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## Multiple locus variable number tandem repeat analysis for the characterization of wild feline *Bartonella* species and subspecies

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1 *Bartonella*-wildcats-MLVA-Vet-Mic

2 **Multiple locus variable number tandem repeat analysis for the characterization**  
3 **of wild feline *Bartonella* species and subspecies**

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27 **Highlights**

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

34 **Abstract**

35 *Bartonella* genus includes an increasing number of species and subspecies, especially among wild

36 felids, the positioning of which, with regards to the zoonotic species *Bartonella henselae*, is

37 important to determine.

38 The aim of this study was to test the ability of a molecular typing technique to distinguish between

39 various *Bartonella* isolates obtained from four different species of free-ranging and captive wild

40 felids and to identify key profiles or markers allowing differentiating them from each other and/or

41 from *B. henselae* or *B. koehlerae*.

42 A molecular typing technique for *B. henselae* based on the polymorphism of variable number

43 tandem repeat units (VNTR) called MLVA (Multiple Locus VNTR Analysis) was applied to 24

44 *Bartonella* isolates from free-ranging or captive wild felids, 19 of which were obtained from

45 California and five from three countries in Southern Africa, and compared with 49 *B. henselae*

46 isolates from cats, dog or humans from the United States including the human ATCC (American

47 Type Culture Collection) reference strain, *B. henselae* Houston 1.

48 MLVA allowed distinguishing *Bartonella* isolates from wild felids from either *B. henselae* or *B.*

49 *koehlerae*. We confirmed infection of semi-captive cheetahs with an isolate similar to a Californian

50 bobcat isolate. MLVA also confirmed the unique profile of a free-ranging cheetah isolate from

51 Namibia.

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

56 **Keywords:** *Bartonella*, Wild felids, MLVA.

## 57 **Introduction**

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

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

78 been used yet to differentiate *Bartonella* species and subspecies and explore their relationships.  
79 Various methods have been proposed for typing *Bartonella* isolates within *B. henselae* species, the  
80 agent of cat scratch disease (Bergmans *et al.*, 1996; especially Pulsed-Field Gel Electrophoresis  
81 (PFGE) (Arvand *et al.*, 2001), Multilocus Sequence Typing (MLST) (Iredell *et al.*, 2003 ; La Scola  
82 *et al.* 2003) and Multispacer Typing (MST) (Li *et al.*, 2006). However, none of them was tested to  
83 easily and reliably differentiate the main species and sub-species within the *Bartonella* genus. Thus,  
84 there was a need for a simple and inexpensive method to study the relationships between *Bartonella*  
85 species and their heterogeneity within each species. MLVA (Multiple Locus Variable number  
86 tandem repeats Analysis) has been developed in our laboratory and is based on the polymorphism  
87 of five main VNTR, called BHV-A to E (for *Bartonella henselae* Variable Number Tandem Repeat  
88 (VNTR) (Monteil *et al.*, 2007). To date, if we exclude whole genome sequencing, MLVA is still the  
89 most discriminatory typing method for *B. henselae* isolates (Bouchouicha *et al.*, 2008). This method  
90 allowed distinguishing 99 profiles among 178 human, feline and canine isolates (Bouchouicha *et*  
91 *al.*, 2009) and to characterize two groups of *B. henselae* isolates/strains, group A including only  
92 isolates from cats, and group B including all the isolates obtained from humans, a dog and the rest  
93 of the cats. Interestingly, groups A and B were particularly characterized by a different distribution  
94 of BHV-D alleles, as all group A isolates harboured only one or two repeats whereas group B  
95 isolates presented a minimum of five repeats, at least at the level of the isolates that were available  
96 at this time. Epidemiological, experimental and molecular arguments favour the hypothesis that  
97 group B isolates are zoonotic while group A isolates are not (Bouchouicha *et al.*, 2009; Gil *et al.*,  
98 2013).

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

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

110

## 111 **Materials and Methods**

### 112 *Bartonella* strains, isolates and DNA extraction

113 Twenty four isolates of *Bartonella* from wild felids were used in this study (Table I): Four isolates  
114 from free-ranging pumas (*Felis oncolor*), seven isolates from free-ranging bobcats (*Lynx rufus*),  
115 mainly from northern California, and eight isolates from semi-captive cheetahs (*Acinonyx jubatus*)  
116 from southern California (San Diego wildlife safari Park); two isolates from cheetahs (one captive  
117 from Zimbabwe (Kelly *et al.*, 1998) and one free-ranging from Namibia) and three isolates from  
118 African lions (*Panthera leo*) from Kruger Park, Republic of South Africa. Five isolates were  
119 determined to be *B. henselae*, 17 were identified as two new subspecies of *B. koehlerae*,  
120 respectively *B. koehlerae* subsp. *boulouisii* isolated from free ranging Californian mountain lions  
121 (n=3), and *B. koehlerae* subsp. *bothieri* isolated from 14 free ranging bobcats (n=6) or semi-captive  
122 cheetahs (n=8) (Chomel *et al.*, 2016; Molia *et al.*, 2016). A lion isolate from Kruger Park was  
123 identified as *B. koehlerae*. Finally, one isolate from a free ranging cheetah from Namibia was close  
124 to *B. henselae* and *B. koehlerae*, but different from all the other wild felid isolates (Molia *et al.*,  
125 2016). In addition, we used *B. henselae* reference strain Houston 1 (ATCC 49882) as a positive  
126 control for MLVA typing.

127 Samples were used as DNA extracts or cell lysates from isolated strains. For DNA extraction,  
128 bacteria were scraped from a 5% rabbit blood agar plate, as previously described (Chomel *et al.*,  
129 1995) and suspended in 500 µl sterile distilled water. These suspensions were boiled for 10 min,

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

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

134 Two genotypes of *B. henselae*, differing for 3 bp located at positions 172 to 175 of 16S rRNA gene  
135 have been described (Bergmans *et al.*, 1996). Type-specific 16S rRNA gene amplification of  
136 *Bartonella* DNA with primer 16SF and either primer BH1 or BH2 was performed with the  
137 following modifications: amplification was carried out in 25 µl reaction volumes and each reaction  
138 mixture contained 3 µl of purified DNA or 5 µl of cell lysate, 2,5 µl of TaKaRa amplification  
139 buffer, 2 µl of dNTP, 2 µl of each primer and 0,2 µl of TaKaRa® Ex taq (RR001 DNA polymerase  
140 TAKARA biomedical group, Shiga, Japan). An initial denaturation step at 95 °C for 3 min was  
141 followed by the amplification programme: DNA was denatured for 20 s at 95 °C, and primers were  
142 annealed for 30 s at 56 °C, and extended at 72 °C for 1 min. After 29 cycles, there was a final  
143 extension programme at 72 °C for 10 min. PCR products were separated by gel electrophoresis in 2  
144 % agarose gels (Invitrogen, Electrophoresis Grade, Ultra pure ref : 15510).

### 145 Multiple Locus Variable number tandem repeat Analysis (MLVA)

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

151 PCR products were separated by gel electrophoresis. 1,5 % agarose gels (Invitrogen,  
152 Electrophoresis Grade, Ultrapure; ref : 15510) were prepared in TBE (Tris Boric acid EDTA (pH  
153 8,3)) running buffer, stained with ethidium bromide and photographed under UV illumination with  
154 an analysis image system (Gel Doc Biorad) and the software Quantity One. Long gels (26cm x  
155 40cm, CBS Scientific® model SGU-2640T-02) and a migration times (2h) at 300 V/cm (Generator

156 PS608 (600V - 800mA – 300W), Apexex ®) 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



181 from North American cats and human patients previously obtained in our laboratory, as most of the  
182 wild felid isolates originated from this continent (Monteil *et al.*, 2006; Bouchouicha *et al.*, 2009).

183 Fragment sizes were converted to repeat units and imported into BioNumerics 7.6.3 (Applied  
184 Maths, Belgium) as a character data.

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

189

## 190 **Results**

### 191 16S rRNA type of *Bartonella* isolates from wild felids

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

196

### 197 Multiple Locus VNTR Analysis of the wild felid isolates

198 The MLVA profiles obtained for the different *Bartonella* isolates from either free-ranging or semi-  
199 captive wild felids are presented in Table II. Fifteen different allelic profiles were identified for the  
200 24 wild felid isolates, of which 23 isolates gave profiles that were considered as complete which  
201 corresponded to 14 MLVA profiles.

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

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

208 In addition, the *B. koehlerae* isolate from a Kruger Park's lion presented a unique profile with only  
209 3 of BHVs being amplified (BHV-A, BHV-D and BHV-E).

210

#### 211 Discriminatory power of MLVA for typing of wild felid isolates

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

217

#### 218 Relationships between the *Bartonella* isolates obtained from wild Felids

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

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

236

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

239 For a few VNTR, some alleles (numbers of repeats) were not previously observed in the 244  
240 *henselae* isolates or strains obtained from domestic cats, dogs or humans previously typed by  
241 MLVA in our laboratory. The *Bartonella* isolates and strains were grouped into three main clusters  
242 G1, G2 and G3 (Figures 1 and 2). Group G1 was made of two subgroups: G1a (17 isolates/strains)  
243 and a G1b (28 isolates/strains). Subgroup G1a was almost exclusively composed of *B. henselae*  
244 genotype I isolates, including the human strain *B. henselae* Houston I and other human strains  
245 associated with most severe clinical forms. The only exception was one feline isolate from North  
246 Carolina (NC61) (Figure 2). Subgroup G1b was mostly constituted of *B. henselae* genotype II  
247 isolates (25 of 28 isolates/strains), associated with less severe clinical forms in humans, *i.e.*  
248 bacillary angiomatosis (Figure 2). Group G2 only contained *B. henselae* 16 S rDNA genotype II  
249 isolates and strains from domestic cats or some wild felids (the lion *B. koehlerae* isolate was also  
250 contained in this group). All the nine *B. henselae* isolates/strains of this group had a repeat score of  
251 1 for BHV-D, and were thus labeled as belonging to the previously described group A. Oppositely,  
252 most isolates/strains (40 of 45, *i.e.* 89% ; Isolate NC112 with the BHV-D repeat score of 3 was not  
253 assimilated to a group A nor to a group B isolate and was not included in the calculation of this  
254 percentage) of group G1 had a BHV-D repeat score of 5 to 10, and were thus labeled as belonging  
255 to the previously described group B.

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

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

## 261 Discussion

262 MLVA has been successfully used for *B. henselae* typing (Azzag *et al.*, 2012; Bouchouicha *et al.*,  
263 2009; Cicuttin *et al.*, 2014; Gil *et al.*, 2013; Podsiadly *et al.*, 2012). In this study, we demonstrated  
264 that this user-friendly and sensitive typing technique is a highly efficient method for discriminating  
265 isolates from different feline species. It was able to differentiate *Bartonella* isolates close to but  
266 distinct from *B. henselae* and *B. koehlerae*, at least for 3 to 4 VNTRs. Our results also confirmed  
267 the phylogenetic proximity between *B. henselae* and *B. koehlerae*, as previously reported (Koehler  
268 *et al.*, 1994; Zeaiter *et al.*, 2002).

269 Of the 24 *Bartonella* isolates obtained from four different species of free-ranging and captive wild  
270 felids, a high level of polymorphism was observed for the 23 ones that could be typed entirely, with  
271 14 different profiles. The Hunter and Gaston D.I. was 0.90, which is high when considering the  
272 relatively small number of isolates (n=23) and the fact that some of these isolates clustered,  
273 according to their host species. By comparison, the D.I. for *Brucella* is 0.87 (Tian *et al.*, 2017 ) and  
274 for *Mycoplasma* the D.I. is 0.84 (Sakmanoglu *et al.*, 2019). By comparison with the *B. henselae*  
275 isolates from cats and humans, the non-*B. henselae* isolates from free-ranging and captive wild  
276 felids, the majority of them identified as *B. koehlerae* subsp. *bothieri* (California bobcats and semi-  
277 captive cheetahs) or *B. koehlerae* subsp. *boulouisii* (pumas), presented specific and unique profiles  
278 not observed in any of the other tested isolates. The absence of amplification of BHV-D was  
279 characteristic of *B. koehlerae* subsp. *bothieri* isolates, while the presence of only one repeat for  
280 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

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

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

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

323

#### 324 **Conflict of interest statement**

325 The authors have no conflict of interest to declare.

326

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467

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

469 **The use of color is desirable but not essential**

470

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

472 “0”: No amplification for the VNT considered

473 <sup>1</sup> The *B. koehlerae* isolate was not included in the trees, as only 3 VNTR/5 were amplified in this  
 474 species.

475 <sup>2</sup> This isolate was not included in the trees and its profile not considered, as typing of BHV-C could  
 476 not be obtained due to lack of bacterial DNA.

477 <sup>3</sup> The isolate was lost and not enough DNA available for further analysis

478 **\* The use of color is desirable but not essential**

479

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

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

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

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

488

489 \* The use of color is essential

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

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

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

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

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

497 ★most severe forms in humans; ✦: ✦acillary angiomas in humans

498 NR: Not reported ; DC: Domestic Cat.

499

500 \* The use of color is essential

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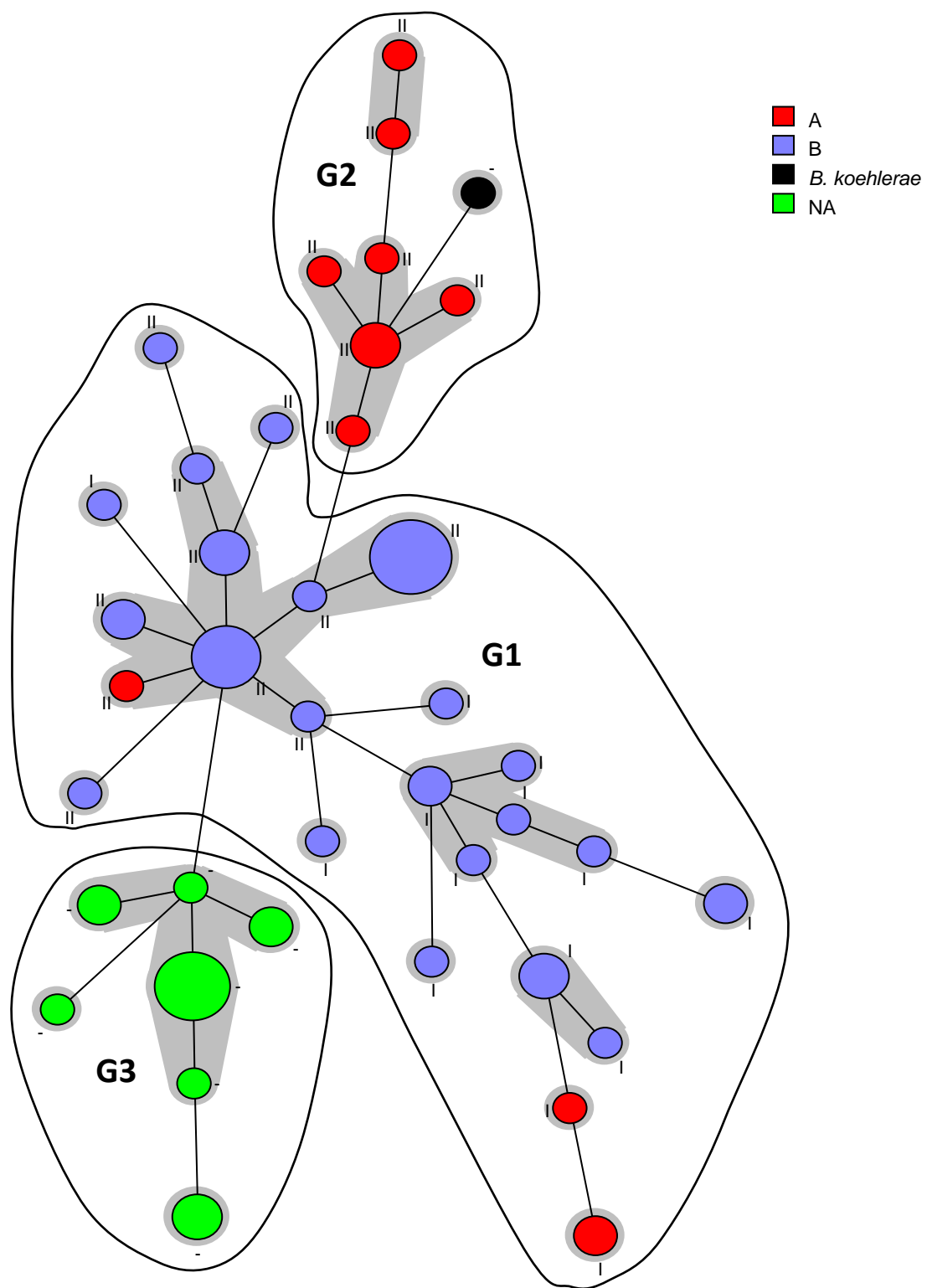
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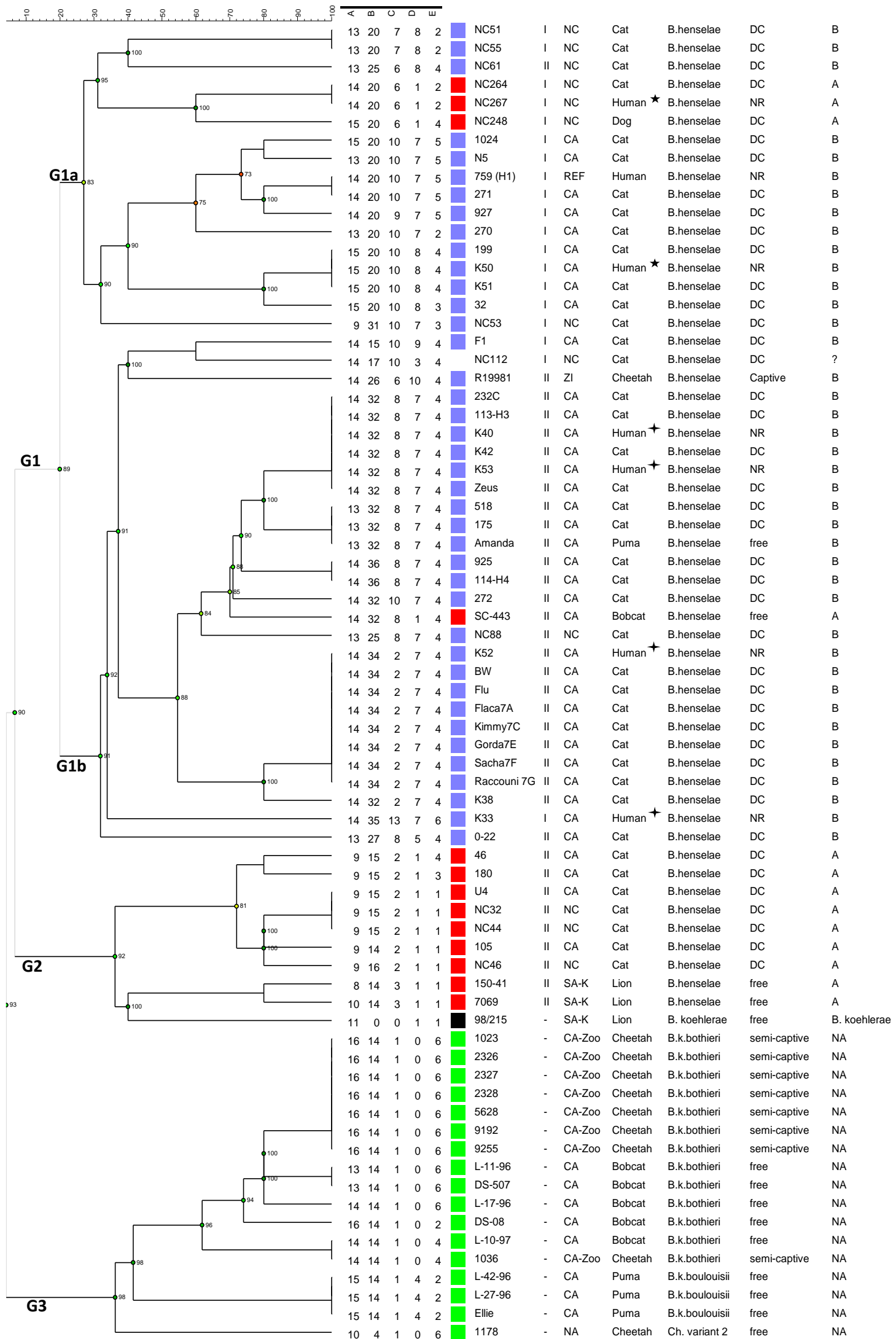
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<b><i>Bartonella</i></b>	<b>Animal species</b>	<b>Geographic origins</b>	<b>Isolate N°</b>	<b>Ref</b>
<i>B. henselae</i>	Lion (2 isolates)	South Africa, Kruger Park (free ranging)	150-41	Molia et al, 2004
			7069	Molia et al, 2004
	Puma (1 isolate)	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
<i>B. koehlerae koehlerae</i>	Lion	South Africa, Kruger Park (free ranging)	98/215	Molia et al, 2004
<i>B. koehlerae</i> subsp <i>boulouisii</i>	Puma (3 isolates)	California (free ranging)	L-42-76	This study
			L-42-96	This study
			Ellie	This study
<i>B. koehlerae</i> subsp <i>bothierii</i>	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
<i>B. koehlerae</i> unknown subsp	Cheetah (1 isolate)	Namibia (free ranging)	1178	Molia et al, 2004



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

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