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Molecular Epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* Isolates Recovered from Wild Animal Species

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Mycobacterial isolates were obtained by radiometric culture from 33 different species of captive or free-ranging animals ($n = 106$) and environmental sources ($n = 3$) from six geographic zones within the United States. The identities of all 109 isolates were confirmed by using mycobactin J dependence and characterization of five well-defined molecular markers, including two integration loci of IS*900* (loci L1 and L9), one *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*)-specific sequence (locus 251), and one *M. avium* subsp. *avium*-specific marker (IS*1245*), as well as *hsp65* and IS*1311* restriction endonuclease analyses. Seventy-six acid-fast isolates were identified as *M. paratuberculosis*, 15 were identified as belonging to the *M. avium-M. intracellulare* complex (but not *M. paratuberculosis*), and the remaining 18 were identified as mycobacteria outside the *M. avium-M. intracellulare* complex. Fingerprinting by multiplex PCR for IS*900* integration loci clustered 67 of the 76 *M. paratuberculosis* strains into a single clade (designated clade A18) and had a Simpson's diversity index (D) of 0.53. In contrast, sequence-based characterization of a recently identified *M. paratuberculosis* short sequence repeat (SSR) region enabled the differentiation of the *M. paratuberculosis* isolates in clade A18 into seven distinct alleles ($D = 0.75$). The analysis revealed eight subtypes among the 33 species of animals, suggesting the interspecies transmission of specific strains. Taken together, the results of our analyses demonstrate that SSR analysis enables the genetic characterization of *M. paratuberculosis* isolates from different host species and provide evidence for the host specificity of some *M. paratuberculosis* strains as well as sharing of strains between wild and domesticated animal species.

Paratuberculosis, or Johne's disease, is a chronic granulomatous gastroenteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) (46, 59). The disease occurs worldwide and is primarily a disease of domesticated ruminants, including cattle (both beef and dairy), sheep, goats, and farmed deer. Paratuberculosis has been reported to occur in wild ruminant species, including deer (10), bison (7), and elk (13, 40), as well as nonruminants, such as wild rabbits (26), their predators, including foxes and stoats (6), and primates, such as mandrills and macaques (41, 60), indicating a wide host range. In addition to the economic impact on food animal production, with losses estimated to be from $200 million to $250 million annually (47), infections in free-ranging and captive wildlife are also of great concern. Up to one-third of zoos accredited by the American Zoo and Aquarium Association have reported at least one culture-confirmed case of paratuberculosis since 1995 (39). In addition, *M. paratuberculosis* is of interest because of its potential association with Crohn's disease in humans (19, 28, 29, 51).

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The existence and importance of wildlife reservoirs of *M. paratuberculosis* in the transmission cycle are still undetermined (17), and few investigations have examined the role of wildlife in the epidemiology of this important disease. There is much to learn about the dynamics of transmission of infection within animal populations and the involvement of specific subtypes in determining the characteristics of the infections and the velocity of their spread. A comprehensive analysis of the molecular diversity within *M. paratuberculosis* strains from various animal species will augment our understanding of the host range, distribution, and natural history of *M. paratuberculosis* infections and also aid in the development of a population genetic framework for this economically important bacterium. DNA-based subtyping techniques such as multiplex PCR for integration loci (MPIL), amplified fragment length polymorphism (AFLP) analyses, and IS*900*-based restriction fragment length polymorphism (RFLP) analyses have been used in an attempt to reveal the genetic variation in *M. paratuberculosis* and differentiate among strains infecting different populations (8, 15, 22, 43, 49, 56). However, these techniques have proved to have a limited ability to discriminate among strains.

The objective of this study was to analyze the distribution and molecular diversity of *M. paratuberculosis* strains isolated

from multiple animal species (captive and free-ranging) from different geographic zones.

MATERIALS AND METHODS

Bacterial isolates. Fecal and tissue samples from both captive and free-ranging animal species throughout the United States were submitted for radiometric mycobacterial culture (12). Samples were collected from animals suspected of infection with *M. paratuberculosis* on the basis of exposure to animals with confirmed infections or animals with clinical signs consistent with Johne's disease (weight loss and/or diarrhea). Water samples ($n = 3$) were also obtained from enclosures housing infected animals. Acid-fast isolates were tested for mycobactin J dependency (a characteristic of *M. paratuberculosis*) and evaluated for the presence of IS*900* by PCR (24). The strains in the collection represented laboratory acquisitions from zoo specimens between 1993 and 2003 (see Table 1). The isolates were assigned a geographic zone on the basis of the locations of the host animals at the time of sample collection. Zones 1 through 6 represent the northwest, southwest, north-central, south-central, northeast, and southeast regions of United States, respectively.

Freezer stocks of the mycobacterial isolates were revived in 7H9 broth (Becton, Dickinson, Sparks, Md.) for molecular identification and diversity analysis by an independent laboratory in a blinded fashion. The identities of the strains were revealed after molecular analyses were complete. Five controls including type strain ATCC 19698, a bovine *M. paratuberculosis* strain, and three deliberately mixed cultures (known to contain *M. paratuberculosis* and *M. paratuberculosis* in one culture and *M. paratuberculosis* and *M. avium* subsp. *avium* in the other two cultures) were also analyzed.

DNA extraction. Approximately 10 ml of turbid 7H9 broth culture was centrifuged at 2,500 rpm (Beckman GP; Beckman Institute, Urbana, Ill.) for 25 min to obtain a pellet. The pellet was resuspended in 500 μl of sterile deionized distilled water and used for DNA extraction by use of the QIAamp DNA Blood Mini kit (Qiagen Inc., Valencia, Calif.), with a few modifications, as described previously (43).

Molecular characterization of the isolates. DNA extracted from the broth cultures was used to confirm the identities of the subspecies by characterizing all isolates with well-defined molecular markers as described previously (43): (i) PCR amplification and hybridization for two of the integration loci of insertion sequence IS*900* regarded as diagnostically definitive for *M. paratuberculosis* (loci L1 [left side integration site of IS*900* into an unknown open reading frame] and L9 [left side integration of IS*900* into *alkA*]); (ii) PCR amplification of a recently identified unique *M. paratuberculosis* sequence (locus 251) (4, 43), and (iii and iv) *hsp65* and IS*1311* restriction endonuclease analyses for polymorphisms in the *hsp65* and IS*1311* genes, respectively, as described previously (43). PCR amplification of an additional DNA sequence that differed between the sheep and cattle types of *M. paratuberculosis* was carried out as described previously (11).

An *M. avium* subsp. *avium*-specific marker (IS*1245*) was used to distinguish the *M. paratuberculosis* and the *M. avium* subsp. *avium* isolates (27, 33). Primers were designed to amplify a 300-bp region of IS*1245* in *M. avium* subsp. *avium* that is similar to but not homologous with a closely related insertion sequence (IS*1311*) found in *M. paratuberculosis* (gene information identification number [GI], 7555405 and 4210754). The DNA samples were amplified with primers 5′-biotin-GGT CGC GTG TCC GCG TGT GG-3′ and 5′-ACT TCC CGG TGG CCC ACT GGA-3′. The primers were used at final concentrations of 0.2 μM in a 50-μl PCR master mixture that also included 1.25 U of Hotstar *Taq* polymerase (Qiagen Inc.), 1× PCR buffer, 2.5 mM $MgCl_2$, a 200 μM concentration of each deoxyribonucleoside triphosphate (Amersham Pharmacia Biotech Inc., Piscataway, N.J.), 5% dimethyl sulfoxide, and 5 μl (approximately 50 ng/μl) of genomic DNA. The thermal cycler parameters used for amplification included an initial incubation step at 95°C for 15 min to activate the Hotstar *Taq* polymerase, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s, with a final extension step at 72°C for 7 min. A microtiter plate-based reverse-phase hybridization assay was carried out with the probe 5′-CTC GCT CTG CTC GAC GTC AGT GAC CAA AGT GCC GAA AC-3′ as described previously (43, 53). A negative control consisting of the PCR master mixture alone, without genomic DNA, was included in all PCR amplifications and hybridizations. The cutoff optical density was set at 0.2 on the basis of analysis of several known negative controls and *M. avium* subsp. *avium* positive controls.

IS*900* PCR was performed as described previously (42). The PCR products were electrophoresed at 100 V for 2 h in a 1.5% agarose gel prestained with ethidium bromide and visualized on a UV transilluminator (Alpha Innotech Corporation, San Leandro, Calif.).

MPIL fingerprinting analysis. Genotyping of all isolates by MPIL fingerprinting analysis was performed by a previously established method (8, 9, 43). Briefly, a total of 28 DNA fragments representing the right and left integration sites of IS*900* were amplified in six sets of multiplex PCRs, referred as 9R1, 5R2, 4L1, 4L2, 3L3, and 3L4, respectively, as described previously (43). The PCR products were electrophoresed at 100 V for 4 h in a 2% agarose gel prestained with ethidium bromide and visualized on a UV transilluminator (Alpha Innotech Corporation). Cluster analysis was done with the Molecular Evolutionary Genetics Analysis (MEGA) program (version 2.1; www.megasoftware.net) by the unweighted pair-group method with arithmetic averages (36). The distance matrix for input into the MEGA program was created from the binary data by using the ETDIV and ETMEGA programs (http://foodsafe.msu.edu/whittam/programs/).

SSR analysis. The results of the recently described multilocus short sequence repeat (MLSSR) analysis (2) for *M. paratuberculosis* strain differentiation indicated that the mononucleotide G-residue repeat locus within the phosphatidylethanolamine-binding domain (GI, 13881618) was the most discriminatory (Simpson's diversity index, 0.7) and was selected for use for fingerprinting in this study. Amplification of this short sequence repeat (SSR) locus was carried out with primers 5′-TCA GAC TGT GCG GTA TGG AA-3′ and 5′-GTG TTC GGC AAA GTC GTT GT-3′. The amplification parameters were as described above for IS*1245* analysis. A PCR master mixture blank was included as a negative control for each batch. A total of 5 μl of the PCR product was electrophoresed at 125 V for 45 min in 1.5% agarose gels prestained with ethidium bromide and visualized on a UV transilluminator (Alpha Innotech Corporation). The PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc., Valencia, Calif.) and sequenced by using standard dye terminator chemistry, and the sequences were analyzed on an automated DNA sequencer (3700 DNA Analyzer; Applied Biosystems). All chromatograms were visually inspected, and sequences were edited with the EditSeq program (DNASTAR, Madison, Wis.) to correct ambiguities and then aligned by use of the MegAlign program (DNASTAR).

A consensus phylogenetic tree was generated with the MEGA program (version 2.1) (36) by use of a maximum-parsimony model with 1,000 bootstrap replications. Numbers were assigned to each clade to reflect the number of G residues in the polymorphic region. Simpson's and Shannon-Wiener's diversity indices were calculated by use of the equations $1 - \Sigma(\text{allele frequency})^2$ and $-1 \times \Sigma(\text{allele frequency} \times \ln \text{allele frequency})^2$, respectively (45).

RESULTS

Molecular characterization of the isolates derived from wildlife species. The results of the molecular target analyses are summarized in Table 1. An isolate was characterized as *M. paratuberculosis* if the profile was L1 and L9 positive, locus 251 positive, and IS*1245* negative and carried no *hsp65* polymorphism, as detected by restriction enzyme analysis (43). An isolate was characterized as belonging to the *M. avium-M. intracellulare* complex if the profile was L1 and L9 negative, locus 251 negative, and IS*1245* positive and carried the polymorphism in *hsp65*, resulting in a truncated band upon restriction digestion with PstI. All other acid-fast isolates that failed to amplify any of the targets were classified as mycobacteria outside the *M. avium-M. intracellulare* complex. On the basis of these classification criteria, 76 isolates were identified as *M. paratuberculosis*, 15 were identified as belonging to the *M. avium-M. intracellulare* complex, and 18 were identified as mycobacteria outside the *M. avium-M. intracellulare* complex. All *M. paratuberculosis* isolates were further classified by using the IS*1311* restriction patterns consistently identified in either cattle (two restriction sites) or sheep strains (one restriction site) (43, 57). Of the 76 isolates classified as *M. paratuberculosis*, 12 isolates from nine different animal species (Table 1) had the IS*1311* restriction profile reported for sheep strains. The 12 *M. paratuberculosis* isolates with IS*1311* profiles typical for sheep strains were further analyzed by using a recently described molecular marker to distinguish sheep and cattle isolates (11).

TABLE 1. Molecular characteristics of acid-fast strains by host and geographic locality

Animal species or sample	Zone[a]	L1–L9[b]	251[c]	IS*1245*[d]	*hsp65*[e]	IS*1311*[f]	DMC[g]	IS*900*[h]	MPIL type	G repeat[i]	Indentification	MJ dep.[j]
Transcaspian urial	2	1	1	0	1	1	−	1	A1	9Gs	MAP[k]	1
Addax	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Angolan springbok	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Angolan springbok	2	1	1	0	1	1	−	1	A18	10Gs	MAP	1
Axis deer	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bay duiker	4	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Bay duiker	4	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Bay duiker	4	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	1	1	A18	7Gs	MAP	1
Bison	3	1	1	0	1	1	1	1	A18	7Gs	MAP	1
Blesbok	2	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	1
Blesbok	2	1	1	1	1/0	1	−	1	A18	2GsC4Gs	MAP	0
British red deer	2	1	1	0	1	1	−	1	A18	13Gs	MAP	1
Chinese Reeve's muntjac	2	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	1
Chinese Reeve's muntjac	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Elk	1	1	1	0	1	1	−	1	A18	13Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	13Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	13Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	13Gs	MAP	1
Elk	3	1	1	0	1	1	−	1	A18	15Gs	MAP	1
Ellipsen waterbuck	2	1	1	1	1/0	1	−	1	A18	9Gs	MAP	0
Environmental water	2	1	1	1	1/0	1	−	1	A18	2GsC4Gs	MAP	1
Environmental water	2	1	1	1	1/0	1	−	1	A18	2GsC4Gs	MAP	0
Gemsbok	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Goat	3	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Goat	3	1	1	0	1	1	1	1	A18	7Gs	MAP	1
Impala	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Impala	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Impala	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Indian axis deer	2	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	0
Indian axis deer	2	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	1
Indian sambar	2	1	1	1	1	0	1	1	A18	2GsC4Gs	MAP	0
Key deer	6	1	1	0	1	1	−	1	A18	11Gs	MAP	1
Malayan sambar	2	1	1	1	1	1	−	1	A18	9Gs	MAP	1
Nyala	4	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Nyala	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Oryx	5	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Sheep	3	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	0
Sika	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Thomson gazelle	6	1	1	1	1	1	−	1	A18	7Gs	MAP	1
Thomson gazelle	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Turkomen markhor	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Turkomen markhor	2	1	1	1	1/0	0	1	1	A18	2GsC4Gs	MAP	1
Transcaspian urial	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Transcaspian urial	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Transcaspian urial	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Transcaspian urial	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Transcaspian urial	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Tule elk	1	1	1	0	1	1	−	1	A18	−	MAP	1
Tule elk	1	1	1	0	1	1	1	1	A18	7Gs	MAP	1
Tule elk	1	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Sitatunga	6	1	1	0	1	1	−	1	A18	7Gs	MAP	1
Waterbuck	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
Waterbuck	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
White-tailed gnu	2	1	1	0	1	1	−	1	A18	9Gs	MAP	1
White-tailed deer	5	1	1	0	1	1	−	1	A18	10Gs	MAP	1
Sheep	3	1	1	0	1	0	2	1	A18 variant	15Gs	MAP	1
Springbok	6	1	1	1	1/0	0	1	1	B4	2GsC4Gs	MAP	1
Springbok	3	1	1	1	1/0	0	1	1	B4	2GsC4Gs	MAP	1
Bison	3	1	1	0	1	1	−	1	Unique	−	MAP	1

Continued on following page

TABLE 1—*Continued*

Animal species or sample	Zone[a]	L1–L9[b]	251[c]	IS*1245*[d]	*hsp65*[e]	IS*1311*[f]	DMC[g]	IS*900*[h]	MPIL type	G repeat[i]	Indentification	MJ dep.[j]
Blesbok	2	1	1	1	1/0	0	1	1	Unique	2GsC4Gs	MAP	1
Dybowski's sika	2	1	1	0	1	1	−	1	Unique	−	MAP	1
Ellipsen waterbuck	2	1	1	1	1/0	0	1	1	Unique	2GsC4Gs	MAP	0
Sika	2	1	1	0	0	Neg	−	1	Unique	−	MAP	1
Angolan springbok	2	0	0	1	0	0	−	1	B11	2GsC4Gs	MAIC[l]	1
Bay duiker	4	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Bighorn sheep	1	0	0	1	0	Neg	−	1	B11	−	MAIC	0
Dama gazelle	3	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Dom water buffalo	2	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Eland	6	0	0	1	0	Neg	−	0	B11	−	MAIC	0
Elk	1	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Giraffe	1	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Giraffe	4	0	0	1	0	Neg	−	0	B11	2GsC4Gs	MAIC	0
Impala	6	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Munjtac	3	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Nubian ibex	2	0	0	1	0	0	−	1	B11	2GsC4Gs	MAIC	0
Sable antelope	2	0	0	1	1/0	0	−	1	B11	2GsC4Gs	MAIC	0
Spanish ibex	2	0	0	1	0	0	−	0	B11	2GsC4Gs	MAIC	0
Oryx	6	0	0	0	0	Neg	−	0	B11	−	Outside MAIC[m]	0
Armenian mouflon	2	0	0	0	0	0	−	1	B11	2GsC4Gs	Outside MAIC	0
Bison	1	0	1	0	0	Neg	−	0	B11	2GsC4Gs	Outside MAIC	0
Springbok	3	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Elk	1	0	0	0	0	0	−	0	B11	2GsC4Gs	Outside MAIC	0
Environmental water	2	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Gnu	6	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Gnu	6	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Goat	3	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Impala	6	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Indian gaur	2	0	0	0	1/0	Neg	−	1	B11	−	Outside MAIC	0
Key deer	6	0	0	0	1	Neg	−	0	B11	−	Outside MAIC	0
Macaque	3	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Thomson gazelle	1	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Thomson gazelle	6	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Toucanet bird	3	0	0	0	Neg	Neg	−	0	B11	−	Outside MAIC	0
Unknown	6	0	0	0	0	Neg	−	0	B11	7Gs	Outside MAIC	0
White-tailed deer	5	0	0	0	0	Neg	−	0	B11	−	Outside MAIC	0
Transcaspian urial	2	0	1	1	0	0	−	0	Unique	2GsC4Gs	MAIC	0
Control (ATCC 19698)	3	1	1	0	1	1	−	1	A18	11Gs	MAP	1
Control (bovine MAP)	3	1	1	0	1	1	−	1	A18	10Gs	MAP	1

[a] Zones 1 through 6 represent the northwest, southwest, north-central, south-central, northeast, and southeast regions of United States, respectively.
[b] L1–L9, PCR amplification and hybridization for IS*900* integration loci L1 (left side integration site of IS*900* into an unknown ORF) and L9 (left side integration of IS*900* into *alk*A); 1, positive; 0, negative.
[c] 251, PCR amplification of *M. paratuberculosis* unique sequence locus 251; 1, positive; 0, negative.
[d] IS*1245*, PCR amplification and hybridization for a region of IS*1245* deleted from *M. paratuberculosis*; 1, positive; 0, negative.
[e] *hsp65*, restriction endonuclease analysis for polymorphisms in the *hsp65* gene (20, 21); 1, no restriction site; 0, one restriction site.
[f] IS*1311*, restriction endonuclease analysis for polymorphisms in IS*1311* gene; 0, two restriction sites; 1, three restriction sites; neg, no amplification.
[g] DMC, a molecular marker based on a recently identified polymorphism used to differentiate cattle and sheep strains (11); —, not analyzed.
[h] IS*900*, IS*900* PCR amplification; 1, positive; 0, negative.
[i] G repeat, amplification and sequencing of a locus containing variable numbers of G-residue repeats (2); −, amplification or sequencing unsuccessful.
[j] MJ dep., mycobactin J growth dependence; 1, growth is dependent on mycobactin J supplementation; 0, growth is not dependent on mycobactin J supplementation.
[k] MAP, *M. paratuberculosis*.
[l] MAIC, *M. avium-M. intracellulare* complex.
[m] Outside MAIC, mycobacteria outside *M. avium-M. intracellulare* complex.

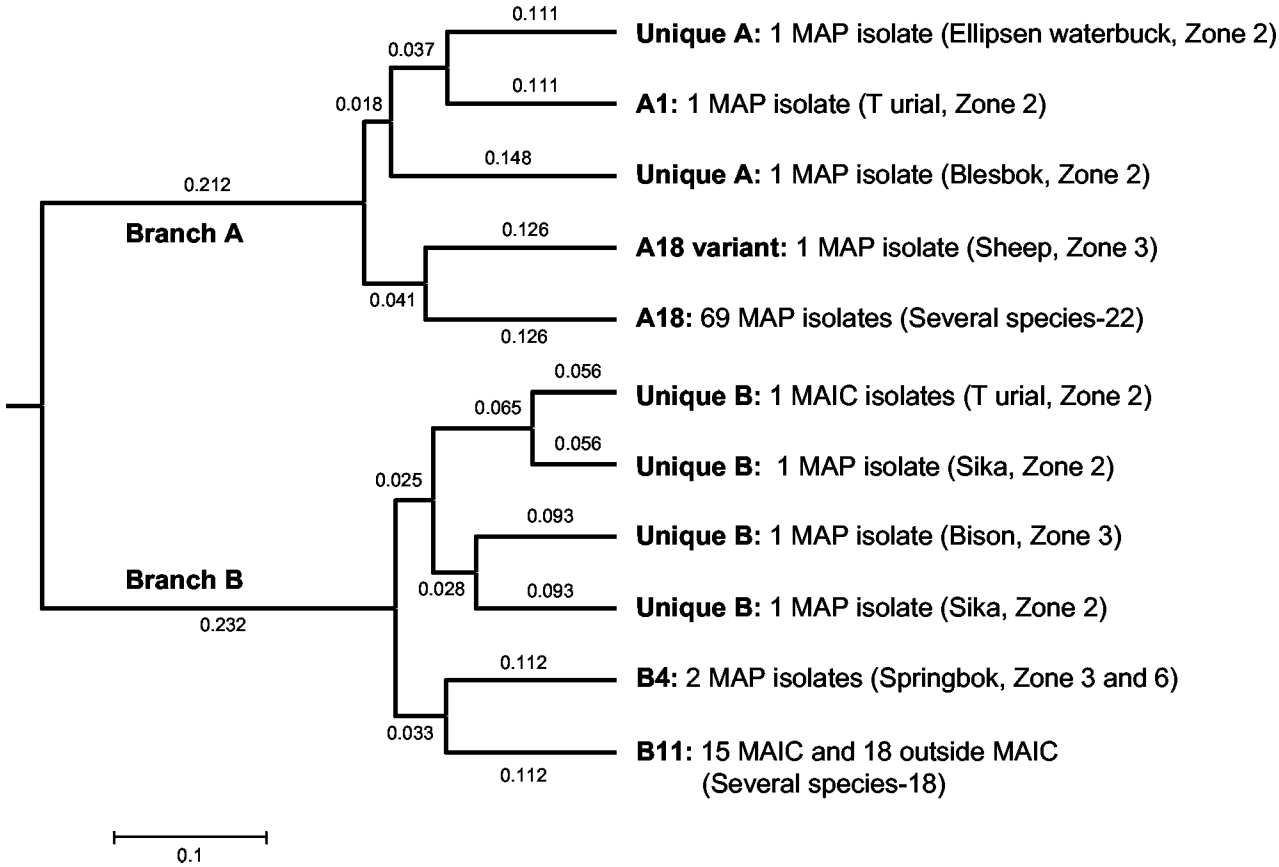

FIG. 1. Dendrogram showing the distribution of MPIL fingerprints. The assigned fingerprints, isolate identities, and host species are shown. The proportion distance of each cluster is also indicated. MPIL analysis had a Simpson's diversity index of 0.533, indicative of a limited degree of strain discrimination capability. Shannon's diversity index was 1.067. MAP, *M. paratuberculosis*; MAIC, non-*M. paratuberculosis M. avium-M. intracellulare* complex; Outside MAIC, mycobacteria outside the *M. avium-M. intracellulare* complex.

That analysis identified 10 isolates derived from several host species with the genotype reported for cattle strains. Of the other two sheep isolates, one had a cattle strain genotype, which indicated that it was a variant. Non-*M. paratuberculosis M. avium-intracellulare* complex isolates (n = 12) and those outside the *M. avium-M. intracellulare* complex (n = 2) also had the IS*1311* profile typical of sheep strains. The IS*1311* region targeted failed to be amplified from 3 *M. avium-M. intracellulare* complex isolates and 16 mycobaterial isolates outside the *M. avium-M. intracellulare* complex. All the blinded controls, including the deliberately mixed cultures, were accurately identified by these molecular analyses.

All isolates classified as *M. paratuberculosis* by molecular analysis were positive by the IS*900* PCR. Additionally, four isolates classified as belonging to the *M. avium-M. intracellulare* complex and two isolates classified as mycobacteria outside the *M. avium-M. intracellulare* complex were positive by the IS*900* PCR.

Seven of the isolates classified as *M. paratuberculosis* by independent molecular methods as well as by IS*900* PCR were apparently not mycobactin J dependent. Two isolates (one non-*M. paratuberculosis M. avium-M. intracellulare* complex isolate and one non-*M. avium-M. intracellulare* complex isolate) were positive for locus 251 by PCR. Sequence analysis of the locus 251 amplicon from the *M. avium-M. intracellulare* complex isolate showed 100% homology to the published sequence (GI, 20152939).

Fifteen of the *M. paratuberculosis* isolates were also positive for the *M. avium* subsp. *avium*-specific marker, IS*1245*. Since attempts were not made to isolate single colonies, it is possible that the *M. paratuberculosis* repository stocks contained a low level of DNA of a non-*M. paratuberculosis* strain whose genetic material was masked for molecular typing purposes by the *M. paratuberculosis* isolate in the aliquot.

MPIL fingerprinting analysis. All isolates studied (n = 109) and two *M. paratuberculosis* control isolates (ATCC 19698 and a field isolate from a cow) were fingerprinted by MPIL analysis. Cluster analysis organized the fingerprints into 30 electrophoretic types. Electrophoretic types that were separated by distances less than 0.1 unit were assigned identical MPIL fingerprints (Fig. 1). The fingerprint nomenclature was based on a previously defined set of *M. paratuberculosis* MPIL fingerprints in our collection (43). Both data sets were merged for cluster analysis and fingerprint assignment. The analysis divided the isolates into two major branches. Ninety-four percent (73 of 76) of the *M. paratuberculosis* isolates from several species clustered in branch A and could be further divided into A18 (n = 69), A18 variant (n = 1), A1 (n = 1), and unique

branch A ($n = 2$) fingerprints. Branch A exclusively clustered only *M. paratuberculosis* isolates. All non-*M. paratuberculosis M. avium-M. intracellulare* complex isolates ($n = 15$) clustered into branch B, with the fingerprints designated B11 ($n = 14$) and unique ($n = 1$). Mycobacterial isolates outside the *M. avium-M. intracellulare* complex ($n = 18$) did not amplify any target and were clustered into branch B, with the fingerprints designated B11. Five *M. paratuberculosis* strains derived from a bison, springbok ($n = 2$), and sika ($n = 2$) also clustered in branch B, with the fingerprints designated B4 ($n = 2$) and unique B ($n = 3$). The Simpson's and Shannon-Wiener's diversity index values for the MPIL fingerprint analysis were 0.533 and 1.067, respectively, indicating limited discriminatory capacity.

SSR analysis. A region within the *M. paratuberculosis* genome that carries various numbers of G residues was amplified from all isolates. Only isolates ($n = 91$) with a detectable 408-bp amplification product were sequenced for further analysis. These included *M. paratuberculosis* ($n = 72$), non-*M. paratuberculosis M. avium-M. intracellulare* complex organisms ($n = 12$), organisms outside the *M. avium-M. intracellulare* complex ($n = 5$), and control *M. paratuberculosis* strains (ATCC 19698 and a field isolate from a cow). Of the 72 *M. paratuberculosis* strains analyzed, 66 had the A18 MPIL fingerprint, while 2 carried unique fingerprints. The remaining four isolates carried the A1, B4, and B11 ($n = 2$) fingerprints. Both of the control *M. paratuberculosis* isolates displayed the A18 fingerprint. Two reference strains (*M. paratuberculosis* K10 and a bovine *M. paratuberculosis* isolate [2]) were also included in the cluster analysis. Each allele was assigned a number congruent with the number of G-residue repeats. A total of eight alleles with 7 to 20 G-residue repeats (7Gs to 20Gs, respectively) were identified among 76 *M. paratuberculosis* isolates (including control and reference strains). Thirty-eight percent of the *M. paratuberculosis* isolates ($n = 28$) had the 7Gs repeat fingerprint. This fingerprint clustered all the bison ($n = 7$), impala ($n = 3$), nyala ($n = 2$), Thomson gazelle ($n = 2$), and goat ($n = 2$) *M. paratuberculosis* strains included in this analysis. This allele also clustered 8 of the 13 *M. paratuberculosis* isolates obtained from elks. The 7Gs alleles were also found in one *M. paratuberculosis* isolate each from an addax, axis deer, oryx, and sitatunga and in one mycobacterial isolate outside the *M. avium-M. intracellulare* complex from an unknown source. Alleles with 9Gs clustered all of the bay duiker ($n = 3$), all of the Transcaspian urial ($n = 6$), and three of the four waterbuck *M. paratuberculosis* isolates. It also included one *M. paratuberculosis* isolate from each of the following: springbok, munjtac, gemsbok, sambar, sika, markhor, and gnu. Alleles with 10Gs were found in *M. paratuberculosis* isolates from the bovine control, springbok, key deer, and white-tailed deer. Alleles with 11Gs were found in *M. paratuberculosis* type strain ATCC 19698 and another key deer isolate. Alleles with 13Gs were found in *M. paratuberculosis* strains isolated from elk ($n = 4$) and a British red deer ($n = 1$). Alleles with 15Gs were found in *M. paratuberculosis* isolates from an elk and a sheep. Isolates with more than 15Gs were clustered into an allele designated >15Gs. Isolates with this allele included the reference strain *M. paratuberculosis* K10, with 20Gs, and a bovine *M. paratuberculosis* isolate, with 17Gs. Allele 2GsC4Gs, in which a C residue replaced the third G residue within the repeat, was also identified. All non-*M. paratuberculosis M. avium-M. intracellulare* complex isolates ($n = 12$), four of five non-*M. avium-M. intracellulare* complex isolates, and some *M. paratuberculosis* strains ($n = 13$) carried this fingerprint. The species whose isolates included this allele are listed in Table 1. The phylogenetic analysis thus divided the *M. paratuberculosis* isolates ($n = 66$) with the A18 fingerprint into seven distinct alleles (Fig. 2). The Simpson's and Shannon-Wiener's diversity index values for the G-residue-repeat analysis were 0.751 and 1.593, respectively, indicating a relatively robust discriminatory capability.

DISCUSSION

Paratuberculosis has been well documented in a majority of domestic ruminant species. It has gained importance in the animal production industry because of the economic losses incurred from herd infections and possible human health hazards associated with *M. paratuberculosis* (3, 29, 51). *M. paratuberculosis* has also been recovered from many captive and free-ranging nondomestic animal species representing virtually all pseudoruminants and ruminants except giraffids (37, 38). The known host range of *M. paratuberculosis* has recently been extended to include nonruminant wildlife species, such as primates (41, 60), wild rabbits (25), and foxes and stoats (6). These reports support the contention that *M. paratuberculosis* has a wide host range and that disease caused by this organism may have an epidemiology more complex than was previously known.

One of the strategies for the control and eradication of paratuberculosis in an infected herd is to eliminate the transmission of *M. paratuberculosis* to susceptible animals. The presence of a wildlife reservoir with the potential to transmit the infection to domestic animals may affect the success of domestic agriculture control programs. Although the frequency of transmission from wildlife to domestic animals has not been documented, several reports suggest that infection may be spread from domestic animals to wildlife (13, 25). Knowledge of the extent of strain sharing across different host species is vital to understanding the dynamics of *M. paratuberculosis* transmission. Methods for differentiation or subtyping of bacterial strains provide important information for molecular epidemiological analyses and help provide an understanding of the population genetics of the organism. This is the first report of a comprehensive molecular analysis conducted to establish the degree of similarity or heterogeneity in *M. paratuberculosis* isolates from taxonomically and spatially diverse host species.

Definitive identification of *M. paratuberculosis* requires confirmation with multiple molecular markers. Present methods for the diagnosis of *M. paratuberculosis* infection include isolation of the organism from fecal and tissue specimens, antibody detection by enzyme-linked immunosorbent assay, and IS*900*-based PCR (55). The culture protocol used at present relies on mycobactin J dependency as a confirmatory test. Our analysis identified seven *M. paratuberculosis* isolates (9%) that were not dependent on mycobactin J for growth. Mycobactin J dependency was determined by two serial subcultures on slants with and without mycobactin J supplementation for each of the seven *M. paratuberculosis* isolates. Although attempts were not made to isolate single colonies, all subcultures were carried out with inocula from slants without mycobactin J to rule out

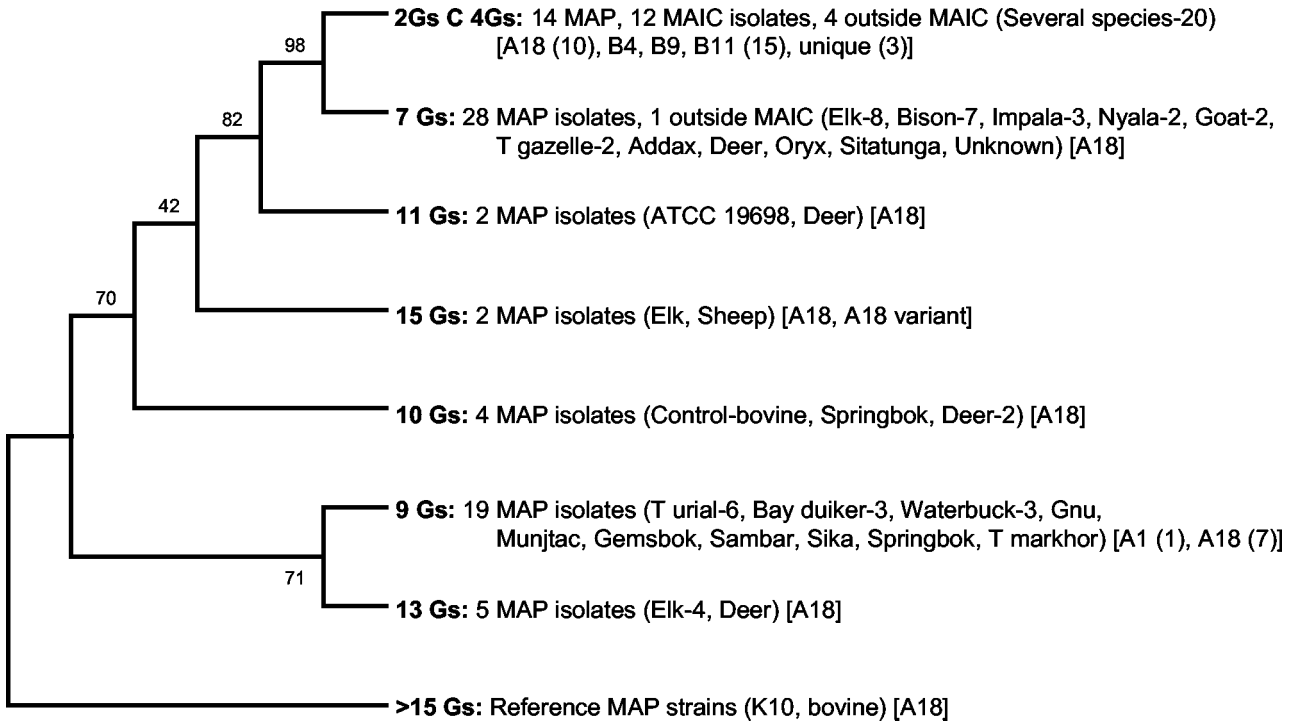

FIG. 2. Phylogenetic tree showing the distribution of strains by the numbers of G-residue repeats. The numbers of G-residue repeats, isolate identities, host species, and major fingerprints obtained by MPIL analysis are shown. Also shown at the clade origins are the bootstrap values generated by 1,000 replications in a maximum-parsimony model. SSR analysis had a Simpson's diversity index of 0.751, indicative of a high degree of strain discrimination capability. Shannon's diversity index was 1.593. MAP, *M. paratuberculosis*; MAIC, non-*M. paratuberculosis M. avium-M. intracellulare* complex; Outside MAIC, mycobacteria outside the *M. avium-M. intracellulare* complex.

potential mycobactin J carryover. This discrepancy indicates that diagnostic tests that rely on mycobactin J dependency alone need to be interpreted with caution.

IS*900*-based PCR identification techniques have routinely been used for the detection of *M. paratuberculosis* (5, 30, 58). However, IS*900*-like elements have been found in *M. avium* subsp. *avium* isolates (44) and in some isolates outside the *M. avium-M. intracellulare* complex (14, 20, 34). In the present study, four isolates that were classified within the *M. avium-M. intracellulare* complex but that were not *M. paratuberculosis* and two isolates classified outside the *M. avium-intracellulare* complex by extensive molecular analyses were identified as *M. paratuberculosis* by IS*900* PCR. Only one of these isolates was apparently mycobactin J dependent. This implies that the detection of *M. paratuberculosis* based on detection of IS*900* alone needs to be evaluated with caution to avoid false-positive results.

Although a previous analysis indicated that locus 251 is unique to *M. paratuberculosis* (43), an *M. avium-M. intracellulare* complex isolate amplified a PCR product of the expected size and sequence analysis revealed 100% homology of that product to the published sequence of locus 251 (GI, 20152939). These discrepancies clearly illustrate the need for multiple molecular markers for confirmation of the identity of *M. paratuberculosis* as the cause of infection. Molecular characterization of acid-fast mycobacterial isolates by use of three specific markers and two polymorphic sites aided in the accurate classification of the isolates. In addition, strains that lacked mycobactin J dependency or that were positive by IS*900* PCR or locus 251 PCR could be correctly identified on the basis of the presence or the absence of multiple molecular targets.

Molecular diversity analysis of *M. paratuberculosis*. Several attempts have been made to identify genetic variation and host specificity in *M. paratuberculosis* strains isolated from different animal species. Until recently, IS*900* has been the marker of choice for most fingerprinting studies that have been reported (5, 15, 50, 57). While the IS*900*-based RFLP analyses are fairly good at discriminating between cattle and sheep *M. paratuberculosis* strains, *M. paratuberculosis* strains from cattle and other hosts such as goats and rabbits are indistinguishable by this method (5, 25, 49). A recent study in our laboratory (43) by alternate fingerprinting techniques, MPIL and AFLP, demonstrated clustering of 73 and 56% of the *M. paratuberculosis* isolates, respectively, from several hosts (cattle, sheep, goats, mice, deer, and humans). These results were consistent with the hypothesis that there is a relatively small amount of genetic heterogeneity between *M. paratuberculosis* isolates obtained from different host species.

Many measures of diversity have been proposed, but those that are most commonly used are Simpson's and Shannon-Wiener's indices. Simpson's diversity index represents the probability that two individual isolates randomly selected from a sample will belong to different species and accounts for both the richness (diversity) and the proportion (percent) of each species. Simpson's diversity indices for RFLP analysis (n = 1,008) (50), MPIL analysis (n = 247) (43), and AFLP analysis (n = 104) (43) are 0.559, 0.597, and 0.711 respectively. The higher diversity index for AFLP analysis indicates that AFLP

analysis offers a better discriminatory ability. However, the clustering by AFLP analysis was random with respect to host species and geographic location and hence was not informative (43). In addition, AFLP analysis is technically demanding, and band profiles cannot be interpreted in terms of loci and alleles.

Restricted diversity among *M. paratuberculosis* isolates is also revealed by MPIL analysis. In the present study, MPIL fingerprinting analysis separated the *M. paratuberculosis* and *M. avium-M. intracellulare* complex strains into two major clusters (Fig. 1). Within the *M. paratuberculosis* cluster, the A18 fingerprint predominated, with 88% of the *M. paratuberculosis* isolates having the A18 fingerprint. The other *M. paratuberculosis* strains exhibited fingerprints designated A1 ($n = 1$), A18 variant ($n = 1$), B4 ($n = 2$), and unique ($n = 5$). MPIL cluster analysis had a Simpson's diversity index of 0.533 and a Shannon-Wiener's diversity index of 1.067, indicating that the MPIL technique has a limited degree of strain discrimination capability.

A previous study has shown that nine of the MPIL types correspond to a distinct PstI and BstEII RFLP types (8). This supports the idea that both MPIL and RFLP analyses address the same genetic variation and suggests that MPIL typing may be used as a substitute for RFLP typing. Hence, we predict that RFLP analysis of these isolates would result in similarly indistinguishable fingerprints. The results of MPIL analysis suggest that only a few *M. paratuberculosis* strains may be responsible for the widespread dissemination of infection across a variety of species. Conversely, the methods available at present lack sufficient sensitivities for the differentiation of *M. paratuberculosis* strains. We thus evaluated the possibility of resolving the A18 fingerprint using an alternate fingerprinting technique.

SSR sequencing enables high-resolution subtyping of *M. paratuberculosis* isolates from domestic and wild animal species. Restricted allelic variation in mycobacteria is well established (54), and given the small genome size (4.83 Mbp), the expected frequency of polymorphism in *M. paratuberculosis* is low (18). However, there may be specific regions within the genome that have higher rates of polymorphisms. If present, these regions could be used to genotype the organisms. Recent studies (16, 52) with *Bacillus anthracis*, a similarly monomorphic organism, demonstrated the usefulness of single nucleotide polymorphisms, variable-number tandem repeats (VNTRs), and inserted or deleted sequences in discriminating strains within the species. VNTRs or SSRs are generated through natural events such as recombination and slipped-strand mispairing during replication (31). These have successfully been used as markers to understand the clonalities and distributions of subtypes in several bacterial species, such as *Mycobacterium tuberculosis* (23), *Yersinia pestis* (1), and *B. anthracis* (32, 35).

The recent completion of the whole-genome sequence of *M. paratuberculosis* strain K10 (L. L. Li et al., unpublished data) allowed the identification of several SSRs in the genome. Eleven of these highly polymorphic SSRs were used in a composite MLSSR analysis (2) for *M. paratuberculosis* strain differentiation. The results indicated that a mononucleotide G-residue-repeat locus within the phosphatidylethanolamine-binding domain (GI, 13881618) was the most discriminatory (Simpson's diversity index, 0.7) and was selected for fingerprinting in this study.

The G-residue-repeat fingerprinting analysis reported here had a Simpson's diversity index of 0.751, indicative of a comparatively higher degree of strain discrimination capability. Shannon-Wiener's diversity index was 1.593. Phylogenetic analysis of G-residue-repeat sequences divided the *M. paratuberculosis* isolates from the A18 cluster ($n = 66$) into seven different alleles (Fig. 2).

SSR analysis provides strong evidence of interspecies strain transmission and host specificity among isolates of *M. paratuberculosis*. Interestingly, there appeared to be a relation between allele type and host species. For example, all the bison ($n = 7$), impala ($n = 3$), nyala ($n = 2$), Thomson gazelle ($n = 2$), and goat ($n = 2$) *M. paratuberculosis* isolates included in this analysis clustered into allele 7Gs. Similarly, the allele designated 9Gs clustered all of the bay duiker ($n = 3$), all of the Transcaspian urial ($n = 6$), and three of the four waterbuck *M. paratuberculosis* isolates. The elk isolates were divided between two alleles: 7Gs ($n = 8$) and 13Gs ($n = 4$). One of the elk isolates carried the 15Gs allele. This finding is significant, since all previous analyses, in which a variety of fingerprinting techniques were used, have failed to find any association between the fingerprint type and the host species (43, 48, 50). Concordance analysis of the G-residue-repeat alleles from each geographic zone showed no correlation between fingerprint types and geographic zones. Although many of the *M. paratuberculosis* isolates from the same host species were from the same geographic zone, kappa analysis for the concordant pairs clearly indicated that the association between allele type and host species was not due to an origin in a similar geographic region. This is not surprising, since many of the captive animals may have been born in geographic zones different from the ones in which the samples were collected. That is, they likely acquired the *M. paratuberculosis* infection in the first 6 months of life but had been transferred to another facility in a different zone as an adult. Animal movement among institutions is common in the zoo industry. The presence of multiple strains within a single facility suggests that more than one source for the organism (and, presumably, more than one source for the hosts) contributed to the infections in the animal collection. While the presence of multiple strains within a facility suggested that the isolates might have originated from diverse locations, the consistency of identical genotypes within host species suggests that transmission may have occurred within facilities because animals are housed in exhibits as single species or herds.

All *M. paratuberculosis* ($n = 30$) isolates from geographic zone 2 were acquired at the same time from the same animal facility during an apparent Johne's disease outbreak. Fingerprinting by MPIL analysis showed the presence of at least five distinct banding patterns: A18, B11, and unique ($n = 3$). Similarly, SSR analysis identified the presence of multiple strains: strains with the 9Gs, 10Gs, 13Gs, and 2GsC4Gs alleles. This clearly indicates the involvement of more than one *M. paratuberculosis* strain in the outbreak. This suggests the acquisition of infection from different sources. Alternately, multiple genotypes could indicate the occurrence of strains with restricted host ranges.

G-residue-repeat analysis clustered the non-*M. paratuberculosis M. avium-M. intracellulare* complex isolates ($n = 12$) and those outside the *M. avium-M. intracellulare* complex ($n = 4$)

into a group with one common allele, the 2GsC4Gs allele. This group also contained 14 *M. paratuberculosis* isolates from several species. We noted that all 11 *M. paratuberculosis* isolates (Table 1) had sheep strain-like IS*1311* restriction profiles, suggesting that isolates with the sheep strain genotypes are likely to be ancestral to those that are of the bovine strain genotype. The three *M. paratuberculosis* isolates from environmental sources ($n = 2$) and a blesbok that clustered into this fingerprint had IS*1311* restriction profiles consistent with those of the cattle strains. An *M. paratuberculosis* isolate from a sheep with an A18 variant fingerprint was the only isolate that did not carry the 2GsC4Gs fingerprint. This indicates that these *M. paratuberculosis* isolates were phylogenetically closer to the *M. avium* complex strains than the cattle strain genotypes. Together the analyses suggest that the sheep strains may have emerged before the bovine host specialists.

Concluding comments. Our analyses document a relationship between fingerprint types and host species. The results also provide strong evidence that SSR analysis with G-residue-repeat sequences can be used to study strain sharing and interspecies spread. The approach can also be applied to investigate the role of multiple or single dominant strains in the dynamics of transmission of *M. paratuberculosis* infections within and between domestic ruminants and wildlife reservoirs. A similar analysis could potentially be used to illustrate the presence or the lack of an association of the human and animal strains. This will allow us to bridge the gap in our understanding of the epidemiology and evolution of *M. paratuberculosis*, which will subsequently lead to more targeted and more robust control strategies.

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