JOURNAL OF CLINICAL MICROBIOLOGY, July 2006, p. 2499–2506 Vol. 44, No. 7
0095-1137/06/$08.00+0 doi:10.1128/JCM.00498-06

Multispacer Typing To Study the Genotypic Distribution of *Bartonella henselae* Populations

Wenjun Li,[1] Bruno B. Chomel,[2] Soichi Maruyama,[3] Lynn Guptil,[4] Anna Sander,[5] Didier Raoult,[1] and Pierre-Edouard Fournier[1]*

Unité des Rickettsies, IFR 48, CNRS UMR 6020, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France[1]; Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616[2]; Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan[3]; Veterinary Clinical Sciences, Purdue University, West Lafayette, Indiana 47907[4]; and Institute for Medical Microbiology and Hygiene, University of Freiburg, 79104 Freiburg, Germany[5]

Received 7 March 2006/Returned for modification 24 April 2006/Accepted 14 May 2006

Bartonella henselae*, a worldwide fastidious bacterium, has a feline reservoir and is pathogenic for humans. However, the relationship between human and cat isolates of *B. henselae*, as well as its population dynamics and geographic heterogeneity, is not fully understood, in part because of the absence of appropriate typing methods. Multilocus sequence typing (MLST), the most discriminatory genotyping method for *B. henselae*, identified seven genotypes and suggested that human isolates arose from a limited number of cat isolates. Herein, we estimated the discriminatory power of multispacer typing (MST) by studying 126 *B. henselae* cat isolates from various areas of Europe, Asia, and the United States. We identified the nine most variable intergenic spacers conserved by both *B. henselae* and *Bartonella quintana* genomes. By comparing the sequences obtained from these nine spacers for each studied isolate, we identified 39 MST genotypes. The distribution of isolates into MST genotypes matched their phylogenetic organization into four clusters. MST showed that European and Asian isolates were different, in contrast with American isolates, but failed to identify pandemic strains. Our study demonstrated that MST is a powerful method for genotyping *B. henselae* at the strain level and may serve in studying the population dynamics of this bacterium and understanding the relationships between cat and human isolates. Finally, we provide a free-access MST-Rick online software program (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst) that investigators may use to compare their own MST sequences to our database.

Bartonella henselae is a gram-negative, fastidious bacterium associated with cats. Its transmission among cats is mediated by the cat flea, *Ctenocephalides felis* (7). Infected cats may remain bacteremic for long periods, thus playing a major role as a reservoir for the bacterium (6, 24). Human infection occurs through cat scratches or bites (22) and presents as cat scratch disease (2), bacillary angiomatosis (23), peliosis hepatis (32), endocarditis (18), or a variety of other, less frequent manifestations (14).

Although criteria exist for classifying *Bartonella* isolates as new species (27), there is a need for a method able to reliably identify *B. henselae* at the strain level. Such a method would allow investigation of the relationships between cat and human isolates, the question of whether epidemic strains occur in cats, and the geographic heterogeneity of *B. henselae* isolates. Various methods have been proposed for typing *Bartonella* isolates (10, 12, 19, 20, 26, 29, 34). Of these, sequence-based methods have the advantages of being applicable to clinical or environmental specimens and producing reproducible and comparable results. On the basis of comparison of 16S rRNA gene sequences, *B. henselae* isolates were classified into two main genotypes, i.e., types I and II. This gene was considered a useful delineation among isolates because the two genotypes also exhibited different serotypes and possessed consistently distinguishable protein profiles (26). Sequences from the *ftsZ* (12), *gltA* (10), 35-kDa protein-encoding (26), *groEL* and *pap31* (34) genes, and from the 16S-23S intergenic spacer (20), later permitted the identification of three, two, two, four, and six genotypes, respectively, that did not exactly match 16S rRNA gene types. To date, the most discriminatory typing method for *B. henselae* isolates is multilocus sequence typing (MLST) incorporating nine genes (21). This method distinguished seven genotypes among 37 human and cat isolates and suggested that lateral gene transfer occurs among *B. henselae* isolates (21). Although these investigators and others suggested that human infection is caused by a limited number of specific *B. henselae* genotypes (4, 10, 21), the discriminatory power of the genotyping methods that they used and the small number of *B. henselae* isolates that they studied were insufficient to allow any statistically significant conclusions to be drawn. Therefore, a genotyping tool with greater discriminatory power for genotyping *B. henselae* at the strain level is needed to investigate the diversity and population structure of this bacterium.

Recently, we applied a new genotyping method to *Bartonella quintana*, i.e., multispacer typing (MST) (13). This method allows genotyping of bacteria at the strain level. MST, initially

* Corresponding author. Mailing address: Unité des Rickettsies, IFR 48, CNRS UMR 6020, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France. Phone: (33) 04.91.38.55.17. Fax: (33) 04.91.83.03.90. E-mail: Pierre-Edouard.Fournier@medecine.univ-mrs.fr.

developed for *Yersinia pestis* (11), was also applied with success to strains of other human pathogens, including *Rickettsia conorii* (15), *Rickettsia prowazekii* (35) and *Coxiella burnetii* (16). MST was developed with the assumption that intergenic spacers are more variable than genes for genotyping bacteria at the strain level. In this study, to estimate the usefulness of MST for studying the population genetics of *B. henselae*, we applied it to a large collection of cat isolates.

MATERIALS AND METHODS

Study design. One hundred twenty-six *B. henselae* cat isolates of various geographic origins were incorporated in this study (Table 1). All 38 European isolates were grown in our laboratory. For the other 88 isolates, from the United States and Asia, we studied DNA extracted by two of the authors (B.B.C. and L.G.) from their isolates.

***Bartonella henselae* culture and DNA extraction.** *B. henselae* isolates were cultivated on Columbia agar with 5% sheep blood (BioMerieux, Marcy l'Etoile, France) at 37°C in 5% CO_2 (Genbag CO_2 system; BioMerieux). Genomic DNA of *B. henselae* strains was extracted by using the Chelex procedure as previously described (9) or the QIAmp Tissue kit following the manufacturer's recommendations (QIAGEN, Hilden, Germany).

Selection of target sequences. We aligned the genomic sequences of *B. henselae* (GenBank accession number BX897699) and *B. quintana* (BX897700) by using the BLASTn (1) and GenomeComp (33) software programs to identify conserved pairs of consecutive genes. Then, intergenic sequences were aligned using the CLUSTAL W program (31). We classified intergenic spacers conserved by both genomes, with sizes ranging from 150 to 600 bp, by degree of similarity and then selected the 20 most variable spacers (detailed in Table 2).

PCR amplification and sequencing. Primers were designed to amplify the 20 most variable spacers fulfilling the above criteria using the Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for amplifying the 20 most variable spacers were selected within genes flanking the selected spacers and are listed in Table 2. All primers were obtained from Eurogentec (Seraing, Belgium). Their specificity was predicted by comparison with GenBank using the BLASTn software (1). PCRs were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, Mass.). One nanomolar concentration of each DNA preparation was amplified in a 25-μl reaction mixture containing 50 pM of each primer; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg, Md.); 1 U of eLONGase polymerase (Invitrogen); 1 μl of eLONGase buffer A; and 4 μl of eLONGase buffer B. The following conditions were used for amplification: an initial 3 min of denaturation at 94°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 1 min at 68°C. Amplification was completed by holding the reaction mixture for 10 min at 68°C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore, Saint-Quentin en Yvelines, France) as recommended by the manufacturer. PCR products were sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit with Amplitaq polymerase FS (Perkin-Elmer, Coignieres, France) as described by the manufacturer. Sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sterile water was used as a negative control in each PCR assay. Sequences from each genotype were checked twice in both directions to ensure the reliability of the MST method.

Sequence analysis and phylogenetic analysis. Nucleotide sequences were edited using the Autoassembler package (Perkin-Elmer). For each intergenic spacer, a genotype was defined as a sequence exhibiting unique mutations. MST genotypes were defined as unique combinations of spacer genotypes. Multiple alignment of sequences was carried out using the CLUSTAL W software (31). Phylogenetic analysis of the studied isolates was obtained using the neighbor-joining and maximum parsimony methods within the MEGA 3 software (25). For this purpose, sequences of the selected spacers were concatenated. To facilitate sequence comparison with our MST sequences, we developed an online site named MST-Rick. This site contains a local BLAST to help scientists compare their sequences to our database.

Statistical tests. The genotypic variability of *B. henselae* isolates according to their geographic origin was estimated using Fisher's exact test. A difference was considered significant when P was <0.05.

Nucleotide sequence accession numbers. The different genotypes for the discriminatory spacers have been deposited in the GenBank database under accession numbers DQ383226 to DQ383270.

RESULTS

MST genotyping. One thousand four hundred thirteen intergenic spacers were found conserved by *B. henselae* and *B. quintana* genomes. Among them, 293 had a size ranging from 150 to 600 bp. We tested the 20 most variable (S1 to S20) of these 293 spacers among the 126 *B. henselae* cat isolates available. Nine of the spacers (S1 to S9) were found highly variable among these isolates (Table 2). The tRNA-Ala/GCA-tRNA-Ile/AUC spacer (S1), flanked by two tRNA genes, was found to be the most variable spacer among the nine tested, with five variable nucleotide positions and a 15-bp sequence fragment presenting either as a single copy or repeated up to five times, depending on the isolate (Fig. 1; Table 3). Sequences from the S1 spacer classified the 126 isolates into nine genotypes. The BH2865724-*dut* spacer (S2), with 14 variable nucleotide positions, was the second most variable spacer and allowed the 126 tested isolates to be classified into seven genotypes (Table 3). The *dnaJ*-related protein–*cobS* spacer (S3) held eight variable nucleotide positions and classified the 126 isolates into six genotypes (Table 3). The *pssA*-oxidoreductase (S4) and *carB*-cold shock protein (S5) spacers had nine and five variable nucleotide positions, respectively, and classified the 126 isolates into five genotypes each (Table 3). The *alr-gcvP* (S6) and *ftsK*-oxidoreductase spacers (S7) contained eight variable nucleotide positions each and classified the 126 isolates into four genotypes each (Table 3). The BH2864883-BH2864884 (S8) and *acpP2*-malate oxidoreductase (S9) spacers harbored eight and four variable nucleotide positions, respectively, and classified the 126 isolates into three genotypes each (Table 3). In total, 69 variable nucleotide positions were found within the nine intergenic spacers (Table 3). Each variable nucleotide was checked three times to ensure the reliability of MST. Only two alleles at each variable position were found, with the exception of position 256 within the *alr-gcvP* spacer. At this position, 117 isolates had a thymine (types 2 and 3), compared to a cytosine in five European isolates (type 1) and a guanine in four American isolates (type 4) (Table 3). By combining the genotypes obtained from each variable spacer, the 126 tested isolates could be classified into 39 MST genotypes (Table 1). Each of the 39 genotypes was identified based on sequence specificities from either a single spacer or a combination of a maximum of seven spacers (Fig. 2). Sequences from each genotype from the nine spacers were added to the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

Among the 39 MST types, 24 MST types (types 1, 3, 4, 6, 8 to 12, 14, 16, 19 to 21, 23, 26, 28 to 31, 34, 36, 37, and 39) contained only one isolate each and five MST genotypes (types 17, 24, 27, 32, and 33) contained only two isolates each (Table 1). The 19 Asian isolates were distributed into 12 MST genotypes, compared to 6 ($P < 0.01$) and 24 ($P = 0.03$) MST types for the 38 European and 69 American isolates, respectively (Fig. 3). Among the 39 MST genotypes, 10 (types 6, 10, 20, 21, 23, 32, and 36 to 39), 21 (types 1, 3, 4, 7 to 9, 11, 14 to 19, 24 to 30, and 34), and 5 (types 4, 9, 12, 22, and 31) genotypes were specific to Asian, American, and European isolates, respectively.

A significant difference in genotypic diversity was found between *B. quintana* (4 MST types out of 71 isolates) (13), and

TABLE 1. List of *B. henselae* isolates incorporated in this study and corresponding genotypes

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
Amber	USA[b]	5	1	1	1	2	2	2	1	1	5
Aron	USA	7	2	5	4	1	2	1	1	3	7
BisQuick	USA	5	2	6	5	2	2	2	1	1	35
Budda	USA	5	1	1	1	2	2	2	1	1	5
Buster Brown	USA	5	1	1	2	2	2	2	1	1	25
Cleo	USA	5	1	1	1	2	2	2	1	1	5
Kody	USA	4	5	5	5	1	2	1	1	3	27
Earl Grey	USA	5	1	1	2	2	2	2	1	1	25
Erick	USA	5	1	1	1	2	2	2	1	1	5
Faleen	USA	5	1	1	1	2	2	2	1	1	5
Gigi	USA	4	5	5	4	1	2	1	1	3	15
Jackie	USA	5	2	1	1	2	2	2	1	1	16
Junior	USA	5	1	1	1	2	2	2	1	1	5
Kelly	USA	8	2	5	4	1	2	1	1	3	8
Kodie	USA	5	1	1	1	2	2	2	1	1	5
Lathious	USA	5	2	6	5	2	2	2	1	1	35
Levi	USA	5	1	1	1	2	2	2	1	1	5
Mew Mew	USA	7	2	5	4	1	2	1	1	3	7
Mitzi	USA	5	1	1	1	2	2	2	1	1	5
Mokka	USA	5	1	1	1	2	2	2	1	1	5
Molly	USA	5	1	1	2	2	2	2	1	1	25
Norman	USA	5	1	1	1	2	2	2	1	1	5
Patches	USA	7	2	5	4	1	2	1	1	3	7
Pyewacket	USA	5	1	1	1	2	2	2	1	1	5
Rafiki	USA	5	1	1	1	2	2	2	1	1	5
Rocket	USA	7	2	5	4	1	2	1	1	3	7
Rum Tum	USA	5	1	1	1	2	2	2	1	1	5
Sabrina	USA	5	2	6	5	2	2	2	1	1	35
Sadie	USA	4	5	5	4	1	2	1	1	3	15
Saki	USA	5	1	1	1	2	2	2	1	1	5
Sam	USA	9	2	5	4	1	2	1	1	3	9
Samantha	USA	5	1	1	1	2	2	2	1	1	5
Sassy	USA	1	1	1	1	2	2	2	1	1	1
Shannon	USA	5	1	1	5	2	2	2	1	1	26
Simba	USA	3	1	1	1	2	2	2	1	1	3
Sinbad	USA	5	1	1	1	4	2	2	1	1	29
Spaz	USA	5	1	1	1	2	2	2	1	1	5
Sunday	USA	5	2	1	5	2	2	2	1	1	28
Sweetie	USA	5	1	1	2	2	2	2	1	1	25
Tabatha	USA	5	1	1	1	2	2	2	1	1	5
Tasha	USA	3	1	6	3	5	4	4	3	2	18
Timothy	USA	5	1	1	1	2	2	2	1	1	5
Toby	USA	5	1	1	1	2	2	2	1	1	5
Tori	USA	4	5	5	5	1	2	1	1	3	27
Zipper	USA	5	2	6	5	2	2	2	1	1	35
Zoe	USA	5	1	1	1	2	3	2	1	1	30
Newmans	USA	4	2	5	4	1	2	2	1	3	33
White	USA	5	2	6	5	2	2	2	1	1	35
Lavery	USA	5	7	6	1	2	2	1	2	1	17
Rae	USA	5	7	6	1	2	2	2	2	1	34
Fairminer	USA	5	2	6	5	2	2	2	1	1	35
Shaw-Lamon	USA	5	2	6	5	2	2	1	1	1	24
Moyle	USA	5	7	6	1	2	2	1	2	1	17
Linnan	USA	5	2	6	5	2	2	1	1	1	24
Silcock	USA	5	2	6	5	2	2	2	1	1	35
Hunt	USA	5	2	6	5	2	2	2	1	1	35
Eichtais	USA	5	2	6	5	2	2	2	1	1	35
Taylor	USA	5	2	6	5	2	2	2	1	1	35
Ramm	USA	5	2	6	5	2	2	2	1	1	35
USA1	USA	5	1	1	1	2	2	2	1	1	5
USA4	USA	4	1	1	1	2	2	2	1	1	4
USA6	USA	4	5	5	4	1	2	1	1	3	15
USA7	USA	3	1	6	3	5	4	4	3	2	18
USA8	USA	5	1	1	1	2	2	2	1	1	5
USA11	USA	4	1	5	4	1	2	1	1	3	19

Continued on following page

TABLE 1—*Continued*

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
USA12	USA	3	3	6	3	5	4	4	1	2	11
USA15	USA	4	2	5	4	1	2	1	1	3	14
USA16	USA	3	1	6	3	5	4	4	3	2	18
USA17	USA	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.32	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.33	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.34-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.35	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.50-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.52-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.54-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.55-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 56-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.57-M	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC.58-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 59-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.67	France	5	1	1	1	2	2	4	2	1	31
UR.BH.M.NHC.72-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 77-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.78-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.79-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 80-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 82-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.84-M	France	2	4	1	1	3	1	3	3	2	2
UR.BHM.M.NHC.128	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.129	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.130	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC 154	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 155	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 156	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 159	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 161	France	2	4	1	1	3	1	3	3	2	2
FR96/BK7	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK26II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK36	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK36II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK75	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK77	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK78	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK79	Germany	2	4	1	1	3	1	3	3	2	2
ZF-1	France	3	5	3	5	2	2	2	1	3	12
FIZZ	Switzerland	5	1	1	1	2	2	2	1	1	5
J1	Japan	5	2	6	5	2	2	2	1	1	35
J4	Japan	5	2	5	4	1	2	1	1	3	23
J5	Japan	8	2	5	4	1	2	2	1	3	32
J6	Japan	5	2	6	5	2	2	2	1	1	35
J7	Japan	5	2	6	5	2	2	2	1	1	35
J8	Japan	5	2	6	5	2	2	2	1	1	35
P1	Philippines	5	2	6	5	2	2	2	2	1	38
P2	Philippines	5	2	6	5	2	2	2	2	1	38
P4	Philippines	5	2	6	5	2	2	2	2	1	38
P5	Philippines	5	2	6	5	2	2	1	2	1	37
P6	Philippines	4	2	5	4	1	2	1	2	3	36
P7	Philippines	3	2	5	5	2	2	2	1	1	10
P8	Philippines	3	2	6	5	2	2	2	1	1	21
T1	Thailand	5	2	6	5	2	2	2	1	3	39
T3	Thailand	8	2	5	4	1	2	2	1	3	32
T5	Thailand	6	2	5	4	1	2	2	1	3	6
T6	Thailand	4	2	5	4	1	2	2	1	3	33
T7	Thailand	5	2	6	5	2	2	2	2	1	38
T8	Thailand	5	1	6	5	2	2	2	2	1	20

[a] The description of intergenic spacers S1 to S9 and the primers used for their amplification and sequencing are given in Table 2.
[b] USA, United States.

TABLE 2. The 20 most variable intergenic spacers conserved by both *B. henselae* and *B. quintana* and primers used for amplification and sequencing

Spacer name[a]	Spacer position on the genome[b]	Spacer size (bp)[b]	PCR product size (bp)[b]	Forward primer	Reverse primer
tRNA-Ala/GCA-tRNA-Ile/AUC (S1)[c]	1412349–1412683	335	414	TTGCAAAGCAGGTGCTCTCC	TAAGCGTGAGGTCGGAGGTT
BH2865724-*dut* (S2)	1685859–1686289	431	602	GGTTTTTGCCACGGGTATTT	GGAAGTTCTAAACCTTGTCCATGG
dnaJ related protein-*cobS* (S3)	1828960–1829320	361	490	CAATGGAGGCAACCGTTCTT	GTGATATCGGGTACATTTTCAACTG
pssA-oxidoreductase (S4)	609654–610228	575	709	GATTTTTCTTCCGTGTAGCTTTGT	TGTGCGTAAAAATCGATTCATG
carB-cold shock protein (S5)	1292681–1293066	386	509	AGAAGCTATCGAAGCACTCACAAA	TGAATGAACCCGAAACCTTTAGT
alr-gcvP (S6)	1431110–1431442	333	540	TCAAAGAGGTGATTGGGTAGAGC	CTGTTTCACGTATTGATAATGTTGC
ftsK-oxidoreductase (S7)	1799482–1799984	503	594	GCGAACCTTGAGAACTCTGCA	GGGTTTTACACCTTCATTGAGATCA
BH2864883-BH2864884 (S8)	1594026–1594377	352	524	TAACCACATCATCCCCTCCTCT	GAAATAATCATGAAACGCATAAGC
acpP2-malate oxidoreductase (S9)	853898–854063	166	296	CAACTTCACTGATTTCTGCGATAA	CGAGGAGTGGTTAATATGACAGCT
BH16140-BH16150 (S10)	1864960–1865467	508	508	CTCATTACAGAGCAAAACGGATATC	TTATCAAGGTTTGCTTCTACAGCG
dapE-hemN (S11)	76032–76228	197	395	ATGCATATGGTGGATGAGTGTGT	GATTTACAACAACAAGGGCTGGT
phoH-BH02260 (S12)	302238–302400	163	327	CTTATTTTCTCTTTAACGCGCTTT	TCACCTTGGCTTTTACCTGTTGT
Glutathione *S*-transferase–*dapB* (S13)	1383473–1383792	320	441	CTTCTTTTCGCCCTCTTTTAACA	TCGCGTCCCATTCTTCCAT
rpmF-ispA (S14)	1751167–1751490	324	394	GATGGAGAGGTTTTTCGTTTAGG	TGGGCGTGTTTTGCAAGAA
asd-BH12900 (S 15)	1441922–1442299	378	636	TACGCGATGCACCAGGCT	CCGTGTTGTGACCTATCCTGCT
recO-panC (S16)	596596–596744	149	438	TTGTGCAAAGAACTGTTCGTCC	ACCAAACCAATCGAAAATCCTAA
BH16010-*rpsP* (S17)	1846327–1846669	343	461	AGACTGGGAAATTAAAGGCCG	CGTATAGCAGCAGCAAAGCAAG
pgk-gap (S18)	1729282–1729787	506	590	GAACACGTTTTCCTGTGACATCA	GTGATACGGCTGTGGCTTTTG
uvrC-BH05560 (S19)	653261–653650	390	532	AGCTTTTCTTGCTCATTTTCGG	AGCTCAGTCCCTTTCTTATCGC
trwL4-trwL5 (S20)	1805508–1805660	153	280	AGATACATTCGTACGGTGGGGA	CCTGTTGTTATTTTTTGATTGGAG

[a] Intergenic spacer names consist of the name of the 5′-flanking gene combined (-) with the name of the 3′-flanking gene. Flanking open reading frames encoding putative proteins of unknown function are named after their open reading frame number within the *B. henselae* genome (GenBank accession number BX897699).
[b] The positions of the spacers on the genome, the spacer size, and the PCR product size were deduced from *B. henselae* (BX897699).
[c] Spacers S1 to S9 were numbered in descending order of variability.

B. henselae (39 MST types among 126 *B. henselae* isolates; $P < 0.01$).

Phylogenetic classification of MST types. Phylogenetic trees obtained from concatenated spacer sequences using the neighbor-joining (Fig. 3) and maximum parsimony methods showed similar phylogenetic classifications. The 126 tested isolates were grouped into four clusters. Asian isolates were grouped into cluster 1. European isolates were grouped into clusters 2 to 4. In contrast, American isolates did not form a coherent cluster but were spread among the four clusters.

DISCUSSION

In this study, we demonstrated that MST is a highly efficient method for genotyping *B. henselae* at the strain level, with 39 genotypes identified among 126 studied isolates using a combination of nine intergenic spacer sequences. Prior to our study, the most discriminatory genotyping method for *B. henselae*, i.e., MLST using nine genes, had identified seven genotypes among cat and human isolates of *B. henselae* (21). Therefore, MST was more discriminatory than MLST for typing *B. henselae*.

We found *B. henselae* to be significantly more genotypically variable than *B. quintana*, a human pathogen previously identified to be mostly clonal (13) ($P < 0.01$). Such a higher genetic diversity of *B. henselae* is as yet unexplained, despite the studies conducted on the relationship between cat and human isolates. In Germany and The Netherlands, a majority of human isolates were of 16S rRNA gene type I whereas cat isolates mostly belonged to type II (3, 4, 10, 28, 30). In contrast, in Switzerland, France, and the United States, investigators have demonstrated that most of the human isolates of *B. henselae* belonged to 16S rRNA gene type II (5, 8, 17). Iredell et al., using MLST identifying seven genotypes, found that human infection is caused by a limited number of genotypes (21). Therefore, the relationship between human and cat isolates of *B. henselae* remains a puzzling problem. We believe that MST may also be a suitable tool for investigating the dynamics of *B. henselae* populations in humans.

Among the 126 isolates analyzed in this study, we found a significantly higher genotypic heterogeneity among Asian isolates than among European ($P < 0.01$) and American ($P = 0.03$) isolates. This may be explained by the fact that most European isolates originate from only two neighboring coun-

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1       CAATCTTTTTAGAAG----------------------------------------------------------       (106)
2       CAATCTTTTTAGAAGCAATCTTTTTAGAAG-------------------------------------------       (5)
3       CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG----------------------------       (11)
4       CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG---------------       (3)
5       CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG      (1)
```

FIG. 1. Description of the 15-bp repeated sequences within the tRNA-Ala/GCA-tRNA-Ile/AUC spacer. The first column contains the copy number of repeats. Numbers in parentheses indicate the numbers of strains that have the corresponding repeat numbers.

TABLE 3. Polymorphism characteristics of the nine variable intergenic spacers

Spacer name	No. of nucleotide variations	No. of genotypes	Spacer polymorphism, with reference to Houston-1 strain[a]
tRNA-Ala/GCA-tRNA-Ile/AUC (S1)	5	9	G9A, C49T, 203inserT, C256T, 294VNTR
BH2865724-*dut* (S2)	14	7	T19C, G31A, C92T, C103T, C113T, C142T, A156G, A162G, C169T, G237T, A289G, C310T, T332dele, T339C
dnaJ-related protein-*cobS* (S3)	8	6	A3G, G12A, A25G, G46A, C84T, T203C, T255C, T264dele
pssA-oxidoreductase (S4)	9	5	A49G, 51inserA, 93inser,[b] G159T, A274G, A306G, T322C, A362G, T484C
carB-cold shock protein (S5)	5	5	51inser,[e] C83A, G145C, T157C, T240C
alr-gcvP (S6)	8	4	C4T, G10A, C60A, G242A, T256G or C, 296dele,[d] A305G, A306G
ftsK-oxidoreductase (S7)	8	4	C324A, G326A, 362inser,[c] G370A, A390C, C432T, A436G, C480T
BH2864883-BH2864884 (S8)	8	3	G19C, A60G, A61G, 69inserT, A88G, C102A, C249A, C282T
acpP2-malate oxidoreductase (S9)	4	3	C28T, A40C, G96A, C114T
Total (9 spacers)	69	39	

[a] The numbers show each variable nucleotide position in reference to the Houston-1 strain. The locus before the number is that within Houston-1, and the locus after the number is a possible variable nucleotide within other strains. inser, insertion; dele, deletion; VNTR, variable number of tandem repeats.
[b] Insertion of CCAGAGTGCTATTCATTAAATAAGTTTGCTTTTAAAAAATATTTCTTG.
[c] Insertion of TTCACCTGTTTCATA.
[d] Deletion of TTTTTG.
[e] Insertion of GTAGGGCA.

tries, France and Germany, and American isolates were mostly obtained from only two states, California and Florida, whereas Asian isolates originate from three countries. However, the phylogenic analysis built by concatenating the nine spacers (Fig. 3) revealed that Asian isolates, despite their apparent genotypic heterogeneity, were phylogenetically homogeneous and were grouped into a single cluster, without any overlap with European isolates. This may suggest that Asian isolates have a more recent common origin. American isolates appeared to be phylogenetically more heterogeneous than other isolates. None of the 39 MST types identified was represented in European, American, and Asian isolates together. Thus, we did not identify any pandemic isolate. However, our data may be updated by future studies incorporating isolates from other geographic origins.

To limit the number of spacers to be sequenced, we propose specific guidelines that facilitate their selection (Fig. 2). In addition, to facilitate usage of MST for genotyping of *B. henselae*, we created an MST-dedicated, free-access online database, i.e., MST-Rick, to which any investigators

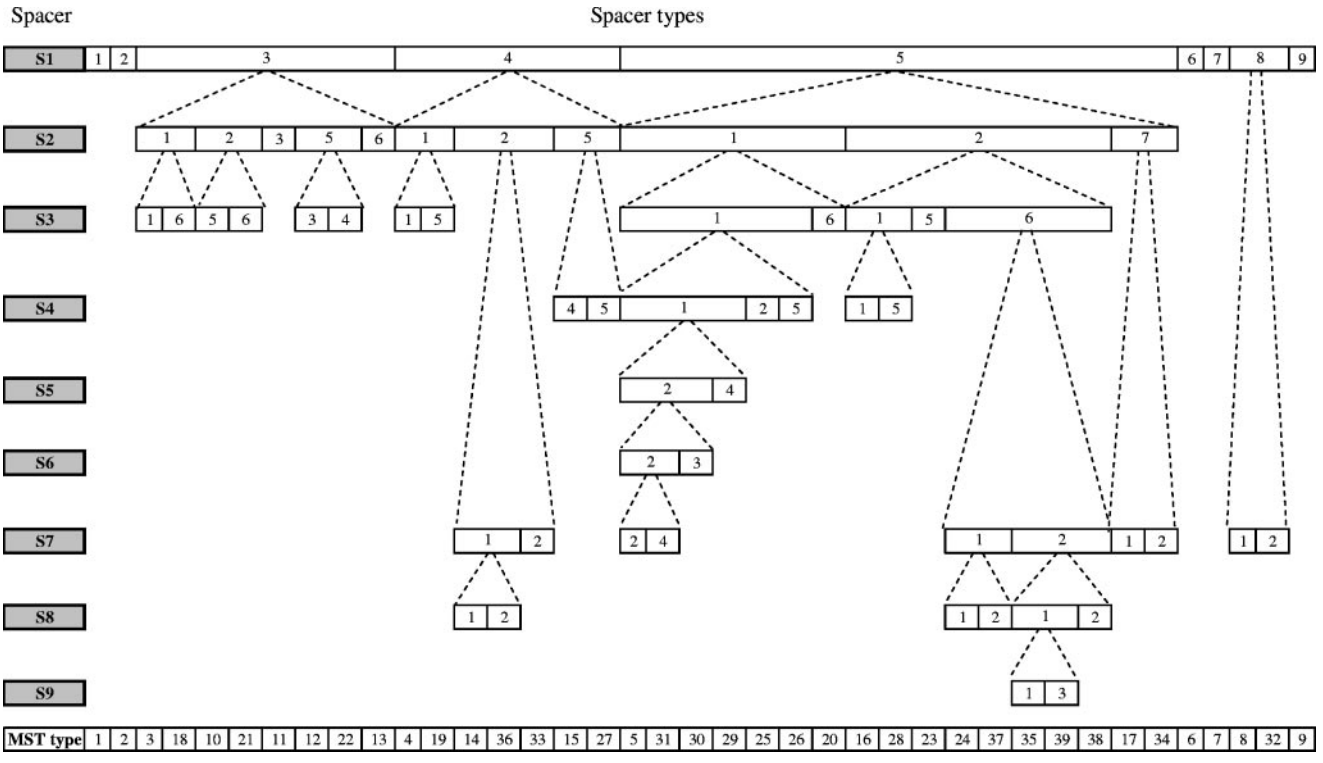

FIG. 2. Guidelines for selection of spacers for MST genotyping of *B. henselae* isolates.

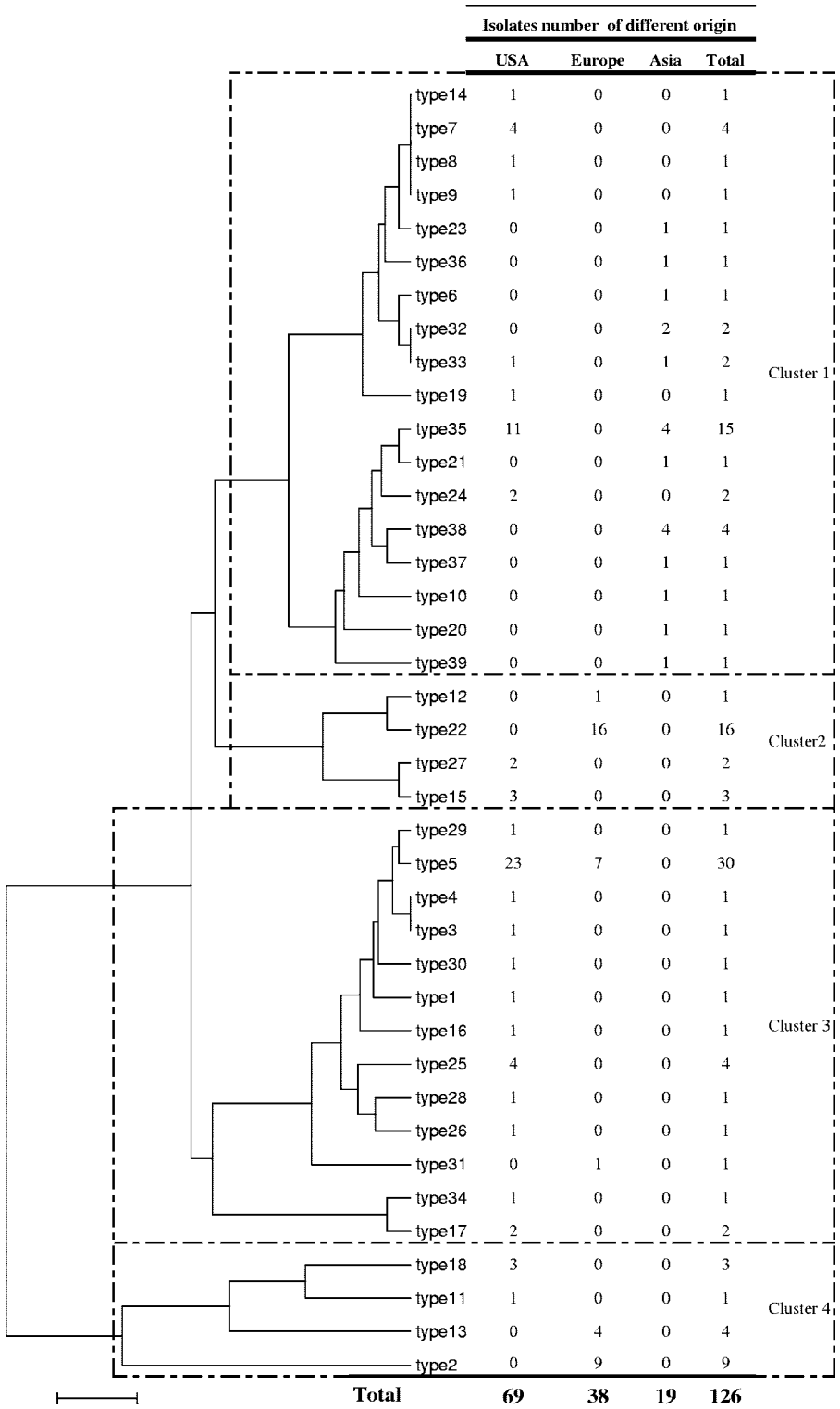

FIG. 3. Dendrogram showing the phylogenetic organization of the 39 MST genotypes, constructed using the neighbor-joining method. Sequences from the nine spacers were concatenated. The scale bar represents a 1% nucleotide sequence variation.

may compare their own spacer sequences (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst). Although our study is preliminary and includes a limited number of strains, we hope that our method and database will be used and implemented by other investigators, which would allow frequent updating of the data.

In conclusion, MST using nine variable intergenic spacers identified 39 genotypes among 126 *B. henselae* cat isolates. As

such, MST is the most discriminatory genotyping method for *B. henselae* isolates to date and may be used to investigate the relationships between human and cat isolates of *B. henselae*. Recently, we successfully used MST for genotyping *B. henselae* isolates within lymph node biopsy samples from patients with cat scratch disease (unpublished data). As *B. henselae* is extremely difficult to grow from human specimens, MST might thus serve as both a detection and a genotyping tool.

ACKNOWLEDGMENTS

We thank Lina Barassi for her technical help.

All authors have read and approved the final version of the manuscript and do not have any conflict of interest related to this research.

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