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Mixed Infections, Cryptic Diversity, and Vector-Borne Pathogens: Evidence from *Polygenis* Fleas and *Bartonella* Species▽

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Coinfections within hosts present opportunities for horizontal gene transfer between strains and competitive interactions between genotypes and thus can be a critical element of the lifestyles of pathogens. *Bartonella* spp. are *Alphaproteobacteria* that parasitize mammalian erythrocytes and endothelial cells. Their vectors are thought to be various biting arthropods, such as fleas, ticks, mites, and lice, and they are commonly cited as agents of various emerging diseases. Coinfections by different *Bartonella* strains and species can be common in mammals, but little is known about specificity and coinfections in arthropod vectors. We surveyed the rate of mixed infections of *Bartonella* in flea vectors (*Polygenis gwyni*) parasitizing cotton rats (*Sigmodon hispidus*) in which previous surveys indicated high rates of infection. We found that nearly all fleas (20 of 21) harbored one or more strains of *Bartonella*, with rates of coinfection approaching 90%. A strain previously identified as common in cotton rats was also common in their fleas. However, another common strain in cotton rats was absent from *P. gwyni*, while a rare cotton rat strain was quite common in *P. gwyni*. Surprisingly, some samples were also coinfected with a strain phylogenetically related to *Bartonella clarridgeiae*, which is typically associated with felids and ruminants. Finally, a locus (*pap31*) that is characteristically borne on phage in *Bartonella* was successfully sequenced from most samples. However, sequence diversity in *pap31* was novel in the *P. gwyni* samples, relative to other *Bartonella* previously typed with *pap31*, emphasizing the likelihood of large reservoirs of cryptic diversity in natural populations of the pathogen.

Most host populations harbor more than one pathogen strain at a given time, leading to mixed infections or "coinfections" in individual hosts (10, 26, 48). Unfortunately, there are gaps in our understanding of within-host pathogen interactions. The problem is particularly acute in vector-borne diseases, where little is known regarding mixed infection interactions in natural populations of the vectors themselves. Rather, with only a few notable exceptions (e.g., reference 25), most population-level or clinical data on mixed infections derive from human studies or other mammalian models. The distinction is crucial because of the role that vectors play in pathogen transmission.

The bacterial pathogen *Bartonella* sp. has become one of a few model organisms for studying the evolution and ecology of vector-borne diseases (28). This is due to diverse efforts to describe *Bartonella* biology at multiple levels, from cells and immune systems (12, 13, 14, 30), to populations and communities (31, 32), to species and clades (36, 44). The recent publication of full genome sequences is obviously key (2). *Bartonella* sp. is a short, gram-negative, fastidious bacterium belonging to the *Alphaproteobacteria* (1). Closely related to *Brucella* spp., *Bartonella* organisms are parasites of mammalian erythrocytes and endothelial cells (12, 13, 14) and are transmitted by blood-feeding insects, such as ticks, fleas, lice, and flies (9, 19, 20, 21, 23, 28). Infection of a host causes chronic bacteremia and creates a reservoir for vectors that can transmit the bacteria to new susceptible hosts. While prolonged bacteremia is normally associated with severe sickness in a susceptible host, *Bartonella*-caused bacteremia typically remains asymptomatic in the reservoir host. Some bartonellae are known to be transmitted by the bite (anterior station transmission) or in the feces (posterior station transmission) of insect vectors. For example, in humans, *Bartonella bacilliformis*, which causes Oroya fever (verruga peruana, or Carrion's disease) in Andean South America is transmitted by the bites of infectious sandflies (5), and *Bartonella quintana*, which causes trench fever in many parts of the world, is transmitted via the feces of infected body lice (21). Fleas infected with *Bartonella henselae* (the causative agent of cat scratch disease and of related conditions such as bacillary angiomatosis [30]) and other bartonellae appear to transmit these agents via their infectious feces (9, 19, 20). Current phylogenetic information indicates six distinct groups worldwide, of which all but one are found in the United States (44). Host and vector affiliations are complex, and the evidence is against strict one-to-one host specificity (28, 32, 33). A consistent trend is that groups of *Bartonella* species tend to be restricted to natural groups of mammalian hosts (rodents, cats, dogs, humans, etc.), indicating a diffuse but long-term coevolutionary history.

We surveyed the incidence of mixed *Bartonella* infections in natural populations of the flea *Polygenis gwyni* parasitizing the Eastern woodrat (*Neotoma floridana*) and the hispid cotton rat (*Sigmodon hispidus*). Previous surveys of mammalian hosts indicated that mixed infections of *Bartonella* can be common (22). An intensive survey of *S. hispidus* in the southeastern United States, for example, revealed that this host exhibits a particularly high infection prevalence overall, as well as non-

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TABLE 1. Loci and GenBank accession numbers used in the present study to reconstruct *Bartonella* phylogenetic relationships and to identify the species relationships of cloned *gltA* amplicons from *Polygenis* fleas

Species (strain)	GenBank accession no. for amplicon				
	GltA	RpoB	GroEL	FtsZ	RibC
B. alsatica (IBS382)	AF204273	AF165987	AF299357	AF467763	AY116630
B. bacilliformis (KC584T)	U28076	AF165988	Z15160	AF007266	AJ236918
B. birtlesii (IBS 325T)	AF204272		AF355773	AF467762	AY116632
B. bovis (91-4T)	AF293394	DQ356078			AY116637
B. clarridgeiae (Houston-2T)	U84386	AF165990	AF014831	AF141018	BCL236916
B. doshiae (R18T)	AF207827	AF165991	AF014832	AF467754	AY116627
B. elizabethae (F9251T)	U28072	AF165992	AF014834	AF467760	AY116633
B. grahamii (V2T)	Z70016	AF165993		AF467753	AY166583
B. henselae (Houston-1T)	L38987	AF171070	AF014829	AF061746	AJ132928
B. koehlerae (C-29T)	AF176091	AY166580	AY116641	AF467755	AY116634
B. quintana (FullerT)	Z70014	AF165994	AF014830	AF061747	AJ236917
B. schoenbuchensis (R1T)	AJ278183	AY167409b	AY116642	AF467765	AY116628
B. taylorii (M6T)	AF191502	AF165995	AF304017	AF467756	AY116635
B. tribocorum (IBS 506T)	AJ005494	AF165996	AF304018	AF467759	
B. vinsonii subsp. *arupensis* (OK 94-513	AF214557	AY166582b	AF304016	AF467758	AY116631
B. vinsonii subsp. *berkhoffii* (93-CO1T)	U28075	AF165989	AF014836	AF467764	AY116629
B. vinsonii subsp. *vinsonii* (BakerT)	Z70015	AF165997	AF014835	AF467757	AY116636
B. bovis (FC7049UT)	AF071190		AF071194	AF467761	
Brucella sp.	AE014291	DQ086137	AE014292	AE014291	

negligible rates of coinfection (33). There is little comparable information on mixed infection rates in *Bartonella* vectors (49). However, with mammalian host populations multiply infected with strains that are vectored by insects with generalist host affiliations (e.g., ticks and fleas), the expectation is that rates of mixed infections in competent vectors should be quite high. The relevance of whether or not this is the case not only bears on the basic natural history and disease dynamics of *Bartonella* but also on the pattern and tempo of disease emergence (5). *Bartonella* has been described as the consummate "versatile pathogen" (27) for the breadth of its host affiliations and plasticity of its lifestyles (2). Population-level data are necessary supplements to evolutionary inferences about the genus, because retrospective analyses of such events as lateral gene transfer (4) can become forward-looking and predictive when accompanied by real-time data on the ecological context for such events (15, 24, 34, 47, 55).

MATERIALS AND METHODS

Trapping and collection methods. Cotton rats and Eastern woodrats were trapped in Bulloch and Screven Counties (one site in each county) in southeastern Georgia, using Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, FL) baited with rolled oats and a trace of peanut butter and set near areas of rodent activity. Trapped animals were lightly anesthetized via intramuscular administration of ketamine hydrochloride and then transferred to a white tray, where they were carefully examined for ectoparasites. Retrieved ectoparasites were transferred to individually labeled cryovials containing 95% ethanol. Fleas were later identified using the methods of Smit (52) and Lewis and Lewis (38). All fleas collected were *Polygenis gwyni*, which is the species that typically parasitizes the cotton rat in the southern United States (38, 52). This flea species has also been reported previously from the Eastern woodrat (16). Following recovery from anesthesia, all cotton rats and woodrats were released at their capture site. Mammals were live trapped under permit 9172 issued by the Georgia Department of Natural Resources, and animal procedures were approved by the IACUC committee at Georgia Southern University (research protocol no. I06003). Voucher flea specimens have been deposited in the Ectoparasite Collection at Georgia Southern University under accession numbers L-358 and L-1102.

DNA methods. Whole genomic DNA from *P. gwyni* was extracted using a DNeasy tissue kit (QIAGEN, Inc.). Each sample was tested for the presence or absence of *Bartonella* by PCR amplification of an approximately 400-bp amplicon from the citrate synthase *gltA* gene, using the universal oligonucleotide primers BhCS781.p and BhCs1137.n (33). *gltA* was chosen because of its high discriminating power for *Bartonella* (36), the existing coverage in GenBank of the genus using this gene, and its prior use in identifying *Bartonella* in the flea host, *S. hispidus* (32). PCR products were visualized by electrophoresis and ethidium bromide staining under UV light on 1.5% agarose gels. Samples yielding successful *gltA* amplicons were then retested with oligonucleotides papn1 and papn2, designed from the bacteriophage-associated gene *pap31* in *B. henselae*. Both PCR amplifications were carried out at 10-μl volumes, containing 1× Invitrogen 10× buffer, 2.0 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 5 pmol of each primer, 1 U of Invitrogen DNA *Taq* polymerase, sterile PCR-grade water, and approximately 5 to 10 ng of whole genomic DNA. Reaction conditions were 1 cycle at 94°C for 2 min and 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s, followed by 1 cycle at 72°C for 15 min. Products from both reactions were cloned via a pCR2.1-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA) and TOPO TA cloning kit and Top10 competent cells, according to the manufacturer's instructions. Positive clones for both genes were PCR amplified at 50-μl volumes, as above, purified with a QIAGEN PCR purification kit (QIAGEN, Inc.) following the manufacturer's instructions, and sequenced at the Vanderbilt University Medical Center Sequencing Core Facility and the University of Arizona Genomic Analysis and Technology Core Facility, with either Invitrogen vector primer *IVT7* or *M13R*. Resulting sequences were then compared against known *Bartonella* sequences in GenBank, using default parameters in BLAST.

Phylogenetic methods. We determined the phylogenetic affinities of the *gltA* amplicons by first constructing a backbone phylogeny of 18 *Bartonella* species, isolated from a wide range of mammalian hosts from each of the five recognized host clusters (Table 1). Initially, species were selected by the availability of sequences in public databases from seven housekeeping genes commonly used in *Bartonella* species delineation (*16S*, *ITS*, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*; not all gene sequences were available for all species). However, *16S* and *ITS* were not used because of strong phylogenetic incongruence and alignment uncertainty in these loci. The remaining genes were first aligned using a partial order alignment algorithm (implemented in the software package POA v.2, using default parameters [37]) and then checked by eye for obvious discrepancies. Individual alignments were then concatenated, yielding a global ca. 4.6-kb alignment.

Maximum parsimony trees were constructed in PAUP 4.0b10 (53), using simple sequence addition, the TBR swapping algorithm, and 10 random addition replicates for each search iteration. Parsimony trees were compared to those generated by a partitioned Bayesian analysis in the software package MrBayes v.3.1.2 (50), under models and parameters separately estimated via Modeltest v.3.6 (45), each conditioned on the same starting tree estimated by maximum likelihood on the entire data set using a general time-reversible model of evo-

TABLE 2. Identification of *Bartonella* spp. isolates cloned from each flea, based on reconstruction of *gltA* or *pap31* phylogenies using GenBank sequences and those derived from the present study[a]

P. gwynii sample no.	Host	Mammal ID	Site	Distinct *gltA* genogroup[b]				n^d	*gltA* GenBank accession no(s)	Distinct pap31 clade[c]			n^d	*pap31* GenBank accession no(s)
				A1/A5	B2/B3	B4	*B. clarridgeiae*			I	II	III		
1	*S. hispidus*	AEA 10-17	A	+	+		+	12	EF616644/655		+	+	8	EF625688/695
2	*S. hispidus*	AEA 10-17	A	+		+		5	EF616656/660				-	
3	*S. hispidus*	AEA 11-20	A					-					-	
4	*S. hispidus*	AEA 11-20	A	+	+			8	EF616661/668	+			3	EF625696/698
5	*N. floridana*	AEA 01-13	B	+		+		12	EF616669/680		+		1	EF625699
6	*N. floridana*	AEA 01-14	B	+				11	EF616681/691				-	
7	*S. hispidus*	LAD-3331	B	+	+			2	EF616692/693	+	+		9	EF625700/708
8	*S. hispidus*	LAD-3331	B	+				10	EF616694/703			+	2	EF625709/710
9	*S. hispidus*	LAD-3331	B					-		+	+		6	EF625711/716
10	*S. hispidus*	LAD-3332	B	+	+			7	EF616704/710	+	+		8	EF625717/724
11	*S. hispidus*	LAD-3332	B	+		+	+	7	EF616711/717	+	+	+	5	EF625725/729
12	*S. hispidus*	LAD-3333	B	+	+			2	EF616718/719	+	+		7	EF625730/736
13	*S. hispidus*	LAD-3333	B	+				7	EF616720/726		+		1	EF625737
14	*S. hispidus*	LAD-3333	B	+				8	EF616727/734	+	+		3	EF625738/740
15	*S. hispidus*	LAD-3333	B	+			+	9	EF616735/742	+	+		2	EF625741/742
16	*S. hispidus*	LAD-3334	B	+				1	EF616643	+	+		7	EF625743/749
17	*S. hispidus*	LAD-3334	B	+	+			7	EF616744/750	+	+		10	EF625750/759
18	*S. hispidus*	LAD-3334	B	+	+		+	15	EF616751/765	+	+	+	15	EF625760/774
19	*S. hispidus*	LAD-3335	B	+			+	6	EF616766/771	+			11	EF625775/785
20	*S. hispidus*	LAD-3335	B	+	+		+	29	EF616772/800	+	+	+	16	EF625786/801
21	*S. hispidus*	LAD-3335	B	+	+			19	EF616801/819	+	+	+	15	EF625802/816

[a] The two collection sites were Candler County, GA (A) and Bulloch County, GA (B). The *gltA* columns represent the full diversity of positive matches based on phylogenetic reconstruction. Most matched previously undescribed "genogroups" from *Sigmodon hispidus*, as shown in Fig. 1 and Fig. 3. The plus signs indicate a positive match. The *pap31* clades are provisional designations based on the topology depicted in Fig. 3.
[b] See Fig. 1.
[c] See Fig. 3.
[d] Number of clones sequenced.

lution (6). Priors were not changed from default values. We ran four simultaneous Metropolis-coupled Monte Carlo Markov chains for 1,000,000 generations, with a heating parameter of 0.1. We sampled every 100 generations and calculated a consensus topology after a "burn-in" of 2,500 trees. The consensus tree was then used as a backbone constraint, and cloned *gltA* amplicons from the *Polygenis* samples of *Bartonella* were grafted onto the tree using a simple distance-based neighbor-joining algorithm. For most positive *Polygenis* samples, this involved replicates of >5 *Bartonella* clones per individual flea. A newly described species, *Bartonella rochalimae*, which is closely related to *B. clarridgeiae* (18), was also grafted onto the tree using a *gltA* sequence from GenBank (accession no. DQ683195).

pap31 sample size was smaller, and there are generally fewer data available on the gene in *Bartonella*. Positive sequences were simply aligned with known *Bartonella* orthologs from GenBank, and both Bayesian and maximum likelihood trees were constructed using the Bayesian methodology described above and for the maximum likelihood tree using a general time-reversible model of nucleotide evolution with parameters estimated from the data. The maximum likelihood analysis was performed with the software program Garli v0.95 (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) (57).

Nucleotide sequence accession numbers. The *gltA* and *pap31* sequences have been deposited in GenBank under the accession numbers EF616644 to EF616819 and EF625688 to EF625816, respectively.

RESULTS

Eight *S. hispidus* and two *N. floridana* rats were trapped from two sites, from which 21 *P. gwyni* fleas were collected. Either *gltA* or *pap31* amplicons of the expected size were detected in 20 of 21 fleas, and replicate sequences were obtained from most samples, such that any *Taq* error or PCR recombination could be identified and not included in the diversity estimates (Table 2). In 17 fleas, we sequenced multiple and divergent *gltA* or *pap31* clones, permitting us to survey the frequency of single or multiple infections. Cloning efficiency varied between individual fleas, resulting in an unequal number of sequences per gene per flea (Table 2). There was some, but not perfect, overlap between *gltA* and *pap31* as positive evidence for mixed infections. Using *gltA* only, mixed *Bartonella* infections were detected in 12 of 16 fleas positive for *Bartonella* and for which five or more sequence replicates were obtained (Table 2). Most *gltA* amplicons exhibited greater than 94% sequence similarity to undescribed *Bartonella vinsonii*-like genogroups previously cultured from *S. hispidus* in the southeastern United States (Fig. 1 and 2) (33). However, in six fleas collected from five different *S. hispidus* isolates, *gltA* amplicons were detected with closest similarity to *B. clarridgeiae*, a species nominally associated with felines and ruminants (44) but which may be closely related to species with broader host ranges (18, 39). BLAST searches with the *pap31* sequences resulted in highest sequence similarity scores with either *B. quintana* or *B. henselae*, although neither of these species was greater than 94% similar to any of the *pap31* sequences (Fig. 3 and 4), reflecting the still-limited survey of *pap31* diversity in *Bartonella* available in GenBank. However, the *pap31* phylogeny revealed three distinct clades of *P. gwyni*-associated *Bartonella*, perhaps mirroring the divergence between strains detected by *gltA*. One clade was characterized by a 1-bp deletion near the boundary between a putative conserved transmembrane domain and an extracellular loop sequence (Fig. 4) (42), producing a UAA stop codon downstream and thus presumably a truncated protein. This deletion was perfectly matched by an alanine-to-valine replacement downstream in a putative inner membrane loop sequence.

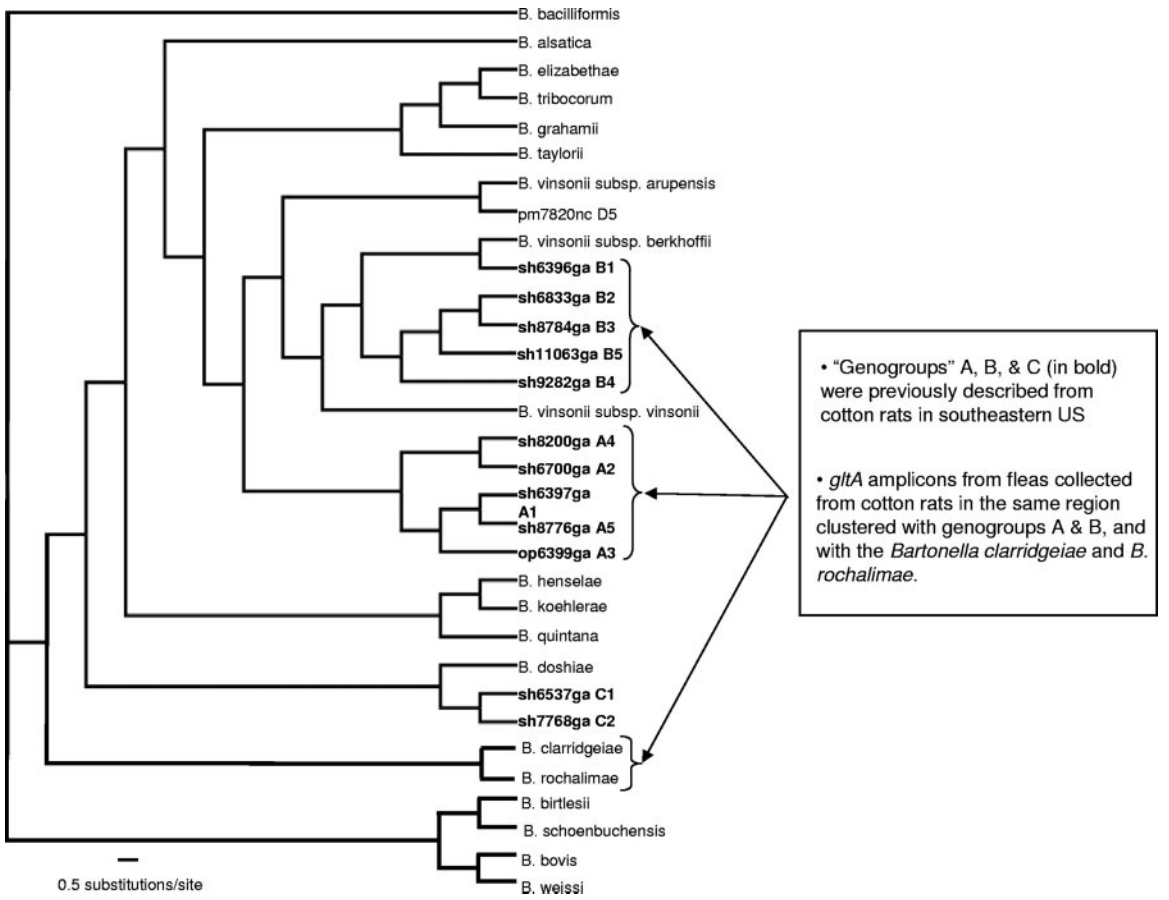

FIG. 1. Bayesian phylogeny of the genus *Bartonella*, including many of the described species. The tree is rooted with *B. bacilliformis* and is based on partial sequences from five concatenated loci, with the exception of those shown in bold (see Table 1 for GenBank accession numbers). The bold taxa represent type isolates of the genogroups (designated A thru D) discovered in previous surveys of cotton rats in the southeastern United States (32, 33). Only *gltA* sequences are available for these. All nonterminal resolved nodes had clade credibility values of >98, based on the Bayesian analysis. The overall topology was supported by parsimony analysis. Arrows indicate the phylogenetic placement of the different *P. gwyni*-derived isolates on the constrained *Bartonella* phylogeny, based on neighbor-joining placement of the amplicons on the tree. With the exception of the isolates similar to *B. clarridgeiae* and *B. rochalimae*, most were >99% similar to the designated A or B genogroup. Most amplicons were confirmed by redundant sequencing of multiple cloned products.

DISCUSSION

We surveyed the prevalence of *Bartonella* in a population of rodent fleas, collected from a general locale in which small mammals had been previously intensively surveyed (31, 32, 33). Because we surveyed in a manner that discriminated between single and mixed infections in fleas, we also estimated the fraction of fleas harboring more than one *Bartonella* isolate and the phylogenetic affinities of coinfecting isolates. We found four noteworthy results.

First, the prevalence of *Bartonella* was surprisingly high, exceeding characteristic records from various putative arthropod vectors (35, 49, 54). Estimating prevalence requires population-level sampling, and only in recent years have such surveys of *Bartonella* in presumed vectors begun to emerge. Cat fleas (*Ctenocephalides felis*) are important agents of zoonotic *Bartonella* transmission and have been examined in a number of studies sufficient to yield population-level data (41, 49). Estimates of cat-associated *Bartonella* prevalence (e.g., *B. henselae*, *B. quintana*, *B. koehlerae*, and *B. clarridgeiae*) have ranged from 20 to 30%, although some *C. felis* populations may exhibit higher rates (35). Less is known about other flea or arthropod vectors from natural populations of mammals. Studies reporting nonnegligible rates of infection in fleas from various small mammals typically have ranged from 10 to 40% (41, 54). Not surprisingly, there are still few studies that report simultaneous estimates of prevalence in vectors and their mammalian hosts (54).

However, we collected fleas from *S. hispidus* in an area that, because of extensive prior work (31, 32, 33), corresponds to an intensively scrutinized regional population (the coastal plain and piedmont of Georgia). In one study, Kosoy et al. (32) found rates of *Bartonella* infection in *S. hispidus* in central and southern Georgia approaching 80%. Thus, the degree of *Bartonella* infection in the *P. gwyni* population we surveyed is consistent with more extensive surveys of *S. hispidus* and lends confidence that these small sample estimates are representative.

Second, we found substantial rates of mixed *Bartonella* infections. More than half of the fleas we surveyed were infected by more than one *Bartonella gltA* genotype (Table 2). If the

	Bartonella genogroups described from the cotton rat, *Sigmodon hispidus* (Kosoy et al. 1997)												*Bartonella gltA* amplicons from fleas, *Polygenis gwyni* (this study)									
	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	*B. clarr.*	*B. rochal.*	#1	#2	#2	#4	#4	#6	#10	#11	#11	#13	#20
S. hispidus A1	-																						
S. hispidus A2	0.970	-																					
S. hispidus A3	0.978	0.970	-																				
S. hispidus A4	0.964	0.988	0.966	-																			
S. hispidus A5	0.997	0.970	0.981	0.964	-																		
S. hispidus B1	0.926	0.920	0.922	0.920	0.926	-																	
S. hispidus B2	0.932	0.926	0.929	0.926	0.932	0.979	-																
S. hispidus B3	0.932	0.926	0.929	0.926	0.932	0.979	0.997	-															
S. hispidus B4	0.938	0.932	0.933	0.926	0.938	0.982	0.991	0.991	-														
S. hispidus B5	0.938	0.932	0.933	0.926	0.938	0.979	0.994	0.994	0.997	-													
B. clarridgeiae	0.869	0.875	0.884	0.872	0.869	0.878	0.878	0.878	0.887	0.884	-												
B. rochalimae	0.887	0.883	0.903	0.880	0.887	0.890	0.890	0.890	0.899	0.896	0.960	-											
P. gwyni #1	0.991	0.961	0.966	0.955	**0.988**	0.917	0.923	0.923	0.929	0.929	0.866	0.880	-										
P. gwyni #2a	0.938	0.932	0.933	0.926	0.938	0.982	0.991	0.991	**1.000**	0.997	0.887	0.898	0.929	-									
P. gwyni #2b	0.970	**1.000**	0.970	0.988	0.970	0.920	0.926	0.926	0.932	0.932	0.875	0.883	0.961	0.932	-								
P. gwyni #4a	**0.997**	0.967	0.974	0.961	0.994	0.923	0.929	0.929	0.935	0.935	0.866	0.883	0.988	0.935	0.967	-							
P. gwyni #4b	**0.997**	0.967	0.974	0.961	0.994	0.923	0.929	0.929	0.935	0.935	0.866	0.883	0.988	0.935	0.967	0.994	-						
P. gwyni #6	**1.000**	0.970	0.978	0.964	0.997	0.926	0.932	0.932	0.938	0.938	0.869	0.887	0.991	0.938	0.970	0.997	0.997	-					
P. gwyni #10	0.929	0.923	0.925	0.923	0.929	0.976	0.994	**0.997**	0.988	0.991	0.875	0.887	0.920	0.988	0.923	0.926	0.926	0.929	-				
P. gwyni #11a	0.938	0.932	0.933	0.926	0.938	0.979	0.994	0.994	0.997	**1.000**	0.884	0.896	0.929	0.997	0.932	0.935	0.935	0.938	0.991	-			
P. gwyni #11b	0.872	0.872	0.888	0.869	0.872	0.878	0.872	0.872	0.881	0.878	**0.947**	**0.942**	0.869	0.881	0.872	0.869	0.869	0.872	0.869	0.878	-		
P. gwyni #13	**0.991**	0.961	0.970	0.955	0.988	0.917	0.923	0.923	0.929	0.929	0.861	0.877	0.982	0.929	0.961	0.988	0.988	0.991	0.920	0.929	0.864	-	
P. gwyni #20	**0.991**	0.961	0.966	0.955	0.988	0.917	0.923	0.923	0.929	0.929	0.861	0.877	0.982	0.929	0.961	0.988	0.988	0.991	0.920	0.929	0.869	0.982	-

FIG. 2. Matrix of genetic similarity (fraction of identical sites) in a 337-bp fragment of *gltA* cloned from *Polygenis gwyni* fleas collected from cotton rats (*Sigmodon hispidus*). Only representative flea-*Bartonella* samples are shown. Many fleas contained mixed infections, and examples are shown (numbers 2, 4, and 11). The grey shading highlights the values that compare the *P. gwyni* strains to the corresponding strains A1 through A5 and B1 through B5 previously cultured from *S. hispidus* (33). The bold values represent the highest similarity values for each flea-associated *Bartonella* sample to the various A or B genogroups described from cotton rats. *B. clarridgeiae* and the newly described *B. rochalimae* (18) are flagellated species distantly related to *Bartonella* species described from cotton rats but nevertheless are genetically similar to amplicons from the fleas of cotton rats.

pap31 screens are included, the rate is even higher. Kosoy et al. (33) originally described four broad genotypic clusters associated with various small rodents from the southeastern United States, designated A through D. Type sequences originally used to define groups A and B together form a diverse but monophyletic group of *B. vinsonii*-like isolates from *S. hispidus*, as originally indicated in the neighbor-joining distance *gltA* tree of Kosoy et al. (33). We detected isolates similar to A and B in the surveyed fleas and, unsurprisingly, did not detect the *Peromyscus*-associated D group. Surprisingly, we did not detect genogroup C, previously cultured from regional samples of *S. hispidus* (31, 32). Cluster A is, by far, the most common *Bartonella* genogroup isolated from cotton rats in the region (31, 32). However, C is more prevalent than B (31, 32), a pattern opposite of what we found in *P. gwyni* from cotton rats (Table 2; Fig. 1). This pattern may simply be an artifact of small sample sizes and may not hold up to more-extensive surveys. However, one possibility is that the different *P. gwyni*/*S. hispidus* isolates exhibit either unequal resident times in the vectors and hosts and/or transmission biases, potentially presenting an opportunity to uncover differential adaptation and specificity in *Bartonella* (M. Kosoy, personal communication).

Third, we successfully amplified a fragment similar to the heme-binding *pap31* in fleas evidently infected with *B. vinsonii*-like isolates (as determined by *gltA*). This is notable, because in both *B. quintana* and *B. henselae*, *pap31* is generally known to be phage-borne and orthologous to a large family of heparin-binding protein-coding genes (*hbp*) critical to heme acquisition, cellular adhesion, and possibly pathogenesis (8, 11, 56). Recent work has described a *pap31* homolog from bacteriophages in *B. vinsonii* subsp. *berkhoffii* (40), a species that was previously thought to lack bacteriophages and *hbpA* protein homologs (8). Assuming the *gltA* results are a reliable guide, we found *pap31*-like sequences in fleas infected by Kosoy et al. genogroups A and B; *pap31* amplicons were cloned in fleas apparently lacking coinfections and harboring either the A or B *gltA* genogroup alone (Table 2). Although there is not yet sufficient coverage of the genus with *pap31* to identify the isolates we detected, three distinct genogroups are evident (Fig. 3). Possibly, the two derived genogroups within the clade correspond to Kosoy et al.'s (33) genogroups A and B.

In *B. quintana*, *pap31* is a member of a five-gene family, composed of three tandemly arrayed paralogs and two other homologs (42). A possible complication is the uncertain copy number of *pap31* homologs across the genus. However, the clade that includes the *P. gwyni* samples is rooted by *hbpA* from *B. quintana*, to the exclusion of other members of the gene family, and includes orthologous sequences from *B. henselae* and *B. vinsonii* subsp. *berkhoffi* (Fig. 3). Moreover, the *pap31* transmembrane protein includes outer membrane loops with the potential to incorporate nearly random in-frame chromosomal sequences. In *B. quintana*, the five homologs are difficult to align at these sites (42) (data not shown). With the exception of some isolates exhibiting distinct similarity to *B. henselae* (Fig. 4), the loop sequences from *P. gwyni* isolates exhibit very little amino acid polymorphism between the conserved transmembrane domains. It is thus likely that the *pap31* topology reflects orthologous sequence variation in the *Bartonella* isolates we surveyed. Because of the sampling design and the high rate of coinfection, it is not possible to determine the significance of the truncated *hbpA* pseudogene. However, possibilities include that some isolates harbor an antigenic variant of the full *hbpA* protein, similar to the *msp2* locus in *Anaplasma* sp. (3, 7), or that the *pap31* pseudogene is a loss-of-function mutant derived from a cryptic strain that has undergone a change in lifestyle (23, 43).

In this vein, the fourth and perhaps most surprising result was the presence in two fleas of an isolate sharing >94% *gltA* similarity to *B. clarridgeiae* and the newly described species *B. rochalimae* (the next closest relative is *Bartonella bovis*, at >87% sequence similarity) (Fig. 1). *B. clarridgeiae* itself has not been described from rodents; rather, felids or canids are the primary reservoirs (49). Species near *B. clarridgeiae* have been reported in various mammalian hosts, however, and recently, a *B. clarridgeiae*-like isolate was identified in rat fleas from Egypt (39). Among the highest BLAST scores for the *B.*

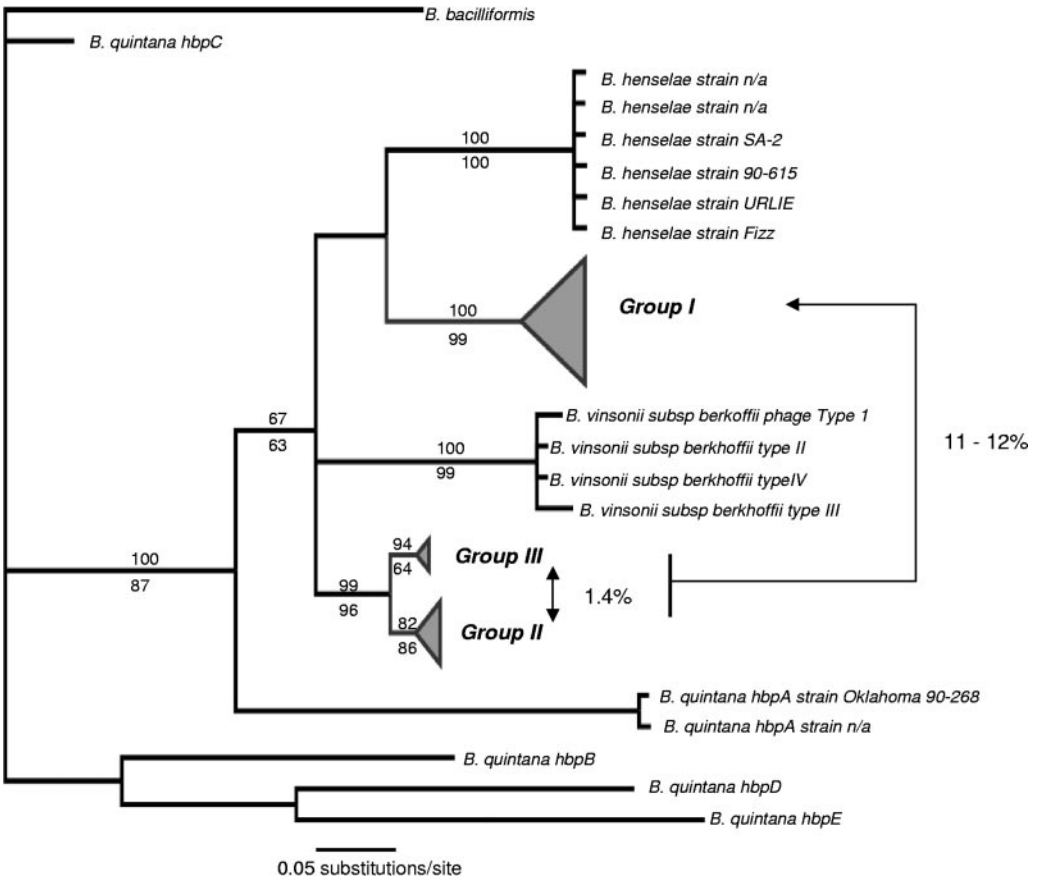

FIG. 3. Consensus tree of *Bartonella* taxa based on partial *pap31* sequences. Numbers above interior branches represent clade credibility values from the Bayesian analysis (above) and 100 maximum likelihood bootstrap replicates (below). Most amplicons were confirmed by redundant sequencing of multiple cloned products. Three distinct genotypic groups are evident from the fleas of cotton rats, as shown. The genetic distance between groups II and III, based on 152 bp of alignable transmembrane domain sequences, was approximately 1.4% (uncorrected *p* distance). Group I differed from both by approximately 11 to 12%. The *Bartonella* isolates in these groups have no clear identity based on BLAST searches of *pap31* sequences. Highest BLAST scores were returned for *B. henselae* or *B. quintana*, but this probably reflects the limited taxonomic sampling of *pap31* across the genus.

clarridgeiae-like isolates in *Polygenis* were uncultured species from rodents and other small mammals (17, 25, 46). Both fleas were coinfected with Kosoy genogroup A or B. In the case of one flea, all three principle *gltA* variants were detected. The significance of a *B. clarridgeiae* species in *Sigmodon* is unknown; because of the size of the survey and the absence of simultaneous information on the competence of *Polygenis* as a *B. clarridgeiae* vector and *B. clarridgeiae* bacteremia in cotton

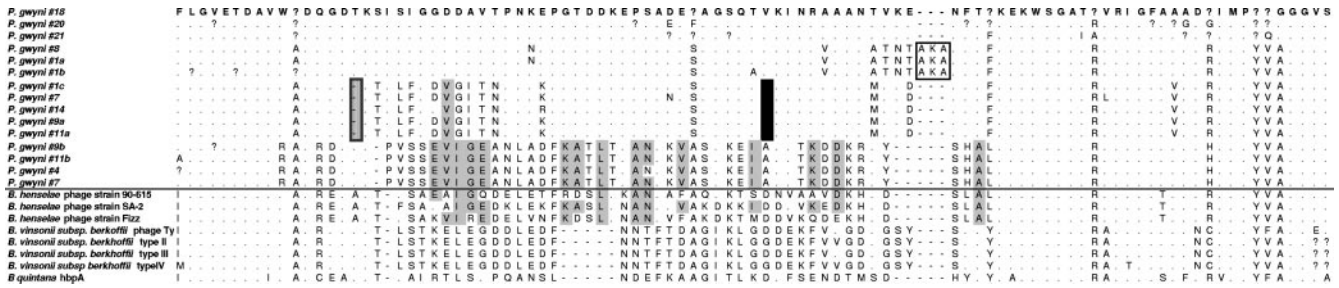

FIG. 4. Amino acid fragments of *pap31* homologs from various taxa and a subset amplified from representative flea samples in the present study. The fragment corresponds to an outer membrane loop sequence between conserved transmembrane domains 3 and 4, as described in reference 42. These loops may be composed of nearly random host chromosomal sequences (56), as evident in the lack of conservation between the related *B. henselae* and *B. quintana*. Seven of the nine *P. gwyni* isolates exhibit strong conservation, likely indicating a recent common ancestor. Some samples align more closely to *B. henselae* strains, as indicated by gray shading. In one group (clade II from Fig. 3), a 1-bp deletion (grey hatched box) causes a UAA stop codon downstream. A gap has been inserted to maintain the alignment in these samples. The black box highlights an insertion of three residues. Dots indicate matches with the topmost sequence, dashes indicate gaps, and question marks indicate uncertainties due to unresolvable ambiguities in the nucleotide sequences. The black line separates the *pap31* amplicons from the present study and those from known species. Dissimilar amplicons were cloned from the same flea samples, as illustrated by *P. gwyni* numbers 1, 9, and 11.

rats, the biological significance is difficult to judge. However, like *B. bacilliformis*, the etiological agent of bartonellosis (Carrion's disease) in humans, and *B. bovis*, *B. clarridgeiae* is one of the few flagellated bartonellae (51) and has long been a problematic species because of its uncertain phylogenetic placement and the odd host range that it shares with *B. bovis* (44). It may not be a coincidence that an isolate resembling these hyper-generalist species has been discovered in *Polygenis*. Efforts to understand the molecular basis of variation in host specificity in the genus (2) would benefit from closer examination of *B. clarridgeiae* and its relatives (18).

Two opposing ecological and evolutionary processes seem to be at work in *Bartonella*. The cryptic diversity in the vectors of *Bartonella*, and the absence of strains common in mammalian hosts, may reflect an evolutionary trend towards differential adaptation to host-specific niches, in either vectors or reservoirs. If so, it seems *Bartonella* possesses a tendency towards fine-scale adaptation, ecological specialization, and divergence between essentially syntopic populations, despite mixed infections, close physical proximity, and generalist lifestyles. The mechanisms by which *Bartonella* genomes are protected during the process of specialization to host-associated niches, while maintaining broad host affiliations and thus mixed infections (29), are presently unknown.

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