon of a suspected clinical case submitted to necropsy. According to the rarefaction curves, no difference in genetic diversity was observed between strains from environmental and individual fecal samples (Fig. 1).

The spatiotemporal distribution of the 6 most common genotypes was analyzed (MapGnt 5, 6, 14, 16, 22, and 23 genotypes). For MapGnt 16, statistically significant spatial ($P = 0.01$) and space-time ($P = 0.04$) clusters were observed (mapped in Fig. 2). The space-time cluster comprised 4 herds with genotype 16 sampled between June 2009 and May 2010. No other clusters in space and/or time were detected (for all, $P \geq 0.07$).

To analyze the genetic relationships of the population, a minimum spanning tree that included a total of 181 strains having a combined VNTR and SSR profile (17 loci) was constructed (Fig. 3). The predominance of genotypes 5 and 6 (red and green, respectively) in the population is illustrated by the sizes of the nodes. Node sizes are proportional to the number of strains with the corresponding genotype. Both genotypes 5 and 6 appeared as consensus sequences to which most alternate genotypes in the population were related (Fig. 3). The population was generally homogeneous, with most genotypes closely related to the 2 principal nodes, differing by a maximum of 3 loci and more generally by only 1. However, a cluster of strains comprised of genotypes 1, 3,

TABLE 3 Simpson's index of diversity with 95% confidence intervals based on different typing methods used separately or in combination[a]

Typing method	No. of different profiles	Simpson's index (95% CI)
Individual typing method		
MLSSR	21	0.829 (0.794–0.865)
MV	3	0.065 (0.014–0.115)
INMV	6	0.381 (0.297–0.464)
Combination of two typing methods		
MLSSR + MV	25	0.836 (0.801–0.872)
MLSSR + INMV	31	0.870 (0.839–0.902)
MV + INMV	7	0.406 (0.321–0.490)
Combination of three typing methods		
MLSS + MV + INMV	33	0.876 (0.845–0.907)

[a] $n = 181$ isolates.

TABLE 4 Number of complete genotypes detected for each sampling point according to the number of isolates tested

No. of isolates per sampling point[a]	Frequency	No. of different genotypes		
		Mean	Minimum	Maximum
1	43	1	1	1
2	18	1.4	1	2
3	5	1.6	1	2
4	1	1	1	1
5	2	2	1	3
6	4	2.3	2	3
7	2	2	1	3
8	1	2	2	2
13	1	4	4	4
14	1	3	3	3

[a] A sampling point represents all isolates collected from a single farm at the same time.

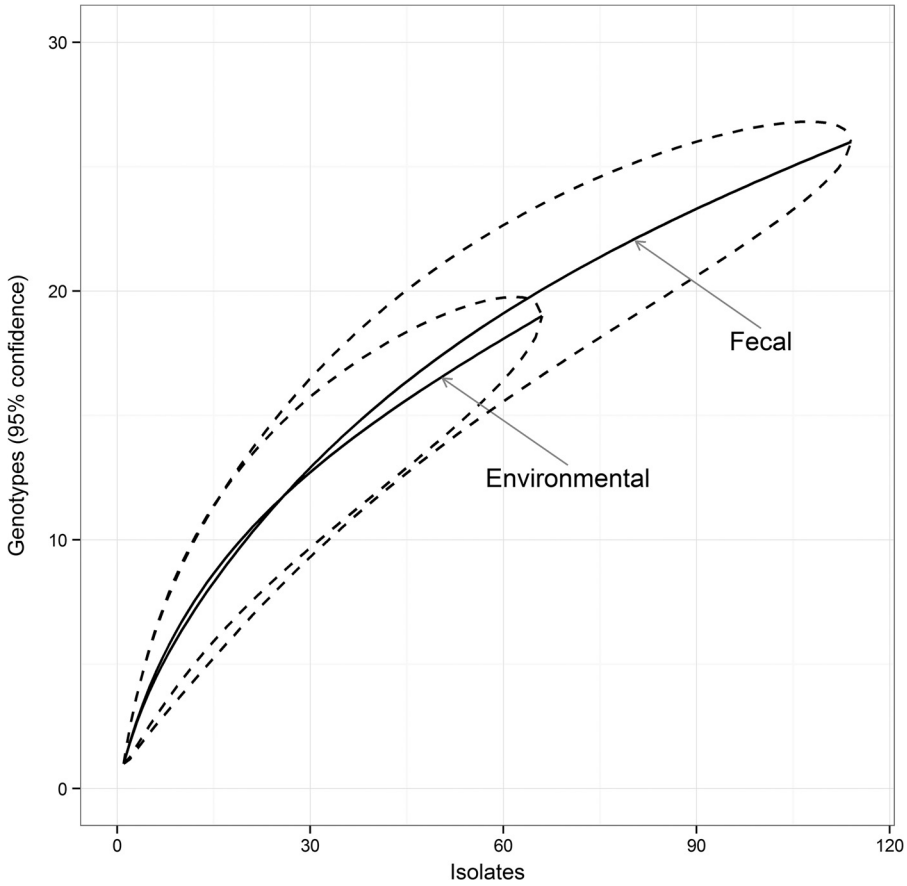

FIG 1 Rarefaction curves with 95% confidence intervals comparing the numbers of genotypes observed in 66 strains (isolates) from environmental sampling and 114 from individual case sampling.

4, 35, and 36, the latter two being from the bison-type strains, were more distantly related to the main cluster.

A second MST was generated to investigate the intra- and interherd genetic diversity using strains from all herds from which more than one strain was recovered. Figure 4 shows the strains grouped by genotypes (nodes) and the corresponding herds from which they were recovered (represented as distinct colors subdividing the nodes and their corresponding number or letter placed outside the nodes). Most herds harbored strains with identical or closely related genotypes (e.g., herd 2 in beige and herd 20 in light green). However, other herds harbored strains with more distantly related genotypes (e.g., herd 53 in dark brown and herd 40 in red). Interestingly, herds 40 and J (red and deep purple, respectively) both harbored strains belonging to two genetically distant genotypes (4 and 6). Also of note, herd 24 (purple) harbored strains belonging to a total of 4 closely related genotypes.

DISCUSSION

This study aimed at investigating the genetic diversity and population structure of *M. avium* subsp. *paratuberculosis* strains isolated from cattle herds in a defined geographical region of Canada (Quebec). A combination of PCR-based typing methods was used to discriminate between *M. avium* subsp. *paratuberculosis* strains. In line with current knowledge, the cattle or C type was the dominant group detected among cattle strains, although three strains of the bison type originating from 2 different herds were also isolated. These two herds were located more than 200 km from each other, and insufficient epidemiological data were available to establish a link between them. To our knowledge, this is the first report of bison-type *M. avium* subsp. *paratuberculosis* strains isolated from domestic species on the American continent. Bison-type strains were first reported from wild animal species (bison) in Montana, USA (38). These strains were subsequently isolated in India and Korea from different domestic and wild host species and identified as carrying the major infecting genotype in some domestic animals (39–41). More recently, bison-type strains have been reported to infect domestic species in Africa (42). The presence of bison-type strains in domestic cattle of Quebec could suggest potential spillover events between unknown reservoirs and dairy herds. Quebec has a considerable population of wild deer, which may serve as a reservoir of infection of bison-type strains for domestic ruminants as previously suggested (13). There are also a small number of bison herds in the province, which could act as a source for these atypical strains, although an epidemiological link remains to be established. Bison-type strains have also been isolated from patients with Crohn's disease, which underscores their potential to colonize the human intestine (43). Collectively, these results underpin the need to better understand the role of these potential reservoirs not only as sources of *M. avium* subsp. *paratuberculosis* strains for domestic cattle, but also as potential sources for human infection.

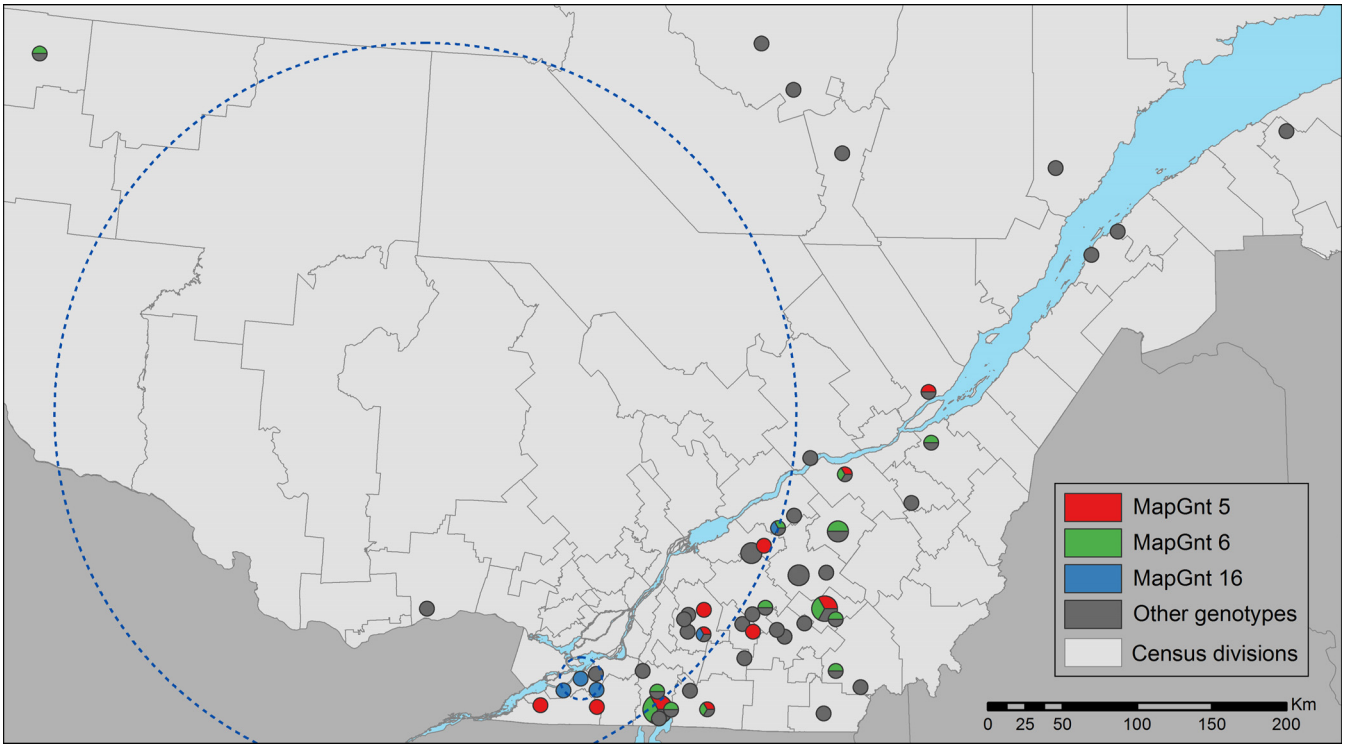

FIG 2 Geographical distribution of the 64 herds included in the study. Point size is proportional to the number of herds (1 to 4) located in the postal code area. Distribution of MapGnt 16 is illustrated in blue, as are the distributions of the two most common genotypes (MapGnt 5 and MapGnt 6, in red and green, respectively). The large blue dotted line circle represents the significant spatial cluster of MapGnt 16, and the small dotted line blue circle represent herds included in the significant spatiotemporal cluster of MapGnt 16.

The relative frequencies of the three main INMV types (INMV1, INMV2, and INMV3) are in slight contrast with other studies from Europe and South America, where INMV 1 and INMV 2 were identified as the two most prevalent genotypes in cattle and INMV 3 represented a minority of strains (26, 44–47). The present results may simply reflect regional specificities/distribution of these types. However, consistent with previous studies, INMV types 7, 8, 13, and 17 were found only in a minority of herds.

The combination of loci MIRU-VNTR 1, 4, 259, 1067, and 3527 has never been used in previous studies. Therefore, a new nomenclature termed "MV type" is proposed. The discriminatory power of MV typing was much lower than INMV or MLSSR typing. Only three distinct MV types were observed for all strains among which the MV 2 type was found to be specific to the bison-type group. Among these 5 loci, only MIRU-VNTR 1067 was found to be polymorphic, in agreement with other studies (30, 48). Considering the results of the present and previous studies, MIRU 1 and MIRU 4 may be excluded from future studies since these loci appear monomorphic for cattle strains from different areas of the world (24, 31). Although the MV typing discriminatory power was somewhat disappointing, it nevertheless subdivided the predominant INMV 2, MLSSR S1, and MLSSR S2 types in distinct clusters, underscoring its advantage in epidemiological investigations. Since INMV 2 has also been reported as the most prevalent *M. avium* subsp. *paratuberculosis* type by others (26, 44–47), MV typing using locus 1067 has the potential to further segregate *M. avium* subsp. *paratuberculosis* strains and should be considered in future studies.

In agreement with other studies, MLSSR typing was found to be the most discriminatory typing method (17, 44, 48). However, different groups have used distinct sets of SSR loci. In addition, different cutoff values were used for mononucleotide repeats: >14 by some authors (49–51) or the more conservative ≥11 as set in this work and other studies (24, 44). It is unfortunate that due to the technical limitations of sequencing long mononucleotide repeats, there is a loss of resolution power, which can potentially misclassify *M. avium* subsp. *paratuberculosis* strains. Nevertheless, using the conservative ≥11 cutoff value, a total of 21 MLSSR types were observed in this population (S1 to S21), of which S1 and S2 were the predominant types and two were novel: S14 and S16. The bison-type strains were segregated into two different MLSSR types, S14 and S20. Although SSR markers are technically more demanding, more difficult to interpret than VNTRs, and less accessible to most laboratories, MLSSR typing subdivided the predominant INMV and MV types into specific MLSSR types, making SSR markers pivotal in *M. avium* subsp. *paratuberculosis* strain typing.

Using all three typing methods in combination allowed the clustering of *M. avium* subsp. *paratuberculosis* strains into 33 distinct genotypes, including MapGnt 5 and MapGnt 6 as the most common. These results are in accordance with previous studies reporting that most strains were found to segregate into specific genotypes presumably due to increased virulence, enhanced transmissibility, or better cultivability (20, 26, 31, 49, 52). Combining the three typing methods increased the overall discriminatory power, although adding MV typing added little advantage based on Simpson's index of diversity. However, it should

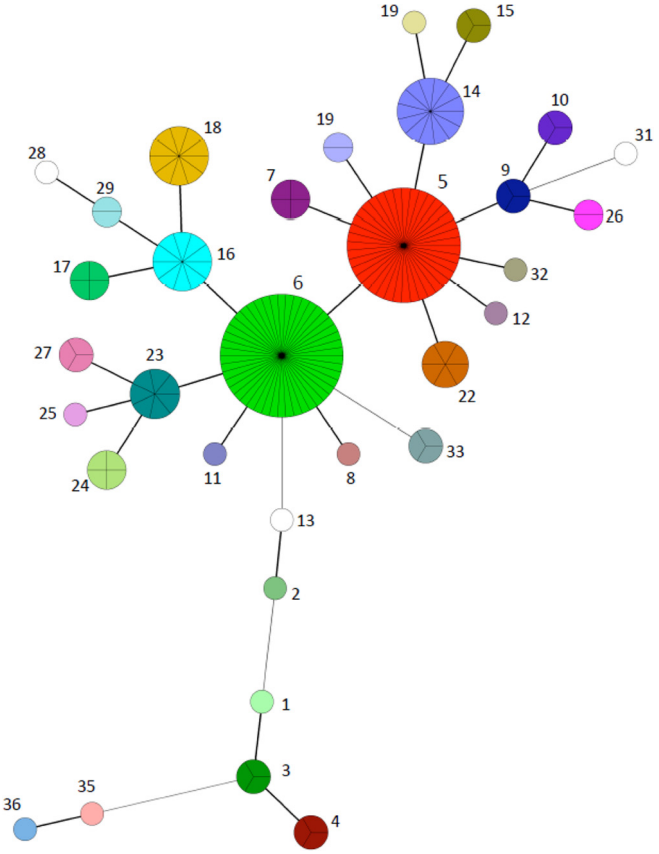

FIG 3 Minimum spanning tree based on combined VNTR and SSR profiles of a set of 17 loci from 181 *M. avium* subsp. *paratuberculosis* strains. Node sizes are proportional to the number of strains sharing a given genotype (cluster). Each cluster is represented as a uniquely colored and numbered pie chart, where the number of subdivisions illustrates the number of strains. The number of loci differing between the nodes is represented by the style of the connecting lines: thick and short, 1 difference; thin and intermediate length, 2 differences; thin and long, 3 differences.

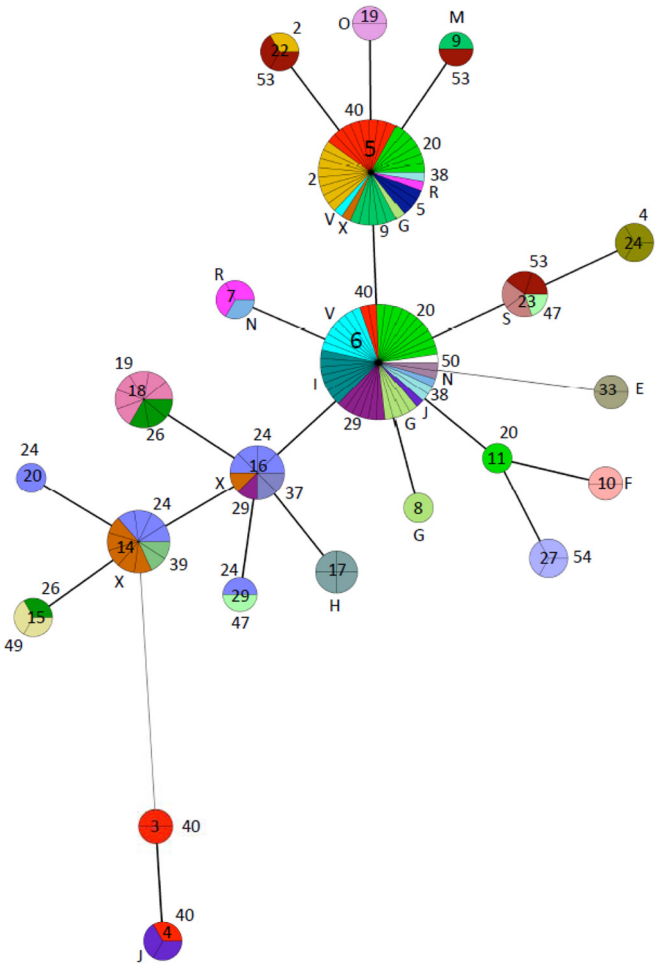

FIG 4 Minimum spanning tree based on combined VNTR and SSR profiles of *M. avium* subsp. *paratuberculosis* strains originating from herds with more than one strain. Node sizes are proportional to the number of strains sharing a given genotype (cluster). A distinct color and its corresponding letter or number placed outside the node represent each herd within a cluster. For each cluster, the number of subdivisions represents the number of strains. The corresponding genotype is indicated inside the node. The number of loci differing between the nodes is represented by the style of the connecting lines: thick and short, 1 difference; thin and intermediate length, 2 differences; thin and long, ≥3 differences.

be noted that Simpson's index is most sensitive to variations in the abundant genotypes and less so to rare ones, which could help explain the results (53). Given its relatively low genetic heterogeneity, our results suggest that using the three methods in combination will be key to study the population structure, the evolution, and the local or global epidemiology of *M. avium* subsp. *paratuberculosis*.

Using environmental or individual fecal samples did not affect the genetic diversity of strains, suggesting that both types of samples could be used interchangeably in epidemiological investigations. Although the number of samples per herd was small, the present results also suggest that typing 5 strains per herds can be used as a proxy to investigate *M. avium* subsp. *paratuberculosis* diversity in a herd at a given point in time. However, it should be noted that a maximum of 30 cows per herd were sampled and rare genotypes could have been missed.

Most herds harbored genetically identical or closely related strains, suggestive of within-herd dispersion and microevolution. In agreement with this finding was the isolation of strains with identical genotypes from both the farm environment and animals on most premises, which suggests that *M. avium* subsp. *paratuberculosis* shedders are likely spreading the disease to herd mates via this passive route, as previously suggested by others (50). The predominance of a single or closely related genotypes within herds could be due to a selective advantage of specific strains caused by increased virulence, persistence, or transmissibility, which has been reported previously for mycobacteria, including *M. avium* subsp. *paratuberculosis* (54–57). Microevolution in patients infected with *Mycobacterium tuberculosis* and along transmission chains has been documented (58–60). Latent infections, such as those caused by mycobacteria, and adaptation to sequential host-to-host transmission are believed to provide the bacterial population sufficient time for microevolution (58–60). Long incubation periods and transmission chains are also a landmark of *M. avium* subsp. *paratuberculosis* infections and might explain the present observations. Interestingly, herd 24 harbored strains belonging to 4 closely related genotypes, suggesting either long-term infection providing sufficient time for microevolution or selective pressure

in that herd. However, we cannot rule out the possibility of inter-herd dispersion of common and genetically closely related strains (e.g., genotypes 5 and 6). On the other hand, a small number of herds harbored genetically distant strains (e.g., herds 40 and 53), which suggests the introduction of multiple *M. avium* subsp. *paratuberculosis* strains.

Regarding the distribution of genotypes between herds, a spatial cluster was observed for MapGnt 16. This cluster was quite large, although most of the herds sharing this genotype within the cluster were located in a smaller area of a linear shape; this could be an artifact of the method, as the spatial scan test searched only for circular clusters. Many of the herds included in this cluster were also temporally clustered, suggesting local dissemination of this genotype within a 1-year period. Interestingly, the two most common genotypes (MapGnt 5 and MapGnt 6) were evenly distributed in space and time. This might be due to random dispersal of the bacteria following large-scale processes such as cattle movements between herds. It has been reported that herds with an elevated turnover of cows show multiple genotypes (25, 61), underscoring the importance of live animal trade in the transmission of *M. avium* subsp. *paratuberculosis* between herds.

Clustering analysis, based on a combination of genetic markers, allowed the formation of subgroups (nodes) generally made up of several herds sharing a common genotype (Fig. 4). It will be interesting, in future epidemiological studies, to investigate if this clustering is a result of increased virulence, animal movements between herds, shared sources of infection (e.g., wildlife or other-farmed-species reservoirs), indirect contacts between herds through mechanical vectors, and/or other factors. Of particular interest in that regard is the possible wild origin of bison-type strains and of genotype 1, 3, and 4 strains, appearing as an outgroup distantly related to the consensus sequences. Domestic livestock and wild animals infected with *M. avium* subsp. *paratuberculosis* can share similar strains (16, 62–64), supporting the transmission of *M. avium* subsp. *paratuberculosis* strains between wildlife and livestock in places where habitats overlap. Likewise, the presence of specific epidemiological links between herds 40 and J should be investigated, as they both harbored strains belonging to genotype 4. Other groups of herds of particular interest are the ones sharing less common genotypes, such as genotypes 17, 18, and 24. At the population level, such molecular epidemiology studies could allow the identification of the most likely transmission pathways and help design control and prevention measures.

Conclusion. The results from this study show that in addition to a majority of cattle-type strains, bison-type strains were isolated for the first time in domestic cattle on the American continent. Further typing of *M. avium* subsp. *paratuberculosis* strains, using a combination of conventional and less conventional markers, detected a total of 33 distinct genetic profiles in the population. Using unconventional markers, such as MIRU-VNTR 1067, proved useful in segregating the predominant genotypes into subtypes, underscoring their usefulness in *M. avium* subsp. *paratuberculosis* epidemiological investigations. The overall typing results suggest that using a combination of VNTR and SSR markers increases the overall discriminatory power and should be used in future studies. The genetic structure of the population was generally homogeneous and suggested the predominance of within-herd dispersion of strains, most likely via environmental contamination, although evidence of strain dissemination between herds was also uncovered. The finding of a cluster of strains, including the bison type, more distantly related to the consensus group suggests the existence of an unknown reservoir. Finally, although a spatiotemporal cluster was observed for a minor genotype, the two most prevalent genotypes were evenly distributed in space and time.

The typing tools that are now available should be used for a better understanding of the epidemiology and control of this important pathogen.

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