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Molecular Identification and Analysis of *Borrelia burgdorferi* Sensu Lato in Lizards in the Southeastern United States

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Lyme borreliosis (LB) group spirochetes, collectively known as *Borrelia burgdorferi* sensu lato, are distributed worldwide. Wild rodents are acknowledged as the most important reservoir hosts. *Ixodes scapularis* is the primary vector of *B. burgdorferi* sensu lato in the eastern United States, and in the southeastern United States, the larvae and nymphs mostly parasitize certain species of lizards. The primary aim of the present study was to determine whether wild lizards in the southeastern United States are naturally infected with Lyme borreliae. Blood samples obtained from lizards in Florida and South Carolina were tested for the presence of LB spirochetes primarily by using *B. burgdorferi* sensu lato-specific PCR assays that amplify portions of the flagellin (*flaB*), outer surface protein A (*ospA*), and 66-kDa protein (*p66*) genes. Attempts to isolate spirochetes from a small number of PCR-positive lizards failed. However, PCR amplification and sequence analysis of partial *flaB*, *ospA*, and *p66* gene fragments confirmed numerous strains of *B. burgdorferi* sensu lato, including *Borrelia andersonii*, *Borrelia bissettii*, and *B. burgdorferi* sensu stricto, in blood from lizards from both states. *B. burgdorferi* sensu lato DNA was identified in 86 of 160 (54%) lizards representing nine species and six genera. The high infection prevalence and broad distribution of infection among different lizard species at different sites and at different times of the year suggest that LB spirochetes are established in lizards in the southeastern United States.

Lyme borreliosis, the most frequently reported arthropod-borne infection in the United States (6), is caused by several species within the *Borrelia burgdorferi* sensu lato genogroup (38). *B. burgdorferi* sensu lato includes at least 11 genospecies worldwide, three of which are present in North America (*Borrelia andersonii*, *Borrelia bissettii*, and *B. burgdorferi* sensu stricto) (16, 28, 33). Thus far, only *B. burgdorferi* sensu stricto has been proven to cause human disease in the United States.

In the northeastern United States, the spirochetes are transmitted to humans by the blacklegged tick, *Ixodes scapularis* (5), and maintained in nature primarily by small rodents (4, 23, 31). In the southeastern and western United States, immature stages of the vector ticks feed primarily on lizards (2, 10, 35, 43). Although *B. burgdorferi* sensu lato has been isolated from birds, rodents, and ticks in southern and western states (9, 31, 33), the organism has never been isolated from wild lizards. Indeed, several studies have shown that strains of two *B. burgdorferi* sensu lato species do not survive in the blood of two lizard species found in California (19, 21, 43), leading to a widely held belief that lizards do not serve as reservoirs of the bacteria. However, a different study (22) showed in laboratory experiments that two common lizards in the southeastern United States, green anoles and southeastern five-lined skinks, were reservoir competent for one strain of *B. burgdorferi* sensu stricto. In the present study, we sought to determine whether lizards in the southeastern United States are naturally infected with *B. burgdorferi* sensu lato by attempting to isolate spirochetes and by using DNA amplification methods to genetically characterize strains present in lizards and to conduct initial experiments to determine if *I. scapularis* ticks could acquire *B. burgdorferi* sensu lato from feeding on naturally infected lizards collected in the wild. Here we present the first reported evidence of *B. burgdorferi* sensu lato among naturally infected wild lizards; these findings demonstrate a broad geographic distribution, three *B. burgdorferi* sensu lato species, and high infection prevalence among multiple lizard species in two southeastern states.

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MATERIALS AND METHODS

Sample collection. Lizards were captured and sampled from national forests and state parks in northern and central Florida and southeastern South Carolina from March 2003 through May 2004. The primary habitats at the collection localities are mixed pine and oak uplands, mixed pine flatwoods, and bay swamps. Prescribed burning of vegetation is conducted regularly at some sites as part of ongoing habitat management programs. Lizards were obtained by stalking and capturing either by hand or via noosing. Attached ticks were immediately removed with forceps and preserved in ethanol. A sample (~50 to 100 μl) of blood was obtained via tail fracture and blotted onto filter paper strips for DNA extraction. The blood from most lizards was obtained by removing the distal portion of the tail by hand, as the tails of most common lizards collected in the study fracture readily, providing a few drops of blood without harming the animals (tail fracturing is a natural escape mechanism for the animals). Most animals were returned to their site of capture immediately after examination and blood collection. Twelve broad-headed skinks (*Eumeces laticeps*) were euthanized humanely to harvest organs and tissues for *Borrelia* isolation attempts. An additional six PCR-positive *E. laticeps* skinks were kept in the laboratory for several months for transmission experiments.

DNA extraction and PCR testing. DNA was extracted from dried filter paper blood samples, tick pools, and cultures using a commercially available kit (MasterPure; Epicentre, Madison, WI) with optimized modifications of the manufacturer's protocols for each starting material. The starting template for filter paper blood samples was an approximately 5- by 5-mm square piece of blood-soaked paper. Culture aliquots of 200 μl were taken from approximately the middle of each conical tube of 4 ml of media suspension in attempts to avoid obtaining dead spirochetes that would presumably settle to the bottom of the tubes. The

TABLE 1. Oligonucleotide primers used in this study

B. burgdorferi sensu lato target gene	Primer	Primer sequence (5′ — 3′)[a]	Base position	Annealing temp (°C)	Amplicon size (bp)	Source or reference
flaB	Outer 1	AAR-GAA-TTG-GCA-GTT-CAA-TC	271–290	52		This study
	Outer 2	GCA-TTT-TCW-ATT-TTA-GCA-AGT-GAT-G	743–767		497	This study
	Inner 1	ACA-TAT-TCA-GAT-GCA-GAC-AGA-GGT-TCT-A	301–328	55		This study
	Inner 2	GAA-GGT-GCT-GTA-GCA-GGT-GCT-GGC-TGT	663–689		389	15
ospA	N1	GAG-CTT-AAA-GGA-ACT-TCT-GAT-AA	334–356	42		13
	C1	GTA-TTG-TTG-TAC-TGT-AAT-TGT	894–874		561	13
	N2	ATG-GAT-CTG-GAG-TAC-TTG-AA	362–381	42		13
	C2	CTT-AAA-GTA-ACA-GTT-CCT-TCT	713–693		352	13
p66	Outer 1	CGA-AGA-TAC-TAA-ATC-TGT	147–158	50		36
	Outer 2	GCT-GCT-TTT-GAG-ATG-TGT-CC	442–461		315	This study
	Inner 1	TGC-AGA-AAC-ACC-TTT-TGA-AT	168–187	50		36
	Inner 2	AAT-CAG-TTC-CCA-TTT-GCA	385–403		236	36

[a] R in a primer sequence signifies A or G; W signifies A or T.

resulting DNA pellets for all extracts were rehydrated in 100 μl of Tris-EDTA buffer. Negative control samples free of any template were included in each round of DNA extractions. Extracts were first screened by nested PCR targeting a 389-bp portion of the conserved 41-kDa chromosomal flagellin gene (*flaB*) of *B. burgdorferi* sensu lato using slight modifications of published primers (Table 1) (15, 32, 42). All *flaB*-positive samples were further tested with a nested PCR assay targeting a 236-bp portion of the chromosomal 66-kDa protein (*p66*) using a combination of published primers (36) and one that we modified (Table 1). The same subset of *flaB*-positive samples was tested with primers (13) targeting a 352-bp portion of the genetically diverse outer surface protein A (*ospA*) gene of *B. burgdorferi* sensu lato.

First-round amplifications contained between 2.5 and 5 μl of DNA extract per individual sample in a total reaction volume of 50 μl. All reactions utilized a hot start master mix (HotMasterMix; Brinkmann-Eppendorf, Westbury, NY), resulting in a final concentration of 1.0 U of *Taq* DNA polymerase, 45 mM KCl, 2.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, and a 0.5 μM concentration of each primer, and were carried out in an automated DNA thermal cycler (PTC 200; MJ Research, Watertown, MA). Outer reaction PCRs consisted of initial denaturation at 95°C for 1 min followed by 40 cycles of 94°C for 30 s, primer annealing at the temperature listed in Table 1 for 30 s, and extension at 68°C for 45 s. Nested reactions included between 1 and 2.5 μl of outer reaction product as template for another 30 cycles with the same parameters and annealing temperature profile as described above and in Table 1.

PCRs were set up in a separate area within a PCR clean cabinet (CleanSpot Workstation; Coy Laboratory Products, Grass Lake, MI) equipped with a germicidal UV lamp. Other precautions to prevent carryover contamination of amplified DNA included different sets of pipettes dedicated for DNA extraction, PCR setup, and postamplification activities; the use of aerosol barrier filter pipette tips; and exposing PCR tubes, pipettes, and tips to UV light prior to PCR setup. Each outer PCR included a negative control sample with sterile water as template and a positive control sample from *B. burgdorferi* sensu stricto strain B31 culture extract. Negative control samples included in each round of extractions were also screened to detect any possible extraction contamination. A portion of the positive and negative control outer reaction samples was carried over as template in each nested reaction, just as for experimental samples. PCR products were electrophoresed in 2% agarose gels, which were stained with ethidium bromide, and visualized and recorded with a digital gel documentation unit.

DNA sequence analysis. PCR products were purified using the MinElute PCR purification kit (QIAGEN, Valencia, CA). DNA templates were sequenced using the fluorescent dideoxy terminator method of cycle sequencing on either a Perkin-Elmer, Applied Biosystems, Inc. 373A or 377 automated DNA sequencer following Applied Biosystems protocols (29). Sequences were generated using the Sequencher Software (Gene Codes Corporation, Ann Arbor, MI). Investigator-derived sequences were compared with those obtained by searching the GenBank database (National Center for Biotechnology Information) using the Basic Local Alignment Search Tool (BLAST) (1) and aligned using Clustal X (40). Phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum parsimony (MP) methods (37, 39) with the tree-building program MEGA version 2.1 (18). Tree topologies and genetic relationships obtained with the two methods were compared for consensus. To estimate node reliability of trees obtained with each method, bootstrap values (11) based on an analysis of either 100 (MP) or 1,000 (NJ) replicates were determined. Pairwise distances were computed by the Kimura two-parameter model (17).

***Borrelia* culture isolation.** We chose 12 *flaB* PCR-positive broad-headed skinks (nine males and three females) for attempts to isolate spirochetes. Within 1 week of testing PCR positive, the animals were euthanized humanely and dissected with sterile technique inside a biosafety cabinet. Whole blood (~100 μl), heart, and liver samples (and testes from males; ~50 mg of each tissue type per sample) were inoculated into tubes containing 4 ml of modified Barbour-Stoenner-Kelly (BSK-H) culture medium supplemented with antibiotics (Sigma, St. Louis, MO) for isolating *Borrelia* (3), incubated at 32°C, and examined weekly for spirochetes by dark-field microscopy for 8 weeks. Samples from *B. burgdorferi* sensu lato reference strains (*B. andersonii* MOK-1c; *B. bissettii* 25015; *B. burgdorferi* sensu stricto B31, JD1, NC92, and WI90; and *Borrelia* sp. strain SCW-30h) were also inoculated and maintained in culture to ensure the ability of the medium to support spirochete growth.

Tick-feeding experiments. Six additional broad-headed skinks (three each from Florida and South Carolina) that tested positive for *B. burgdorferi* sensu lato in initial PCR tests were chosen for a tick-feeding and infection experiment. Individual animals were placed inside a ~1.5-in.-diameter tube constructed of 1/4-in.-mesh hardware cloth, with PVC end caps modified from published designs (14, 22). Approximately 100 *I. scapularis* tick larvae from a laboratory-reared colony at Colorado State University were introduced to each lizard's tube. Feeding tubes were suspended over white plastic photo trays with wet paper towel substrate in separate aquaria. The rims of the trays and aquaria were coated with petroleum jelly to prevent escape of ticks that dropped off. Trays were checked daily for detached ticks. Lizards were fed one or two crickets every other day by removing the PVC end cap on one end of the tube. Water was provided via a moistened cotton ball kept inside the tube at all times. After blood-fed ticks dropped off, they were rinsed in a 5% bleach solution, followed by a rinse in sterile distilled water, and then maintained in glass vials with cloth mesh lids in a glass desiccator jar at ~97% rH and 82°F until they molted to nymphs (~1 month later). The resultant nymphs were tested by PCR to determine if they acquired *B. burgdorferi* sensu lato and maintained infection through the molt. All procedures involving capturing, maintaining, and testing lizards were conducted in accordance with protocols approved by the University of North Florida Institutional Animal Care and Use Committee.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *B. burgdorferi* sensu lato *flaB* gene sequences generated in the present study are AY662999 to AY663008, AY663010 to AY663017, AY823241, AY823242, and AY823244 to AY823249. The *B. burgdorferi* sensu lato *ospA* gene sequences reported here are AY663018 to AY663021, AY663023, AY663024, AY823229, AY823231, and AY823232. The *B. burgdorferi* sensu lato *p66* gene sequences reported here are AY823233 to AY823240. The sequences used for comparison are listed in Table 2.

RESULTS

***B. burgdorferi* sensu lato-specific PCR.** We captured and obtained blood samples from 160 lizards representing seven

TABLE 2. *Borrelia* species reference strains used in this study

Species	Strain	Biological source	Geographic origin	GenBank sequence accession number		
				flaB sequence	*ospA* sequence	*p66* sequence
B. andersonii	19857	Cottontail rabbit	NY	D83762	A24008	
	21038	*Ixodes dentatus*	NY	D83763		
	MOK-1c	*Ixodes dentatus*	MO	AY654915	AY654919	AY654939
	SI-10	*Ixodes scapularis*	GA	AF264883		
B. bissettii	25015	*Ixodes scapularis*	NY	L29245	U65802	AY654938
	DN127	*Ixodes pacificus*	CA	D82857	Y10897	
	FL18	Cotton mouse	FL		AY654922	
	FL27	Cotton rat	FL			AY654935
	FL35	Cotton rat	FL		AY654923	AY654937
	FL42	Cotton rat	FL		AY654924	
	IA1	*Ixodes affinis*	FL		AY654925	
	IS-19	*Ixodes spinipalpis*	CO			U96243
	MI8	Cotton rat	FL	AF264892		
	SCGT8a	*Ixodes minor*	SC	AF264894		
B. burgdorferi sensu lato	SCW-30h	*Ixodes minor*	SC	AF264886	AY654920	AY654940
B. burgdorferi sensu stricto	AA4Pool	*Amblyomma americanum*	FL			AY654928
	AA15Pool	*Amblyomma americanum*	FL			AY654929
	B31	*Ixodes scapularis*	NY	X15661	AY030279	AE001161
	CA8	*Ixodes pacificus*	CA		L23144	
	FLCL3	*Ixodes affinis*	FL			AY654933
	JD1	*Ixodes scapularis*	MA		AF369944	U96240
	KC9	Golden mouse	FL			AY654934
	MI2	Cotton rat	FL	AF264889		
	SCI2	Cotton mouse	GA	AF264885		
	Tr293	*Ixodes ricinus*	Turkey	AB091813		
Borrelia afzelii	ACA1	Human subject	Sweden	X75202		AY090473
	VS461	*Ixodes ricinus*	Switzerland		Z29087	
Borrelia garinii	Ip90	*Ixodes persulcatus*	Russia	X75203		X87727
	PBi	Human subject	Germany		X80257	
Borrelia japonica	HO14	*Ixodes ovatus*	Japan	D82852		
	IKA2	*Ixodes ovatus*	Japan		Y10892	
Borrelia lusitaniae	PotiB2	*Ixodes ricinus*	Portugal	D82856	Y10838	
Borrelia valaisiana	VS116	*Ixodes ricinus*	Switzerland	D82854	Y10840	
Borrelia sinica	CMN3	White-bellied rat	China	AB022138		
Borrelia hermsii	HS1	*Ornithodoros hermsii*	United States			AF016408
Borrelia lonestari	TX	*Amblyomma americanum*	TX	U26704		

genera and 10 species from sites in Florida and South Carolina. Most (77%) of the animals were captured in March and April of 2003 and 2004, which is before most *I. scapularis* larvae and nymphs become active, or during the period of initial activity in the spring (35). Most (93%) animals were free of ticks when they were captured. Only 18 *I. scapularis* larvae and 30 nymphs were observed and removed (all during April and May), and all came from three lizard species: broad-headed and southeastern five-lined skinks and eastern glass lizards.

To determine if the lizards' blood contained *B. burgdorferi* sensu lato DNA, we first screened blood sample extracts with the *B. burgdorferi* sensu lato-specific nested *flaB* PCR assay. Eighty-six (53.8%) of the samples tested positive, including those from nine lizard species (six genera) and nine sites in Florida and South Carolina (Table 3); positive blood samples were obtained from juvenile, subadult, and adult animals. None of the negative control extracts tested positive. The infection prevalence among different positive lizard species

TABLE 3. Prevalence of *B. burgdorferi* sensu lato flagellin (*flaB*) gene DNA among lizards from Florida and South Carolina

Location	Number of PCR-positive animals/number of each species tested (%)[a]										
	Broad-headed skink	Brown anole	Fence lizard	Glass lizard	Scrub lizard	Green anole	Ground skink	Gecko	Race-runner	Five-lined skink	Total
Florida	8/18 (44)	2/4 (50)	3/9 (33)	1/1 (100)	6/14 (43)	7/17 (41)	5/7 (71)	0/3 (0)	2/11 (18)	3/8 (38)	37/92 (40)
South Carolina	13/18 (72)	NT[b]	NT	1/1 (100)	NT	22/33 (67)	1/1 (100)	NT	NT	12/15 (80)	49/68 (72)
Total	21/36 (58)	2/4 (50)	3/9 (33)	2/2 (100)	6/14 (43)	29/50 (58)	6/8 (75)	0/3 (0)	2/11 (18)	15/23 (65)	86/160 (53.8)

[a] Species tested were the broad-headed skink (*Eumeces laticeps*), brown anole (*Anolis sagrei*), eastern fence lizard (*Sceloporus undulatus*), eastern glass lizard (*Ophisaurus ventralis*), Florida scrub lizard (*Sceloporus woodi*), green anole (*Anolis carolinensis*), ground skink (*Scincella lateralis*), Mediterranean gecko (*Hemidactylus turcicus*), six-lined racerunner (*Cnemidophorus sexlineatus*), and southeastern five-lined skink (*Eumeces inexpectatus*).

[b] NT, none tested.

ranged from 18 to 100%. The prevalence among animals from South Carolina (72%) was higher than for those from Florida (40%), but a greater variety of species from Florida was tested. The only species for which no positives were obtained was the Mediterranean gecko, which, along with two brown anoles that did test positive, came from suburban residences. Positive results were obtained for several species that are not commonly, if ever, parasitized by *I. scapularis*: brown and green anoles, Florida scrub lizards, and six-lined racerunners (2, 10). *flaB* PCR-positive samples were obtained from lizards in February through May, September, and December, which includes months (February and December) when *I. scapularis* larvae and nymphs do not typically parasitize hosts (35).

All 86 *flaB*-positive samples were then tested with the *p66* and *ospA* nested PCR assays. Thirty (34.9%) of these 86 tested positive with the *p66* assay, and 17 (19.8%) were also positive with the *ospA* PCR assay. Only 10 of 86 (11.6%) *flaB*-positive samples tested positive with both *p66* and *ospA* assays. Most PCR-amplified target gene fragments were visible in ethidium bromide-stained gels only in the nested reaction products, indicating generally low target copy number in the extracts. Nevertheless, many lizard blood extracts were tested several times for each of the three target gene fragments, with consistent results. Some samples were positive only with the *flaB* assay. Some were consistently *flaB* and *p66* positive but *ospA* negative, and others were consistently *flaB* and *ospA* positive but *p66* negative.

***B. burgdorferi* sensu lato *flaB*, *p66*, and *ospA* sequences.** We sequenced PCR-amplified *flaB*, *ospA*, and *p66* gene fragments from many individual lizard samples and compared the sequences to those obtained via BLAST (1) searching the GenBank database. Most of the *flaB* and *ospA* DNAs analyzed in this study were sequenced in one direction only, with the forward primers used in the PCRs. A few of the *flaB* DNAs and all of the *p66* DNAs were sequenced in both directions with the forward and reverse PCR primers. For a few samples, the initially derived sequences contained some unresolved nucleotides caused by multiple signal polymorphisms at those positions. When these were resequenced, or the sequences obtained with both forward and reverse primers were compared, the unresolved bases in all but a few of these samples could be determined. Based on the BLAST scores and phylogenetic comparisons we conducted, all *flaB*, *ospA*, and *p66* DNAs obtained from lizards or *I. scapularis* ticks fed upon them in the lab belonged to *B. burgdorferi* sensu lato.

Figure 1 shows an NJ phylogenetic tree based on 362 bp of the *flaB* gene, which has been used to reliably differentiate *B. burgdorferi* sensu lato strains to the species level (12, 15, 32). *flaB* sequences derived from 28 lizards and four *I. scapularis* nymph pools (from larvae fed on infected lizards in the lab) clustered into three clades defined by reference strains of the *B. burgdorferi* sensu lato species previously identified in the United States; these included 19 *B. burgdorferi* sensu stricto strains in eight lizard species and the four *I. scapularis* nymph pools, 11 strains of *B. andersonii* in five lizard species, and 2 *B. bissettii* strains in two lizard species (Fig. 1; Table 4; not all data shown). Based on pairwise distances, the *flaB* sequences from lizards and ticks that clustered with *B. burgdorferi* sensu stricto strains were between 98.0 and 100% identical and between 98.6 and 100% similar to the *B. burgdorferi* sensu stricto reference strains included in the analysis. *flaB* sequences from several other lizards clustered with those from *B. andersonii* reference strains (Fig. 1). All except one (SC194) of these sequences from the lizards were nearly identical to that for strain SI-10, a *B. andersonii* isolate from a blacklegged tick in Georgia. The similarity among all of the *B. andersonii flaB* sequences ranged between 98.0 and 100%. The lizard *flaB* sequences from FL60 and FL203 were 99.7% identical and between 98.9 and 99.7% identical to *B. bissettii* reference strain sequences included in the comparison.

The *ospA* sequences (334 bp analyzed) derived from lizards clustered into three different clades defined by the previously described North American strains (Fig. 2). The sequences were heterogeneous, and their phylogenetic clustering did not agree with that obtained from the *flaB* sequence analysis for several individual lizard templates. For example, the *flaB* sequence from lizard samples SC89, SC167, and FL187 clustered with *B. andersonii* strains (Fig. 1), but the *ospA* sequences from these samples clustered with *B. bissettii* sequences (Fig. 2). The FL121 and FL126 *flaB* sequences both clustered with *B. burgdorferi* sensu stricto strains (Fig. 1; FL126 not shown), yet while the FL121 *ospA* sequence clustered along with strain SCW-30h most closely to *B. burgdorferi* sensu stricto reference strain sequences, the FL126 *ospA* sequence clustered with *B. bissettii* strains.

The *ospA* sequences from lizards FL126, FL187, SC89, SC167, and SC194 were between 99.1 and 100% identical and between 93.4 and 100% similar to all *B. bissettii* reference strain sequences included in the comparison and most similar to previously described *B. bissettii* strains from the southeastern United States. The *ospA* sequence from lizard FL121 was 99.7% similar to that for strain SCW-30h and between 93.6 and 95% similar to *B. burgdorferi* sensu stricto reference strains included in the analysis. The sequences from lizards SC87, SC106, and SC107 were 99.7 to 100% identical and between 97.2 and 98.7% similar to the *B. andersonii* reference strains.

All of the *p66* sequences derived from lizards were also very similar to *B. burgdorferi* sensu lato reference strains. The phylogenetic tree based on 233 bp of data showed that all but one of the lizard-derived *p66* sequences analyzed in this study clustered most closely with *B. burgdorferi* sensu stricto reference strains (Fig. 3). The phylogenetic placement of some lizard *p66* sequences also did not correlate with the *B. burgdorferi* sensu lato species clustering produced by the *flaB* sequence analysis for those lizard samples. The sequences from lizards FL71, FL118, FL131, FL139, FL187, SC87, and SC194 were 98.3 to 100% identical to each other as well as to the *B. burgdorferi* sensu stricto reference strains that they were most similar to. The *p66* sequence from lizard SC152 was 99.6% identical to that from *B. bissettii* strain 25015, 98.3% identical to that from FL187, 97.6% identical to that from *B. bissettii* IS-19 from Colorado, and 96.8% identical to that from *B. andersonii* MOK-1c.

***Borrelia* isolation.** Although all lizard blood and tissue cultures were examined weekly for spirochetes for 8 weeks, no spirochetes were detected microscopically. During that time, we extracted and tested DNA from cultures of different starting materials at different times postinoculation using the nested *flaB* PCR assay. At least one template type culture sample from each of the 12 animals tested positive via PCR.

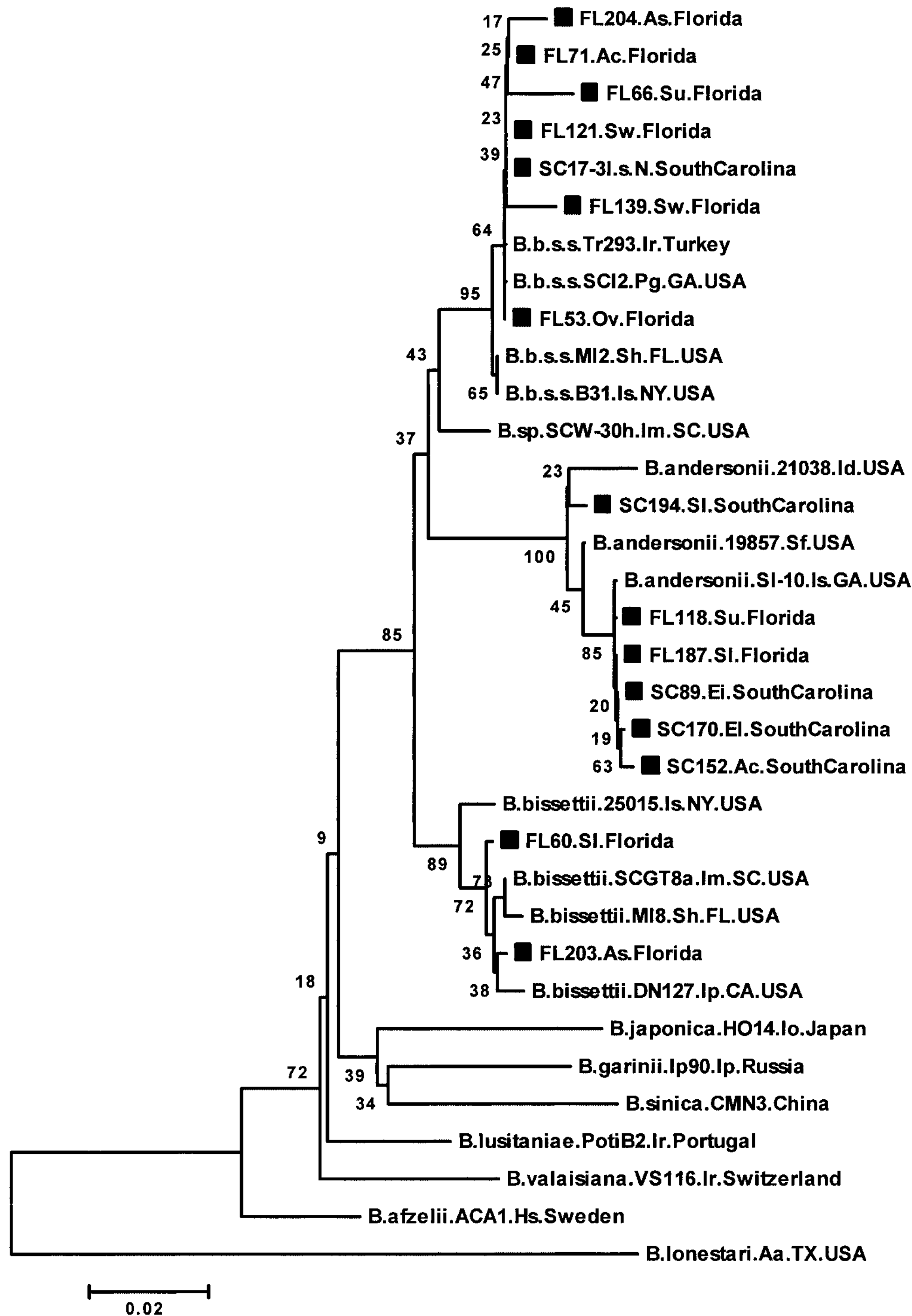

FIG. 1. Unrooted neighbor-joining phylogenetic tree based on 362 bp of the *flaB* gene obtained from lizards in Florida and South Carolina and *B. burgdorferi* sensu lato reference strains. The relapsing fever group species *Borrelia lonestari* was included as an outgroup. Numbers at the branch nodes represent bootstrap values as percentages of 1,000 replications. All sequences obtained in this study are identified by a square symbol preceding the sequence name, and names end with the state, either Florida or South Carolina. Reference strain sequences end with the country name. The strain name for reference sequences follows the species name (e.g., B.bissettii.DN127). Letter symbols representing the specific host and/or source follow the sequence and species or strain name. Abbreviations: Aa, *Amblyomma americanum*; Ac, *Anolis carolinensis*; As, *Anolis sagrei*; B.b.s.s., *B. burgdorferi* sensu stricto; B.sp., *Borrelia* species; Ei, *Eumeces inexpectatus*; El, *Eumeces laticeps*; Hs, human subject; Id, *Ixodes dentatus*; Im, *Ixodes minor*; Io, *Ixodes ovatus*; Ip, *Ixodes pacificus* or *Ixodes persulcatus*; Ir, *Ixodes ricinus*; I.s.N., *Ixodes scapularis* nymphs; Ov, *Ophisaurus ventralis*; Pg, *Peromyscus gossypinus*; Sf, *Sylvilagus floridanus*; Sh, *Sigmodon hispidus*; Sl, *Scincella lateralis*; Su, *Sceloporus undulatus*; and Sw, *Sceloporus woodi*. Examining the tree from top to bottom, the first major clade, beginning with strain FL204 and ending with B.b.s.s.B31, delineates *B. burgdorferi* sensu stricto strains. Below this group, the next major clade groups *B. andersonii* strains. Below this is a clade grouping *B. bissettii* strains.

TABLE 4. Representative Lyme borreliosis group spirochete strains identified in the present study based upon phylogenetic analysis of *flaB*, *ospA*, and *p66* gene sequences[a]

Host ID	Host or tick species	Collection locality (site, county, state)	Genospecies[a]
FL 53	Eastern glass lizard	University of North Florida, Duval Co., FL	*B. burgdorferi* sensu stricto
FL 60	Ground skink	Guana River State Park, St. Johns Co., FL	*B. bissettii*
FL 66	Eastern fence lizard	University of North Florida, Duval Co., FL	*B. burgdorferi* sensu stricto
FL 71	Green anole	Appalachicola National Forest, Leon/Wakulla Co., FL	*B. burgdorferi* sensu stricto
SC 87	Green anole	Francis Marion National Forest, Berkley/Charleston Co., SC	*B. andersonii*
SC 107	Broad-headed skink	Francis Marion National Forest, Berkley/Charleston Co., SC	*B. andersonii*
FL 121	Florida scrub lizard	Ocala National Forest, Marion Co., FL	*B. burgdorferi* sensu stricto
FL 131	Six-lined racerunner	Ocala National Forest, Marion Co., FL	*B. burgdorferi* sensu stricto
FL 203	Brown anole	Fort Pierce Residence, St. Lucie Co., FL	*B. bissettii*
FL 204	Brown anole	Atlantic Beach Residence, Duval Co., FL	*B. burgdorferi* sensu stricto
SC17-3I.s.N[b]	Blacklegged tick	Colony-reared samples	*B. burgdorferi* sensu stricto

[a] See figures for phylogenetic trees based on *flaB*, *ospA*, and *p66* sequences.
[b] This strain was identified in DNA extracted from a pool of three nymph ticks fed as larvae in the laboratory on a broad-headed skink collected in Francis Marion National Forest, Berkley/Charleston Co., SC.

Target products were seen only in the nested reactions, indicating a very low copy number of target DNA template in the culture extracts. One of 12 whole-blood cultures and 1 of 12 liver tissue cultures tested at 1 week postinoculation were PCR positive. At 4 weeks postinoculation, 7 of 12 blood culture extracts tested positive. At 5 weeks, 5 of 12 heart tissue cultures and 3 of 9 testes cultures were positive. However, upon testing again at 8 weeks, only 2 of 12 new extracts from previously positive culture samples (seven blood samples, three heart samples, and two testes samples) were positive. Most of the lizard samples were visibly contaminated with other bacteria. PCR amplification of lizard blood and tissue culture DNA extracts using broad-range 16S rRNA gene primers and DNA sequencing confirmed the presence of *Mycoplasma* spp. Reference strain cultures of several *B. burgdorferi* sensu lato species grew extremely well and did not become contaminated. During the follow-up, *flaB* PCR-positive lizard culture samples were subcultured multiple times into fresh medium; however, the contaminating bacteria persisted and continued to grow in the medium.

Tick-feeding experiments. In August 2003, blood sample extracts from 9 of 20 (45%) broad-headed and southeastern five-lined skinks that had been kept in the laboratory since being captured in April of that year tested positive via *flaB* PCR, and 6 of these (three broad-headed skinks each from Florida and South Carolina) were used in the tick-feeding and transmission experiment. Approximately 100 *I. scapularis* larvae were placed on each animal, and 393 attached and fed to repletion. The minimum and maximum numbers that fed on different lizards were 11 and 124, respectively, with an average of 66 per animal. Blood-fed ticks were kept in the lab until they molted to nymphs. Unfortunately, due to fungal growth in the tubes, only 28 ticks survived through the molt, which was deemed too few to attempt further transmission studies. However, DNA was extracted from the nymphs in six pools of three to six ticks per pool to aim for maximum sensitivity of detection and tested by nested *flaB* PCR to determine whether they acquired *B. burgdorferi* sensu lato and maintained detectable *B. burgdorferi* sensu lato DNA through the molt. All six pools tested positive, indicating a minimum estimated "infection" prevalence of 6 of 28 (21%); the actual number of infected individual ticks may have been higher. The *flaB* products were sequenced from four of the pools. They were not identical, but all were found to be >99% similar to lizard-derived and reference strain *B. burgdorferi* sensu stricto sequences as described above.

DISCUSSION

Studies have shown that two lizard species in California, the western fence lizard (*Sceloporus occidentalis*) and the southern alligator lizard (*Elgaria multicarinata*), have poor reservoir potential for some strains of *B. burgdorferi* sensu lato (19, 20, 21, 41, 43). Another study (22) showed that green anoles and southeastern five-lined skinks, both common in the southeastern United States, were reservoir competent under laboratory-controlled conditions for one strain of *B. burgdorferi* sensu stricto. In the present study, we were unable to obtain pure cultures of *B. burgdorferi* sensu lato from wild caught lizards, including those containing strains belonging to *B. burgdorferi* sensu lato species that have been cultured in BSK medium. We did, however, demonstrate the presence of *B. burgdorferi* sensu lato DNA in cultures of different sample types by PCR testing up to 8 weeks postinoculation.

These results may be explained by the fact that culturing in BSK medium is selective for specific genotypes of *B. burgdorferi* sensu lato (26, 30). Oliver et al. (31) found relatively low *Borrelia* culture isolation prevalence (6.5%) among 200 specimens of three reservoir-competent small mammal species sampled from several sites in Florida. Attempts to isolate spirochetes in BSK medium from a smaller number ($n = 65$) of small mammals from northeast Florida in a different study conducted by one of us (K. Clark) also failed, even though PCR testing showed the *B. burgdorferi* sensu lato prevalence to be very high (7). An alternative explanation for the failure to isolate *B. burgdorferi* sensu lato from lizards in the present study is that the number of spirochetes present in the lizard blood and tissues may have been below the sensitivity level of this detection method, especially if the spirochetes were competing with other bacteria that may have been present in the samples. Additional attempts at isolating spirochetes from more PCR-positive lizards are necessary to determine whether such strains are cultivable in BSK medium. These efforts could include filtering inoculated cultures in attempts to remove bac-

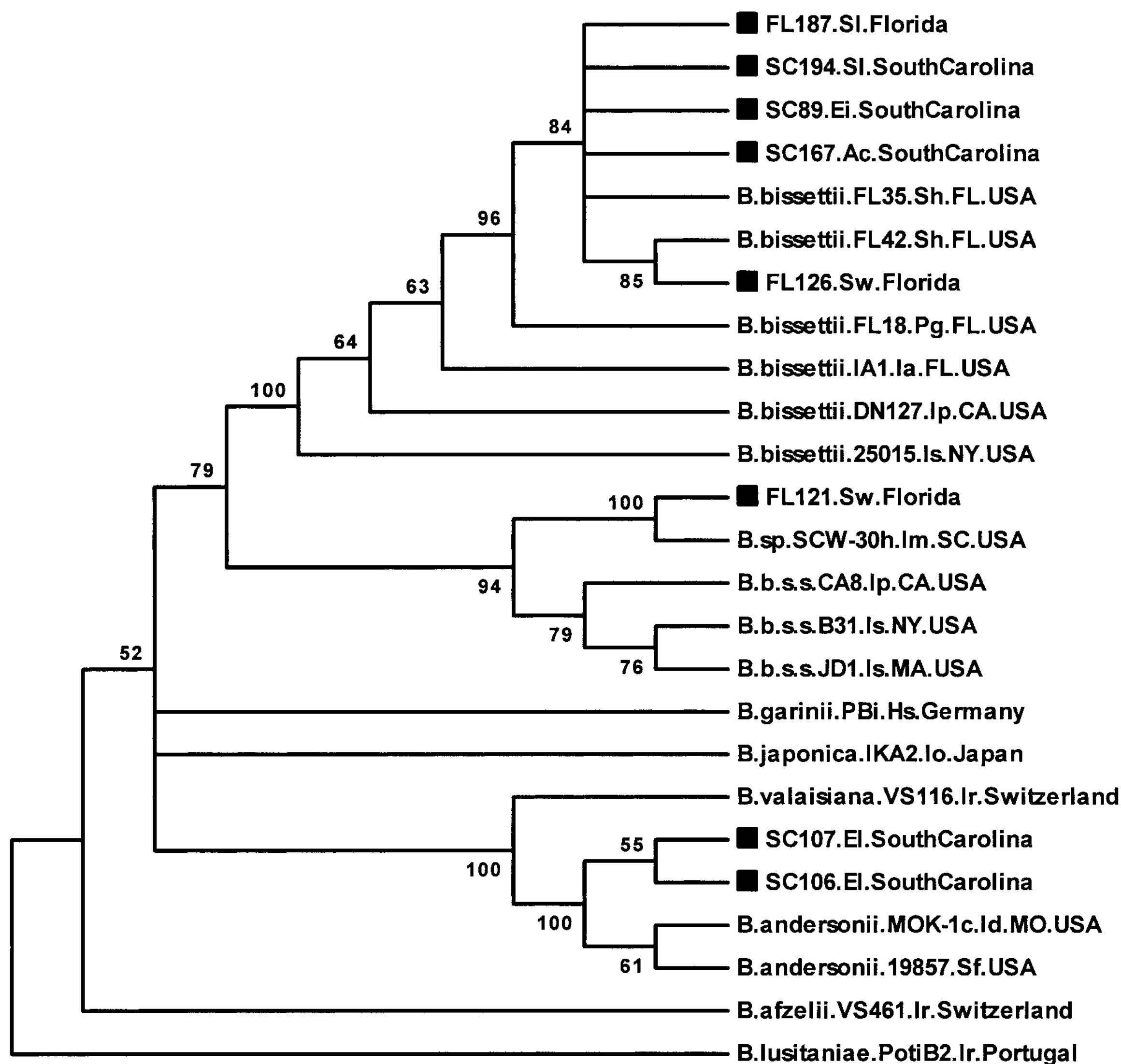

FIG. 2. Unrooted maximum parsimony bootstrap consensus phylogenetic tree based on 334 bp of the *ospA* gene obtained from lizards in Florida and South Carolina and *B. burgdorferi* sensu lato reference strains. Numbers at the branch nodes represent bootstrap values as percentages of 100 replications. Sequences obtained in this study are identified by a square symbol preceding the sequence name. Ia, *Ixodes affinis*. See the legend to Fig. 1 for other abbreviations used in descriptive information for the sequences.

teria other than spirochetes that may be present in lizard blood and tissues. Attempting to isolate these *Borrelia* organisms in tick cell lines is another potentially successful strategy.

Enzootic cycles of *B. burgdorferi* sensu lato transmission in the southeastern United States are more complex than those in the northeastern United States due to the involvement of a greater number of vertebrate and tick species in the South (2, 8, 31). This ecologic diversity may explain the genetic heterogeneity among southern *B. burgdorferi* sensu lato strains and the variation between southern strains and those found elsewhere (24, 25). This heterogeneity may explain why the *p66* and *ospA* primers we used did not amplify products from some of the *flaB*-positive samples we tested. Primer mismatch could have led to a complete lack of amplification or simply reduced the sensitivity of the PCR assays below the level needed for detection. Numerous strains of *B. burgdorferi* sensu lato were described previously from ticks and small mammals in Florida by using similar techniques (7). Many of the *B. burgdorferi* sensu stricto *flaB*-positive samples in that study also failed to test positive with primers designed to amplify portions of several genes, including *p66* and *ospA*, indicating either low target gene copy number in the samples or primer mismatch. BSK culture attempts with human tissue and fluid samples also fail to isolate spirochetes from many samples that test positive via PCR assays. Interestingly, the *p66*- and *ospA*-positive prevalences among *flaB*-positive lizards in the present study (34.9 and 19.8%, respectively) are comparable to the *p66* and *ospA* PCR-positive prevalences among human spinal fluid (39 and 23%, respectively) and urine (42 and 24%, respectively) samples from Lyme arthritis patients in one study (34).

Based on the findings of several studies (12, 15, 32), we considered the phylogenetic clustering of lizard-derived *B.*

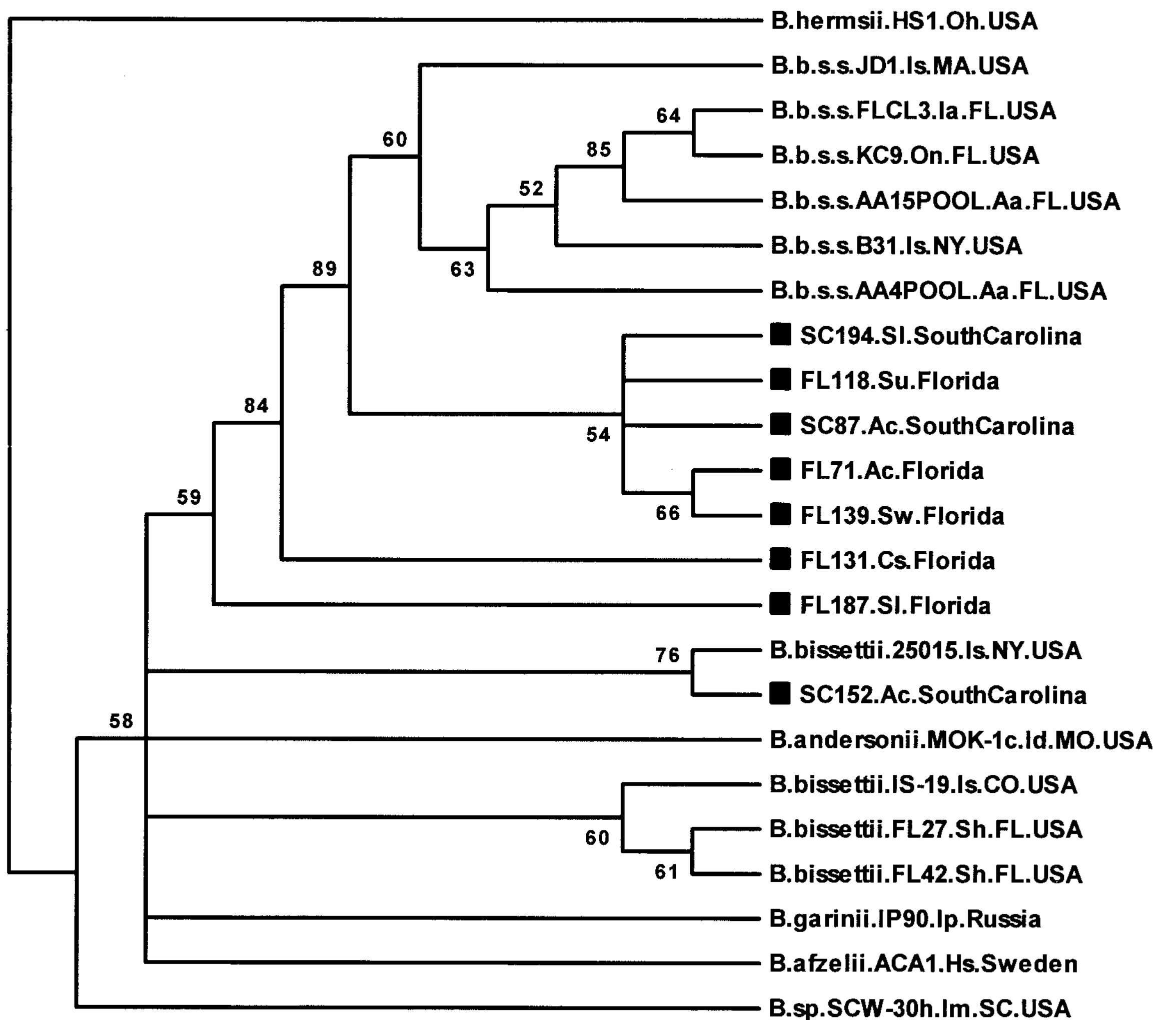

FIG. 3. Unrooted neighbor-joining bootstrap consensus phylogenetic tree based on 233 bp of the *p66* gene obtained from lizards in Florida and South Carolina and *B. burgdorferi* sensu lato reference strains. The relapsing fever group species *Borrelia hermsii* was included as an outgroup. Numbers at the branch nodes represent bootstrap values as percentages of 1,000 replications. Sequences obtained in this study are identified by a square symbol preceding the sequence name. Abbreviations: Cs, *Cnemidophorus sexlineatus*; Is, *Ixodes scapularis* or *Ixodes spinipalpis*; Oh, *Ornithodoros hermsii*; and On, *Ochrotomys nuttalli*. See the legend to Fig. 1 for other abbreviations used in descriptive information for the sequences.

burgdorferi sensu lato strains from partial sequences of the highly conserved *flaB* gene as indicative of species-specific groupings. Our analyses showed much more variability in the partial *ospA* and *p66* gene sequences derived from lizards. For some templates, the *ospA* and *p66* sequences were very similar to those of reference strains of the same species. For other animals, however, the *ospA* and *p66* sequences derived from them clustered with reference strains of a different species group. There are several possible explanations for this. There may be significant genetic variability in the *ospA* and *p66* genes of southern *B. burgdorferi* sensu lato strains. This might include, for example, genetic exchange of the plasmid *ospA* gene among strains of different species, similar to that shown for *ospC* (27). It is also possible that multiple *B. burgdorferi* sensu lato species were present in the blood of many individual lizards, representing a relatively high rate of multiple infection. Additional testing of our samples with an adequately sensitive, PCR-based method that employs *B. burgdorferi* sensu lato species-specific DNA probes could determine this.

Our DNA sequencing did produce some unresolved bases indicative of possible polymorphisms at some locations in the *flaB* and *p66* sequences from a small number of animals. Most of these were resolved upon resequencing those templates with the same primers or the complementary primers used in the PCRs. However, in the *flaB* sequences for a couple of samples, polymorphisms were still evident, and these were located at positions where nucleotide variation occurs between *B. andersonii* and *B. burgdorferi* sensu stricto strains.

We took extensive measures to prevent and identify any contamination of our DNA samples. There was no evidence of contamination of our DNA extracts with reference strain DNA. We used a *B. burgdorferi* sensu stricto reference strain (B31) as a positive control in our PCR assays, and although we did identify several *B. burgdorferi* sensu stricto strains in lizards,

none of their *flaB*, *ospA*, or *p66* sequences were identical to those for B31. The significant genetic variability among the lizard-derived *B. burgdorferi* sensu lato strains, compared to *B. burgdorferi* sensu lato reference strains of *B. andersonii*, *B. bissettii*, and *B. burgdorferi* sensu stricto, therefore renders contamination from reference strains a very unlikely explanation of our findings.

In the present study, we confirmed the presence of DNA of three *B. burgdorferi* sensu lato species in over 50% of tested lizards representing several genera and nine species from Florida and South Carolina. We detected the spirochetes in some lizards sampled during months of the year when *I. scapularis* larvae and nymphs do not normally parasitize hosts, and the duration of the PCR-positive status of several lizards kept in the lab exceeded 4 months. Furthermore, *I. scapularis* nymphs from larvae that fed on PCR-positive lizards also tested positive for *B. burgdorferi* sensu lato via PCR, evidence that the ticks acquired the bacteria from the lizards and maintained them after molting to nymphs. All of these findings suggest, but do not prove, the presence of live spirochetes in these samples.

If lizards serve as reservoirs of *B. burgdorferi* sensu lato, two factors affecting their significance as such would be the duration of infection and their longevity. The duration of *B. burgdorferi* sensu lato infection of lizards in the wild is not known, and reliable estimates of lizard longevity in the wild are difficult to find in the published literature. However, one website that contains information provided by 234 institutions and 425 private collections (F. Slavens and K. Slavens, *Reptiles and amphibians in captivity—longevity—home page*. Last updated 20 March 2003. Retrieved 8 November 2004. http://www.pond-turtle.com/longev.html) provides longevities of captive specimens of many species. The figures provided for representatives of the species included in the present study ranged from approximately 5 to 7 years for green anoles, 2 to 7 years for various *Eumeces* spp. skinks, 1 to 4 years for *Cnemidophorus* spp., 8 years for the Mediterranean gecko (*Hemidactylus turcicus*), 3 to 14 years for the eastern glass lizard (*Ophisaurus ventralis*), 1 to 8 years for the eastern fence lizard (*Sceloporus undulatus*) and other *Sceloporus* spp., and 2 years for the ground skink (*Scincella lateralis*). These figures agree with those provided at another site that contains detailed information about brown and green anoles (*Under the leaves*: *complete anole care*. Last updated 23 July 2003. Retrieved 8 November 2004. http://www.kingsnake.com/anolecare/5.htm). Although it is doubtful that most specimens live as long in the wild, it is possible that many wild specimens live for several years.

The minimal number of ticks removed from the lizards in this study, the distribution of infection among animals of different ages (including juveniles), and the diversity of infected lizard species, including some not commonly parasitized by ticks and a few individuals (brown anoles) from residential areas, all suggest the possibility of alternate transmission pathways besides the tick-borne route. Some other possible means of transmission to be investigated include transmission by mosquitoes, flies, or mites through blood feeding or mechanical transmission; by lizards ingesting infected arthropods; and from lizard to lizard vertically via transovarial transmission and horizontally during mating.

The present study's findings show that lizards in the southeastern United States are naturally infected with Lyme borreliae and suggest that they may play a role in the enzootic maintenance of *B. burgdorferi* sensu lato in the region. It remains to be conclusively shown whether the strains infecting lizards in the southeastern United States are cultivable in BSK medium and infectious or pathogenic to humans and whether ticks or other hematophagous arthropods acquire and maintain viable *B. burgdorferi* sensu lato spirochetes from feeding on lizards. Therefore, the relevance of this discovery to human disease risk in the region is not yet known.

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