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Stable Transmission of *Borrelia burgdorferi* Sensu Stricto on the Outer Banks of North Carolina

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Summary

The spirochaete (*Borrelia burgdorferi*) associated with Lyme disease was detected in questing ticks and rodents during a period of 18 years, 1991–2009, at five locations on the Outer Banks of North Carolina. The black-legged tick (*Ixodes scapularis*) was collected at varied intervals between 1991 and 2009 and examined for *B. burgdorferi*. The white-footed mouse (*Peromyscus leucopus*), house mouse (*Mus musculus*) marsh rice rat (*Oryzomys palustris*), marsh rabbit (*Sylvilagus palustris*), eastern cottontail (*Sylvilagus floridanus*) and six-lined racerunner (*Cnemidophorus sexlineatus*) were live-trapped, and their tissues cultured to isolate spirochaetes. *Borrelia burgdorferi* isolates were obtained from questing adult *I. scapularis* and engorged *I. scapularis* removed from *P. leucopus, O. palustris* and *S. floridanus*. The prevalence of *B. burgdorferi* infection was variable at different times and sites ranging from 7 to 14% of examined questing *I. scapularis*. Mitochondrial (16S) rRNA gene phylogenetic analysis from 65 adult *I. scapularis* identified 12 haplotypes in two major clades. Nine haplotypes were associated with northern/Midwestern *I. scapularis* populations and three with southern *I. scapularis* populations. Sixteen isolates obtained from tick hosts in 2005 were confirmed to be *B. burgdorferi* by amplifying and sequencing of 16S rRNA and 5S–23S intergenic spacer fragments. The sequences had 98–99% identity to *B. burgdorferi* sensu stricto

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strains B31, JD1 and M11p. Taken together, these studies indicate that *B. burgdorferi* sensu stricto is endemic in questing *I. scapularis* and mammalian tick hosts on the Outer Banks of North Carolina.

Keywords

Borrelia burgdorferi; *Ixodes scapularis*; *Peromyscus leucopus*; *Oryzomys palustris*; *Sylvilagus palustris*; Outer Banks; North Carolina

Introduction

Borrelia burgdorferi sensu stricto, the spirochaete associated with Lyme disease, has been consistently linked to the distribution of *Ixodes scapularis* in the Eastern United States (Steere and Malawista, 1979; Steere, 2006). White-tailed deer (*Odocoileus virginianus*) serve as the primary host for adult *I. scapularis*. Transovarial transmission rarely occurs (Piesman et al., 1986; Rollend et al., 2012), and ticks acquire the spirochaete when feeding on infected reservoir competent hosts (Piesman et al., 1986). Immature *I. scapularis* have a wide host range that includes white-footed mice, other rodents, reptiles, birds and medium-sized mammals (Spielman et al., 1984, 1985). White-footed mice, rice rats and other rodents are reservoir competent for the spirochaete and sustain the sylvatic cycle and maintain transmission in endemic areas where human cases are most prevalent (Levine et al., 1985; Anderson, 1988; Mather et al., 1989). Several species of birds are also reservoir competent and can transport infected larvae and nymphs to new locations (Richter et al., 2000; Ogden et al., 2008; Scott et al., 2010; Brinkerhoff et al., 2011).

The majority of cases of Lyme disease are reported in the Northeast and north central United States (Bacon et al., 2008), but confirmed cases of Lyme disease are also regularly reported in the Southeast (Pegram et al., 1983; Oliver, 1996; Centers for Disease Control and Prevention (CDC), 2015). Few areas in the Southeast currently meet Centers for Disease Control and Prevention criteria, which requires two indigenous cases to be reported in the same county for the disease to be considered endemic (CDC, 2015). Human cases are less numerous and more widely geographically dispersed in North Carolina and other southeastern states CDC (2015). However, infected questing *I. scapularis* have been collected in North Carolina (Levine et al., 1989; Qiu et al., 2002) and at various locations in the Southeast (Sonenshine et al., 1995; Clark et al., 2001, 2002; Oliver et al., 2008; Leydet and Liang, 2014). In addition, white-footed mice and other species of rodents considered to be reservoir competent are present throughout the Southeast (Apperson et al., 1993; Clark et al., 2001) and *B. burgdorferi* has been identified in numerous hosts in Virginia (Sonenshine et al., 1995), North Carolina (Ouellette et al., 1997; Ryan et al., 2000), South Carolina (Clark et al., 2002) and other southeastern states (Leydet and Liang, 2014). In this article, we report the results of a series of studies of questing ticks and live-trapped rodents and reptiles on the Outer Banks of NC that span 18 years and document persistent sylvatic maintenance of *B. burgdorferi* sensu stricto on these barrier islands.

Materials and Methods

Questing ticks and rodents were collected at varied intervals between 1991 and 2009 and tested to determine whether they were infected with *B. burgdorferi* sensu lato. Tick and rodent collection methods were relatively consistent with minor differences in chemical restraint of collected mammals. However, pathogen detection technologies for identifying *B. burgdorferi* in ticks and rodents changed markedly during the 18-year span over which these studies were conducted and these differences are noted below.

Outer Banks sampling sites

The Outer Banks are a group of barrier islands distributed in a north–south 320 km chain along the northeastern Coast of NC that separate the Albemarle, Pamlico and Currituck sounds from the Atlantic Ocean (Inman and Dolan, 1989). Questing ticks were initially collected at six sites in October 1991 (Fig. 1) (GPS coordinates are provided below). Additional adult tick collections were made at Audubon Pine Island Sanctuary (Pine Island) during 1992–1993 from September through March, encompassing the yearly period of activity of adult questing *I. scapularis*. Questing ticks were collected again in 2009, from four sites previously sampled between 1991 and 1993. Rodents were trapped and examined for ticks and spirochaetes at Pine Island and Buxton Woods between 1992–1994 and 2005.

The sites spanned the full length of the Outer banks in North Carolina along the main connecting highway, NC 12 (Fig. 1). Soils were consistently sandy, supporting predominately xeric vegetation typical of barrier islands that receive salt spray from the Atlantic, with two exceptions noted below at Pine Island and Nags Head Woods Ecological Preserve (Nags Head Woods). Marked within-site variation in plant species were apparent; example representative species and approximate site coordinates for collection sites are provided below. Corolla (36°22′52.00″N, 75°50′00.00″W), the most northern site is a small residential community with sandy soils and predominately mixed deciduous and pinewoods. Pine Island (36°13′42.14″N, 75°46′26.06″W) is an Audubon wildlife sanctuary located on the western side of highway NC-12 adjacent to Currituck Sound and the Intracoastal Waterway. The sanctuary is separated by a wide pedestrian greenway/road that is periodically mowed. Vegetation on the western side of the road is predominately maritime wet grassland (Schafale and Weakley, 1990). The eastern side of the sanctuary is predominately covered by a xeric maritime shrub thicket or salt shrub (Schafale and Weakley, 1990) that is dominated by bayberry (*Myrica pensylvanica*), live oak (*Quercus virginiana*) and wax myrtle (*Morella cerifera*) interspersed with loblolly pine (*Pinus taeda*), cedar (*Juniperus virginiana*) and mixed beach grasses (e.g. *Ammophilia breviligulata, Uniola paniculata*). Nags Head Woods (35°59′28.06″N, 75°39′46.95″W), which is protected by the relatively high dunes of Jockey's Ridge State Park, a maritime deciduous forest of approximately 1000 acres in size with a diverse mixed flora of about 300 plant species. The forest supports 84 species of trees and shrubs, including loblolly pine, hickory (*Carya* spp.) oak (*Querus* spp), cedar (*J. virginiana*) and magnolia (*Mangolia grandiflora*). The understory includes 11 fern species (e.g. *Amphicarpaea bracteata*), goldenrod (*Solidago canadensis*), milkweed (*Asclepias* spp.), 45 species of vines and numerous other plant species (Krings, 2010). Ticks were also collected south of Nags Head Woods, adjacent to Coquina Beach

(35°49′55.56″N, 75°33′41.82″W and at Bodie Island (35°53′39.62″N, 75°35′22.61″W), a connected peninsula covered by similar xeric salt shrub thicket and beach grasses. In Buxton Woods (35°15′41.16″N, 75°32′19.56″W) located at approximately 56 miles south of Nags Head Woods, sampling for questing adults was conducted in vacant lots with mixed grasses that were dispersed between residences. The sandy soils supported mixed grasses (e.g. *Panicum amarum*) and xeric scrub brush (e.g. *Helianthemum canadense*) (Stalter and Lamont, 1997). Sampling in Cape Hatteras was conducted at sites in Cape Hatteras National Seashore National Park (35°41′03.55″N, 75°18′04.43″W) in salt scrub thickets comprised of wax myrtle (*M. cerifera*), eastern baccharis (*Baccharis halimifolia*) and lanceleaf greenbrier (*Smilax smallii*). A single *I. scapularis* adult was collected at Pea Island National Wildlife refuge (35°41′30″N 75°32′45″W), a site with a vegetation shrub thicket and beach grasses. Sampling at the site was abruptly curtailed due to severe biting fly strike.

Tick collection and handling

Adult questing *I. scapularis* were collected from vegetation with corduroy cloth. Approximately 1 × 1.5 m of corduroy suspended from a wooden base was dragged across the top of and through vegetation while walking. Actual dragging/flagging technique varied with the collector and the density and type of vegetation. On Pine Island, collections were predominately made along pedestrian hiking paths by dragging the cloth across the edge vegetation bordering the main access road and probing with the cloth along deer and rabbit trails. Ticks collected for culture or indirect immunofluorescence microscopy (IFA) were placed in a glass vial, containing a moistened Plaster of Paris/charcoal base, covered with a Nitex® (Houston, TX, USA) mesh screen. The vial was placed in Whirl-Pak® plastic pouch (Nasco, Fort Atkinson, WI, USA) with a lightly moistened cotton ball and then transferred to a cooler, containing reusable cooler packs or held at ambient temperature for transport to the laboratory. In the laboratory, ticks retained for culture or IFA testing were held in humidified plastic chambers below ambient temperature at approximately 20–21°C. Ticks collected for PCR testing were placed in 95% ethanol. Collected ticks were morphologically identified to species using standard dichotomous keys (Cooley and Kohls, 1945; Sonenshine, 1979).

A representative sample of adult tick*s* (*n* = 28), submitted for species validation to James Keirans (Georgia Southern University, Statesboro, GA), was confirmed to be *I. scapularis* and retained in the US National Tick collection as voucher specimens (Accession Numbers: 46104-10, 46116-46119, 46121-27).

Rodent, rabbit and reptile collection

All rodent collections were made at Pine Island and Buxton Woods. In all studies, rodents were trapped in Sherman live traps placed in lines approximately 7 m apart or in 7 × 7 grids. Traps were baited with a mixture of peanut butter and dry rolled oats, and cotton was included in the traps during colder months for insulation (Adler and Wilson, 1983). Trap locations were marked with flagging to aid in trap recovery.

Traps were set before dusk in the evening and then checked for rodents the next morning. Rodent trapping during January 1992–September 1993 on Pine Island was conducted with

49–100 Sherman live traps for four consecutive nights (2546 trap-nights). Additional trapping sessions were conducted for 1–3 consecutive nights with 50–57 traps in 1993–1994 (1461 trap-nights), and 2002 (799 trap-nights). Rodent trapping was conducted again in March–May 2005 (350 trap-nights). In these studies, 100 Sherman live traps were set the first night. Cold weather resulting in some rodent mortality prompted early curtailment of trapping after one night in March. High trapping success also prompted a reduction in the number of traps/night to 60 traps, for two nights, to accommodate handling by a single investigator.

Rabbits were trapped by placing two-door funnel wooden box traps or Havahart® traps (Lititz, PA, USA) in lines in tall vegetation in readily distinguishable rabbit runs. No bait was used. Traps were set in the evening, checked in the morning and kept closed throughout the day.

Lizards were trapped using coverboards (0.6 m × 1.2 m × 1 cm exterior grade plywood) (Grant et al., 1992) and funnel traps placed at the end of drift fences (50 m length × 30.5 cm high) (Gibbons and Semlitsch, 1981). Drift fences were deployed in three lines in an H-shaped pattern. Funnel traps were opened in the morning, checked 2–3 times during the day and closed at night. Coverboards were placed in 7 × 7 grids (n = 49 per grid) and checked 2–3 times each day.

Restraint, handling, euthanasia and sampling protocols of trapped animals varied during the 18-year study period based on the samples to be collected, and changes in recommended anaesthetic protocols for rodents (IACUC-05-038-0). During studies conducted in 1991 and the spring of 1993, trapped animals were released into a bucket and restrained. Ticks were removed and placed in glass mesh-covered vials (noted above). A blood sample was obtained from the tail vein of each animal, after which the animals were identified with numbered ear tags, and released at the exact site of capture.

Captured lizards were identified and marked by toe-clipping (Ferner, 1979). Blood samples were placed in coolers containing reusable cold packs for transport to the laboratory and refrigerated upon arrival.

During August 1993, and monthly during October 1993 through September 1994, trapped rodents were transported to the laboratory for tick collection, euthanasia and blood culture. Rodents were transferred to stainless cages with a mesh bottom suspended over water to collect ticks dropping off of hosts. The ticks were collected from the water and identified to species. After the ticks had dropped off their hosts, the rodents were euthanized with carbon dioxide. Samples of cardiac blood, ear skin and bladder were aseptically obtained and inoculated into 8-ml tubes of BSK-II media and antibiotics (8 mg/ml kanamycin sulphate and 230 mg/ml of 5-fluoruracil (Barbour, 1984). Embryos were obtained from two white-footed mice and one rice rat during necropsy. Embryonated tissues were inoculated into BSK-II after rinsing in 70% alcohol and sterile PBS and dissecting in sterile petri dishes.

Additional rodent trapping was conducted during May and July 2002. Animals were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture and euthanized via cervical dislocation in accordance with the AVMA Guidelines

for the Euthanasia of Animals (AVMA 2001). Blood (obtained by cardiac puncture) and bladder tissue were collected for spirochaete culture.

During March–May 2005, trapped rodents were anesthetized with midazolam (5 mg/kg), and medetomidine (0.5–1 mg/kg) via intraperitoneal injection. After sampling, the rodents were administered Flumazenil (0.5 mg/kg) and Atipamezole (2 mg/kg) intramuscularly for reversal of sedation and then released after recovery. Trapped rabbits were anesthetized using a combination of midazolam 0.5 mg/kg and butorphanol 0.2 mg/kg. Once sedated, a 1 ml blood sample was obtained from the jugular vein for serum. Atipamezole 1 mg/kg intramuscularly was given to reverse the anaesthesia, and rabbits were released in the same area that they were trapped after full recovery from anaesthesia. Ear punch biopsies (Sinsky and Piesman, 1989) were obtained to attempt culture of *B. burgdorferi*. The pinnae were cleaned with 95% ethanol, and an ear punch biopsy was obtained from one pinna using a 1-mm stainless steel biopsy tool. The specimen was then rinsed in sterile phosphate buffered saline (pH 7.38) (Sinsky and Piesman, 1989). The tissue was minced with a pair of sterile scissors and then placed in BSK-II medium for culture as described below.

Borrelia burgdorferi culturing, detection and characterization

Protocols used to culture *B. burgdorferi* relied on variations of BSK-II media (Barbour, 1984). During 1991–1993, BSK-II was used for attempted culture using procedures originally developed by Barbour (1984) and modified by Steere et al. (1983). During 2002 and 2005, BSK-H (Pollack et al., 1993) was used for attempted *B. burgdorferi* culture. During 1991–1994, we attempted to culture spirochaetes from questing ticks and hosts. Prior to dissection, ticks were cleansed and disinfected by passing them through a series of baths: Betadine solution [Purdue Pharma, Stamford, CT], PBS, hydrogen peroxide, PBS, 70% alcohol and PBS. Culture tubes were held at 35°C and examined for spirochaete growth by darkfield microscopy at 3 days post-culture and then at 1 week, and then consecutively at weekly intervals thereafter for 4–5 weeks post-inoculation.

Detection of *Borrelia burgdorferi* by IFA

Indirect immunofluorescence microscopy was used to examine tick specimens after dissection when culture was not attempted. Antigen for IFA was prepared by growing *B. burgdorferi* stock cultures of strain ATCC 35210 / B31 grown in BSK-II medium in glass tubes in an incubator maintained at 35°C. *Borrelia* were harvested, centrifuged (International Equipment Co. Model CL, Needham Heights, MA, USA) at approximately 1500 rpm (352***g***) for 10 min and spirochaetes suspended above the sediment were removed with a sterile glass Pasteur pipette. An aliquot of the pellet was diluted 1 : 1 in PBS, centrifuged (Sorvall RC 5B Plus SS-34 rotor (Thermo Scientific, Walthan, MA, USA) at 10,000 rpm (7818***g***) for 10 min and the pellet resuspended in 1 ml PBS. Serial dilutions were prepared and a dilution selected that facilitated visualization of the spirochaetes without them overlapping in a glass well on Teflon-coated slides. The slides were air-dried and then frozen and stored at −20°C prior to use.

Suspect cultures and the gastrointestinal contents of dissected ticks were examined for *B. burgdorferi* by IFA (Levine et al., 1989) using monoclonal antibody H5332 (provided by

Alan Barbour, University of Texas Health Science Center, San Antonio, TX, USA). Optimal working dilutions for each batch of conjugate were determined by draughtboard dilutions to accommodate for variations in the activity of each lot of antibody. Working dilutions of the monoclonal antibody ranged from 1/5 to 1/25. Working dilution for the fluorescein isothiocyanate conjugated anti-mouse immunoglobulin G antibody (Kirkegard and Perry Laboratories, Gaithersburg, MD, USA) was consistently 1/200 (Ouellette et al., 1997).

DNA extraction from ticks

In 2005, ticks and tissues were ground with a sterile plastic pestle, resuspended in 100 μl of TE buffer and an IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Inc., Bothwell, WA, USA) was used for DNA extraction (Levin et al., 2002). In 2009, ticks collected from three NC field sites were stored at −20°C until DNA extraction. Ticks were first individually washed with 70% ethanol. Extraction was carried out using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) using the modified, total DNA from ticks for the detection of *Borrelia* DNA protocol. The DNA samples were stored at −20°C until PCR testing was performed.

Detection of *Borrelia burgdorferi* in questing ticks collected in 2005 and 2009 by PCR

Genomic DNA extracted from the ticks collected and examined in 2009 was initially tested using tick-specific actin primers, to confirm that DNA was not degraded (Gao et al., 2011). Briefly, species-specific PCR primer sets were designed for *B. burgdorferi, B. andersonii* and *B. bissettii* with PRIMERQUEST software (http://scitools.idtdna.com/Primerquest/) for amplification of the *ospA* gene (Table 1). Primers were purchased from IDT (http://www.idtdna.com). Polymerase chain reactions were run in 25 μl volumes with 12.5 μl AmpliTaq Gold® Master Mix (Applied BioSystems, Foster City, CA, USA), forward and reverse primers at a final concentration of 250 nM,1 μl of template DNA and RT-PCR water (Ambion, Austin, TX, USA) to final volume. Amplifications were performed in an ABI GeneAmp 9700 (Applied BioSystems, Foster City, CA) thermal cycler with the following conditions: 95°C for 5 min; 40 cycles of 95°C for 60 s, 59°C for 60 s, 72°C for 120 s; 72°C for 7 min followed by a 4°C hold for the duplex reaction containing *B. burgdorferi* and *B. andersonii* primers. The uniplex PCR reaction contained *B. bissettii* primers only and was run as above except the annealing temperature was increased to 70°C. Polymerase chain reaction products were electrophoresed in 2% agarose gels stained with SYBR® Safe (Invitrogen, Carlsbad, CA,USA) and visually recorded with a BioDoc-It (UVP, Upland, CA, USA) gel documentation unit. The PCR amplicons were excised from agarose gels, purified (Qiaquick Gel Extraction kit; Qiagen, Valencia, CA, USA) and sequenced (McLab, S. San Francisco, CA, USA). NCBI BLAST searches were performed with sequenced amplicons to verify identities of the genes.

DNA extraction from presumed ear punch biopsy *Borrelia* cultures

Sixteen *Borrelia* isolates obtained in 2005 from ear punch biopsies and held at −80°C were thawed and placed in BSK-H culture medium as described above. Although other isolates had been obtained previously to 2005, they were no longer available for extraction and sequencing. Extraction of DNA from presumed *Borrelia* cultures was performed using DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's

protocol. A maximum of 2×10^9 cells/ml were used for DNA extraction. Concentration and quality of the extracted DNA was determined with a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The extracted DNA was stored at −20°C prior to preparation for amplification.

Prokaryotic 16S rRNA sequencing library preparation

To verify that each of the 16 *Borrelia* culture only contained a single genospecies, prokaryotic 16S rRNA gene amplicons were prepared following the 16S Metagenomic Sequencing Library Preparation protocol for the Illumina MiSeq system with some modifications. Briefly, primers were designed to amplify the V3 and V4 regions of 16S rRNA (Muyzer et al., 1993; Sim et al., 2012) and with overhang adapter sequences compatible with the Illumina index and sequencing adapters (Table 2). Polymerase chain reaction amplification components were NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 1 μM of each amplicon PCR primer and 5 ng/μl template DNA. The amplification PCR was performed using: one cycle of 95°C for 3 min; 25 cycles each of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; 1 cycle of 72°C for 5 min; and hold at 4°C. Clean-up of the PCR amplicon products to remove free primers and primer dimers was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Following amplicon clean-up, second set of PCR was performed to attach barcoding indexes to the amplicon PCR products. Dual-index primers were designed so that samples could be multiplexed in one MiSeq lane. The index PCR was performed as follows: one cycle of 95°C for 3 min; eight cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; one cycle of 72°C for 5 min; and hold at 4°C. Clean-up of the PCR index products was performed as described above using Agencourt AMPure XP beads. The indexed amplicon libraries were normalized and pooled. The pooled library was checked for quality and quantified using a High Sensitivity DNA chip on the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). The library was diluted and combined with a PhiX Control library (v3) (Illumina, San Diego, CA, USA) at 10% and sequenced on two lanes on an Illumina v3 300PE MiSeq sequencer (Illumina), using standard sequencing protocols. Base calls were generated on instrument during the sequencing run using the MiSeq Real-Time Analysis (RTA 1.18.54) software. FastQ generation, demultiplexing and adapter trimming were performed by the MiSeq Reporter software (2.4 and 2.5.1).

Bioinformatics and statistical analyses of *Borrelia* isolates from 2005

The resulting demultiplexed reads were processed using the QIIME 1.8.0 toolkit (Caporaso et al., 2010). Briefly, the paired end reads were joined together and open reference-based OTU picking was performed where Uclust (Edgar, 2010) was applied to search using Greengenes 13_8 reference database (DeSantis et al., 2006) filtered at 97% identity. Reads not matching a reference sequence were removed from analysis. OTUs were assigned based on a database hit of 97% or greater sequence identity, and taxonomy was assigned against the appropriate database.

PCR assays for *Borrelia* 5S–23S IGS and *Ixodes* mitochondrial 16S rRNA gene

Genomic DNA from 65 randomly selected *Ixodes* ticks from Nags Head Woods Preserve (n = 25), Corolla (n = 21) and Pine Island (n = 19) collected in October 2009 were reexamined

for *Borrelia* by amplifying 5S–23S intergenic spacer (IGS) region of *Borrelia* spp. using the specific primers 23SN1 and 23SC1 (Rijpkema et al., 1995). PCR reactions were each done in a 20 μl volume comprised of 10 μl of AmpliTaq Gold PCR Master Mix, 1 μl (10 μM) each of respective forward and reverse primers, 1 μl of template DNA and 7 μl of nuclease water. Thermocycler conditions for amplification of 5S–23S IGS fragments were as follows: 95°C for 10 min, 39 cycles of 94°C for 1.00 min, 50°C for 1.30 min, and 72°C for 1.30 min, and a final cycle of 72°C for 5 min. Genomic DNA from 16 *Borrelia* cultures isolated from ear punches of *O. palustris* and *P. leucopus* in 2005 were also amplified using the above-mentioned primers and PCR conditions.

Concurrently, species identifications of the field-collected *I. scapularis* adults were confirmed by amplifying mitochondrial 16S rRNA gene using the primers 16S+1 and 16S−1 (Black and Piesman, 1994). PCR reactions were prepared as previously described for the *Borrelia* 5S–23S intergenic spacer. The mitochondrial 16S rRNA gene of ticks was amplified using the following thermocycler conditions: Initial denaturation of 95°C for 10 min, 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1.00 min, and a final cycle of 72°C for 10 min. PCR products were electrophoresed in 1.2% agarose gels containing ethidium bromide and visualized through transillumination.

Additional sequence and phylogenetic analyses

Amplicons from 16 *Borrelia* cultures from rodent ear punch biopsies taken in 2005 and four DNA samples from *Ixodes* collected in 2009 that produced expected sized bands of ~375 bp in *Borrelia*-specific 5S–23S PCR assay, and ~460 bp in 65 samples from tick mitochondrial 16S PCR assay were sequenced by Eton BioScience, Inc. (Research Triangle Park, NC, USA), using the forward primer that was used in amplification. Individual sequences from 5S–23S and mitochondrial 16S amplicons were analysed by BLASTN for sequence match analysis in NCBI database. The mitochondrial 16S rRNA gene sequences were multiple-aligned with ClustalX software package (Larkin et al., 2007) along with reference 16S rRNA gene sequences of *I. scapularis* strains retrieved from GenBank (2016). Haplotypes were identified by nucleotide sequence variation with each variant designated as a unique haplotype even if its sequence differed by only one substituted base (Norris et al., 1996).

A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) with bootstrap analyses consisting of 1000 iterations with MEGA 6 software package (Tamura et al., 2013), and evolutionary distances were calculated using Kimura 2 parameter model. All the sequences were submitted to GenBank under the accession numbers: *Borrelia* 23S: (ticks) KT821557–KT821560; (rodents) KX419243–KX419258; *I. scapularis* 16S: KT821561–KT821624.

Results

Prevalence of *Borrelia burgdorferi* in questing ticks

Questing adult *I. scapularis* were collected at eight sites on the Outer Banks of North Carolina during 21 October 2011–March 1992, November–January 1993, November 1993, and October 2009 (Fig. 1). Sample collections traversed 134 km from Corolla, NC, to Cape

Hatteras National seashore. *Borrelia burgdorferi*-infected *I. scapularis* were detected at four of eight (50%) sites in October 1991 (Table 3). Isolates were obtained from 34 questing adult *I. scapularis* collected from these four locations and confirmed to be *B. burgdorferi* by IFA with a species-specific monoclonal antibody (H5332). During February–March 1992, November 1992–January 1993, and November 1993, 541 questing ticks were collected at six locations on the Outer Banks, dissected and examined for spirochaetes by dark-field microscopy (Table 4). Seven percent of the questing adult *I. scapularis* were found infected, and the isolated spirochaetes were confirmed to be *B. burgdorferi* by IFA conducted with a species-specific monoclonal antibody (H5332). In October 2009, 71 adult *I. scapularis* were collected from three locations on the Outer Banks that had been previously sampled in the early 1990s (Table 5). *Borrelia burgdorferi* was detected by PCR (*OspA*) in 14% of the *I. scapularis* collected. The spirochaete was detected in two of the 24 (8%) adult questing *I. scapularis* collected in Corolla, and eight of the 23 (35%) adult questing *I. scapularis* collected on Pine Island. However, none of the 24 *I. scapularis* adults collected in Nags Head Woods were infected. All ticks tested were negative for *B. andersonii* and *B. bissettii*.

Attempted culture of *Borrelia burgdorferi* from rodents, rabbits, and lizards

During 1991–1993, 304 rodents were live-trapped in 2546 trap-nights, and 58 rabbits (*Sylvilagus floridanus, S. palustris*) were live-trapped in 2301 trap-nights at Pine Island and Buxton Woods and examined for ticks. One hundred fifty-four six-lined racerunners lizards (*Cnemidophorus sexlineatus*) were also live-trapped in 13,573 coverboard checks, and 3,505 drift fence pit trap checks. Blood from 51 lizards, 58 rabbits and 45 rodents was inoculated into BSK-II medium. Spirochaetes were isolated from *P. leucopus, O. palustris* and *S. palustris,* but not *C. sexlineatus* trapped on Pine Island (Table 6). Isolates were obtained from 20% of the *O. palustris* and 50% of the *P. leucopus,* and 4% of *S. palustris*. During these studies, a small number of *I. scapularis* that survived removal from trapped hosts were examined for spirochaetes by IFA. No spirochaetes were observed in 8 larvae removed from *P. leucopus* trapped at Pine Island. However, spirochaetes were detected in one of two nymphs removed from *P. leucopus*, and one of three larvae and one of four nymphs removed from *O. palustris* trapped on Pine Island during October and November 1992. No spirochaetes were observed in one nymph and six larvae removed from *P. leucopus* or in a nymph removed from a south-eastern five lined skink (*Eumeces inexpectatus*) trapped at Buxton Woods.

In another study conducted during August 1993–September 1994, three species of small rodents ($n = 123$) trapped in 1491 trap-nights were examined for *Borrelia. Borrelia burgdorferi* was detected in 36.6% of the trapped rodents. The majority of rodents trapped were white-footed mice ($n = 106$) (Table 7), and with the exception of January 1993, *Borrelia*-infected *P. leucopus* were trapped throughout the 13 consecutive months in which trapping was conducted. Eighty-nine *B. burgdorferi* isolates were obtained from the various tissues collected and confirmed by IFA. *Borrelia* were predominately isolated from bladders and skin samples. No spirochaetes were found in the embryonated tissues; however, *B. burgdorferi* was isolated from one of the two pregnant white-footed mice from which these tissues were obtained. One of three (33.3%) *Mus musculus* a juvenile, trapped in April 1994 was infected. Fifty per cent of the *O. palustris* that were examined ($n = 14$) were infected

with the spirochaete. *Borrelia burgdorferi* was detected in one adult male *O. palustris*; but not a juvenile male trapped in November 1993. Two adult male *O. palustris* (100%) were found infected in December 1993, but the spirochaete was not observed in two adult females. In addition, the spirochaete was detected in one adult male *O. palustris* that was one of four rice rats trapped (25%) in February 1994, an adult male rice rat trapped in April and three (100%) adult male rice rats trapped in August 1994. *Borrelia burgdorferi* was detected in 37 of 106 (35%) *P. leucopus* (Table 7). The spirochaete was detected in *P. leucopus* in all months except January and was most prevalent in the mice in June. Four engorged ticks *I. scapularis* nymphs and 25 *I. scapularis* larvae were collected from infected rodents, but the ticks were not examined for spirochaetes.

Rodents, *P. leucopus* and *O. palustris*, were trapped at Pine Island again in May and July 2002. Fifty-five animals were trapped in 799 trap-nights. Blood and bladder tissue from 32 *P. leucopus*, and 11 *O. palustris* was placed in BSK-H culture. Spirochaete isolates were obtained from 4 (9%) of the animals examined. Spirochaetes were isolated from the bladders of three of 15 (20%) *P. leucopus* trapped in May 2002, but not in 17 *P. leucopus* trapped in July. The three infected animals were adult males. Spirochaetes were also isolated from one of 11 (9%) *O. palustris* trapped in July 2002. The animal was an adult male.

During March–May 2005 at Pine Island, 140 rodents and two small rabbits were trapped. Attached partially engorged *I. scapularis* were also collected from 18 rodent hosts [*O. palustris* ($n = 3$), *P. leucopus* ($n = 15$)]. *Borrelia burgdorferi was* detected by PCR (*OspA*) in 10 (69%) of 15 nymphal *I. scapularis* and 1 (20%) of 5 larval *I. scapularis* removed from hosts. *Borrelia burgdorferi* was not detected in two larvae removed from an adult *O. palustris* in March, but was detected in one larval *I. scapularis* removed from an adult male *O. palustris* in April. *Borrelia burgdorferi* was detected in one nymph removed from each of two adult female *P. leucopus,* and a third nymph from an adult male *P. leucopus* collected in April. The remaining ticks were removed from animals trapped in May. One infected nymph was found on a juvenile female *P. leucopus* and three adult male *P. leucopus*. Two infected nymphs were found on an adult female *P. leucopus*. Ear punch biopsies were obtained at the time of capture for culture. At approximately 72 h after culture preparation, *Borrelia* were observed via dark-field microscopy in ear punch cultures from 10 (29%) of 35 *O. palustris* and 15 (14%) of 105 *P. leucopus*. No spirochaetes were detected in blood cultures from two *S. palustris*. Twenty-nine animals were recaptured; four animals were trapped three times. One juvenile male white-footed mouse yielded positive cultures for spirochaetes both times it was trapped, first in April and then 21 days later in May.

Sequence results, *Borrelia* isolated in 2005 and detected in 2009

The OTU profiles of 17 of the 26 spirochaete isolates obtained from rodent ear punch biopsies in 2005 were investigated using UCLUST consisting of 383,704 quality filtered MiSeq 16S rDNA sequencing reads. Using a sequence similarity threshold of 97% resulted in identification of one dominant OTU containing 380,430 reads (99.1%) across all of the 17 samples, indicating that the samples contained only a single *Borrelia* sp. Subsequent, nucleotide BLAST results for the GenBank database indicated that the sequences (GenBank Accession numbers: KU896130, KU896131, KU896132, KU896133) had 99–100% identity

to *B. burgdorferi* strain B31 (Accession No. CP009656.1 accessed 2 February 2015; e-value <0.001). Sequence analysis of 5S–23S IGS amplicons from 16 of these isolates revealed that the *Borrelia* were 98–99% identical to GenBank sequences of the B31, JD and M11p strains of *B. burgdorferi* (Accession Nos. CP009656, CP002312.1 and, KM269459.1, respectively).

Of the 65 *I. scapularis* collected in 2009 and tested for *Borrelia*, four ticks from Pine Island were positive. BLAST analysis of the 5S–23S sequences showed that the ticks were infected with *B. burgdorferi* sensu stricto that exhibited 99% sequence similarity to the JD1 and B31 strain of *B. burgdorferi* (Accession Nos. CP009656.1 and CP009656, respectively).

Sequence and phylogenetic analyses, ticks collected in 2009

The mitochondrial 16S rRNA gene fragments were successfully amplified by PCR from all 65 tick DNA samples. The sequences were 99% similar to the GenBank sequence of *I. scapularis* (Fig. 2), confirming the taxonomic identification of ticks collected on the Outer Banks as *I. scapularis*. The reconstructed neighbour-joining tree further showed that these 65 sequences of the 16S rRNA gene and GenBank reference sequences formed two major clades (Fig. 2). The Outer Banks *I. scapularis* sequences clustered into 12 haplotypes with nine haplotypes (58 ticks) and three haplotypes (seven ticks) represented in the two clades with significant bootstrap values. A majority of the *I. scapularis* ticks were assigned to Clade I containing sequences from northern and midwestern tick populations. Additionally, 18 of 19 *I. scapularis* from the Pine Island collection site, including four ticks infected with *B. burgdorferi* sensu stricto, were placed in Clade I. Notably, 16S rRNA gene sequences varied by 16–19 nucleotides for ticks collected at the same site (e.g. Nags Head Woods: Clusters C and P) as well as different sites [e.g. Pine Island (Cluster B) versus Corolla (Cluster R)] on the Outer Banks.

Discussion

Transmission of *B. burgdorferi* sensu stricto is stable on Pine Island. The spirochaetes were consistently detected in questing adult ticks at the site in collections made during an 18-year period. It has also been repeatedly isolated from rodents at the site, as well as sporadically from lagamorphs (Ryan et al., 1998). White-tailed deer and medium-sized mammals that serve as hosts for adult *I. scapularis* are present, and known rodent reservoirs are abundant. A path through the sanctuary is a routine route for recreational walking, jogging and bird viewing; both are activities that pose a potential risk of seasonal human exposure to *B. burgdorferi*-infected questing ticks. *Borrelia burgdorferi* sensu stricto was also detected at other sites traversing most of the Outer Banks from Cape Hatteras to Corolla.

Human cases of Lyme disease have been diagnosed in NC since 1983 (Pegram et al., 1983), but until recently, the disease was not considered endemic in NC. Case reporting and what is considered a case of Lyme disease have changed as the case definition has changed Centers for Disease Control and Prevention (CDC), 2016. Indeed, what were previously considered cases of Lyme disease in NC two decades ago based on the then current CDC case definition (Levine et al., 1991) may not be considered cases today. Lyme disease was not considered endemic in any county in NC until 2009, when the illness of two individuals met the case definition for Lyme disease, and the disease was recognized as endemic in Wake County in

the Piedmont of NC (Oliver, 1996; North Carolina Department of Health and Human Services, 2014). Lyme disease is now also considered endemic in Alleghany, Guilford, Haywood and Wake counties in NC (NCDHHS 2014). Although transmission of *B. burgdorferi* on the Outer Banks appears stable, cases meeting the current CDC Lyme disease definition have not been diagnosed and reported to the NC Department of Public Health (Bacon et al., 2008). Although, adult *I. scapularis* have been found on dogs in the coastal plain and on the Outer Banks, serologic studies of NC dogs have also documented limited exposure to the spirochaete (Duncan et al., 2005). In one study, *B. burgdorferi* was identified in *I. affinis,* but not *I. scapularis,* collected in four counties (Beaufort, Gates, Lenoir and Martin) in the NC coastal plain (Maggi et al., 2010). *Borrelia burgdorferi* transmission is clearly occurring at multiple locations in North Carolina; however, based on the current CDC definition (Centers for Disease Control and Prevention, 2015), endemic Lyme disease transmission is not occurring in counties in which many infected *I. affinis* were identified. Because suitable reservoir competent hosts are present in these counties, introduction of the northern variant of *I. scapularis* could markedly increase the risk of Lyme disease to residents.

We isolated *B. burgdorferi* from both *P. leucopus* and *O. palustris*. An isolate from an *S. floridanus* was also previously characterized as *B. burgdorferi* (Ryan et al., 2000). Our understanding of the genus *B. burgdorferi* species complex has markedly broadened since the initial isolation of *B. burgdorferi* and its recognized association with Lyme disease (Steere et al., 1983). More than 12 genospecies have been identified and include several species well documented in the southeastern United States (Oliver et al., 2008; Rudenko et al., 2009; Clark et al., 2014). Oliver et al. (2003) have also documented the role that other species of ticks and hosts may play in the maintenance of *B. burgdorferi* transmission as well as other genospecies of *Borrelia* in the Southeast. Rudenko et al. (2014) have identified genetically diverse strains of *Borrelia* in individual *I. minor* that fed on a single Carolina Wren (*Thryothorus ludovicianus*). The 16 isolates that we sequenced had 98–99% homology with *B. burgdorferi* sensu stricto. Additionally, *B. burgdorferi* sensu stricto was identified in *I. scapularis* ticks by PCR amplification and sequencing of *ospA* gene and 5S–23S IGS fragments. *Borrelia burgdorferi* sensu stricto has been extensively isolated from *I. scapularis* collected at numerous sites in the Eastern United States and is the primary vector of Lyme disease spirochaetes in the United States.

The cotton mouse, *P. gossypinus* and the hispid cotton rat (*Sigmondon hispidus*) were documented to be abundant and playing potential roles as reservoir hosts for *B. burgdorferi* in South Carolina, Georgia and Florida (Oliver et al., 1995; Clark et al., 2002). Infection of these species was sustained for many months. At the Pine Island site, *P. leucopus* and *O. palustris* both sustain *B. burgdorferi* transmission. In these studies, we have focused on the primary pathogen of public health interest, but there is a great deal more that we need to learn to clearly understand the dynamics of spirochaete transmission. Although vector competent ticks and reservoir competent hosts are abundant in the state, the vectorial capacity of resident *I. scapularis* for transmission to humans needs further study. Arsnoe et al. (2015) documented differences in the questing behaviour of nymphal *I. scapularis* acquired at Lyme disease endemic sites in Wisconsin and Rhode Island and *I. scapularis* collected in at non-endemic sites in Tennessee and Florida. Two distinct or potentially

complementary hypotheses could be postulated about the local dynamics of *Borrelia* spp. transmission. The paucity of human Lyme disease cases diagnosed in the Southeast is the consequence of differences in the human host-seeking behaviour of northern and southern *I. scapularis* genotypes and/or it is determined by the abundance of fossorial hosts available in the southeast, which divert questing contact from humans (Apperson et al., 1993; Qiu et al., 2002; Arsnoe et al., 2015).

We trapped *P. leucopus* at Pine Island and Buxton Woods. Shipp-Pennock et al. (2005) examined the systematic relationship of *P. leucopus* at sites along the Outer Banks and other mid-Atlantic coastal areas. They confirmed the presence of the mouse on the Outer Banks but also identified various subspecies. Specimens they acquired that were previously identified as *P. gossypinus* were determined to be *P. leucopus buxtoni* and a new subspecies.

Sequencing technology has markedly broadened the ability to ask questions about the relatedness of species and strains. There is still a great deal to learn about these *Borrelia* and rodents and other potential reservoirs in the southeast. How do the various variants in the genus *Borrelia* and host genetic variation potentially alter the transmission and maintenance of Lyme disease spirochaete transmission? Is there competitive inhibition when ticks and hosts are coinfected? Does infection with one genospecies infer immunity to infection with another, and is there variation in the ability of different subspecies of *P. leucopus* to support spirochaete infection of attached ticks.

Our mt16S rDNA sequences of *Ixodes* ticks from the Outer Banks separated into two clades, similar to previous studies (Rich et al., 1995; Norris et al., 1996; Qiu et al., 2002; Brinkerhoff et al., 2014; Sakamoto et al., 2014); an all American clade described by Norris et al. (1996) consisting of populations from the north-east, Midwest and south and a southern clade limited to southern populations. Notably, two ticks from Pine Island exhibited 100% similarity to Virginia *I. scapularis* collected in an area of emerging Lyme disease (Brinkerhoff et al., 2014). Is the occurrence of the northern variant of *I. scapularis,* the tipping point that increases the number of Lyme disease cases that will be diagnosed, and how does the competition between northern and southern variants of the tick alter the maintenance of transmission? Other tick species such as *I. minor* and *I. affinis* that potentially contribute to the maintenance of spirochaete transmission (Clark et al., 2001; Nadolny et al., 2011) and the variety of alternative hosts that appear to be competent (Anderson, 1988; Levin et al., 1995; Richter et al., 2000) heighten the need to thoroughly explore the local vectorial capacity of individual tick species and capacity (potential) of resident tick hosts (Mather et al., 1989).

The presence of *B. burgdorferi* may reflect the movement of infected ticks on birds. At individual sites, such as Nags Head Woods, *I. scapularis* may have been resident for centuries without the presence of the spirochaete. At other sites, such as Pine Island, migrating birds may have more recently introduced the tick and the spirochaete. *Borrelia burgdorferi* has been detected in other bird-feeding tick species removed from birds (Levine et al., 1991) and in particular in *I. affinis* collected in several NC counties (Harrison et al., 2010; Maggi et al., 2010). The spirochaete has also been detected in birds in Georgia and South Carolina (Durden et al., 1997). Passerine birds may serve as numerically important

reservoirs of spirochaete presence as well as transport hosts for infected ticks (Richter et al., 2000). Similar studies have supported the role of birds as transport hosts in the Midwest (Brinkerhoff et al., 2011), Canada (Ogden et al., 2008; Scott et al., 2010) and Europe (Olsen et al., 1995; Marie-Angèle et al., 2006; Kjelland et al., 2010). More than 170 species of birds have been identified at Pine Island, including numerous migratory species that could have potentially introduced the tick and the spirochaete as the birds moved along the Atlantic flyway (National Audubon Society 2015). Early isolated cases in the 1980s and 1990s in NC (Pegram et al., 1983; Harshbarger et al., 1984) may have been associated with individual ticks that dropped from migrating birds and moulted to the next stage and acquired a human host. Other Atlantic coastal and coastal plain sites where *B. burgdorferi* has been detected in ticks, rodents and other vertebrates fall within the eastern flyway. When local reservoir competent hosts for the tick are present, a new endemic nidus of spirochaete transmission may be established and sustained when infected ticks are introduced into new locations by birds (Morshed et al., 2005).

Marked changes were apparent on the Outer Banks during the 18-year span within which the studies were conducted. New residential and commercial construction along Highway NC 12 that is adjacent to the study sites has reduced available wildlife habitat and residential subdivisions are now numerous. Much of the residential construction is devoted to accommodating seasonal visitors between April and September. Transmission is most stable when tick-feeding activity is concentrated on the primary reservoir host (Spielman et al., 1984) and the transmission of *B. burgdorferi* to *I. scapularis* in endemic areas in the Northeast is predominately anchored by *P. leucopus* (Levine et al., 1985; Anderson, 1988; Piesman and Happ, 1997). In the Southeast, maintenance of *B. burgdorferi* transmission in areas with substantial host diversity may include other *I. scapularis* hosts and be relatively complex, with alternative species serving as key reservoirs (Clark et al., 2002; Brown et al., 2006). *Ixodes scapularis* has a broad host range and feeding on a wild range of hosts, but not all are reservoir competent (LoGiudice et al., 2003). Keesing et al. (2010) have argued that habitat loss associated with residential and other development may displace species such as opossum that dilute transmission and favour the perpetuation of species such as the white-footed mouse that serve as reservoirs. A comparatively large proportion of *I. scapularis* that attach to opossums die during attachment, and opossums are not considered reservoir competent (Keesing et al., 2009). Robust modelling of the potential consequent changes in the density of infected nymphal ticks suggests a decline in the prevalence of *B. burgdorferi* in nymphs as non-reservoir competent hosts (e.g. squirrels) are added to collective potential host population (LoGiudice et al., 2008).

Differences in host behaviour and habitat preference apparently also may play a role in the potential for *B. burgdorferi* to be maintained by different species at individual sites. Shaw et al. (2003) found that at a site in Duchess County New York that larval *I. scapularis* burdens on white-footed mice were three times the burdens observed on chipmunks (*Tamia striatus*), which equates to a greater opportunity for the mice to become infected. Pine Island has a rich diversity of hosts, which includes rodents, lagomorphs, birds, reptiles and medium-sized mammals. Several of these are viable potential reservoirs (e.g. mice, rice rats) for the spirochaete; others (e.g. six-lined racerunner) may be less reservoir competent. Geographic and temporally sympatric, environmental factors, tick-stage-specific biting rate, tick

survival, host abundance, host access, host immunity and the other determinants of vectorial capacity make the stability of Lyme disease spirochaete transmission locally dynamic (Spielman et al., 1984; Bertrand and Wilson, 1997). Substantial further study will be needed to understand why few cases of Lyme disease are being diagnosed on the Outer Banks and in the south-east.

Our studies spanned 18 years. The methods used to collect questing ticks remained the same throughout this series of studies. Rodent trapping methods were also relatively consistent; however, direct handling and tissue sampling protocols for the rodents did change. A combination of euthanasia and whole blood and organ testing and ear punch testing was used, which reflected improvements in anaesthetic and euthanasia methods. Testing protocols to detect *B. burgdorferi*, however, changed markedly throughout the 18-year period. Initial techniques during the 1990s focused on the use of culture methods and direct observation using immunofluorescence microscopy visualization techniques using monoclonal antibodies. Studies after the turn of the century took advantage of molecular PCR-based technologies. Each of these techniques introduces its own unique bias affecting the potential interpretation of the proportion of ticks and rodents infected. Although these changes in pathogen detection techniques may prevent the exact comparison of tick and host pathogen prevalence in the collected specimens, they do not negate the primary goal of presenting the results of these studies, documenting that *B. burgdorferi* sensu stricto transmission has apparently remained stable on the Outer Banks of NC for more than 18 years.

Documentation of questing adult *I. scapularis* at Pine Island and other sites on the Outer Banks, and isolation of the spirochaete ticks collected on the upper barrier islands (Corolla and Pine Island), at Buxton woods in the relative middle of the Outer Banks and at Cape Hatteras National Sea shore at the southern end, suggest that transmission may be stable along the entire island chain. Although we did not extend our sampling outside of the Outer Banks, *I. scapularis* infected with *B. burgdorferi* have been detected at other sites in Coastal NC, SC and Georgia. *Borrelia burgdorferi*-infected nymphs have been collected in the Piedmont of the state in Chatham County (Levine et al., 1989; Smith et al., 2010), and Magnarelli et al. (1992) detected antibody to *B. burgdorferi* in rodents in three of four counties located in North Carolina's coastal plain (Gates, New Hanover, Onslow). *Borrelia burgdorferi* has also been detected in questing ticks and ticks removed from hosts in the Coastal Plain and on Assateaque Island in coastal Maryland (Oliver et al., 1999; Anderson et al., 2016), and coastal Virginia (Levine et al., 1991; Sonenshine et al., 1995; Oliver et al., 1999). The spirochaete has also been detected in ticks and rodents near Charleston South Carolina (Oliver et al., 2000) removed from birds on St. Catherine Island, and ticks removed from rodents on Sapelo Island off the coast of Georgia (Oliver et al., 1993; Durden et al., 1997). Documentation of infected *I. scapularis* at Virginia Beach, on the Outer Banks of NC and on barrier islands along the South Carolina coast, suggests that Lyme disease spirochaete transmission is occurring in a nearly continuous chain along the coasts of these three Mid-Atlantic States. Indeed, a near continuum of sites of *B. burgdorferi* sensu stricto transmission is evident along the East Coast from Maine to Georgia. Recent studies, however, have documented the presence of *Borrelia burdorferi* in ticks and human cases of Lyme disease in Western Virginia (Brinkerhoff et al., 2014). A paradoxical contrast is

apparent. Although reservoir competent tick hosts and *B. burgdorferi* transmission are occurring in both Western Virginia and Coastal Virginia and Coastal North Carolina, human cases are primarily being diagnosed in the western section of these states. Our limited study of the population genetics of *I. scapularis* suggested that northeastern genotypes are established on the Outer Banks. An understanding of biotic and abiotic factors constraining the appearance of human-biting phenotypes will require further research.

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Impacts

- *Borrelia burgdorferi*, the spirochaete associated with Lyme disease, was identified in the black-legged deer tick and rodents sampled at five sites on the Outer Banks of North Carolina.
- The spirochaete was isolated from white-footed mice, rice rats and marsh rabbits. Sampling dates spanned 18 years indicating that transmission of the spirochaete is stable and endemic in the sampled tick and rodent populations.
- Sequence analysis confirmed that spirochaete isolates were *B. burgdorferi* and that ticks from both northern and southern populations are found on the Outer Banks of North Carolina.

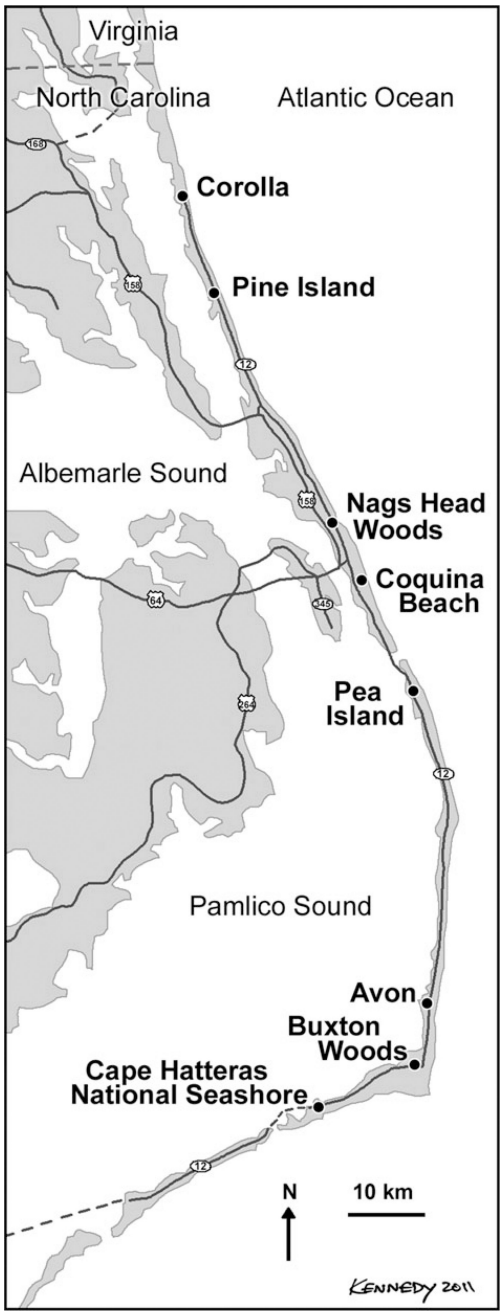

Fig. 1.
Study sites on the Outer Banks of North Carolina.

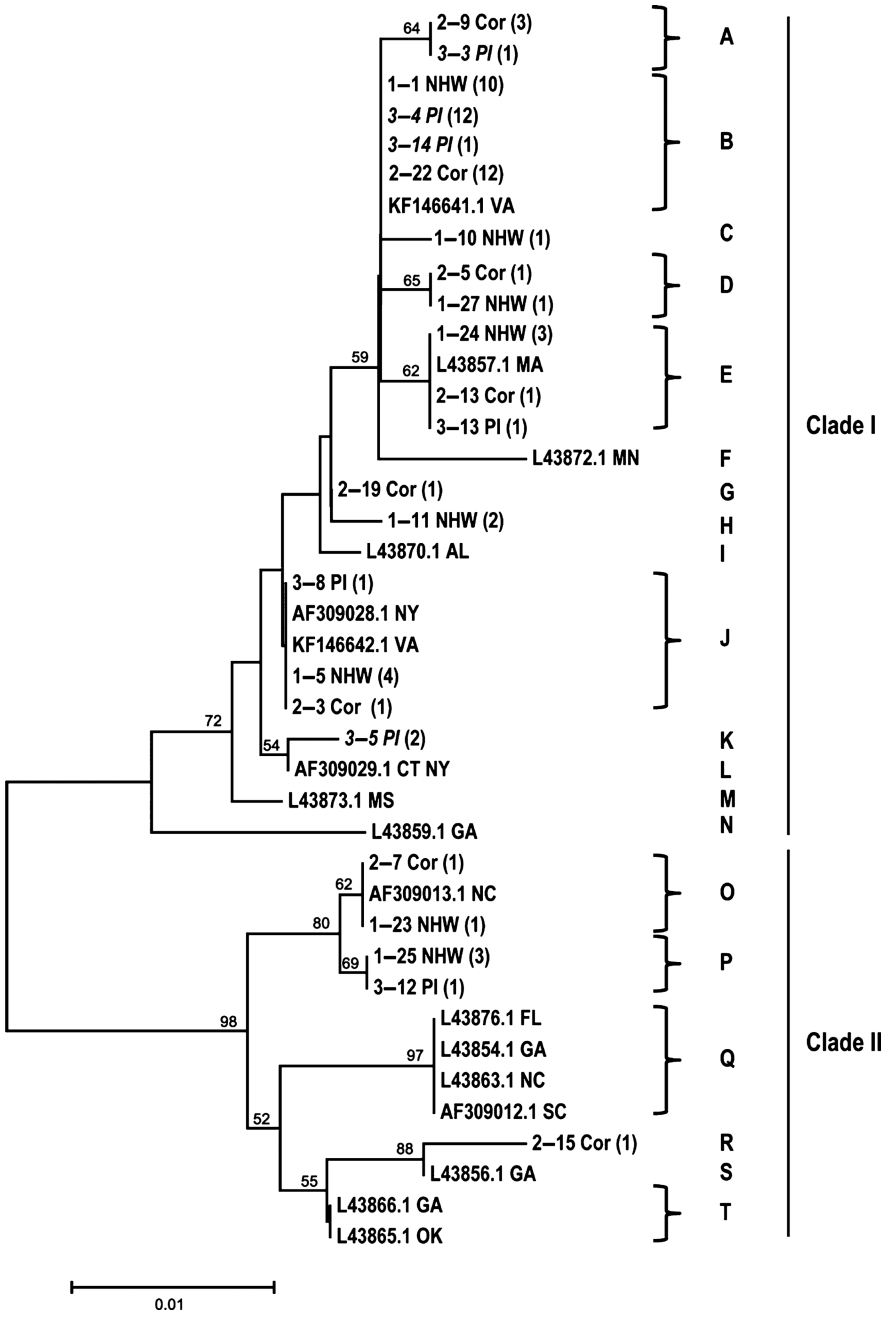

Fig. 2.

Neighbour-joining tree showing phylogenetic relationship of partial 16S mtDNA sequences of known *Ixodes scapularis* (*n* = 65) collected on the Outer Banks of North Carolina. Sequences preceded by numbers were generated in the present study. Numbers in parentheses after each sequence represent the number of ticks examined from the same collection site with 100% nucleotide sequence identity. Letters mark each distinct haplotypes with each exhibiting 100% sequence identity. Sequences printed in italics each represent one tick infected with *Borrelia burgdorferi* sensu stricto. Abbreviations for tick

collection sites on Outer Banks: PI, Pine Island; Cor, Corolla; NHW, Nags Head Woods Preserve.

Table 1

Borrelia PCR primers

Primers[a]	Nucleotide sequence (5′to 3′)	Tm (°C)	Location within target[b]	Amplicon size (bp)
B. burgdorferi/bissettii forward[c]	GCGTTTCAGTAGATTTGCCTGGTG	58.3	86–109	575
B. burgdorferi reverse	AAGTGCCTGAATTCCAAGCTGC	58.3	640–661	
B. andersonii forward	CCAAGGACAAGTCAACAACAGAAGC	58.1	347–371	174
B. andersonii reverse	CGAGCTTTCCTTCAAACTTAATGCCG	58.3	497–521	
B. bissettii reverse	AGTATGGTTCCGTTTGCTCTTGCC	59.4	434–411	349
B. burgdorferi sensu stricto[d] 23SN1	ACCATAGACTCTTATTACTTTGAC	50.0	469–446	~380
B. burgdorferi sensu stricto[d] 3SC1	TAAGCTGACTAATACTAATTACCC	50.0	92–115	

[a] Species-specific primers were designed using IDT Primer Quest™ software (Integrated DNA Technologies, 2016).

[b] Positions of the oligonucleotides are listed relative to the numbering of the *Borrelia burgdorferi ospA* gene (M57248). Nucleotide sequences were retrieved from GenBank™ (http://www3.ncbi.nlm.nih.gov) under accession numbers AY654919 (*B. andersonii*), and AF230516 (*B. bissettii*) for comparison to *B. burgdorferi* using ClustalW function in BioEdit® software.

[c] This forward primer was used for amplification of both *B. burgdorferi* and *B. bissettii*.

[d] The positions and sequences of the primers from Rijpkema et al. (1995).

Table 2

Amplicon primers designed to amplifying the V3 and V4 regions of 16S rRNA gene

Primer	Primer sequence
Round1 Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG
Round1Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGTCAATTCMTTTRAGT
Index primers	
N501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC
N502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
N503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
N504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC
N505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
N506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC
N507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
N508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
N720	CAAGCAGAAGACGGCATACGAGATCATTCCGTGTCTCGTGGGCTCGG
N721	CAAGCAGAAGACGGCATACGAGATTTGGCAGTGTCTCGTGGGCTCGG
N722	CAAGCAGAAGACGGCATACGAGATGCGAATCTGTCTCGTGGGCTCGG

Table 3

Borrelia burgdorferi isolated from adult questing *Ixodes scapularis* collected on the Outer Banks, 22 October 1991–13 March 1992

Sampling site	Gender	Number examined	Number of isolates	Percent positive
Avon	Males	9	0	0
	Females	0	0	0
Buxton Woods	Males	12	0	0
	Females	14	2	14
Cape Hatteras	Males	2	0	0
	Females	3	2	33
Coquina Beach/ Bodie island	Males	20	2	5
	Females	30	1	3
Corolla	Males	8	0	0
	Females	19	0	0
Nags Head Woods Preserve	Males	15	0	0
	Females	33	0	0
Pea Island	Males	1	0	0
	Females	0	0	0
Pine Island	Males	28	10	36
	Females	44	17	39
Total Outer Banks		238	34	14

Individual ticks were dissected and cultured in BSK-II, and isolates were confirmed by indirect immunofluorescence microscopy with a species-specific monoclonal antibody (H5332).

Table 4

Borrelia burgdorferi detected by indirect immunofluorescence microscopy in adult questing *Ixodes scapularis* collected on the Outer Banks, North Carolina February–March 1992, November 1992–January 1993 and November 1993

Sampling site	Gender	Number examined	Number positive	Percent positive
Bodie Island/Coquina Beach	Males	34	2	6
	Females	38	0	0
Buxton Woods	Males	17	2	12
	Females	25	1	0
Corolla	Males	80	0	0
	Females	61	0	0
Hatteras	Males	0	0	0
	Females	6	0	0
Nags Head Woods Preserve	Males	48	0	0
	Females	43	0	0
Pine Island	Males	99	22	22
	Females	90	12	13
Total		541	39	7

Table 5

Borrelia burgdorferi detected[a] in adult questing *Ixodes scapularis* collected on the Outer Banks of North Carolina October 2009

Sampling site	Number examined	Number positive	Percent positive
Corolla	24	2	8
Nags Head Woods Preserve	24	0	0
Pine Island	23	8	35
Total	71	10	14

[a]PCR amplification of *Borrelia burgdorferi OspA* gene fragments.

Table 6

Isolation of *Borrelia burgdorferi* from vertebrate hosts collected at Pine Island during July 1992–August 1993

Species	Number examined	Number of isolates	Percent positive
Six-lined racerunner (*Cnemidophorus sexlineatus*)	51	0	0
House mouse (*Mus musculus*)	2	0	0
Rice rat (*Oryzomys palustris*)	15	6	20
White-footed mouse *(Peromyscus leucopus)*	28	14	50
Eastern cottontail *(Sylvilagus floridanus)*	11	0	0
Marsh rabbit (*Sylvilagus palustris*)	47	2	4
Total	154	22	14

Table 7

Percent of *Peromyscus leucopus* trapped at Pine Island infected with *Borrelia burgdorferi* during August 1993–September 1994

Sampling Date	Number trapped	Number infected	Percent infected
1993			
August	12	7	58
October	13	2	15
November	6	3	50
December	7	1	14
1994			
January	4	0	0
February	14	1	7
March	7	1	14
April	13	6	46
May	6	3	50
June	7	5	71
July	6	2	33
August	5	3	60
September	6	3	50
Total	106	35	33