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Highlights

- Specific *Bartonella* profiles were associated to specific feline reservoirs hosts
- The *B. koehlerae* subspecies seem highly adapted to their wild felid hosts
- These *B. koehlerae* subspecies cluster with non-zoonotic rather than with zoonotic *B. henselae*
- *B. henselae* isolates, likely from domestic cats, can be transmitted to wild felids
- MVLA is a useful identification tool for discriminating these *Bartonella* subspecies

Abstract

Bartonella genus includes an increasing number of species and subspecies, especially among wild felids, the positioning of which, with regards to the zoonotic species *Bartonella henselae*, is important to determine.

The aim of this study was to test the ability of a molecular typing technique to distinguish between various *Bartonella* isolates obtained from four different species of free-ranging and captive wild felids and to identify key profiles or markers allowing differentiating them from each other and/or from *B. henselae* or *B. koehlerae*.

A molecular typing technique for *B. henselae* based on the polymorphism of variable number tandem repeat units (VNTR) called MLVA (Multiple Locus VNTR Analysis) was applied to 24 *Bartonella* isolates from free-ranging or captive wild felids, 19 of which were obtained from California and five from three countries in Southern Africa, and compared with 49 *B. henselae* isolates from cats, dog or humans from the United States including the human ATCC (American Type Culture Collection) reference strain, *B. henselae* Houston 1.

MLVA allowed distinguishing *Bartonella* isolates from wild felids from either *B. henselae* or *B. koehlerae*. We confirmed infection of semi-captive cheetahs with an isolate similar to a Californian bobcat isolate. MLVA also confirmed the unique profile of a free-ranging cheetah isolate from Namibia.

Specific profiles were observed making MVLA a useful identification/classification tool of these wild felid isolates and suggesting that they are highly adapted to a specific feline reservoir. Finally, circulation of *B. henselae* isolates between domestic cats, wild felids and humans is likely occurring, based on the close allelic profiles of some isolates.

Keywords: *Bartonella*, Wild felids, MLVA.

Introduction

Bartonella species are emerging pathogens that have been isolated from a wide range of terrestrial mammals, including humans (Boulouis et *al.*, 2005; Kosoy & Goodrich, 2019); Reguer *et al.*, 2016) and bats (Corduneanu *et al.*, 2018; Stuckey *et al.*, 2017). An increasing number of new species have been described during the past twenty years and at least 17 *Bartonella* species or subspecies have been reported to be pathogenic for humans and animals (Breitschwerdt, 2017). A growing number of human (and animal) diseases, including cat-scratch disease, bacillary angiomatosis, peliosis hepatitis, endocarditis, chronic lymphadenopathy, meningoencephalitis, stellar retinitis and osteomyelitis are associated to the already well known *Bartonella* species (like *B. henselae*) and to some of the new *Bartonella* species and subspecies (Chomel et *al.*, 2004). Domestic cat, free-ranging and captive wild felids represent a large reservoir of *Bartonella* species and usually display asymptomatic chronic bacteremia (Bevins et *al.*, 2012; Chomel et *al.*, 1995; Chomel et *al.*, 2006; Filoni et *al.*, 2012; Molia et *al.*, 2004). However, the population dynamics and the species tropisms of *Bartonella* species are not completely understood (Kosoy & Goodrich, 2019). Possible exchanges of ectoparasites between domestic cats, free-ranging and captive wild felids could occur in the wild or accidentally in zoological parks, causing potential exchanges of vector-borne pathogens including *Bartonella* sp. between felids (Carver *et al.,* 2016). New *Bartonella* subspecies were recently described in wild felids native (free-ranging) or introduced (captive) in California (Chomel *et al.,* 2016; Molia *et al.,* 2016).

Different molecular methods allow to distinguish and determine *Bartonella* genotypes at the species level, as reported by Kosoy *et al.* (2018). Next Generation Sequencing (NGS) techniques have not

been used yet to differentiate *Bartonella* species and subspecies and explore their relationships. Various methods have been proposed for typing *Bartonella* isolates within *B. henselae* species, the agent of cat scratch disease (Bergmans et *al.*, 1996; especially Pulsed-Field Gel Electrophoresis (PFGE) (Arvand et *al.*, 2001), Multilocus Sequence Typing (MLST) (Iredell et *al.*, 2003 ; La Scola *et al.* 2003) and Multispacer Typing (MST) (Li et *al.*, 2006). However, none of them was tested to easily and reliably differentiate the main species and sub-species within the *Bartonella* genus. Thus, there was a need for a simple and inexpensive method to study the relationships between *Bartonella* species and their heterogeneity within each species. MLVA (Multiple Locus Variable number tandem repeats Analysis) has been developed in our laboratory and is based on the polymorphism of five main VNTR, called BHV-A to E (for *Bartonella henselae* Variable Number Tandem Repeat (VNTR) (Monteil et *al.*, 2007). To date, if we exclude whole genome sequencing, MLVA is still the most discriminatory typing method for *B. henselae* isolates (Bouchouicha et *al.*, 2008). This method allowed distinguishing 99 profiles among 178 human, feline and canine isolates (Bouchouicha et *al.*, 2009) and to characterize two groups of *B. henselae* isolates/strains, group A including only isolates from cats, and group B including all the isolates obtained from humans, a dog and the rest of the cats. Interestingly, groups A and B were particularly characterized by a different distribution of BHV-D alleles, as all group A isolates harboured only one or two repeats whereas group B isolates presented a minimum of five repeats, at least at the level of the isolates that were available at this time. Epidemiological, experimental and molecular arguments favour the hypothesis that group B isolates are zoonotic while group A isolates are not (Bouchouicha et al., 2009; Gil *et al.,* 2013).

MLVA has since been used by different teams in different countries (Azzag *et al.,* 2012; Cicuttin *et al.,* 2014; Gil *et al.,* 2013; Podsiadly *et al.,* 2012). The aim of this study was to apply MLVA to various *Bartonella* isolates obtained from four different species of free-ranging and captive wild felids from California and from three South-African countries, in order to characterize their mutual relationships, their relationships with *B. henselae* and *B. koehlerae* (a species phylogenetically close

to *B. henselae* and which reservoir host is also the cat), and to identify key profiles or markers allowing to differentiate them from each other and / or from *B. henselae* or *B. koehlerae*. This study gave us the opportunity to test the presence of five main VNTR developed for *B. henselae* typing in other *Bartonella* species or subspecies. It supported the concept of strains highly adapted to a given species of feline reservoir, as strains from pumas, bobcats and a cheetah were easily differentiated with the method.

Materials and Methods

Bartonella strains, isolates and DNA extraction

Twenty four isolates of *Bartonella* from wild felids were used in this study (Table I): Four isolates from free-ranging pumas (*Felis oncolor*), seven isolates from free-ranging bobcats (*Lynx rufus*), mainly from northern California, and eight isolates from semi-captive cheetahs (*Acinonyx jubatus)* from southern California (San Diego wildlife safari Park); two isolates from cheetahs (one captive from Zimbabwe (Kelly *et al.,* 1998) and one free-ranging from Namibia) and three isolates from African lions (*Panthera leo*) from Kruger Park, Republic of South Africa. Five isolates were determined to be *B. henselae*, 17 were identified as two new subspecies of *B. koehlerae*, respectively *B. koehlerae* subsp. *boulouisii* isolated from free ranging Californian mountain lions (n=3), and *B. koehlerae* subsp. *bothieri* isolated from 14 free ranging bobcats (n=6) or semi-captive cheetahs (n=8) (Chomel *et al.,* 2016; Molia *et al.,* 2016). A lion isolate from Kruger Park was identified as *B. koehlerae*. Finally, one isolate from a free ranging cheetah from Namibia was close to *B. henselae* and *B. koehlerae*, but different from all the other wild felid isolates (Molia *et al.,* 2016). In addition, we used *B. henselae* reference strain Houston 1 (ATCC 49882) as a positive control for MLVA typing.

Samples were used as DNA extracts or cell lysates from isolated strains. For DNA extraction, bacteria were scraped from a 5% rabbit blood agar plate, as previously described (Chomel *et al.,* 1995) and suspended in 500 µl sterile distilled water. These suspensions were boiled for 10 min,

and centrifuged at 3000 *g* for 15 min. Nucleo Spin Tissue kit (Macherey-Nagel) was used for the purified DNA preparation, according to the manufacturer's instructions. The DNA solutions were stored at – 20 °C before testing.

Partial sequence typing of 16S rRNA gene of *Bartonella* isolates

Two genotypes of *B. henselae*, differing for 3 bp located at positions 172 to 175 of 16S rRNA gene have been described (Bergmans et *al.*, 1996). Type-specific 16S rRNA gene amplification of *Bartonella* DNA with primer 16SF and either primer BH1 or BH2 was performed with the following modifications: amplification was carried out in 25 µl reaction volumes and each reaction mixture contained 3 µl of purified DNA or 5 µl of cell lysate, 2,5 µl of TaKaRa amplification buffer, 2 µl of dNTP, 2 µl of each primer and 0,2 µl of TaKaRa ® Ex taq (RR001 DNA polymerase TAKARA biomedical group, Shiga, Japan). An initial denaturation step at 95 °C for 3 min was followed by the amplification programme: DNA was denatured for 20 s at 95 °C, and primers were annealed for 30 s at 56 °C, and extended at 72 °C for 1 min. After 29 cycles, there was a final extension programme at 72 °C for 10 min. PCR products were separated by gel electrophoresis in 2 % agarose gels (Invitrogen, Electrophoresis Grade, Ultra pure ref : 15510).

Multiple Locus Variable number tandem repeat Analysis (MLVA)

PCR reactions were performed in a volume of 25 µl containing 1 µg of bacterial DNA, with each of the five pairs of primers (for respective amplifications of five polymorphic VNTR called BHV for *Bartonella henselae* VNTR, *i.e.* BHV-A, BHV-B, BHV-C, BHVD and BHV-E), according to the PCR protocol described previously by Monteil et *al.* (2007). Sterile water was used as a negative control in each PCR assay.

PCR products were separated by gel electrophoresis. 1,5 % agarose gels (Invitrogen, Electrophoresis Grade, Ultrapure; ref : 15510) were prepared in TBE (Tris Boric acid EDTA (pH 8,3)) running buffer, stained with ethidium bromide and photographed under UV illumination with an analysis image system (Gel Doc Biorad) and the software Quantity One. Long gels (26cm x 40cm, CBS Scientific® model SGU-2640T-02) and a migration times (2h) at 300 V/cm (Generator

156 PS608 (600V - 800mA – 300W), Apelex ®) were used, in order to allow a good estimation of the
157 band size.
158 New primer design for BHV D amplification: For those isolates for which BHV-D was not
159 amplified using the already available primers, a new reverse (R: GCCATAGGAGGATAAGAAG)
160 primers was designed. With this modified pair of primers, the annealing temperature was slightly
161 modified (53°C); all the other parameters were identical to those described by Monteil et *al*. (2007).
162 Diversity index
163 For the evaluation of the polymorphism and of the discriminatory power of the MLVA, the Hunter
164 and Gaston discrimination index (HGDI) was used (Hunter & Gaston, 1988), as recommended by
165 the European Society of Clinical Microbiology and Infectious Diseases Study Group on
166 Epidemiological Markers. Polymorphism is considered high when this index is higher than 95%
167 (Struelens, 1996).
168 Profiles Analysis and construction of the dendrogram
169 For each isolate, an MLVA profile was defined as a unique combination of the repeat numbers for
170 the five BHV (BHV-A to -E). A profile is considered as complete when three to five amplified
171 VNTR by isolate/strain are obtained according to the bacterial species or subspecies. For more
172 convenience, the different MLVA profiles obtained were arbitrarily given a number. The distance-
173 based method UPGMA (unweighted group pair method analysis using average linkages) was then
174 performed to compute the tree, as already described (Bouchouicha *et al.,* 2009). With this method,
175 the character states are considered to be unordered and, for a given BHV, the same weight is given
176 to a small or a large difference of the number of repeats. To investigate the relationships between
177 the *Bartonella* species isolated from free-ranging,captive wild felids, domestic cats, dog and human
178 patients a minimum spanning tree (MST) and a phenogram (based on UPGMA cluster analysis)
179 were constructed. Both included all allelic profiles obtained from the different *Bartonella* species of
180 free-ranging and captive wild felids and the others allelic profiles of 49 *B. henselae* isolates/strains

from North American cats and human patients previously obtained in our laboratory, as most of the wild felid isolates originated from this continent (Monteil *et al.,* 2006; Bouchouicha *et al.,* 2009). Fragment sizes were converted to repeat units and imported into BioNumerics 7.6.3 (Applied Maths, Belgium) as a character data.

Based on the topology of the phenogram, several groups were defined, named G1 to G3. In addition, based on BHV-D, the isolates/strains were labeled as belonging to the previously described group A (BHV-D score of 1-2 repeats, supposed non-zoonotic isolates) or B (BHV-D score> 4 repeats, zoonotic isolates/strains) (Bouchouicha et al., 2009; Gil et al., 2013).

Results

16S rRNA type of *Bartonella* isolates from wild felids

The expected size of the PCR products on this genomic DNA segment is approximately 185 bp for *B. henselae*. All *Bartonella* isolates resulted only in a 16S rDNA gene amplicon with primers BH16SF & BH2, indicating a classification as genotype II, including *B. henselae* isolates from wild cats and the lion *B. koehlerae* subsp. *koehlerae* isolate.

Multiple Locus VNTR Analysis of the wild felid isolates

The MLVA profiles obtained for the different *Bartonella* isolates from either free-ranging or semi-captive wild felids are presented in Table II. Fifteen different allelic profiles were identified for the 24 wild felid isolates, of which 23 isolates gave profiles that were considered as complete which corresponded to 14 MLVA profiles.

All *B. henselae* VNTRs (BHVs) were easily amplified from wild felid *B. henselae* isolates and the three free-ranging pumas from California. On the contrary, one VNTR, BHV-D, could not be amplified for isolates from California bobcats (n=6) and cheetahs (n=9) not identified as infected with *B. henselae*, as well as for two BHV-C from one Californian bobcat and one lion from Kruger

Park. We were still unable to amplify BHV-D in these isolates even when using two newly designed primers.

In addition, the *B. koehlerae* isolate from a Kruger Park's lion presented a unique profile with only 3 of BHVs being amplified (BHV-A, BHV-D and BHV-E).

Discriminatory power of MLVA for typing of wild felid isolates

The *B. koehlerae* subsp. *koehlerae* isolate from a Kruger Park's lion for which only 3 BHVs could be amplified was excluded for the calculation of the discriminatory power of MLVA, which was thus estimated in the context of the 23 *Bartonella henselae* from wild felids isolates with complete MLVA profiles obtained from the different species of free-ranging and captive wild felids, based on the HGDI value. The global DI for these 23 isolates was 0.90.

Relationships between the *Bartonella* isolates obtained from wild Felids

Two major sets of isolates/strains were distinguished by MLVA. The first set included all *B. henselae* isolates (MLVA profiles 2 to 6) and *B. koehlerae* subsp. *koehlerae* (MLVA profile 14). The second set was made of *B. koehlerae* subspecies isolates from bobcats, cheetahs or pumas. The California free-ranging bobcat strains harboured limited diversity (MLVA profiles 8 to 11), while all those from captive cheetahs, living in a southern Californian zoo, presented a unique or almost unique profile (MLVA profiles 12 and 8) very close to that of the Californian free-ranging bobcat isolates, as shown in Table II and figures 1, 2 and 3 (additional file). All *B. koehlerae* subsp. *bothieri* isolates were characterized by the absence of amplification of BHV-D. An additional cheetah isolate, collected from a free-ranging cheetah living in Namibia, had a unique and specific MLVA profile (MLVA profile 13) different from the other cheetah isolates. Nevertheless, as for *B. koehlerae* subsp. *bothieri* isolates, BHV-D could not be amplified. The small group of California free-ranging puma isolates was quite divergent from the Californian bobcats and cheetah isolates

and corresponded to the newly identified *B. koehlerae* subsp. *boulouisii*, with a unique profile (MLVA profile 7) (Table II and figure 2). Both subspecies shared an atypical number of repeats for BHV-C (1 repeat only), as compared to the usual number of repeats in *B. henselae* (between 2 and 25). In the wild felid strains that had been previously identified as *B. koehlerae* subspecies, one or two MLVA markers were found to be missing.

Comparison of VNTR alleles and profiles of *Bartonella* isolates from wild felids to those of *B. henselae* isolates from cats and human patients

For a few VNTR, some alleles (numbers of repeats) were not previously observed in the 244 *henselae* isolates or strains obtained from domestic cats, dogs or humans previously typed by MLVA in our laboratory. The *Bartonella* isolates and strains were grouped into three main clusters G1, G2 and G3 (Figures 1 and 2). Group G1 was made of two subgroups: G1a (17 isolates/strains) and a G1b (28 isolates/strains). Subgroup G1a was almost exclusively composed of *B. henselae* genotype I isolates, including the human strain *B. henselae* Houston I and other human strains associated with most severe clinical forms. The only exception was one feline isolate from North Carolina (NC61) (Figure 2). Subgroup G1b was mostly constituted of *B. henselae* genotype II isolates (25 of 28 isolates/strains), associated with less severe clinical forms in humans, *i.e.* bacillary angiomatosis (Figure 2). Group G2 only contained *B. henselae* 16 S rDNA genotype II isolates and strains from domestic cats or some wild felids (the lion *B. koehlerae* isolate was also contained in this group). All the nine *B. henselae* isolates/strains of this group had a repeat score of 1 for BHV-D, and were thus labeled as belonging to the previously described group A. Oppositely, most isolates/strains (40 of 45, i.e. 89% ; Isolate NC112 with the BHV-D repeat score of 3 was not assimilated to a group A nor to a group B isolate and was not included in the calculation of this percentage) of group G1 had a BHV-D repeat score of 5 to 10, and were thus labeled as belonging to the previously described group B.

All *B. koehlerae* subspecies isolates from free-ranging and captive wild felids grouped together, forming a distinct group, named G3.

The allelic profile (MLVA profile 4) of the *B. henselae* isolate from one California puma (Amanda) was almost identical to the profile of a *B. henselae* isolate from a North Carolina domestic cat (NC88).

Discussion

MLVA has been successfully used for *B. henselae* typing (Azzag *et al.,* 2012; Bouchouicha et *al.*, 2009; Cicuttin *et al.,* 2014; Gil *et al.,* 2013; Podsiadly *et al.,* 2012). In this study, we demonstrated that this user-friendly and sensitive typing technique is a highly efficient method for discriminating isolates from different feline species. It was able to differentiate *Bartonella* isolates close to but distinct from *B. henselae* and *B. koehlerae*, at least for 3 to 4 VNTRs. Our results also confirmed the phylogenetic proximity between *B. henselae* and *B. koehlerae*, as previously reported (Koehler *et al.*, 1994; Zeaiter *et al.*, 2002).

Of the 24 *Bartonella* isolates obtained from four different species of free-ranging and captive wild felids, a high level of polymorphism was observed for the 23 ones that could be typed entirely, with 14 different profiles. The Hunter and Gaston D.I. was 0.90, which is high when considering the relatively small number of isolates (n=23) and the fact that some of these isolates clustered, according to their host species. By comparison, the D.I. for *Brucella* is 0.87 (Tian *et al,* 2017) and for *Mycoplasma* the D.I. is 0.84 (Sakmanoglu *et al*, 2019). By comparison with the *B. henselae* isolates from cats and humans, the non-*B. henselae* isolates from free-ranging and captive wild felids, the majority of them identified as *B. koehlerae* subsp. *bothieri* (California bobcats and semi-captive cheetahs) or *B. koehlerae* subsp. *boulouisii* (pumas), presented specific and unique profiles not observed in any of the other tested isolates. The absence of amplification of BHV-D was characteristic of *B. koehlerae* subsp. *bothieri* isolates, while the presence of only one repeat for BHV-C seems to be shared by *B. koehlerae* subsp. *boulouisii* and *B. koehlerae* subsp. *bothieri* at

281 least at the level of our study. This absence of amplification of one or two VNTR was an additional
282 reason to maintain their temporary positioning in the *B. koehlerae* group.
283 Our data (the MLVA profiles of isolates from semi-captive cheetahs were either identical to profile
284 #8 found in a bobcat or differed by one allele only from the profile #9 also obtained from this
285 Californian wild species), also strongly supported the possible infection of semi-captive cheetahs at
286 the Safari Wildlife Park in San Diego by bobcat isolates, as previously reported (Chomel *et al.,*
287 2016). The MLVA profiles were almost identical to the ones seen in free-ranging bobcats from
288 California with a diversity of profiles in free-ranging bobcats and only two almost identical profiles
289 in semi-captive cheetahs and quite different from the only free-ranging cheetah isolate from
290 Namibia (MLVA profile 13). This is not surprising, as possible interactions between free-ranging
291 and semi-captive wild felids could occur in open-air zoological parks, causing potential exchanges
292 of bacteria between felids, probably through flea exchanges (Carver *et al.,* 2016). The isolate from
293 the other cheetah from Africa was from a pet cheetah from Zimbabwe, which was infected with *B.*
294 *henselae*. Because of its captive status and the fact that cat fleas (*Ctenocephalides felis*) are able to
295 infect a very broad range of wild mammals (Clark *et al.,* 2018) infection with a domestic cat *B.*
296 *henselae* isolate is fully plausible. Such exchanges through cat flea bites could lead to the infection
297 of wild felids by zoonotic *B. henselae* isolates.
298 Interestingly, three puma isolates clustered together, supporting the recognition of a highly host
299 adapted *Bartonella*, which was recently described as *B. koehlerae* subspecies *boulouisii*. The same
300 was observed for *B. koehlerae* subspecies *bothieri*, which appears to be adapted to bobcats, but with
301 the possibility to infect at least one other felid species, the cheetah. We previously showed that in
302 experimental conditions, a mountain lion isolate could easily infect domestic cats (Yamamoto *et al.,*
303 1998). Spillover from domestic cats to wildlife, likely through flea exchange, could also be
304 documented, as the allelic profile of one *B. henselae* isolate from a Californian puma was identical
305 to the allelic profile of a previously typed domestic cat from California. Similarly, a lion *B.*
306 *koehlerae* isolate (98/215) from Kruger Park had a MLVA profile (11-0-0-1-1) very close to the

feline reference strain of *B. koehlerae* previously typed by MLVA (11-0-0-1-2). In contrast, no wild felid isolates have been detected so far in domestic cats.

All the wild felid isolates belonging to newly identified subspecies (or not yet characterized for one cheetah isolate) clustered in a single MLVA group G3. Further phylogenetic analyses based on whole genome sequencing are necessary to better understand the relationships between these three groups.

All *B. henselae* VNTRs were easily amplified in wild felids *B. henselae* isolates, whereas Group G3 isolates were characterized by specific signatures, either the absence of amplification of BHV-D (for *B. koehlerae* subsp. *bothieri*), and/or only one repeat for BHV-C (*B. koehlerae* subsp. *boulouisii* and *B. koehlerae* subsp. *bothieri*). Lack of amplification of BHV-D may be related to either a true BHV-D deletion or, if present, the flanking regions are too different for these isolates compared to *B. henselae* to allow annealing of one or both primers. Application of the MLVA technique to other *Bartonella* species and subspecies and to more *B. henselae* isolates from different host and geographic origins could help to investigate their relationships and their biodiversity, and to determine some potential markers of bacterial pathogenicity, including particular highly virulent genotypes.

Conflict of interest statement

The authors have no conflict of interest to declare.

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References

1. Arvand, M., Klose, A. J., Schwartz-Porsche, D., Hahn, H., Wendt, C., 2001. Genetic variability and prevalence of *Bartonella henselae* in cats in Berlin, Germany, and analysis of its genetic relatedness to a strain from Berlin that is pathogenic for humans. J. Clin. Microbiol. 39, 743-746.
2. Arvand, M. & Viezens, J., 2007. Evaluation of pulsed-field gel electrophoresis and multi-locus sequence typing for the analysis of clonal relatedness among *Bartonella henselae* isolates. *Int J Med Microbiol* 297, 255-262.
3. Azzag, N., Haddad, N., Durand, B., Petit, E., Ammouche, A., Chomel, B., Boulouis, H.J., 2012. Population structure of *Bartonella henselae* in Algerian urban stray cats. PLoS One. 7, e43621. doi: 10.1371/journal.pone.0043621. Epub 2012 Aug 30
4. Bergmans, A.M., Schellekens, J, van Embden, J., Schouls, L.M., 1996. Predominance of two *Bartonella henselae* variants among cat scratch disease patients in the Netherlands. J Clin Microbiol, 34, 254–260.
5. Bevins, S.N., Carver, S., Boydston, E.E., Lyren, L.M, Alldredge, M., Logan, K.A., Riley, S.P., Fisher, R.N., Vickers, T.W., Boyce, W., Salman, M., Lappin, M.R., Crooks, K.R., VandeWoude, S., 2012. Three pathogens in sympatric populations of pumas, bobcats, and domestic cats: implications for infectious disease transmission. PLoS One. 7, e31403. doi: 10.1371/journal.pone.0031403.

6. Bouchouicha, R., Berrich, M., Monteil, M., Chomel B., Kasten, R., Boulouis, H.J., Haddad, N., 2008. Comparison of MLVA and other typing techniques for *Bartonella henselae*. Clin. Microbiol. Infect. 15, 104-105.

7. Bouchouicha, R., Durand, B., Monteil, M., Chomel, B., Berrich, M., Birtles, R., Breitschwerdt, E., Koehler, J., Kasten, R., Petit, E., Maruyama, S., Arvand, M., Boulouis, H.J., Haddad, N., 2009. Epidemiological applications of Multi-locus Variable number tandem repeat Analysis (MLVA) for *Bartonella henselae* of Human and Feline origins. Emerg. Infect. Dis. 15, 813-816.

8. Boulouis, H.J., Chang, C. C., Henn, J. B., Kasten, R. W., Chomel, B.B., 2005. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. Vet. Res. 36, 383-410.

9. Breitschwerdt, E.B., 2017. Bartonellosis, One Health and all creatures great and small. Vet Dermatol. 28, 96-e21. doi: 10.1111/vde.12413.

10. Carver, S., Bevins, S.N., Lappin, M.R., Boydston, E.E., Lyren, L.M., Alldredge, M., Logan, K.A., Sweanor, L.L., Riley, S.P., Serieys, L.E., Fisher, R.N., Vickers, T.W., Boyce, W., Mcbride, R., Cunningham, M.C., Jennings, M., Lewis, J., Lunn, T., Crooks, K.R., Vandewoude, S., 2016. Pathogen exposure varies widely among sympatric populations of wild and domestic felids across the United States. Ecol Appl. 26, 367-81.

11. Chomel, B.B., Abbott, R.C., Kasten, R.W., Floyd-Hawkins, K.A., Kass, P.H., Glaser, C.A., 1995. *Bartonella henselae* prevalence in domestic cats in California: risk factors and association between bacteremia and antibody titers. J. Clin. Microbiol. 33, 2445-2450.

12. Chomel, B.B., Boulouis, H.J., Breitschwerdt, E.B., 2004. Cat scratch disease and other zoonotic *Bartonella* infections. J Am Vet Med Assoc. 224, 1270-1279.

13. Chomel, B.B., Kasten, R.W., Henn, J.B., Molia, S., 2006. *Bartonella* Infection in Domestic Cats and Wild Felids. Ann. N.Y. Acad. Sci. 1078, 410-415.

14. Chomel, B.B., Molia, S., Kasten, R.W., Borgo, G.M., Stuckey, M.J., Maruyama, S., Chang, C.C., Haddad, N., Koehler, J.E., 2016. Isolation of *Bartonella henselae* and two new *Bartonella* subspecies, *Bartonella koehlerae* subspecies *boulouisii* subsp. nov. and *Bartonella koehlerae* subspecies *bothieri* subsp. nov. from free-ranging californian mountain lions and bobcats. PLoS One. 11, e0148299. doi:10.1371/journal.pone.0148299.

15. Cicuttin, G.L., Brambati, D.F., De Gennaro, M.F., Carmona, F., Isturiz, M.L., Pujo,l L.E., Belerenian, G.C., Gil, H., 2014. *Bartonella* spp. In cats from Buenos Aires, Argentina. Vet. Microbiol. 168, 225-8.

16. Clark, N.J., Seddon, J.M., Šlapeta, J., Wells, K., 2018. Parasite spread at the domestic animal - wildlife interface: anthropogenic habitat use, phylogeny and body mass drive risk of cat and dog flea (*Ctenocephalides* spp.) infestation in wild mammals. Parasit. Vectors. 11, 8 doi: 10.1186/s13071-017-2564-z.

17. Corduneanu, A., Sándor, A.D., Ionică, A.M., Hornok, S., Leitner, N., Bagó, Z., Stefke, K., Fuehrer, H.P., Mihalca, A.D., 2018. *Bartonella* DNA in heart tissues of bats in central and eastern Europe and a review of phylogenetic relations of bat-associated Bartonellae. Parasit Vectors. 11, 489.

18. Filoni, C., Catão-Dias, J.L., Cattori, V., Willi, B., Meli, M.L., Corrêa, S.H., Marques, M.C., Adania, C.H., Silva, J.C., Marvulo, M.F., Ferreira Neto, J.S., Durigon, E.L., de Carvalho, V.M., Coutinho, S.D., Lutz, H., Hofmann-Lehmann, R., 2012. Surveillance using serological and molecular methods for the detection of infectious agents in captive Brazilian neotropic and exotic felids. Vet. Diagn. Invest. 24, 166-173.

19. Gil, H., Escudero, R., Pons, I., Rodríguez-Vargas, M., García-Esteban, C., Rodríguez-Moreno, I., García-Amil, C., Lobo, B., Valcárcel, F., Pérez, A., Jiménez, S., Jado, I,J., Segura, F., Anda, P., 2013. Distribution of *Bartonella henselae* variants in patients, reservoir hosts and vectors in Spain. PLoS One.8, e68248. doi: 10.1371/journal.pone.0068248.

20. Hunter, P.R. & Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.

21. Iredell, J., Blanckenberg, D., Arvand, M., Grauling, S., Feil, E.J., Birtles, R.J., 2003. Characterization of the natural population of *Bartonella henselae* by multilocus sequence typing. J. Clin. Microbiol. 41, 5071-5079.

22. Kelly, P.J., Rooney, J.J., Marston, E.L., Jones, D.C., Regnery, R.L., 1998. *Bartonella henselae* isolated from cats in Zimbabwe. Lancet. 351, 1706.

23. Koehler, J.E., Glaser, C.A., Tappero, J.W., 1994. *Rochalimaea henselae* infection. A new zoonosis with the domestic cat as reservoir. JAMA. 271, 531-535.

24. Kosoy, M. & Goodrich, I., 2019. Comparative Ecology of *Bartonella* and *Brucella* Infections in Wild Carnivores. Front. Vet. Sci. 5, 322. doi: 10.3389/fvets.2018.00322.

25. Kosoy M, McKee C, Albayrak L, Fofanov Y. 2018. Genotyping of *Bartonella* bacteria and their animal hosts: current status and perspectives. Parasitology. 145(5):543-562. doi: 10.1017/S0031182017001263.

26. La Scola B, Zeaiter Z, Khamis A, Raoult D. 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. Trends Microbiol. 11(7):318-21.

27. Li, W., Chomel, B. B., Maruyama, S., Guptill, L., Sander, A., Raoult, D., Fournier, P. E., 2006. Multispacer typing to study the genotypic distribution of *Bartonella henselae* populations. J. Clin. Microbiol., 44, 2499-2506.

28. Monteil, M., Durand B., Bouchouicha R., Petit E., Chomel B., Arvand M., Boulouis H.-J., Haddad, N., 2007. Development of discriminatory Multiple locus VNTR Analysis (MLVA) for *Bartonella henselae*. Microbiol., 153, 1141-1143.

29. Molia, S., Chomel, B.B., Kasten, R.W., Leutenegger, C.M., Steele, B.R., Marker, L., Martenson, J.S., Keet, D.F., Bengis, R.G., Peterson, R.P., Munson, L., O'Brien, S.J., 2004. Prevalence of *Bartonella* infection in wild African lions (*Panthera leo*) and cheetahs (*Acinonyx jubatus*).Vet. Microbiol. 20, 31-41.

30. Molia, S., Kasten, R.W., Stuckey, M.J., Boulouis, H.J., Allen, J., Borgo, G.M., Koehler, J.E., Chang, C.C., Chomel, B.B., 2016. Isolation of *Bartonella henselae, Bartonella koehlerae* subsp. *koehlerae, Bartonella koehlerae* subsp. *bothieri* and a new subspecies of *B. koehlerae* from free-ranging lions (Panthera leo) from South Africa, cheetahs (*Ac inonyx jubatus*) from Namibia and captive cheetahs from California. Epidemiol. Infect. 144, 3237-3243.

31. Podsiadly, E., Haddad, N., Bouchouicha, R., Berrich, M., Durand, B., Monteil, M., Boulouis, H.J., Tylewska-Wierzbanowska, S., 2012. Characterization of Polish Feline *Bartonella henselae* isolates by Multiple-Locus-Tandem repeat Analysis and Pulse Field Electrophoresis. Annals Agric. Environm. Med. 19, 39-43.

32. Reguer, Y., O'Rourke, F., Kemp, V.A.J., 2016. Bartonella spp. - a chance to establish One Health concepts in veterinary and human medicine. Parasit Vectors. 9, 261. doi: 10.1186/s13071-016-1546-x

33. Sakmanoglu A, Sayin Z, Pinarkara Y, Uslu A, Ucan US, Erganis O. Evaluation of random amplified polymorphic DNA and multiple-locus variable number tandem repeat analyses for *Mycoplasma cynos*. J Microbiol Methods. 2019 Jun;161:1-7. doi: 10.1016/j.mimet.2019.04.004.

34. Struelens, M. J., 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin. Microbiol. Infect. 2, 2–11.

35. Stuckey, M.J., Chomel, B.B., de Fleurieu, E.C. Aguilar-Setién, A., Boulouis, H.J., Chang, C.C., 2017. *Bartonella*, bats and bugs: A review. Comp. Immunol. Microbiol. Infect. Dis. 55, 20-29.

36. Tian GZ, Cui BY, Piao DR, Zhao HY, Li LY, Liu X, Xiao P, Zhao ZZ, Xu LQ, Jiang H, Li ZJ. Multi-locus variable-number tandem repeat analysis of Chinese *Brucella* strains isolated from 1953 to 2013. Infect Dis Poverty. 2017 May 2;6(1):89. doi: 10.1186/s40249-017-0296-0.

37. Yamamoto, K., Chomel, B.B., Kasten, R.W., Chang, C.C., Tseggai, T., Decker, P.R., Mackowiak, M., Floyd-Hawkins, K.A., Pedersen, N.C., 1998. Homologous protection but lack of heterologous-protection by various species and types of *Bartonella* in specific pathogen-free cats. Vet. Immunol. Immunopathol. 65, 191-204.

38. Zeaiter, Z., Liang, Z., Raoult, D., 2002. Genetic Classification and Differentiation of *Bartonella* Species Based on Comparison of Partial ftsZ Gene Sequences. J. Clin. Microbiol. 40, 3641-3647.

Table I*: Animal species and geographic origins of the studied isolates

The use of color is desirable but not essential

Table II*: Characteristics and MLVA profiles of the studied isolates from wild felids

"0": No amplification for the VNT considered

[1] The *B. koehlerae* isolate was not included in the trees, as only 3 VNTR/5 were amplified in this species.

[2] This isolate was not included in the trees and its profile not considered, as typing of BHV-C could not be obtained due to lack of bacterial DNA.

[3] The isolate was lost and not enough DNA available for further analysis

* The use of color is desirable but not essential

Figure 1*: Minimum spanning tree (MST) of *Bartonella* isolates and strains from cats, human patients, one dog and 22 free-ranging and captive wild felids

A: Group A *B. henselae* isolates (Red circles); **B:** Group B *B. henselae* isolates/strains (Blue circles); **G1**: Group G1 isolates; Group **G2** isolates/strains; **G3**: Group G3 wild cat isolates (Green circles).

I = Genotype I; **II =** Genotype II

In gray: Clonal complexes of isolates, defined by a maximum distance of 1 between nodes in the same partition.

* The use of color is essential

Figure 2*: Clustering analysis of *Bartonella* isolates and strains from cats, human patients, one dog and 22 free-ranging and captive wild felids

A: BHVA; **B:** BHVB; **C:** BHVC; **D:** BHVD; **E:** BHVE.

Orig: Origin; **CA=**California; **ALG=**Algeria **NA=**Namibia; **NC=**North Carolina; **REF=**Reference strain; **SA-K=**South Africa-Kruger; **ZI =** Zimbabwe.

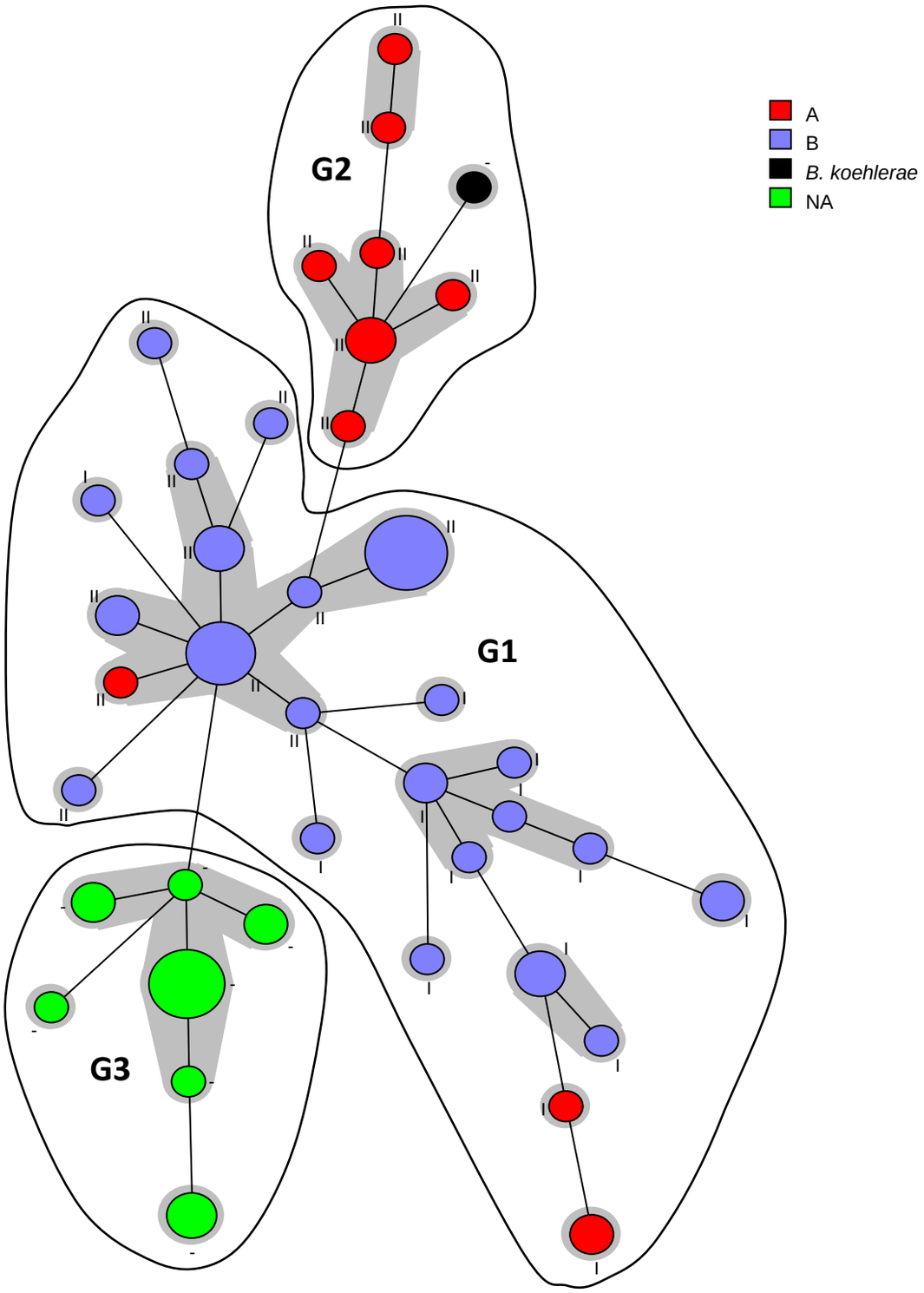
G1: Group G1; **G1a:** Group G1a; **G1b:** Group G1b; **G2**: Group G2; **G3**: Group G3.

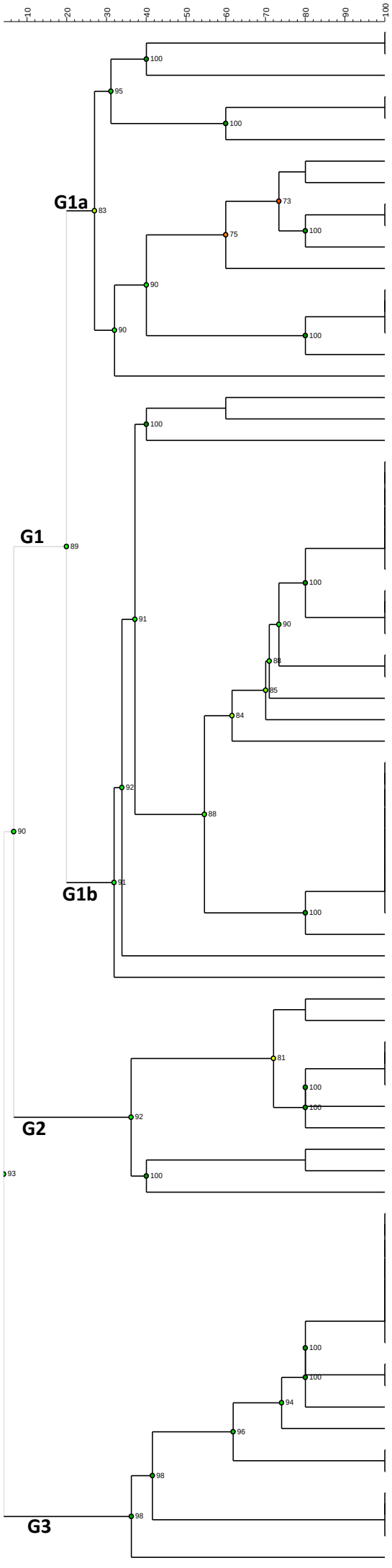
The groups have been color coded: Red = Group A; Blue = Group B; Green = Group G3.

★most severe forms in humans; : bacillary angiomatosis in humans

NR: Not reported ; DC: Domestic Cat.

* The use of color is essential


A
B
B. koehlerae
NA
G1
G2
G3



A	B	C	D	E							
13	20	7	8	2		NC51	I	NC	Cat	B.henselae	DC	B
13	20	7	8	2		NC55	I	NC	Cat	B.henselae	DC	B
13	25	6	8	4		NC61	II	NC	Cat	B.henselae	DC	B
14	20	6	1	2		NC264	I	NC	Cat	B.henselae	DC	A
14	20	6	1	2		NC267	I	NC	Human ★	B.henselae	NR	A
15	20	6	1	4		NC248	I	NC	Dog	B.henselae	DC	A
15	20	10	7	5		1024	I	CA	Cat	B.henselae	DC	B
13	20	10	7	5		N5	I	CA	Cat	B.henselae	DC	B
14	20	10	7	5		759 (H1)	I	REF	Human	B.henselae	NR	B
14	20	10	7	5		271	I	CA	Cat	B.henselae	DC	B
14	20	9	7	5		927	I	CA	Cat	B.henselae	DC	B
13	20	10	7	2		270	I	CA	Cat	B.henselae	DC	B
15	20	10	8	4		199	I	CA	Cat	B.henselae	DC	B
15	20	10	8	4		K50	I	CA	Human ★	B.henselae	NR	B
15	20	10	8	4		K51	I	CA	Cat	B.henselae	DC	B
15	20	10	8	3		32	I	CA	Cat	B.henselae	DC	B
9	31	10	7	3		NC53	I	NC	Cat	B.henselae	DC	B
14	15	10	9	4		F1	I	CA	Cat	B.henselae	DC	B
14	17	10	3	4		NC112	I	NC	Cat	B.henselae	DC	?
14	26	6	10	4		R19981	II	ZI	Cheetah	B.henselae	Captive	B
14	32	8	7	4		232C	II	CA	Cat	B.henselae	DC	B
14	32	8	7	4		113-H3	II	CA	Cat	B.henselae	DC	B
14	32	8	7	4		K40	II	CA	Human ✦	B.henselae	NR	B
14	32	8	7	4		K42	II	CA	Cat	B.henselae	DC	B
14	32	8	7	4		K53	II	CA	Human ✦	B.henselae	NR	B
14	32	8	7	4		Zeus	II	CA	Cat	B.henselae	DC	B
13	32	8	7	4		518	II	CA	Cat	B.henselae	DC	B
13	32	8	7	4		175	II	CA	Cat	B.henselae	DC	B
13	32	8	7	4		Amanda	II	CA	Puma	B.henselae	free	B
14	36	8	7	4		925	II	CA	Cat	B.henselae	DC	B
14	36	8	7	4		114-H4	II	CA	Cat	B.henselae	DC	B
14	32	10	7	4		272	II	CA	Cat	B.henselae	DC	B
14	32	8	1	4		SC-443	II	CA	Bobcat	B.henselae	free	A
13	25	8	7	4		NC88	II	NC	Cat	B.henselae	DC	B
14	34	2	7	4		K52	II	CA	Human ✦	B.henselae	NR	B
14	34	2	7	4		BW	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Flu	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Flaca7A	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Kimmy7C	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Gorda7E	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Sacha7F	II	CA	Cat	B.henselae	DC	B
14	34	2	7	4		Raccouni 7G	II	CA	Cat	B.henselae	DC	B
14	32	2	7	4		K38	II	CA	Cat	B.henselae	DC	B
14	35	13	7	6		K33	I	CA	Human ✦	B.henselae	NR	B
13	27	8	5	4		0-22	II	CA	Cat	B.henselae	DC	B
9	15	2	1	4		46	II	CA	Cat	B.henselae	DC	A
9	15	2	1	3		180	II	CA	Cat	B.henselae	DC	A
9	15	2	1	1		U4	II	CA	Cat	B.henselae	DC	A
9	15	2	1	1		NC32	II	NC	Cat	B.henselae	DC	A
9	15	2	1	1		NC44	II	NC	Cat	B.henselae	DC	A
9	14	2	1	1		105	II	CA	Cat	B.henselae	DC	A
9	16	2	1	1		NC46	II	NC	Cat	B.henselae	DC	A
8	14	3	1	1		150-41	II	SA-K	Lion	B.henselae	free	A
10	14	3	1	1		7069	II	SA-K	Lion	B.henselae	free	A
11	0	0	1	1		98/215	-	SA-K	Lion	B. koehlerae	free	B. koehlerae
16	14	1	0	6		1023	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		2326	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		2327	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		2328	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		5628	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		9192	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
16	14	1	0	6		9255	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
13	14	1	0	6		L-11-96	-	CA	Bobcat	B.k.bothieri	free	NA
13	14	1	0	6		DS-507	-	CA	Bobcat	B.k.bothieri	free	NA
14	14	1	0	6		L-17-96	-	CA	Bobcat	B.k.bothieri	free	NA
16	14	1	0	2		DS-08	-	CA	Bobcat	B.k.bothieri	free	NA
14	14	1	0	4		L-10-97	-	CA	Bobcat	B.k.bothieri	free	NA
14	14	1	0	4		1036	-	CA-Zoo	Cheetah	B.k.bothieri	semi-captive	NA
15	14	1	4	2		L-42-96	-	CA	Puma	B.k.boulouisii	free	NA
15	14	1	4	2		L-27-96	-	CA	Puma	B.k.boulouisii	free	NA
15	14	1	4	2		Ellie	-	CA	Puma	B.k.boulouisii	free	NA
10	4	1	0	6		1178	-	NA	Cheetah	Ch. variant 2	free	NA

Bartonella	**Animal species**	**Geographic origins**	**Isolate N°**	Ref
B. henselae	Lion (2 isolates)	South Africa, Kruger Park (free ranging)	150-41	Molia et al, 2004
			7069	Molia et al, 2004
	Puma (1 isolate)	California (free ranging)	Amanda	This study
	Bobcat (1 isolate)	California (free ranging)	SC-443	Chomel et al,2016
	Cheetah (1 isolate)	Zimbabwe (captive)	R19981	This study
B. koehlerae koehlerae	Lion	South Africa, Kruger Park (free ranging)	98/215	Molia et al, 2004
B. koehlerae subsp *boulouisii*	Puma (3 isolates)	California (free ranging)	L-42-76	This study
			L-42-96	This study
			Ellie	This study
B. koehlerae subsp *bothierii*	Bobcat (6 isolates)	California (free ranging)	L-08-96	Chomel et al,2016
			L-10-97	Chomel et al,2016
			L-11-96	
			L-17-96	Chomel et al,2016
			DS-08	Chomel et al,2016
			DS-507	Chomel et al,2016
	Cheetah (8 isolates)	California, San Diego Zoo (semi-captive)	1023	This study
			1036	This study
			2326	This study
			2327	This study
			2328	This study
			5628	This study
			9192	This study
			9255	This study
B. koehlerae unknown subsp	Cheetah (1 isolate)	Namibia (free ranging)	1178	Molia et al, 2004

Isolate N°	*Animal species*	*Bartonella*	BHV A	BHV B	BHV C	BHV D	BHV E	MLVA profile number
150-41	Lion	*B. henselae*	8	14	3	1	1	2
7069	Lion	*B. henselae*	10	14	3	1	1	3
Amanda	Puma	*B. henselae*	13	32	8	7	4	4
SC-443	Bobcat	*B. henselae*	14	32	8	1	4	5
R19981	Cheetah	*B. henselae*	14	26	6	10	4	6
98/215[1]	Lion	*B. koehlerae*	11	0	0	1	1	14
L-42-96	Puma	*B. koehlerae* subsp.	15	14	1	4	2	7
L-27-96	Puma	*boulouisii*	15	14	1	4	2	7
Ellie	Puma		15	14	1	4	2	7
L-08-96[2]	Bobcat	*B. koehlerae* subsp.	12	14	0	0	3,5	0
L-10-97	Bobcat	*bothieri*	14	14	1	0	3,5	8

L-11-96	**Bobcat**		**13**	**14**	**1**	**0**	**5,5**	**9**
L-17-96	**Bobcat**		**14**	**14**	**1**	**0**	**5,5**	**10**
DS-08	**Bobcat**		**16**	**14**	**1**	**0**	**2**	**11**
DS-507	**Bobcat**		**13**	**14**	**1**	**0**	**5,5**	**9**
1023	**Cheetah**	***B. koehlerae* subsp.**	**16**	**14**	**1**	**0**	**5,5**	**12**
1036	**Cheetah**	***bothieri***	**14**	**14**	**1**	**0**	**3,5**	**8**
2326	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
2327	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
2328	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
5628	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
9192	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
9255	**Cheetah**		**16**	**14**	**1**	**0**	**5,5**	**12**
1178[3]	**Cheetah**	***B. koehlerae***	**9,5**	**4**	**1**	**0**	**6**	**13**
		unknown subsp.						