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Novel Single Nucleotide Polymorphism-Based Assay for Genotyping *Mycobacterium avium* subsp. *paratuberculosis*

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Typing of *Mycobacterium avium* subspecies *paratuberculosis* strains presents a challenge, since they are genetically monomorphic and traditional molecular techniques have limited discriminatory power. The recent advances and availability of whole-genome sequencing have extended possibilities for the characterization of *Mycobacterium avium* subspecies *paratuberculosis*, and whole-genome sequencing can provide a phylogenetic context to facilitate global epidemiology studies. In this study, we developed a single nucleotide polymorphism (SNP) assay based on PCR and restriction enzyme digestion or sequencing of the amplified product. The SNP analysis was performed using genome sequence data from 133 *Mycobacterium avium* subspecies *paratuberculosis* isolates with different genotypes from 8 different host species and 17 distinct geographic regions around the world. A total of 28,402 SNPs were identified among all of the isolates. The minimum number of SNPs required to distinguish between all of the 133 genomes was 93 and between only the type C isolates was 41. To reduce the number of SNPs and PCRs required, we adopted an approach based on sequential detection of SNPs and a decision tree. By the analysis of 14 SNPs *Mycobacterium avium* subspecies *paratuberculosis* isolates can be characterized within 14 phylogenetic groups with a higher discriminatory power than mycobacterial interspersed repetitive unit–variable number tandem repeat assay and other typing methods. Continuous updating of genome sequences is needed in order to better characterize new phylogenetic groups and SNP profiles. The novel SNP assay is a discriminative, simple, reproducible method and requires only basic laboratory equipment for the large-scale global typing of *Mycobacterium avium* subspecies *paratuberculosis* isolates.

Mycobacterium avium subspecies *paratuberculosis* causes Johne's disease, a chronic infectious enteritis principally of ruminants. The disease occurs worldwide and is responsible for significant losses to the livestock industry. *M. avium* subspecies *paratuberculosis* also has been detected in a subset of human patients with Crohn's disease (1), although the zoonotic role of the bacterium remains controversial.

Strain typing is a prerequisite for tracing the sources of infection and for studying the epidemiology, population structure, and evolutionary relationships between isolates. It can also reveal the genetic diversity underlying important phenotypic characteristics, such as host specificity, pathogenicity, antibiotic resistance, and virulence. Typing of *M. avium* subspecies *paratuberculosis* strains presents a challenge, since *M. avium* subspecies *paratuberculosis*, like *Mycobacterium tuberculosis*, is genetically monomorphic (2). Genetic diversity among *M. avium* subspecies *paratuberculosis* strains has been investigated using molecular techniques, such as restriction fragment length polymorphism (RFLP) and IS*900* analysis (IS*900* RFLP) (3), pulsed-field gel electrophoresis (PFGE) (4), amplified fragment length polymorphism (AFLP) analysis (5), random amplified polymorphic DNA (RAPD) analysis (6), mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis (7), and short-sequence repeat (SSR) analysis (8). However, these techniques have limited discriminatory power when applied to *M. avium* subspecies *paratuberculosis*; although this power can be increased by combining complementary genotyping techniques, it is often insufficient for accurately determining relationships among isolates or global epidemiological studies (9, 10).

Whole-genome sequencing (WGS) provides the ultimate resolution of isolates, and, unlike the techniques listed above, it can provide a phylogenetic context to facilitate global epidemiology studies and affirm epidemiological connections (10, 11, 12). Although WGS is becoming cheaper, it is still too expensive to be used for routine genotyping of *M. avium* subspecies *paratuberculosis* isolates and requires robust data handling and analysis processes. Single nucleotide polymorphisms (SNPs) have been used successfully to type several genetically monomorphic pathogens, including *M. tuberculosis* (13), *Mycobacterium bovis* (14), *Salmonella enteritica* serovar Typhi (15), and *Yersinia pestis* (16). SNP assays have been used to discriminate between *M. avium* subspecies *paratuberculosis* strain types I, II, and III (17, 18) and between strains derived from animal and human hosts (19). However, these assays were based on a limited number of SNPs, many of which were not informative when applied to a wider wild-type population. The purpose of this study was to develop an SNP assay that is discriminative, practicable, and reproducible for

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the large-scale global typing of *M. avium* subspecies *paratuberculosis* isolates. Hence, we developed an SNP typing method based on PCR and restriction enzyme digestion which would minimize costs and require only basic laboratory equipment. Additionally, we adopted an approach based on sequential detection of SNPs and a decision tree to reduce the number of SNPs and PCRs required.

MATERIALS AND METHODS

Selection of genome sequences and *M. avium* subspecies *paratuberculosis* strains. Genome sequences from 133 *M. avium* subspecies *paratuberculosis* isolates generated in a previous study (10) were selected for SNP and phylogenetic analyses. This panel was chosen to maximize genetic diversity and reduce phylogenetic discovery bias (2), since it comprised isolates with different genotypes (determined by multiplex PFGE and MIRU-VNTR), which were selected from 17 different countries and isolated from 8 different host species. The panel also included isolates representing the major strain types that have been described previously (reviewed by 20, 21). The sequence reference numbers are given in Fig. 1 and 2. The field isolates ($n = 26$) for which genome sequence data were not available and that were used to validate the SNP assay are shown in Table 1.

Preparation of genomic DNA. Genomic DNA from the United Kingdom field isolates ($n = 10$) (Table 1) was extracted from plugs used to perform the PFGE analysis. Briefly, half of each plug was washed three times with 2 ml of Tris EDTA buffer (pH 8) (10 mM Tris HCl, 1 mM EDTA) for 10 min with shaking. After washing the plugs, 0.5 to 1 ml of sterile deionized water was added, and the agarose was melted at 70°C. The suspension was stored at −20°C until required for PCR. DNA from the Portuguese field isolates ($n = 16$) (Table 1) was extracted from pure cultures grown in Middlebrook 7H9 medium supplemented with 10% oleic albumin dextrose catalase (OADC) and 2 mg/liter of mycobactin, as previously described (10), using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions, with minor modifications. Briefly, the bacterial suspension was centrifuged at 5,000 × *g* for 10 min, and the pellet was resuspended in 180 μl of ATL buffer. Zirconium beads (0.1 mm) were added to the mixture, and mechanical disruption of the cells was performed twice with a FastPrep FP120 Bio101 bead shaker (Savant Instruments, Inc., Holbrook, NY) at 6.5 ms^{-1} for 45 s. The disrupted mixture was cooled on ice for 15 min, and 20 μl of proteinase K was added. The remaining procedure was completed according to the manufacturer's instructions. The DNA was eluted with 200 μl of AE buffer and stored at −20°C until required for PCR.

SNP analysis and phylogenomics. The genome sequence of *M. avium* subspecies *paratuberculosis* K10 (22) (GenBank accession number NC_002944.2) was used as the reference genome for the phylogenetic analyses. Raw genome sequence data for the *M. avium* subspecies *paratuberculosis* isolates are available from the European Nucleotide Archive under accession number PRJEB2204. The assembly of the reads into contigs was performed using the Velvet assembler program (freely available at https://www.ebi.ac.uk/~zerbino/velvet/), and the alignment of the sequences and positioning of SNPs were executed using the MUMmer package (freely available at http://mummer.sourceforge.net/) with *M. avium* subspecies *paratuberculosis* K10 as the reference sequence. SNPs were then extracted and concatenated, and a phylogenetic tree was calculated using phyML (freely available at http://atgc.lirmm.fr/phyml). This phylogenetic tree was then imported into R using the ade4 package to explore the paths which exist between the genomes. These paths are a description of the strains which group on the same branch as the structure of the phylogenetic tree descends (i.e., all strains are included on the root branch, which then bifurcates to include a subset of strains on one branch and the remaining strains on the other, and so on). Using these paths to assign groups of genomes, SNPs which were shared by all strains on each branch were compiled. These data then allowed comparison of the SNPs present in reducing sized groups of genome sequences. (As the tree descends through specific branches, the number of genomes remaining in each subsequent group becomes progressively smaller.) These data permitted the detection of discriminating SNPs which are present in one group of sequences yet absent in others. A set cover analysis was then performed on the data set to calculate the minimum number of SNPs which were required to discriminate between the groups of strains at each selected level of the tree (see below). These SNPs were then selected and taken forward for validation, as described below. SNPs were named according to their base positions in the revised *M. avium* subspecies *paratuberculosis* K10 genome sequence.

Set cover approximation and decision tree construction. The set cover problem asks, given a universe of elements to be covered (in this case, the universe was a series of pairwise combinations of all genome groups) and a number of sets which contain various combinations of the elements to be covered (in this case, these sets are specific SNPs which are found to discriminate between one or more of the pairwise combinations of genome groups), what is the minimum number of sets required to cover all the elements of the universe. In this case, the set cover asks the following: what is the minimum number of SNPs required to fill all the pairwise combinations of genome groups? In this way, the defined set of SNPs would discriminate between every possible pairwise combination of genome groups. The set cover problem is a class of decision problem known to be NP-complete, meaning the time taken to reach an exact solution increases exponentially with problem size. Due to the large number of total SNPs and genome groups to consider, we opted to use an approximation algorithm known as the greedy solution to estimate the set cover. Greedy algorithms work by iteratively selecting the set which covers the largest number of uncovered universe elements. The greedy approach is considered a suitable polynomial time approximation for the set cover problem. This analysis resulted in the identification of a set of SNPs which could distinguish between all pairwise combinations of genome groups.

The chosen SNPs which comprised the set cover formed the basis of the decision tree for determining group assignment of genomes, since every bifurcation of the phylogenetic tree could be discriminated by at least one SNP present in the set cover. The structure of the phylogenetic tree was then manually investigated in light of the SNPs within the set cover to minimize the number of SNPs required to determine between phylogenetically relevant branches. These SNPs were then validated, as outlined below.

SNP validation and primer design. Primers for PCR amplification of the selected SNPs were designed using the online software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and the revised *M. avium* subspecies *paratuberculosis* K10 genome sequence (23) (GenBank accession number AE016958.1) (Table 2). Primers were designed to be 18 to 20 mer, with a melting temperature between 63°C and 67°C.

PCRs were carried out in 50 μl containing 200 μM each deoxynucleotide triphosphate (Invitrogen), 0.5 μM each primer (Table 2), 1 U of Phusion high-fidelity DNA polymerase, 1× Phusion GC buffer (New England BioLabs), and 4 μl of extracted DNA (5 ng/μl). Amplification was performed in a TC-Plus Thermal cycler (Techne), with an initial step at 98°C for 3 min, followed by 35 cycles at 98°C for 30 s, 63°C to 67°C for 30 s (annealing temperatures provided in Table 2), and 72°C for 40 s, and ending with a step at 72°C for 10 min. The amplified products were electrophoretically analyzed in a 1.5% (wt/vol) agarose gel stained with SYBR safe DNA gel stain (Life Technologies) in 0.5× Tris-borate-EDTA (TBE) buffer. Gel electrophoresis images were acquired with an Alphaimager 2200 (Alpha Innotech). DNA ladder IV (Bioline) and *M. avium* subspecies *paratuberculosis* K10 (positive control) were included on each gel.

Restriction endonuclease analysis of PCR products was performed according to the manufacturer's instructions, using 1 unit of restriction enzyme and 10 μl of amplified product in a total reaction volume of 25 μl. All restriction endonucleases were purchased from New England BioLabs (Table 2). Restricted products were detected by electrophoresis on 1.5% (wt/vol) agarose gels, as described above.

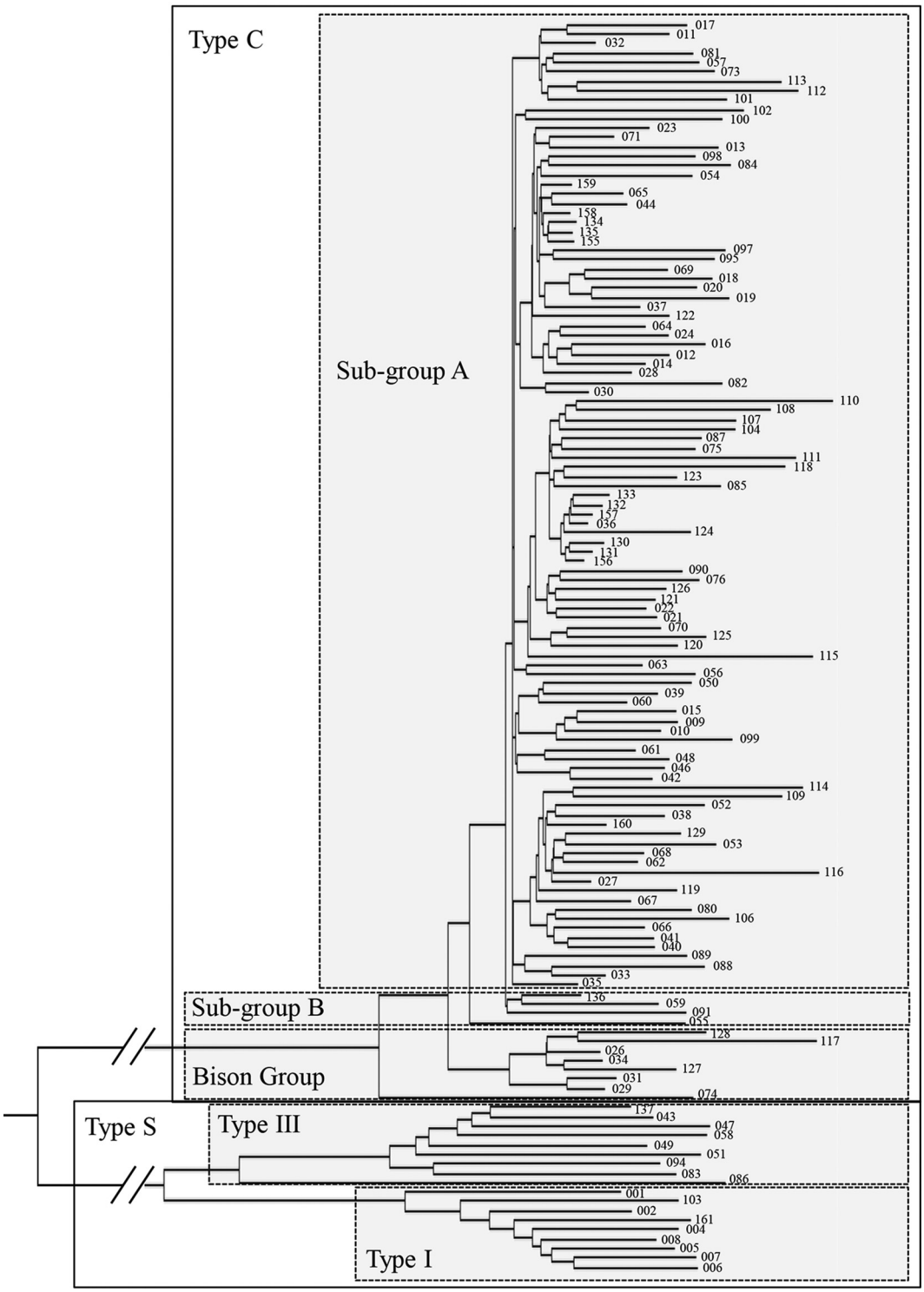

FIG 1 Whole-genome SNP-based phylogenetic tree of 133 *M. avium* subspecies *paratuberculosis* isolates included in this study (strain sequence reference *Mycobacterium avium* subsp. *paratuberculosis* Moredun Research Institute [MAPMRI] numbers are indicated). Previously described lineages and subgroups A and B described in this study are highlighted in gray.

Confirmation of the presence of the SNPs was obtained by sequencing the PCR products. PCR product (40 μl) was purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Sequencing of PCR products was carried out by Eurofins Genomics (MWG-Biotech) using the same primers used for the amplification of the fragments. Confirmation of the presence or absence of the SNP in the expected position of the genome was achieved using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The

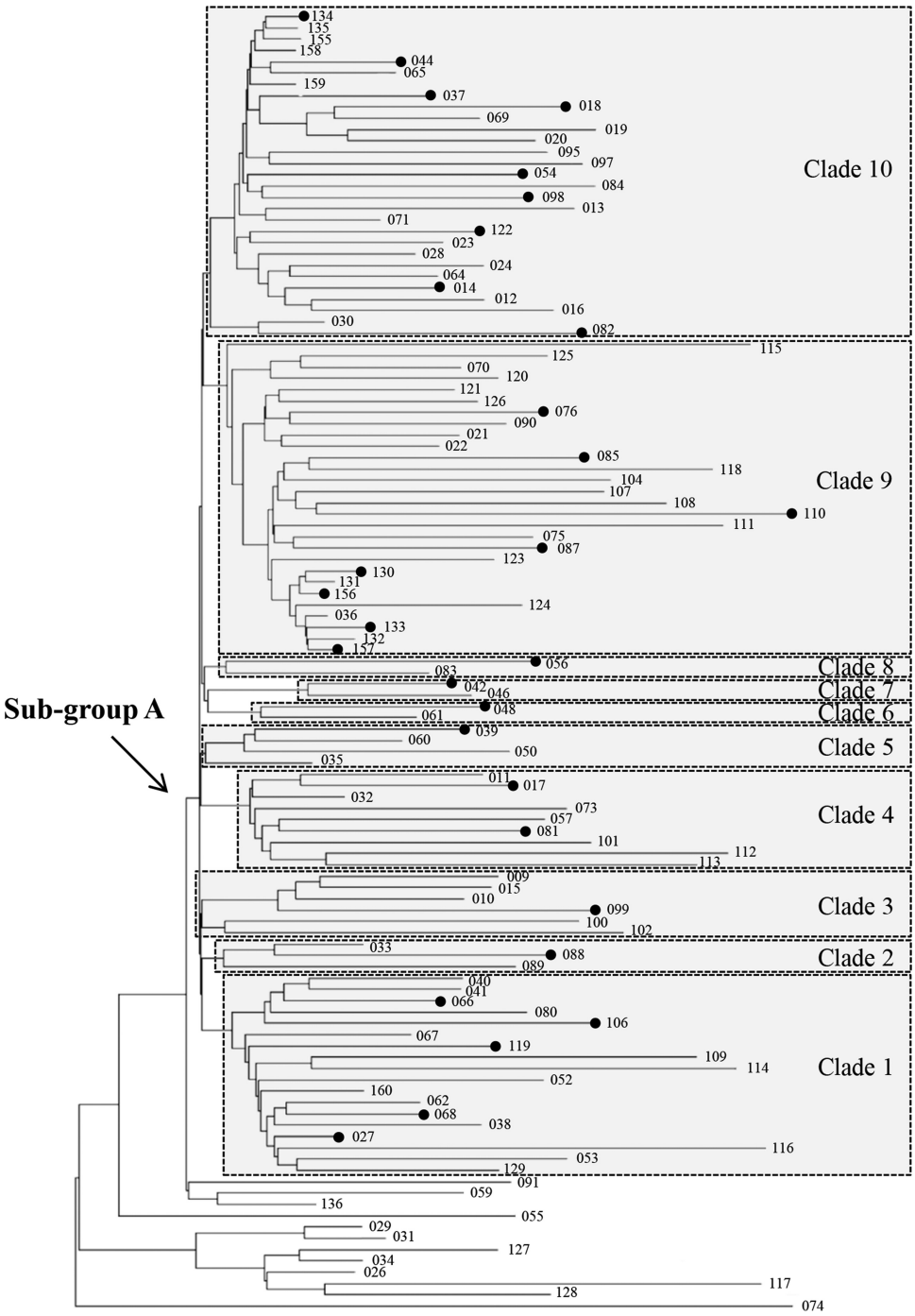

FIG 2 Whole-genome SNP-based phylogenetic tree of 115 type C *M. avium* subspecies *paratuberculosis* isolates (strain sequence reference MAPMRI numbers are indicated). Ten clades within the phylogenetic subgroup A can be distinguished by PCR and sequencing following the analysis of 10 SNPs (gray boxes). Black circles indicate the 30 strains used for the validation of the method.

phylogenetic profile for each isolate was obtained by combining the results for all SNPs.

Discriminatory power of SNP-based genotyping assay. The discriminatory power of the assay was calculated using the Hunter-Gaston discriminatory index (HGDI), according to Hunter and Gaston (24).

RESULTS

The genome sequence data from 133 *M. avium* subspecies *paratuberculosis* strains were compared to the reference *M. avium* subspecies *paratuberculosis* strain K10 to identify SNPs. A total of 28,402 SNPs were identified among all of the isolates. A phylogenetic tree was generated based on the SNP analysis, and distinct phylogenetic groups were identified (Fig. 1 and 2), which conformed to the broadly recognized phylogenetic structure of *M. avium* subspecies *paratuberculosis* (10). By using an adaptation of the set cover problem, the minimum number of SNPs required to discriminate between all the isolates was calculated to be 93. To

TABLE 1 Additional *M. avium* subspecies *paratuberculosis* field isolates used for validation of SNP assay

Isolate	Host	Geographic location	Multiplex PFGE profile	INMV profile[a]	SNP profile (this study)
F043531	Cattle	Northern Ireland (U.K.)	2.1	1	1
F012398	Cattle	Cumbria (U.K.)	2.1	1	11
F005713	Cattle	Wiltshire (U.K.)	2.1	1	11
C217551	Cattle	Selkirkshire (U.K.)	2.1.	1	9
C216785/2	Cattle	Mid Glamorgan (U.K.)	2.1	1	9
C219376	Cattle	Shropshire (U.K.)	2.1	1	9
C221325	Cattle	Gwynedd (U.K.)	2.1	1	9
C524656	Cattle	Aberdeenshire (U.K.)	2.1	1	1
C326442	Cattle	Ayr (U.K.)	2.1	1	9
C216962/6	Cattle	Leicestershire (U.K.)	2.1	1	9
C1	Goat	São Miguel (Azores, PT[b])	ND[c]	ND	3
C2	Goat	São Miguel (Azores, PT)	ND	ND	3
C4	Goat	São Miguel (Azores, PT)	ND	ND	3
C7	Goat	São Miguel (Azores, PT)	ND	ND	3
C9	Goat	São Miguel (Azores, PT)	ND	ND	3
C11	Goat	São Miguel (Azores, PT)	ND	ND	3
C13	Goat	São Miguel (Azores, PT)	ND	ND	3
C14	Goat	São Miguel (Azores, PT)	ND	ND	3
C16	Goat	São Miguel (PT)	ND	ND	3
C4A4	Goat	São Miguel (Azores, PT)	ND	ND	3
B1	Cattle	Vila do Conde (PT)	ND	2	9
B3	Cattle	Vila do Conde (PT)	ND	2	9
B13	Cattle	Póvoa do Varzim (PT)	ND	2	11
B18	Cattle	Póvoa do Varzim (PT)	ND	2	11
B21	Cattle	Póvoa do Varzim (PT)	ND	2	11
B22	Cattle	Póvoa do Varzim (PT)	ND	2	11

[a] Profile number assigned by INRA and published in an online database http://mac-inmv.tours.inra.fr.
[b] PT, Portugal.
[c] ND, not determined.

refine the number of SNPs to a number manageable for routine laboratory procedures, we considered a strategy based on sequential detection of SNPs and a decision tree (Fig. 3).

The phylogenetic analysis distinguished two major strain groups corresponding to those previously designated type C and type S (Fig. 1). We identified an SNP (snp3842359) which could be detected using BsmB1 (Table 2) that would discriminate between these two groups. Type C strains have an A, and type S strains have either a G or complement, at base position 3842359. This constituted the first step in the decision tree; the next step was determined according to whether the isolate was type C or type S (Fig. 3).

For further analysis of type S strains, we identified an SNP (snp343677) which could be detected using AvaII that discriminated the type S subgroups, type I and type III (Table 2, Fig. 1 and 3). Additionally, snp3842359 also could be used to distinguish type I and type III strains, since type I strains have a G and type III have a C at base position 3842359 (Fig. 3), which could be detected by PCR amplification and sequencing of the product.

The type C group comprised the majority of the isolates, and the high homogeneity within this group posed a challenge for identification of clade-specifying SNPs. First, SNP analysis was repeated with only the sequence data from the 115 type C isolates (Fig. 2), and the minimum number of SNPs required to distinguish between these 115 isolates was determined to be 41. We then considered 3 principal subgroups designated bison, as reported previously (10), A, and B (as shown in Fig. 1 and 2). We identified an SNP (snp50173) which could distinguish the bison group from subgroups A and B using ApoI (Table 2, Fig. 1 and 3). This constituted step 2 in the decision tree (Fig. 3).

Within the bison group, Indian bison type could be differentiated from U.S. bison type using snp2327379, and further differentiation of the Indian bison type was possible using snp305277 (Table 2; Fig. 3). Due to the limited number of bison type strains available, extensive verification of these SNPs was not possible in this study.

To further discriminate between type C isolates in subgroups A and B, we identified an SNP (snp4111202) which could be detected using FatI (Table 2, Fig. 1 and 3). Due to the small number of isolates in subgroup B, we did not, at this stage, seek additional SNPs to further discriminate the isolates within this group. This constituted step 3 in the decision tree (Fig. 3).

Within the larger subgroup A, SNPs were identified that could discriminate 10 groups (snp3879247, snp2939977, snp1932058, snp1327872, snp3844632, snp1966028, snp305277, snp4339946, snp2087274, and snp1686154; Tables 2 and 3; Fig. 2 and 3). These SNPs were verified by sequencing of the PCR products and comparison of the sequences with the reference *M. avium* subspecies *paratuberculosis* K10 strain. It was not possible to identify SNPs with specific restriction endonuclease sites for the clades differentiated using snp2939977 and snp1932058, but all other SNPs could be detected by restriction endonuclease analysis (Table 2).

SNP validation and genotyping. For the validation of the selected SNPs comprising the decision tree (Fig. 3), DNA from isolates belonging to type C (bison group: MAPMRI029, MAPMRI031, MAPMRI127, MAPMRI034, MAPMRI117, and MAPMRI026; subgroup A: MAPMRI110, MAPMRI120, and MAPMRI027; subgroup B: MAPMRI059, MAPMRI136, and MAPMRI091) and type S (type I: MAPMRI007and MAPMRI001; type III: MAPMRI051, MAPMRI045, and MAPMRI047) (Fig. 2) were used for amplifying products containing SNPs (as indicated in Fig. 3), and the PCR products were digested with the correspondent restriction enzymes (Table 2). PCR products were also purified and sequenced to confirm SNPs. To assess the validity of the 10 SNPs for discriminating the clades within subgroup A, 30 isolates from the original panel of 115 sequenced strains were retested. These were selected to be representative of the 10 different phylogenetic clades, as shown in Figure 2, and were subjected to analyses for all 14 SNPs (Fig. 3), all of which were confirmed to be present. The *M. avium* subspecies *paratuberculosis* isolates were grouped into SNP profiles, as shown in Table 3 and Figure 3.

A further 26 *M. avium* subspecies *paratuberculosis* isolates (Table 1) not previously sequenced or typed using this SNP assay were genotyped using the 14 SNPs. These isolates belonged to 4 phylogenetic groups within subgroup A. Significantly, 10 isolates from different geographic regions of United Kingdom with the same MIRU-VNTR and PFGE profiles were classified into 3 different SNP profiles, one of which was not identified in the original phylogenetic analysis and, therefore, represented a new SNP profile (SNP11) (Table 3). Two United Kingdom isolates were identified to belong to the SNP1 profile, 6 isolates, to SNP9, and 2 isolates, to profile SNP11. The 16 Portuguese isolates were distributed among 3 phylogenetic groups: all of the isolates from Azores were present in the same group identified by profile SNP3; 2 isolates from the same region in the north of Portugal were identified in the same

TABLE 2 PCR primers and restriction endonucleases used in the SNP assay for this study

SNP name	Primer name	Primer sequence (5′ to 3′)	Annealing temp (°C)	Product size (bp)	Enzyme	Restriction results[a]
snp3842359[b]	MAP_F1	CACCTGGCCAAGTACTACCA	63	528	BsmBI	(A) Type C; 528 bp
	MAP_R1	GCGATGTCATGATGCTGCTG				(G/C) Type S; 312 and 216 bp
snp343677	TypeS_F	AACACCAGGATCGCGTTCTT	65	511	AvaII	(G) Type I; 297, 152, and 62 bp
	TypeS_R	CAATTAGCGGTCGAGTCGTC				(A) Type III; 293 and 218 bp
snp50173	BisonF	GGACGATTACTCGGTTCCAG	63	469	ApoI	(T) Bison group; 226, 192, and 51 bp
	BisonR	ACCCGTGTTCGGCTACCT				(G) Cattle group; 277 and 192 bp
snp4111202	SNP4_F	GTCAGAAACATCCCGCCTTC	65	461	FatI	(G) Type C subgroup A; 284 and 177 bp
	SNP4_R	GTATTGAGTGAGGCAAGCGG				(C) Type C subgroup B; 461 bp
snp3879247	SNP5_F	GTTGATCGACAGCGAGTGC	64	465	BlpI/DdelI	(C) 227 and 238 bp
	SNP5_R	GTGGTGTCCGAGGTGAACTT				(T) 465 bp
snp2939977	SNP6_F	TATCTCCAAGGACGCATTCC	64	516	NA[c]	NA
	SNP6_R	CTGCCATGTCCGTCCTTAAT				
snp1932058	SNP7_F	GGCTTGAAACTCCAACTGCT	63	452	NA	NA
	SNP7_R	CGTCGTACATCCTCGTGGT				
snp1327872	SNP8_F	GCGCTTGTTGTACAGGTTGA	64	528	AvaI/BsoBI	(G) 292, 171, and 65 bp
	SNP8_R	TACGACGAAGACCCCGACTA				(T) 463 and 65 bp
snp3844632	SNP9_F	GATCGATGCGGAGCTCGT	64	457	FatI	(G) 457 bp
	SNP9_R	TGACAGGAAGGTCCATAGCC				(C) 241 and 217 bp
snp1966028	SNP10_F	GTCGAGGGCTTCCAGGTT	67	427	SapI/EarlI	(A) 427 bp
	SNP10_R	GTCTGAGGCCAGCGACAC				(C) 246 and 182 bp
snp305277[d]	SNP11_F	CCATCCCGAGTTCAACAAGT	64	461	BspMI/BfuAI	(G) 310 and 151 bp
	SNP11_R	ACTTGTCGGGGTTGTAGCTG				(A) 461 bp
snp4339946	SNP12_F	AACCGCTCAAGGCGAAAG	64	464	BstEII	(T) 292 and 172 bp
	SNP12_R	TCCCTTATCTGCGAAGTGCT				(A) 464 bp
snp2087274	SNP13_F	CAGACCGAGCACCTCCTG	65	453	HpyAV	(C) 453 bp
	SNP13_R	CCGCGTTGAAGGATCTCAAG				(A) 227 and 226 bp
snp1686154	SNP14_F	GAATCCCCGGAACTGGTG	65	525	MscI	(G) 525 bp
	SNP14_R	GCAGTCCAGATAACGGAACG				(A) 284 and 241 bp

[a] Letter in parentheses represents the expected base at the correspondent SNP position.
[b] SNP can also distinguish between types I and III; type I has a G, and type III has a C, at base position 3842359, detectable by sequencing the PCR product.
[c] NA, not applicable.
[d] SNP can also distinguish between two phylogenetic subgroups of bison group isolates (Fig. 3).

phylogenetic group as 6 isolates from the United Kingdom (profile SNP2); and the remaining 4 Portuguese isolates from the same region were found to belong to the new phylogenetic group together with the 2 DNAs from the United Kingdom (profile SNP11) (Table 1).

Discriminatory power of SNP-based genotyping assay. The discriminatory power of the SNP-based assay was 0.8390 for the 56 isolates that were used for the validation of the assay. In order to compare the discriminatory power of the SNP assay with MIRU-VNTR analysis, we used the typing results for 46 isolates, which had been typed using both methods. The HGDI was calculated to be 0.8135 for the SNP assay and 0.6386 for MIRU-VNTR.

DISCUSSION

Several methods have been used to characterize *M. avium* subspecies *paratuberculosis* strains, but they have some limitations. Techniques based on the analysis of total genomic DNA, such as RFLP and PFGE, require culture of the isolates to prepare moderate amounts of high-quality DNA and, therefore are slow and can be technically demanding, labor intensive, hard to standardize, and expensive. Furthermore, RFLP and PFGE can clearly distinguish between types C and S but do not give sufficient discrimination within these strain types for detailed epidemiological studies. Techniques such as AFLP and RAPD employ PCR to detect smaller genomic DNA fragments but are less utilized for epidemiological studies due to difficulties in standardization and reproducibility and limited discriminative power. Other typing methods based on repetitive sequences, such as SSR and MIRU-VNTR, are popular due to their ease of use and rapidity but, again, are limited with respect to their ability to discriminate within the two major strain types, and the typing results may not reflect the evolutionary relationships between isolates (10, 12, 25, 26).

In this study, a novel typing assay based on SNP analysis by PCR and restriction or sequencing of the amplified products was developed. This technique is easy to perform, is applicable to a small quantity of genomic DNA, and is based on standard PCR and restriction endonuclease analysis. It was possible to refine the number of SNPs to a number manageable for routine laboratory procedures by adopting an approach based on sequential detection of SNPs via a decision tree. The SNP assay was highly discriminative, possessing a higher discriminatory power than MIRU-VNTR when applied to 46 *M. avium* subspecies *paratuberculosis* isolates.

SNP-based typing assays are particularly useful for monomorphic pathogens that exhibit limited genetic diversity. Furthermore, they have the advantage that they can be used to determine phylogenetic relationships, unlike techniques based on mobile or repetitive DNA elements, which interrogate a relatively small proportion of the mycobacterial genome and can exhibit homoplasy. However, SNP discovery is subject to phylogenetic discovery bias

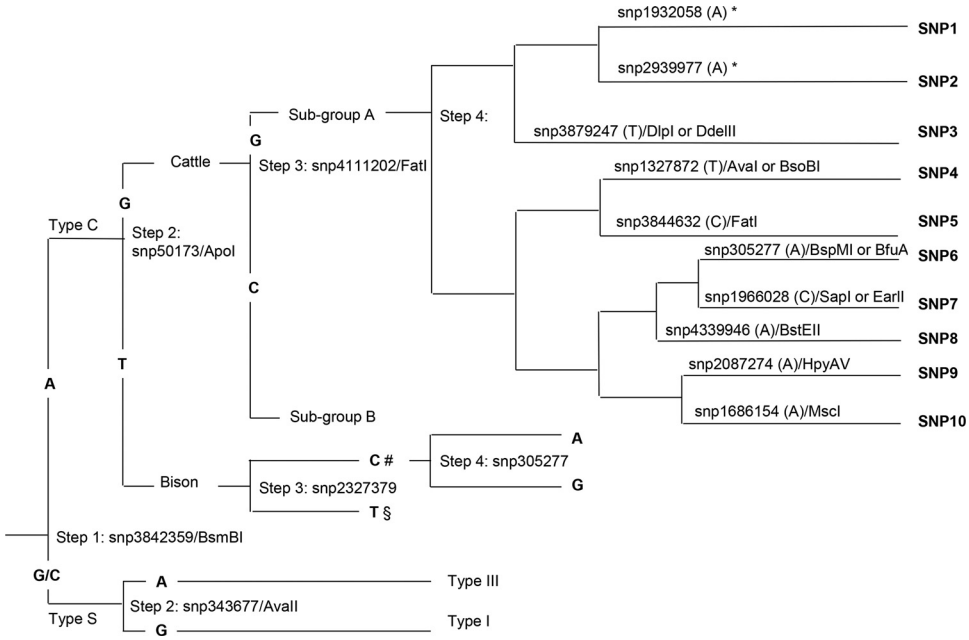

FIG 3 Work flow with a schematic representation of the decision tree with the sequential numbered steps and restriction enzymes, SNP positions, expected bases, and SNP profiles obtained based on the SNP analysis. *, SNP that can be detected only by sequencing of the amplified product; #, bison type strains from India; §, bison type strains from the United States.

(2), a phenomenon well described for *M. tuberculosis* (27) and *Bacillus anthracis* (28), and is most likely to be encountered when information is missing on strains geographically restricted or belonging to rare phylogenetic groupings. For this reason, we utilized a large collection of global isolates, which had been previously genotyped by classical molecular tools (PFGE and MIRU-VNTR), to maximize genetic diversity and include representatives of all previously reported strain types. The SNPs identified in this study should provide the necessary means to unambiguously classify *M. avium* subspecies *paratuberculosis* strains within this global framework. Even so, the composition of any panel of SNPs needs to be reviewed or augmented when additional groups of strains that were not included in the initial analysis are discovered. This has been illustrated in this study, with the discovery of a new phylogenetic group represented by profile SNP 11 comprising 6 isolates, when an additional uncharacterized 26 isolates were screened using the SNP assay. WGS needs to be performed, and the sequence comparisons and SNP analysis needs to be repeated to determine the positions of these isolates within the phylogenetic tree and to determine any additional SNPs that could be used to define the group. In this study, we concentrated on finding SNPs to differentiate within type C strains in subgroup A. The SNP assay needs to be expanded to differentiate between strains within type S, bison type, and type C subgroup B; however, WGS data from more strains belonging to these phylogenetic groups are required for SNP discovery to pro-

TABLE 3 SNP profiles of type C *M. avium* subspecies *paratuberculosis* isolates in phylogenetic subgroup A used in this study

Phylogenetic group	SNP profile	No. of isolates verified	Base at SNP position[a,b]													
			3842359	343677	50173	4111202	3879247	2939977	1932058	1327872	3844632	1966028	305277	4339946	2087274	1686154
Reference base (K10)			A	A	G	G	C	G	G	G	G	A	G	T	C	A
Clade 1	1	7	A	A	G	G	C	G	**A**	G	G	A	G	T	C	G
Clade 2	2	1	A	A	G	G	C	**A**	G	G	G	A	G	T	C	G
Clade 3	3	11	A	A	G	G	**T**	G	G	G	G	A	G	T	C	G
Clade 4	4	2	A	A	G	G	C	G	G	**T**	G	A	G	T	C	G
Clade 5	5	1	A	A	G	G	C	G	G	G	**C**	A	G	T	C	G
Clade 6	6	1	A	A	G	G	C	G	G	G	G	A	**A**	T	C	G
Clade 7	7	1	A	A	G	G	C	G	G	G	G	**C**	G	T	C	G
Clade 8	8	1	A	A	G	G	C	G	G	G	G	A	G	**A**	C	G
Clade 9	9	16	A	A	G	G	C	G	G	G	G	A	G	T	**A**	G
Clade 10	10	9	A	A	G	G	C	G	G	G	G	A	G	T	C	**A**
New clade	11	6	A	A	G	G	C	G	G	G	G	A	G	T	C	**G**
Total		56														

[a] SNP position in the revised *M. avium* subspecies *paratuberculosis* K10 sequence (GenBank accession number AE016958.1).
[b] Defining SNP base is marked in bold type.

vide a phylogenetically robust framework for strain differentiation combined with sufficient discriminatory power for detailed genetic studies.

SNPs have been described in previous studies that differentiate between the two major phylogenetic groups, type S and type C, and between *M. avium* subspecies *paratuberculosis* strain types I and III. A PCR-restriction endonuclease analysis (REA) assay described by Whittington et al. (29) based on an SNP at base position 223 in the IS*1311* insertion sequence has been used extensively for discriminating between type S and type C strains. However, in a recent study (10), the IS*1311*-REA incorrectly identified strain MAPMRI074 as a type S strain when WGS and SNP analysis clearly confirmed it to be type C, suggesting that the C-to-T allelic variation at base pair position 223 in IS*1311* occurred after the initial divergence of type C from type S strains. The SNP identified in this study, snp3842359, and the corresponding restriction endonuclease BsmBI for PCR-REA SNP detection could provide a more reliable alternative assay for differentiating type S and C strains.

IS*1311* SNP analysis has also been used to distinguish bison type strains from non-bison type C and type S strains (30). In bison-type strains, all copies of IS*1311* have a T at base pair position 223, whereas the non-bison type C strains have one or more copies with a C or T at the same position. Copy number with respect to this allele is not always easy to assess and can be variable (10). Snp50173 and the corresponding restriction endonuclease ApoI for PCR-REA SNP detection could provide an easier, alternative assay for discriminating bison-type strains from other type C strains.

A study published by Castellanos and colleagues (17) developed a PCR-REA assay to detect an SNP present on the *gyrB* gene at base position 1626 that allowed discrimination of type III from type I and II strains. Additional SNPs in the *gyrA* gene were identified that could differentiate types I and III from type II. In our study, it is possible to use only a single SNP to discriminate type I, type II, and type III based on the amplification and sequencing of a fragment containing the snp3842359 where, in the same position of the genome, type I strains have a G, type II strains have an A, and type III strains have a C. This is an improvement compared with the system previously reported.

In conclusion, we developed a novel SNP-based genotyping assay based on the analysis of 14 SNPs that can be used to characterize *M. avium* subspecies *paratuberculosis* isolates within 14 phylogenetic groups, with a higher discriminatory power than MIRU-VNTR assay and other typing methods. We adopted an approach based on sequential detection of SNPs and a decision tree based on PCR restriction enzyme digestion to reduce the number of SNPs and required PCRs. This novel assay can overcome some issues regarding the genotyping of isolates characterized as type I, type III, and bison type. Continuous updating of genome sequences are needed in order to better characterize new phylogenetic groups and SNP profiles. The novel SNP assay is a discriminative, simple, reproducible method and requires only basic laboratory equipment for the large-scale global typing of *M. avium* subspecies *paratuberculosis* isolates.

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