

Bartonella Prevalence and Genetic Diversity in Small Mammals from Ethiopia

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Abstract

More than 500 small mammals were trapped at 3 localities in northern Ethiopia to investigate *Bartonella* infection prevalence and the genetic diversity of the *Bartonella* spp. We extracted total DNA from liver samples and performed PCR using the primers 1400F and 2300R targeting 852 bp of the *Bartonella* RNA polymerase beta subunit (*rpoB*) gene. We used a generalized linear mixed model to relate the probability of *Bartonella* infection to species, season, locality, habitat, sex, sexual condition, weight, and ectoparasite infestation. Overall, *Bartonella* infection prevalence among the small mammals was 34.0%. The probability of *Bartonella* infection varied significantly with species, sex, sexual condition, and some locality, but not with season, elevation, habitat type, animal weight, and ectoparasite infestation. In total, we found 18 unique *Bartonella* genotypes clustered into 5 clades, 1 clade exclusively Ethiopian, 2 clades clustered with genotypes from central and eastern Africa, and the remaining 2 clades clustered with genotypes and species from Africa and Asia. The close relatedness of several of our *Bartonella* genotypes obtained from the 3 dominant rodent species in Tigray with the pathogenic *Bartonella elizabethae* from *Rattus* spp. in Asia indicates a potential public health threat.

Key Words: *Bartonella*—Prevalence—Genotypes—Small mammals—Ethiopia.

Introduction

A PART FROM BEING OF SERIOUS CONCERN for agriculture in Africa, rodents have a major veterinary and public health importance because they act as natural reservoirs and hosts of several pathogens infectious to humans and animals. In Africa, the role of rodents in the epidemiology of rodent-associated diseases, such as plague, Lassa fever, and leptospirosis, has been well established (Gratz 1997). However, much remains to be understood regarding other emerging and reemerging diseases and pathogens, such as *Bartonella*. In recent years, a growing number of studies have targeted identification and characterization of *Bartonella* species from mammalian hosts worldwide, because they are considered as emerging infectious pathogens affecting humans and animals (e.g., Raoult et al. 1998, Kosoy et al. 2003, Pretorius et al. 2004, Gundi et al. 2010, Yuan et al. 2011, Gundi et al. 2012).

Bartonella spp. are Gram-negative bacteria belonging to the α 2-subclass of Proteobacteria (Houpikian and Raoult 2001). They are facultative intracellular parasites of mammalian

endothelial and red blood cells (Just et al. 2008, Kosoy 2010). To date, at least 20 species and subspecies are recognized worldwide, of which about 10 are reported as pathogenic to humans (Boulouis et al. 2005, Just et al. 2008). Although their mode of infection and associated risk factors are poorly known (Boulouis et al. 2005, Morway et al. 2008), infections caused by *Bartonella* have been associated with human diseases such as cat scratch disease, trench fever, endocarditis, and neuroretinitis. Transmission of *Bartonella* infections between hosts is believed to be mediated by a range of ectoparasites, including fleas, ticks, lice, and sandflies (Reeves et al. 2007, Reis et al. 2011). The sandfly *Lutzomyia verrucarum*, the body louse *Pediculus humanus humanus*, the fleas *Ctenocephalides felis* and *Ctenophthalmus nobilis nobilis* (Billeter et al. 2008), and quite recently the tick *Ixodes ricinus* (Reis et al. 2011) have been confirmed as arthropod vectors. Furthermore, several other fleas, sand flies, lice, and ticks are suspected vectors.

Given the large diversity of rodent species in Africa (Musser and Carleton 2005), there are relatively few reports

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on the detection, characterization, and prevalence of *Bartonella* species among African rodents. The first report on *Bartonella* presence and dynamics in African rodent species came from a population of the fat sand rat *Psammomys obesus* in Tunisia (Fichet-Calvet et al. 2000). Later *Bartonella* infections were reported among murine rodents in South African: *Aethomys namaquensis*, *Mastomys natalensis*, *Rhabdomys pumilio*, and *Tatera leucogaster* (Pretorius et al. 2004), and other rodent species, including *Arvicanthis neumanni*, *Lophuromys rita*, *Mus minutoides*, and *Rattus rattus* from Democratic Republic of Congo (DRC) and Tanzania (Gundi et al. 2012). From these studies we may infer that: (1) A very diverse group of rodent species in the continent is infected with the bacteria (and many more species wait investigation), (2) the *Bartonella* infecting the rodents are very diverse, with a high discovery rate for potentially novel species or genotypes, and (3) some of the *Bartonella* genotypes detected from the African rodents are genetically closely associated with species already known to be pathogenic to humans.

The objectives of this study were to: (1) Investigate *Bartonella* infection prevalence among small mammals trapped from domestic and peridomestic habitats and “scrublands” in Tigray, northern Ethiopia and (2) identify the genetic diversity of *Bartonella* species parasitizing these small mammals and estimate the phylogenetic relationship of the Ethiopian *Bartonella* with previously described species or genotypes in Africa and elsewhere in the world.

Materials And Methods

Trapping localities and sampling

We trapped small mammals from three localities, Golgolnaele (N13°52, E39°43, 2700 meters above sea level [a.s.l.]),

Mahbere Silassie (N13°39, E39°08, 2600 meters a.s.l.), and Aroresha (N12°25, E39°33, 1600 meters a.s.l.), in Tigray, northern Ethiopia (Fig. 1). The first two localities are from the Ethiopian highlands and the third locality from a lowland area. Trapping was conducted during the dry (March and May, 2010), wet (July and August, 2010), and early dry (November and December, 2010) seasons to represent the 3 main seasons as far as rodent population dynamics in Tigray are concerned (Y. Meheretu et al., unpublished data). Trapping was carried out in a total of 30 randomly selected household compounds (homesteads), 10 from each locality. We set 8 traps per household compound, 4 inside houses (near beds, food, clothing cabinets, holes, and hide or jute sacks) and 4 outside the houses (near walls and fences within the compound). Additional trapping was made in “scrublands” (a mixture of fallow field and bushy vegetation) for the whole study period in Golgolnaele, once (March, 2010) in Mahbere Silassie, but never in Aroresha. In the “scrublands,” we set a 60- \times 60-meter permanent square grid consisting of 7 parallel lines, 10 meters apart, with trapping stations also 10 meters apart (*i.e.*, a total of 49 trapping stations per grid). Trapping was conducted for 2 consecutive days using Sherman LFA live traps (7.5 \times 9.0 \times 23.0 cm, HB Sherman Trap, Tallahassee, FL) baited with peanut butter mixed with wheat flour.

Captured animals were handled following the ethical policies and guidelines approved by the committee for Animal Care and Use (Mekelle University, Ethiopia). All captured animals were sacrificed using an appropriate dose of ether. External morphological measurements (body weight, head-body, tail, hind foot, and ear lengths) were recorded for further taxonomic identification. Sexual condition was determined as follows: (male, testes abdominal [reproductively inactive], testes scrotal [reproductively active]; female, vagina

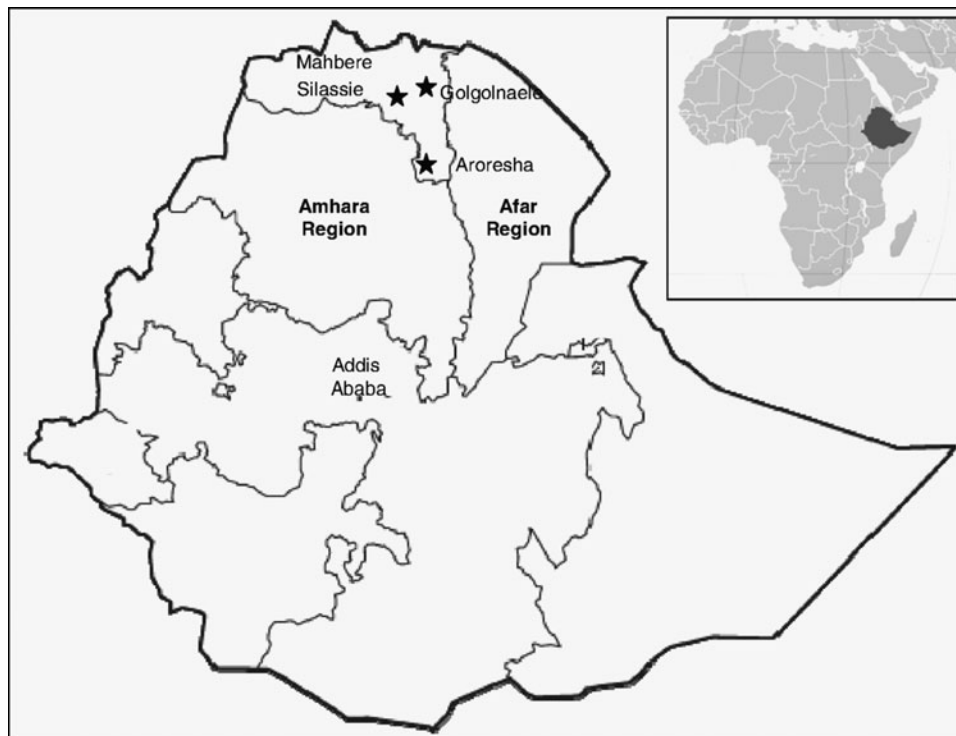


FIG. 1. Map of Ethiopia showing the 3 study localities—Mahbere Silassie, Golgolnaele, and Aroresha—in Tigray Region (*top*).

plugged [reproductively inactive], vagina perforated, vagina perforated and pregnant, and vagina perforated and lactating [reproductively active]). Ectoparasites were removed while combing the fur and preserved in 70% ethanol. Tissue samples were taken from liver and kidney and preserved in 70% ethanol and/or in RNAlater (an RNA stabilization reagent).

The small mammals were initially identified in the field to genus level and later confirmed to species level at the Laboratory of the Evolutionary Ecology Group, University of Antwerp (Belgium), by combining craniometric measurements and mitochondrial DNA cytochrome *b* sequencing of a subset of the samples using L7 (5'-ACCAATGACATGAAAATCATCGTT-3') and H6 primers (5'-TCTCCATTTC TGGTTTACAAGAC-3') (Montgelard et al. 2002). The genotyping revealed that the species tagged "*Mastomys awashensis*" during the initial field identification was in fact composed of two morphologically cryptic species, *M. awashensis* and *Stenocephalemys albipes*. Thus, we genotyped all individuals initially tagged as "*M. awashensis*." Some representative sequences of each small mammal species are available in GenBank (AN: JQ956464-JQ956479).

Detection of *Bartonella* in host samples

Total DNA was extracted from liver samples using buffers ATL, AL, AW1, AW2, AE, and proteinase K (QIAGEN) with silica columns (Zymo Research) following the QIAamp DNA Mini Kit instructions. DNA was eluted in 60 μ L of buffer AE. PCR was performed using the primers 1400F (5'-CGCATGGCTFACTTCGATG-3') and 2300R (5'-GTAGACTGATTAGAACGCTG-3') (Renesto et al. 2001) targeting 852 bp (positions 1456–2307 without primers) of the *Bartonella* RNA polymerase β -subunit (*rpoB*) gene, which has been shown to be useful for discriminating different *Bartonella* species (La Scola et al. 2003). Amplifications were performed in a 25- μ L reaction volume containing 0.2 μ M of each primer, 1 \times DreamTaq Buffer (containing 2 mM MgCl₂), 0.8 mM MgCl₂, 0.2 mM of each dNTP, and 1 U DreamTaq DNA Polymerase (Fermentas). The thermal profile started with an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were visualized on 1.4% agarose gels. A sample was considered as positive for the prevalence study when a band of ~900 bp was visible. For the phylogeny study, 77 of the positive PCR products were purified and sequenced by VIB Genetic Service Facility (University of Antwerp, Belgium) using the same primers as for the PCR.

Ecological analysis

We calculated *Bartonella* infection prevalence (proportion of *Bartonella*-positive individuals) and the 95% confidence intervals (95% CI) for each small mammal species in Quantitative Parasitology 3.0 using the Sterne exact method (Rozsa et al. 2000, Reiczigel 2003).

To analyze the correlates of host and environmental factors with the probability that an individual host was infected with *Bartonella*, we used a generalized linear model (GLM) with binomial family and a logit link to construct a multiple logistic model in R (R Development Core Team 2010). The GLM was fitted to relate the logit of *Bartonella* occurrence to host species (6 levels: *Arvicanthis dembeensis*, *Crocidura olivieri*, *M. awa-*

shensis, *Mus* spp., *R. rattus*, and *S. albipes*), season (3 levels, wet, early dry, and dry), locality (3 levels, Golgolnaele, Mahbere Silassie, and Aroresha), habitat (2 levels, domestic versus peridomestic), sex (2 levels, male versus female), sexual condition (2 levels, reproductively active versus inactive), flea infection (2 levels, infected versus not infected), mite infection (2 levels, infected versus not infected), tick infection (2 levels, infected versus not infected), weight (continuous), and biologically relevant interactions. Note that data for "scrublands" were not included in the GLM analysis. Model selection was based on the Akaike Information Criterion (AIC) and likelihood-ratio tests (LRT), and we used a top-down protocol as described in Zuur et al. (2009). Statistical significance was declared at *p* values of less than 0.05.

In summary, the generalized linear starting model included the following sets of variables: *Bartonella* infection, host species, season, interaction between season and locality, habitat nested within locality, sex, sexual condition, flea infestation, mite infestation, tick infestation, and weight. First, we tested if the probability of *Bartonella* infection varied with habitat type by nesting habitat within locality. It turned out that the test was not significant ($\chi^2=5.0$, degrees of freedom [df]=3, *p*=0.1708), hence habitat type was dropped from the analysis. Second, we looked at the interaction between locality and season and found out that the interaction was not significant ($\chi^2=5.9$, df=4, *p*=0.2086), hence it was dropped from the analysis. Third, we tested for among locality variations (to see if the three localities can be grouped in a 2-level factor [highland and lowland] or treated separately), and the test was significant ($\chi^2=12.4$, df=1, *p*=0.0004), indicating that the data fit best when the 3 localities are treated separately than when grouped into highland and lowland. Then, we began to drop each variable from the full model separately to reveal their importance in the analysis. Hence, our final model included species ($\chi^2=141.5$, df=6, *p*<0.0001), locality ($\chi^2=17.6$, df=2, *p*=0.0001), sex ($\chi^2=8.7$, df=1, *p*=0.0031), and sexual condition ($\chi^2=7.8$, df=1, *p*=0.0052).

Genetic analyses

Sequences were checked and aligned using Geneious Pro 5.5.3 (Drummond et al. 2010). During the checking process, clear double peaks (distinct from the baseline noise) were visible on the sequencing chromatograms of half of the samples. These were likely the result of mixed infections. The uncertainty thus introduced could be encoded by replacing double-peak positions by undetermined nucleotides (e.g., R for A or G, Y for T or C, etc.). However, because several mixed sequences seemed to result from co-infections with very different *Bartonella* genotypes, this encoding could produce substantial noise during phylogenetic reconstruction. Therefore, the only mixed infection sequences entered in the phylogeny were those where there was clearly one set of dominant peaks, and these were entered without uncertainty. Sequences were compared to other Bartonellae using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990) on the National Center for Biotechnology Information (NCBI) website. The new sequences have been deposited in GenBank (accession numbers JQ425623–JQ425636 and JQ425638–JQ425644).

Sequences were aligned with a representative subset of *rpoB* sequences of *Bartonella* isolated from rodents together

with reference species, all available in Genbank. *Brucella melitensis* 16M^T was used as an outgroup. The final alignments included 825 sites. Estimates of evolutionary divergence between sequences were computed using the p-distance method in MEGA 5.05 (Tamura et al. 2011). We estimated the phylogenetic relationship between our sequences and other *Bartonella* sequences in two ways. First, for comparison with existing literature, we used neighbor-joining with the Kimura 2-parameter (K2P) model of nucleotide substitution in MEGA 5.05 (Tamura et al. 2011), as used in several recent studies of small mammal *Bartonella* (Inoue et al. 2008; Welc-Faleciak et al. 2008; Inoue et al. 2009; Gil et al. 2010; Gundi et al. 2010). Second, we used jModelTest 0.1.1 (Guindon and Gascuel 2003; Posada 2008) to evaluate the fit of 24 nested models of nucleotide substitution to the data using the AIC. The AIC indicated that the sequence evolution model best describing the data is HKY85 with a proportion of invariables sites and rate variation among sites with 4 rate categories (HKY85+I+G). This model of sequence evolution was then used for a maximum likelihood (ML) estimation of the phylogeny in PhyML 3.0 (Guindon et al. 2010). Branch supports were evaluated by both nonparametric bootstrap analysis (1000 replicates and 200 replicates for neighbor joining [NJ] and ML analyses, respectively) and approximate likelihood-ratio tests (aLRT) relying on a nonparametric Shimodaira–Hasegawa (SH)-like procedure (ML analysis only; Guindon et al. 2010). Where appropriate, tree branches are annotated with both support estimates. We compared the goodness of fit of the NJ and ML tree topologies using the SH test (Shimodaira and Hasegawa 1999) implemented in TREE-PUZZLE 5.2 (Schmidt et al. 2002) using both the K2P and HKY85+I+G models of sequence evolution.

Result

Small mammal community

In total, 529 small mammals belonging to at least 6 different species were trapped from the 3 localities and tested for *Bartonella* infection. An overview of the number of samples tested for *Bartonella* infection and the subsequent result are summarized in Table 1. In Golgolnaele and Mahbere Silassie, 2 species, *A. dembeensis* and *S. albipes*, comprised 81.9% and 67.7% of the tested small mammals, respectively. In Aroresha, *R. rattus* and *M. awashensis* comprised 66.7% of the tested small mammals. Note that, while the proportion of tested *R. rattus* in Golgolnaele and Mahbere Silassie was 2.3% and 4.5%, respectively, the proportion in Aroresha was 46.5%.

Host species and Bartonella infection

We found 34.0% prevalence of *Bartonella* infection in the small mammals overall (CI 30.1– 38.2%). This prevalence estimate is conservative because we did not use an internal control to prove the efficiency of DNA extraction and/or possible PCR inhibitors. The highest *Bartonella* prevalence was observed in *S. albipes* (64.6%) (CI 55.3–73.0%), followed by *A. dembeensis* (37.9%) (CI 31.7–44.6%), *M. awashensis* (37.5%) (CI 24.8–52.0%), *Mus* spp. (5.6%) (CI 1.0–19.0%), *Crociodura olivieri* (2.4%) (CI 0.13–12.7%), and *R. rattus* (1.5%) (CI 0.08–8.1%) (Table 1). The GLM analysis showed significant differences in the probability of *Bartonella* infection among the small mammal species, with higher frequency of infection in *S. albipes* and lower frequency of infection in *R. rattus* and *C. olivieri* (Table 2). Except for 1 *R. rattus* trapped from a domestic area in Golgolnaele and 1 *C. olivieri* trapped from a peridomestic

TABLE 1. SEASONAL PREVALENCE OF *BARTONELLA* INFECTION IN SMALL MAMMALS TRAPPED FROM 3 LOCALITIES IN TIGRAY, ETHIOPIA

Locality	Host	Wet			Early dry			Dry			Total		
		Tested	Positive	%	Tested	Positive	%	Tested	Positive	%	Tested	Positive	%
Aroresha	<i>A. dembeensis</i>	7	2	28.6	7	3	42.9	2	0	0	16	5	31.3
	<i>C. olivieri</i>	—	—	—	6	0	0	4	0	0	10	0	0
	<i>M. awashensis</i>	1	1	100	18	4	22.2	4	1	25	23	6	26.1
	<i>Mus</i> spp.	3	1	33.3	5	0	0	4	0	0	12	1	8.3
	<i>R. rattus</i>	16	0	0	16	0	0	21	0	0	53	0	0
	Total	27	4	14.8	52	7	13.5	35	1	2.9	114	12	10.5
Golgolnaele	<i>A. dembeensis</i>	40	13	32.5	25	10	40	71	17	23.9	136	40	29.4
	<i>C. olivieri</i>	—	—	—	2	0	0	1	0	0	3	0	0
	<i>M. awashensis</i>	4	2	50	2	1	50	10	5	50	16	8	50
	<i>Mus</i> spp.	8	1	12.5	9	0	0	5	0	0	22	1	4.5
	<i>R. rattus</i>	1	1	100	2	0	0	3	0	0	6	1	16.7
	<i>S. albipes</i>	15	9	60	37	23	62.2	25	15	60	77	47	61.0
	Total	68	26	38.2	77	34	44.2	115	37	32.2	260	97	37.3
Mahbere Silassie	<i>A. dembeensis</i>	9	5	55.6	17	11	64.7	46	24	52.2	72	40	55.6
	<i>C. olivieri</i>	—	—	—	28	1	3.6	1	0	0	29	1	3.4
	<i>M. awashensis</i>	2	1	50	—	—	—	7	3	42.9	9	4	44.4
	<i>Mus</i> spp.	1	0	0	—	—	—	1	0	0	2	0	0
	<i>R. rattus</i>	2	0	0	2	0	0	3	0	0	7	0	0
	<i>S. albipes</i>	6	3	50	25	21	84	5	2	40	36	26	72.2
	Total	20	9	45	72	33	45.8	63	29	46.0	155	71	45.8
	Overall	115	39	33.9	201	74	36.8	213	67	31.5	529	180	34.0

Columns for each season indicate the number of individuals tested, those positive for *Bartonella* infection and the prevalence (%). (Wet season is represented by July and August, Early Dry season by November and December, and Dry season by March and May).

TABLE 2. RESULTS OF THE GENERALIZED LINEAR MODEL (FINAL MODEL) FOR THE EFFECT OF HOST SPECIES, SEASON, LOCALITY, SEX, AND SEXUAL CONDITION ON LIKELIHOOD OF *BARTONELLA* INFECTION

Effects	Estimate	Std. Error	Pr(> z)
<i>A. dembeensis</i>	-1.6429	0.4976	0.000962
<i>C. olivieri</i>	-5.3647	1.1664	4.24e-06
<i>M. awashensis</i>	-1.1912	0.4477	0.007797
<i>Mus</i> spp.	-3.7334	0.8801	2.21e-05
<i>R. rattus</i>	-4.9135	1.0705	4.44e-06
<i>S. albipes</i>	0.7192	0.5811	0.215861
Early dry season	0.5164	0.2715	0.057153
Wet season	0.3349	0.2897	0.247586
Golgolnaele	0.6236	0.4471	0.163061
Mahbere Silassie	1.5930	0.4654	0.000619
Male	-0.6450	0.2231	0.003847
Sexually active	0.6004	0.2247	0.007552

area in Mahbere Silassie, the remaining individuals of these two species ($n=66$ and $n=42$, respectively) tested negatively for *Bartonella* infection.

Spatial variation of *Bartonella* infection

Bartonella-positive hosts were found from each of the three localities (Table 1). The highest prevalence was observed in Mahbere Silassie (45.8%, CI 38.1–53.9%), followed by Golgolnaele (37.3%, CI 31.5–43.5%) and Aroresha (10.5%, CI 6.0–17.5%). In the first two localities, *Bartonella* prevalence was predominantly observed in *S. albipes* (72.2% and 61.0%, respectively). In Aroresha, *Bartonella* prevalence was predominantly detected in *A. dembeensis* (31.3%). Despite its abundance in Aroresha, all *R. rattus* individuals ($n=53$) tested from this locality were negative for *Bartonella* infection.

The results of the GLM analysis indicated that the chance of small mammals being infected with *Bartonella* was significantly higher in Mahbere Silassie than in Aroresha ($p=0.0006$), but not significantly different in Golgolnaele compared with Aroresha ($p=0.16$). However, within localities the chance of being infected with *Bartonella* was not significantly different between peridomestic and domestic habitats ($p=0.1708$). We note that data for the “scrublands” were not included in the GLM analysis since this habitat type was not investigated in Aroresha. However, *Bartonella*-positive rodents were found in this habitat in both Mahbere Silassie and Golgolnaele localities.

Seasonality of *Bartonella* infection

Overall, *Bartonella* prevalence within a locality appears stable across seasons (Table 1). We detected a trend in the probability of *Bartonella* infection in the small mammals during the early dry season compared to the dry season ($p=0.06$). However, the probability of infection was not significantly different between wet and dry seasons ($p=0.25$). It is worth mentioning here that we detected *Bartonella* infection in each of the months distributed across the 3 seasons.

Host characteristics and *Bartonella* infection

The male-to-female sex ratio of the tested small mammals was 50.9% (males 269, females 260). *Bartonella* prevalence was

28.6% (CI 23.4–34.4%) in the males and 39.6% (CI 33.8–45.8%) in the females. The GLM analysis confirmed that the likelihood of being infected with *Bartonella* is significantly less in the captured males compared to the females ($p=0.004$).

The ratio of reproductively active small mammals to reproductively inactive ones was 52.6%. *Bartonella* prevalence in the reproductively active individuals was 40.6% (CI 34.9–46.6%) and in reproductively inactive ones the prevalence was 26.7% (CI 21.5–32.6%). The likelihood of being infected with *Bartonella* was significantly higher in reproductively active individuals compared to reproductively inactive ones ($p=0.008$).

Ectoparasite infestation and *Bartonella* infection

In total, 1882 fleas, 1484 mites, and 219 ticks were recovered from the small mammals tested for *Bartonella*. The mean number of fleas per host (infested plus noninfested) was 3.6 (standard error [SE]=0.23; range, 0–48), mites 2.8 (SE=0.29; range, 0–57), and ticks 0.4 (SE=0.08; range, 0–23). Overall prevalence of flea infestation among the small mammals was 66.9% (CI 62.8–70.8%), mites 32.7% (CI 28.8–36.9%), and ticks 15.1% (CI 12.3–18.4%). However, infestation with any of the ectoparasites did not significantly correlate with the probability of *Bartonella* infection in the small mammals.

Genetic characterization of *Bartonella*

Eighteen unique partial *rpoB* genotypes (designated as A–T, Table 3) of 852 bp were obtained from 66 sequenced PCR products. Eleven PCR products produced complex sequence chromatograms, likely due to mixed infections, which were not further interpreted. To relate the Ethiopian *Bartonella* genotypes to those known to be pathogenic to humans or found in small mammals in other parts of the world, we included 115

TABLE 3. LOCALITY AND HOST SPECIES OF THE 18 UNIQUE *BARTONELLA* GENOTYPES FROM NORTHERN ETHIOPIA

Genotypes	Number of individuals			Total
	Golgolnaele	Mahbere Silassie	Aroresha	
A	10 SA, 1AD	5SA		16
B	7SA, 1RR	5 SA		13
C		2SA, 1AD		3
E			3MA	3
F			1MA	1
G	1SA			1
H			1AD	1
I	3AD			3
J		2AD		2
K			1AD	1
L	2SA			2
M		1AD		1
N	2AD			2
P		1AD		1
Q		3SA	1MA	4
R	2MA			2
S		2AD		2
T	8AD			8
Total	37	22	7	66

SA, *S. albipes*; MA, *M. awashensis*; AD, *A. dembeensis*; RR, *R. rattus*. Note that letters “D” and “O” are not used to represent genotypes.

Bartonella sequences from GenBank in the phylogenetic analyses. The SH test showed that the ML tree topology was significantly better than the topology constructed by NJ, irrespective of which model of sequence evolution is assumed in the comparison (K2P and HKY85+I+G: $\Delta \log L=93.82$, $p=0.02$, and $\Delta \log L=79.55$, $p=0.004$, respectively). We present the ML phylogeny estimate in Figure 2, and the NJ tree as supplementary information in Appendix 1 (Supplementary Data are available at www.liebertpub.com/vbz/.) The deeper ancestry of the tree is not well resolved, with low aLRT and bootstrap support. More shallow nodes are, however, well supported, allowing us to describe 5 clades in which the Ethiopian *Bartonella* genotypes fall.

Clade 1 contained *Bartonella* genotypes exclusively from our samples, grouping together genotypes obtained from *S. albipes*, *A. dembeensis*, and *R. rattus* from Golgolnaele and Mahbere Silassie. Two of these genotypes (A and B) were the most frequent in our dataset (Table 3). The sequence similarities of these genotypes and other *Bartonella* were relatively low (mean = $90.6\% \pm 0.64\%$ [range, 75.6–95.9%]).

Clade 2 is divided in 2 subclades: Subclade 2a grouped together genotypes I, J, H, and K obtained from *A. dembeensis* from each of our sampling localities with a sequence found in Cairo spiny mouse *Acomys cahirinus* from Egypt, a sequence from the cosmopolitan brown rat (*Rattus norvegicus*) from China, and the reference sequence of *Bartonella elizabethae* from *R. norvegicus*. Subclade 2b grouped together genotype G from *S. albipes* from Golgolnaele locality and genotypes E and F obtained from *M. awashensis* from Aroresha locality with two sequences of *Bartonella* found in black rats *R. rattus* in Nepal. The outgroup of clades 2a and 2b was a sequence from *A. neumanni* from the DRC. The sequence similarities of genotypes I, J, H, K and G, E, F to *B. elizabethae* (human pathogen) were very high, 99.5–99.8% and 98.2–98.4%, respectively.

Clade 3 was comprised of purely African genotypes. Genotype L obtained from *S. albipes* from Golgolnaele grouped with *Bartonella* sequences from *Lophuromys* spp. from Tanzania and DRC, and with a sequence from *Mus minutoides* from the DRC. Genotype L has 99.3% sequence similarity with the genotype from *M. minutoides* and the within-clade mean similarity was 97.8%. Sequence similarity between the sequences of clade 3 and other *Bartonella* was 90.25%.

Clade 4 also was comprised of purely African genotypes, genotypes M, N, and P obtained from *A. dembeensis* from Golgolnaele and Mahbere Silassie grouped with *Bartonella* from *A. neumanni* from DRC. Sequence similarity between the Ethiopian genotypes and the DRC genotype was on average 97.7%. Placing this clade in a wider context, its sister clade was also purely African, grouping *Bartonella* from *Praomys delectorum* and *Lophuromys* spp. from Tanzania together with a

sequence obtained from *R. rattus* from DRC. Sequence similarity between the two clades was on average 94.2%.

Clade 5 grouped genotypes Q, R, S, and T with sequences from Tanzania and Egypt, and with *B. birtlesii* from *Apodemus* spp. in Japan. It is again more informative to consider the wider context—Clade 5 together with its sister clade formed an African or, perhaps Afro-Caucasian clade with the exception of the *B. birtlesii*. (The Netherlands origin of fat-tailed gerbils, *Pachyuromys duprasi*, in Fig. 2 refers to the location of the animals before being imported to Japan [see Inoue et al. 2009]; this gerbil species has a Northern Sahara distribution range.) The Caucasian aspect of the clade was a sequence obtained from the Libyan jird *Meriones libycus* from Georgia, the range of which spans North Africa, Arabia, and the Caucasus. The sequence similarities of genotypes Q, R, S, T, and the other *Bartonella* were relatively low (mean = $87.94 \pm 0.8\%$, ranging from 74.0% to 93.7%).

Discussion

Bartonella prevalence

This study is the first to report presence of *Bartonella* in Ethiopian small mammals. We report that the overall prevalence of *Bartonella* infection in the small mammals was 34.0%. *Bartonella* infection was found in domestic and peridomestic areas in the 3 localities of different elevation and in “scrublands” in 2 of the high-elevation localities. We detected highly diverse *Bartonella* genotypes, some of which were novel and others closely related to a pathogenic species.

Among the small mammals, *Bartonella* infection was low in *R. rattus*, *Mus* spp., and *C. olivieri*. Such low *Bartonella* prevalence has also been reported in *R. rattus* (1/25) and *C. olivieri* (0/8) in DRC (Gundi et al. 2012), whereas in South Africa *Bartonella* was undetected in *R. rattus* (but the sample size was only $n=2$) (Pretorius et al. 2004). In contrast to these data from Africa, in Asia as high as 43% and 64% *Bartonella* prevalence was reported in *Rattus* spp. and the house shrew *Suncus murinus*, respectively (Gundi et al. 2010). The lower level of *Bartonella* infection in *Rattus* spp. appears to be a common pattern in Africa and may be an instance of host escape during colonization (Torchin et al. 2003).

Several of our genotypes in clade 2 were closely related to *B. elizabethae*, a worldwide known rodent associated zoonotic *Bartonella* species, infection of which has been associated with endocarditis and neuroretinitis in humans (Daly et al. 1993, O’Halloran et al. 1998). Finding such closely related *Bartonella* sequence raises a potential public health significance of the bacteria, emphasizing the need for large-scale ecological and epidemiological studies in Ethiopia. To strengthen our assertion, we would like to draw attention in to three important

FIG. 2. Phylogenetic tree of *Bartonella* based on the *rpoB* gene showing the position of the genotypes found in Ethiopian rodents compared to other rodent’s strains or to reference *Bartonella*. The tree is a maximum likelihood (ML) estimate given the HKY85 substitution model and an estimated proportion 0.541 of invariant sites with 4 gamma-distributed rate categories to account for rate heterogeneity across sites. Support for internal branches was assessed both by approximate likelihood-ratio test (aLRT) tests (minimum of Shimodaira–Hasegawa [SH]-like and chi-squared-based parametric, black below branches) and bootstrap (200 replicates, red above branches). *Brucella melitensis* was used as an outgroup. Underlined sequences represent *Bartonella* known to be pathogenic for humans (Chomel and Kasten 2010). Each sequence taken from GenBank is labeled with its accession number, the name of the rodent host species and the country of origin where available. For the reference sequences, the information about the host is taken from (Chomel and Kasten 2010). Scale bar, number of substitutions per site. (Color images available online at www.liebertpub.com/vbz/).

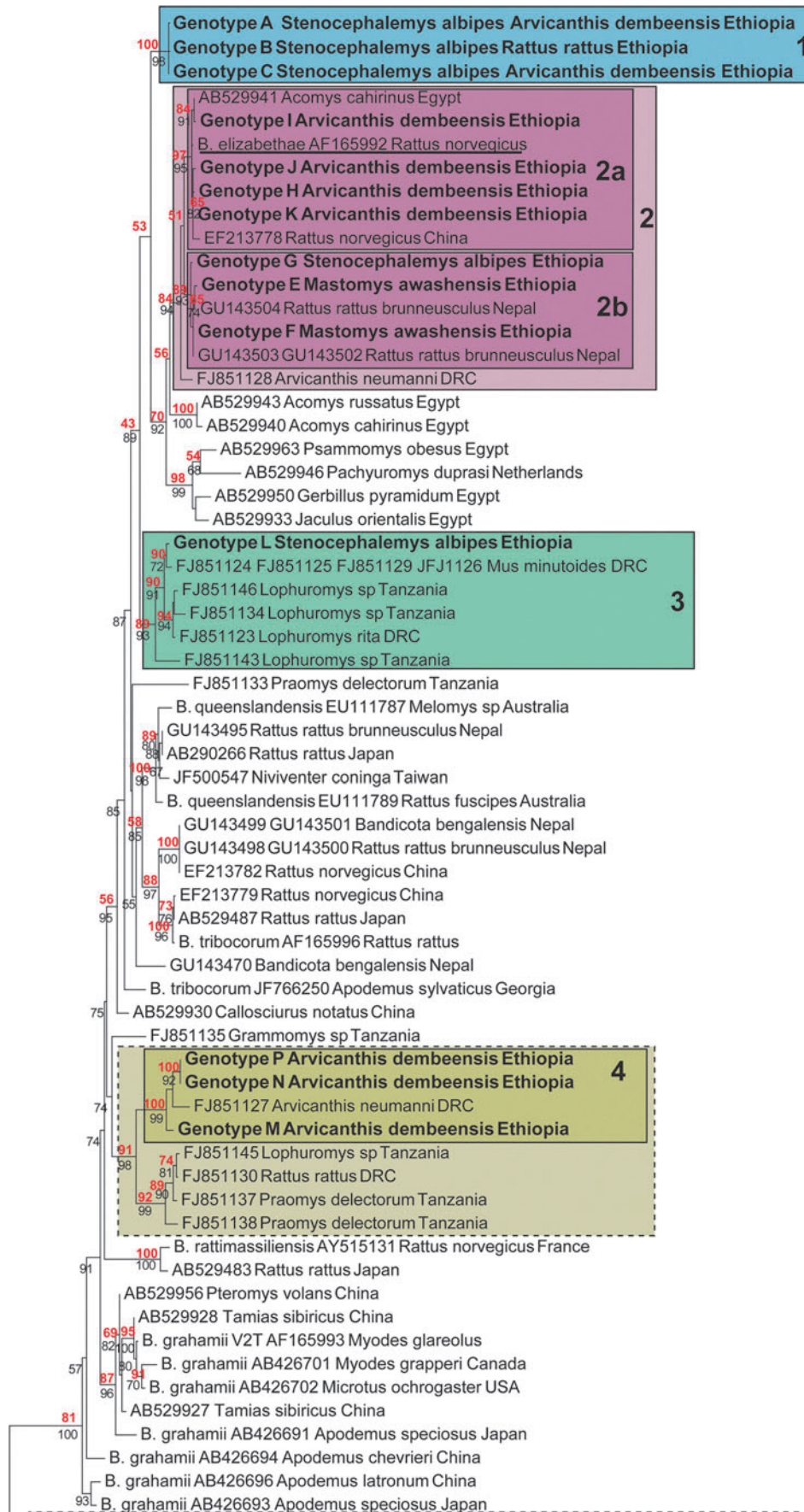


FIG. 2. (Continued)



FIG. 2. (Continued)

points here: (1) A substantial proportion (38.9%) of our *Bartonella* genotypes appear closely related to the pathogenic *B. elizabethae*, (2) our *Bartonella* genotypes associated with *B. elizabethae* were obtained from the 3 dominant rodent species (*M. awashensis*, *S. albipes*, and *A. dembeensis*) in agricultural fields (where farming activities such as weeding and harvest are still performed manually) and in rural inhabitations (where the housing conditions and activities around houses favor contact between humans and rodents), and (3) these *Bartonella* genotypes were detected in rodents trapped from each of the 3 study localities (at 1600-, 2600-, and 2700-meter elevations). The strong association of *B. elizabethae* with *Rattus* spp. in Asia, but with native rodents in Africa (in our result and Gundi et al. 2012), suggests that *B. elizabethae* is not specific to *Rattus* spp. in Africa, adding to the public health significance of the native rodents in Africa.

Seasonality of *Bartonella* infection

We have detected *Bartonella* infection in the small mammals in the study localities in each of the months distributed across the 3 seasons. However, we did not find significant seasonal difference in *Bartonella* infection in the small mammals. We suspected an increase in *Bartonella* infection during the early dry season in response to the dynamics of the host populations, rodent reproduction and abundances in Tigray highlands peak during early dry season (Y. Meheretu et al., submitted for publication). Similar correlated fluctuation in *Bartonella* prevalence and host density has been reported in woodrats (Morway et al. 2008) and in cyclic populations of field voles (Telfer et al. 2007).

Host characteristics and *Bartonella* infection

The GLM analysis showed significantly less chance of *Bartonella* infection among male individuals compared to the females. This may appear surprising given the generally accepted wisdom that males show increased susceptibility to infections due to reduced immune response compared to females (Klein 2000, Moore and Wilson 2002). However, behavioral differences between the sexes, for example, in home range and mobility, can also result in differential exposure and contact with infections. A better understanding of the sex differences would assist in modeling the potential spread of *Bartonella*.

Our results also indicated increased probability of *Bartonella* infection in reproductively active individuals. *Bartonella* infection has been shown to persist for several months in rodents. For instance, *Bartonella* infection has been reported to persist for more than 1 year in deer mice (Bai et al. 2011). This means that if the probability of infection is cumulative over time, the proportion of *Bartonella*-positive individuals will increase with the age of individuals (Gundi et al. 2004) and would explain why a heavier individuals or the class of reproductively active individuals show a higher prevalence. Contrary to our findings significantly lower infections in reproductively active male cotton rats than inactive males has been reported in the United States (Kosoy et al. 2004). However, they did not find a significant difference between reproductively active and inactive females. Jardine et al. (2006) also reported a decrease in the prevalence of *Bartonella* infection in ground squirrels (*Spermophilus richardsonii*) with age.

Ectoparasite infestation and *Bartonella* infection

Although the exact transmission mechanism of *Bartonella* among hosts is not fully understood (Morway et al. 2008), a growing number of studies have implicated several ectoparasites as potential vectors of *Bartonella* transmission in rodents (Billeter et al. 2008; Sackal et al. 2008; Reis et al. 2011). Several species of *Bartonella*, including the human pathogen *B. elizabethae*, have been detected in the Oriental rat flea *Xenopsylla cheopis* (Reeves et al. 2007; Tsai et al. 2010; Billeter et al. 2011), the cat flea *C. felis* (Billeter et al. 2008), and several species of *Ixodes* ticks (Kim et al. 2005). Recently, the vector competence of *I. ricinus* in *Bartonella* transmission has been confirmed experimentally (Reis et al. 2011). We did not find increased probability of *Bartonella* infection with increasing ectoparasite infestation in our data. In a previous study, we reported *X. cheopis* and *Ixodes* spp. as the most abundant fleas and ticks infesting rodents in Tigray highlands, respectively (Meheretu et al. 2011), thus we might have expected *Bartonella* infection to be associated with the load of these ectoparasites.

In a study investigating the dynamics of 4 closely related *Bartonella* species and their flea vectors in cyclic populations of field voles, it was shown that only 1 species of *Bartonella* was influenced by the presence of fleas and only in spring time (Telfer et al. 2007). It seems likely that the relevant ectoparasites are present in significant numbers such that host density rather than vector abundance plays the more important role in the dynamics of flea-transmitted *Bartonella* spp. (Telfer et al. 2007).

In our study, we did not investigate the correlation with respect to the different ectoparasite species. It is possible that at the species level, there might be a correlation between some *Bartonella* strains with specific ectoparasites. The presence of known *Bartonella* vectors on the small mammals in our localities calls for investigation of the bacteria in these ectoparasites. Detection of *Bartonella* in the ectoparasites would be a fundamental step toward establishing a link between the potential vectors and the bacteria (Reeves et al. 2007).

Detection of *Bartonella* in host samples

The pattern of clustering in the phylogenetic tree revealed that our *Bartonella* genotypes fall into 5 clades, 1 clade so far exclusively Ethiopian, 2 clades from Central and Eastern Africa, and the remaining 2 clades with genotypes from Africa and Asia. Recently, La Scola et al. (2003) suggested a cutoff point of sequence similarity <95.4% for delimitation of *Bartonella* species when using the *rpoB* gene. Although such choices are entirely arbitrary, this does allow the levels of sequence similarity observed between our genotypes and described *Bartonella* species to be set somewhat in context. Four of our clades would appear sufficiently diverged to satisfy this species description cutoff: Clades 1 and 5 (genotypes Q, R, S, T), which have been found only in Ethiopia, and clades 3 and 4 examples of which are restricted to Africa.

Three of our genotypes obtained from *A. dembeensis* were closely related to a genotype from *A. neumanni* from DRC in clade 4, hinting that the genotypes might be specific to *Arvicanthis* spp. On the other hand, it is interesting to note that on 4 occasions (in clades 1 and 5), the same *Bartonella* genotype was detected from 2 different rodent species (see genotypes A, B, C, and Q), suggesting low host-specificity of these genotypes. Previously low host specificity among *Bartonella* isolates has

been reported in South Africa where *rpoB*-identical isolates were detected in *M. natalensis* and *Tatera leucogaster* (Pretorius et al. 2004).

The discovery of 18 different *rpoB* genotypes in a very restricted sampling area indicates a high level of *Bartonella* diversity among Ethiopian small mammals, some of which are of potential public health concern. The phylogenetic analyses, including representative *Bartonella* samples from other rodents, illustrate well that Muridae (e.g., rats, gerbils, mice) are likely an important reservoir of *Bartonella*. Given that ~272 species of Muridae exist in Africa (37% of the world total) (Musser and Carleton 2005; IUCN 2011), it seems clear that many more *Bartonella* genotypes/species are waiting to be discovered.

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No competing financial interests exist.

References

- Altschul SF, Gish W, Miller W, Myers EW, et al. Basic local alignment search tool. *J Mol Biol* 1990; 215:403–410.
- Bai Y, Calisher CH, Kosoy MY, Root JJ, et al. Persistent infection or successive reinfection of deer mice with *Bartonella vinsonii* subsp. *arupensis*. *Appl Environ Microbiol* 2011; 77: 1728–1731.
- Billeter SA, Levy MG, Chomel BB, Breitschwerdt EB. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Med Vet Entomol* 2008; 22:1–15.
- Billeter SA, Gundi VAKB, Rood MP, Kosoy MY. Molecular detection and identification of *Bartonella* species in *Xenopsylla cheopis* fleas (Siphonaptera: Pulicidae) collected from *Rattus norvegicus* rats in Los Angeles, California. *Appl Environ Microbiol* 2011; 76:7850–7852.
- Boulouis H-J, Chang C-C, Henn JB, Kasten RW, et al. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. *Vet Rec* 2005; 36:383–410.
- Chomel BB, Kasten RW. Bartonellosis, an increasingly recognized zoonosis. *J Appl Microbiol* 2010; 109:743–750.
- Daly JS, Worthington MG, Brenner DJ, Moss CW, et al. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J Clin Microbiol* 1993; 31:872–881.
- Drummond AJ, Ashton B, Buxton S, Cheung M, et al. *Geneious v5.5*. 2010. Available from <http://www.geneious.com/>. Accessed November 15, 2012.
- Fichet-Calvet E, Jomaa I, Ismail RB, Ashford RW. Patterns of infection of haemoparasites in the fat sand rat, *Psammomys obesus*, in Tunisia, and effect on the host. *Ann Trop Med Parasitol* 2000; 94:55–68.
- Gil H, Garcia-Esteban C, Barandika JF, Peig J, et al. Variability of *Bartonella* genotypes among small mammals in Spain. *Appl Environ Microbiol* 2010; 76:8062–8070.
- Gratz NG. The burden of rodent-borne diseases in Africa south of the Sahara. *Belg J Zool* 1997; 127(Suppl):71–84.
- Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 2003; 52:696–704.
- Guindon S, Dufayard J-F, Lefort V, Anisimova M, et al. New algorithms and methods to estimate Maximum-Likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst Biol* 2010; 59:307–321.
- Gundi VAKB, Davoust B, Khamis A, Boni M, et al. Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European *Rattus norvegicus*. *J Clin Microbiol* 2004; 42:3816–3818.
- Gundi VAKB, Kosoy MY, Myint KSA, Shrestha SK, et al. Prevalence and genetic diversity of *Bartonella* species detected in different tissues of small mammals in Nepal. *Appl Environ Microbiol* 2010; 76:8247–8254.
- Gundi VAKB, Kosoy MY, Makundi RH, Laudisoit A. Identification of diverse *Bartonella* genotypes among small mammals in equatorial Africa. *Am J Trop Med Hyg* 2012; 87:319–326.
- Houpikian P, Raoult D. Molecular phylogeny of the genus *Bartonella*: What is the current knowledge? *FEMS Microbiol Lett* 2001; 200:1–7.
- Inoue K, Maruyama S, Kabeya H, Yamada N, et al. Prevalence and genetic diversity of *Bartonella* species isolated from wild rodents in Japan. *Appl Environ Microbiol* 2008; 74:5086–5092.
- Inoue K, Maruyama S, Kabeya H, Hagiya K, et al. Exotic small mammals as potential reservoirs of zoonotic *Bartonella* spp. *Emerg Infect Dis* 2009; 15:526–532.
- IUCN. IUCN Red List of Threatened Species. Version 2011.2. 2011. Available at www.iucnredlist.org/. Accessed November 15, 2012.
- Jardine C, Waldner C, Wobeser G, Leighton FA. Demographic features of *Bartonella* infections in Richardson's ground squirrels (*Spermophilus richardsonii*). *J Wildl Dis* 2006; 42:739–749.
- Just FT, Gilles J, Pradel I, Pfalzer S, et al. Molecular Evidence for *Bartonella* spp. in cat and dog fleas from Germany and France. *Zoonoses Public Health* 2008; 55:514–520.
- Kim CM, Kim JY, Yi YH, Lee MJ, et al. Detection of *Bartonella* species from ticks, mites and small mammals in Korea. *J Vet Sci* 2005; 6:327–334.
- Klein SL. Hormones and mating system affect sex and species differences in immune function among vertebrates. *Behav Process* 2000; 51:149–166.
- Kosoy M, Murray M, Gilmore RD, Bai Y, et al. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. *J Clin Microbiol* 2003; 41:645–650.
- Kosoy M, Mandel E, Green D, Marston E, et al. Prospective studies of *Bartonella* of rodents. Part I. Demographic and temporal patterns in population dynamics. *Vector Borne Zoonotic Dis* 2004; 4:285–295.
- Kosoy MY. Ecological associations between bacteria of the genus *Bartonella* and mammals. *Biol Bull* 2010; 37:716–724.
- La Scola B, Zeaiter Z, Khamis A, Raoult D. Gene-sequence-based criteria for species definition in bacteriology: The *Bartonella* paradigm. *TIM* 2003; 11:318–321.
- Meheretu Y, Welegerima K, Laudisoit A, Bauer H, et al. Preliminary investigation on rodent-ectoparasite associations in the highlands of Tigray, northern Ethiopia: Implications for potential zoonoses. *Integ Zool* 2011; 6:366–374.

- Montgelard C, Bentz S, Tirard C, Verneau O, et al. Molecular systematics of Sciurognathi (Rodentia): the mitochondrial cytochrome b and 12S rRNA genes support the Anomaluroidea (Peptidae and Anomaluridae). *Mol Phylogenet Evol* 2002; 22:220–233.
- Moore SL, Wilson K. Parasites as a viability cost of sexual selection in natural populations of mammals. *Science* 2002; 297:2015–2018.
- Morway C, Kosoy M, Eisen R, Montenieri J, et al. A longitudinal study of *Bartonella* infection in populations of woodrats and their fleas. *J Vector Ecol* 2008; 33:353–364.
- Musser GG Carleton MD. Superfamily Muroidea. In Wilson DE, Reeder DA, ed. *Mammal Species of the World: A Geographic and Taxonomic Reference*. Baltimore: The John Hopkins University Press, 2005:894–1531.
- O'Halloran HS, Draud K, Minix M, Rivard AK, et al. Leber's neuroretinitis in a patient with serologic evidence of *Bartonella elizabethae*. *Retina* 1998; 18:276–278.
- Posada D. jModelTest: Phylogenetic model averaging. *Mol Biol Evol* 2008; 25:1253–1256.
- Pretorius A-M, Beati L Birtles RJ. Diversity of *Bartonella* associated with small mammals inhabiting Free State province, South Africa. *Int J Syst Evol Micr* 2004; 54:1959–1967.
- R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. ISBN 3-900051-07-0. 2010.
- Raoult D, Ndihokubwayo JB, Tissot-Dupont H, Roux V, et al. Outbreak of epidemic typhus associated with trench fever in Burundi. *Lancet* 1998; 352:353–358.
- Reeves WK, Rogers TE, Durden LA, Dasch GA. Association of *Bartonella* with the fleas (Siphonaptera) of rodents and bats using molecular techniques. *J Vector Ecol* 2007;32:118–122.
- Reiczigel J. Confidence intervals for the binomial parameter: Some new considerations. *Stat Med* 2003;22:611–621.
- Reis C, Cote M, Le Rhun D, Lecuelle B, et al. Vector competence of the tick *Ixodes ricinus* for transmission of *Bartonella birtlesii*. *PLoS Negl Trop Dis* 2011; 5:e1186. doi:10.1371/journal.pntd.0001186.
- Renesto P, Gouvernet J, Drancourt M, Roux V, et al. Use of rpoB Gene Analysis for Detection and Identification of *Bartonella* Species. *J Clin Microbiol* 2001; 39:430–437.
- Rozsa L, Reiczigel J, Majoros G. Quantifying parasites in samples of hosts. *J Parasitol* 2000; 86:228–232.
- Sackal C, Laudisoit A, Kosoy M, Massung R, et al. *Bartonella* spp. and *Rickettsia felis* in Fleas, Democratic Republic of Congo. *Emerg. Infect Dis* 2008; 14:1972–1974.
- Schmidt HA, Strimmer K, Vingron M, von Haeseler A. Tree-puzzle: Maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* 2002; 18:502–504.
- Shimodaira H, Hasegawa M. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol* 1999; 16:1114–1116.
- Tamura K, Peterson D, Peterson N, Stecher G, et al. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28:2731–2739.
- Telfer S, Begon M, Bennett M, Bown KJ, et al. Contrasting dynamics of *Bartonella* spp. in cyclic field vole populations: The impact of vector and host dynamics. *Parasitology* 2007; 134:413–425.
- Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, et al. Introduced species and their missing parasites. *Nature* 2003; 421:628–630.
- Tsai Y-L, Chuang S-T, Chang C-C, Kass PH, et al. *Bartonella* species in small mammals and their ectoparasites in Taiwan. *Am J Trop Med Hyg* 2010; 83:917–923.
- Welc-Faleciak R, Paziewska A, Bajer A, Behnke JM et al. *Bartonella* spp. infection in rodents from different habitats in the Mazury Lake District, northeast Poland. *Vector Borne Zoonotic Dis* 2008; 8:467–474.
- Yuan C, Zhu C, Wu Y, Pan X, et al. Bacteriological and molecular identification of *Bartonella* species in cats from different regions of China. *PLoS Negl Trop Dis* 2011; 5:e1301. doi:10.1371/journal.pntd.0001301.
- Zuur AF, Ieno EN, Walker NJ, Saveliev AA, et al. *Mixed Effects Models and Extensions in Ecology*. New York: R. Springer-Verlag, 2009:596.

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APPENDIX 1.

Phylogenetic tree based on partial *rpoB* sequences of 18 Ethiopian and 114 known *Bartonella* using the neighbor joining method with the Kimura 2-parameter model of sequence evolution. GenBank accession numbers are indicated when relevant. A *Brucella melitensis* sequence was used as an outgroup. Numbers represent percentage bootstrap support (1000 replicates) for values "superior or equal" 50%. Scale bar, nucleotide substitutions per site.