Multi-Locus Sequence Typing of *Ehrlichia chaffeensis* Reveals Extensive Genotypic Variation across the United States

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Abstract. *Ehrlichia chaffeensis* causes human monocytic ehrlichiosis, and its principal vector is the *Amblyomma americanum* tick. The most frequently identified cases of ehrlichiosis come from the southeastern and south central states of the United States. In this study, a molecular typing system was developed that allows for the genetic differentiation of *E. chaffeensis* isolates. This multi-locus typing system included sequencing and analyzing intergenic regions ECH0033–ECH0035 and ECH0217–ECH0218, plus, variable genes variable length PCR target, 28-kDa, 120-kDa, and *hemE*. We examined a total of 31 unique isolates from humans and white-tailed deer, and eight DNA samples extracted from infected *A. americanum* collected from multiple states. This is the largest evaluation of *E. chaffeensis* isolates and their genotypes. Our findings show that when sequences of all six loci were concatenated and compared, the 39 samples could be separated into 23 genotypes and further grouped into six phylogenetic clades. The data in this study show no clear pattern between the geographic alignment with the genetic differentiation between the strains. As a result, this poses a challenge to understanding the spread of *E. chaffeensis* in the United States. Interestingly, our findings indicate that multiple strains from distant geographic origins share the same mutations, which suggests that the strains are being moved from one site to another by their hosts or vectors. In addition, we are seeing a northward shift in the lone star tick distribution in the United States. Last, some data also suggest minimal genetic mutations have occurred over time among strains that are within geographical proximity.

INTRODUCTION

Ehrlichia chaffeensis, a tick-borne bacterium that causes human monocytic ehrlichiosis, is transmitted to humans and various species of domesticated and wild animals by *Amblyomma americanum* (the lone star tick).¹ *Amblyomma americanum* is an abundant and widely distributed human-biting hard tick that occurs throughout a large expanse of the eastern United States.^{1,2} Human ehrlichiosis was first described in the United States in 1987, and the first isolation of the pathogen in cell culture was reported in 1991.^{3–5} The organism was designated a new species, *E. chaffeensis*, based on its 16 S rRNA gene sequence, which was significantly different from those of previously described *Ehrlichia* species.^{4,6} Since then, two additional species, *Ehrlichia ewingii* and *Ehrlichia muris euclairensis*, have been identified as pathogens of humans in the United States, although infections with *E. chaffeensis* remain the most important in terms of magnitude and severity.^{7–9} In the United States, cases of ehrlichiosis are most frequently identified from the southeastern and central states.^{4,10–12} The severity of infections varies greatly, from moderately severe to fatal disease.¹³ Strain determinants for differences in clinical presentation are poorly understood.^{14,15}

A few studies have examined one or two genetic loci, including the variable length PCR target (VLPT), the 120-kDa, and the 28-kDa antigen genes to distinguish isolates of *E. chaffeensis*, and some of these investigations have identified genetic patterns among loci to suggest geographic clustering of certain strains.^{1,3,15–19} To more fully characterize the genetic variability of *E. chaffeensis* and identify molecular patterns that potentially correlate with geographic origin, we examined genomic DNA from 39 isolates of *E. chaffeensis* obtained from humans and white-tailed deer, as well as DNA samples extracted from infected *A. americanum*.

MATERIALS AND METHODS

Isolates and DNA extraction. Thirty isolates of *E. chaffeensis*, obtained from humans ($n = 28$) or white-tailed deer ($n = 2$) during 1991–2013, were included in the analysis (Table 1). These isolates originated from a minimum of 22 distinct locations in nine southeastern and Midwestern states.^{3,5,18,20–27} All *E. chaffeensis* isolates were cultured in continuous canine macrophage DH82 cells maintained at 37°C in a 5% CO₂ atmosphere using minimal essential medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM MEM nonessential amino acids (Gibco), 0.1 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), and 10 mM sodium pyruvate.^{3,28} Infected cultures were monitored for *Ehrlichia* morulae using cytopsin slide preparations stained with Diff-Quick. When the *E. chaffeensis* cultures were harvested for cryopreservation, a 0.5-mL aliquot was set aside for DNA extraction using the KingFisher Cell & Tissue DNA kit (Thermo Fisher Scientific, Waltham, MA) and KingFisher mL magnetic particle processor. In addition, DNA extracted from nine, *E. chaffeensis*-infected *A. americanum* ticks, collected from four separate locations in New York and Connecticut, were also used in the analyses. DNA from the infected *A. americanum* ticks was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD).

Selection of loci. A subset of four isolates that originated from widely separated geographical areas (Arkansas^T, Heartland, Jax, and Wakulla) was selected to evaluate candidate genes and intergenic regions for nucleotide differences. Six loci were selected for analysis: VLPT, 120-kDa protein gene, 28-kDa protein gene, *hemE*, and intergenic regions between ECH0217–0218 and ECH0033–ECH0035. The VLPT, 120-kDa, and 28-kDa genes have been used in previous studies to distinguish between a smaller set of *E. chaffeensis* isolates.^{1,17,19} In addition, intergenic regions between ECH0217–0218 and ECH0033–ECH0035 were identified because of differences found in the whole genome alignments between the following isolates: Arkansas^T, Heartland, Jax,

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TABLE 1
Ehrlichia chaffeensis isolates and DNA samples from *Amblyomma americanum* ticks used in this study

Isolate or tick DNA	Source	Year	Location	Reference
Arkansas ^T	Human	1991	Sebastian County, Arkansas	3
91HE17	Human	1991	Arkansas	25
MO-18	Human	2013	Holt County, Missouri	This study
Heartland	Human	1999	Nebraska	30
Jax	Human	1996	Nassau County, Florida	26
MO-19	Human	2013	Nodaway County, Missouri	This study
Liberty	Human	1998	Liberty County, Florida	4
MO-13	Human	2009	Andrew County, Missouri	This study
MO-17	Human	2011	Doniphan County, Kansas	This study
MO-3	Human	2009	Missouri	This study
MO-6	Human	2009	Missouri	This study
Osceola	Human	1997	Baker County, Florida	4
Sapulpa	Human	1995	Tulsa County, Oklahoma	16
St. Vincent	Human	1996	Bibb County, Georgia	26
V1	Human	1998	Tennessee or Kentucky	27
V3	Human	1998	Tennessee or Kentucky	27
V4	Human	1998	Tennessee or Kentucky	27
V5	Human	1998	Tennessee or Kentucky	27
V6	Human	1998	Tennessee or Kentucky	27
V7	Human	1998	Tennessee or Kentucky	27
V8	Human	1999	Unknown	20
V9	Human	1999	Unknown	20
V10	Human	1999	Unknown	22
V19	Human	1999	Unknown	This study
Wakulla	Human	1997	Wakulla County, Florida	4
West paces	Human	1998	Tennessee	4
CT17-107	<i>A. americanum</i>	2017	Fairfield County, Connecticut	24
CT17-121	<i>A. americanum</i>	2017	Fairfield County, Connecticut	24
CT17-126	<i>A. americanum</i>	2017	Fairfield County, Connecticut	24
CT17-143	<i>A. americanum</i>	2017	Fairfield County, Connecticut	24
CT17-174	<i>A. americanum</i>	2017	Fairfield County, Connecticut	24
2018NY-Aa95M	<i>A. americanum</i>	2018	Suffolk County, New York	This study
2018NY-Aa140M	<i>A. americanum</i>	2018	Suffolk County, New York	This study
2018NY-Aa171M	<i>A. americanum</i>	2018	Suffolk County, New York	This study
Lithonia	Human	2000	DeKalb County, Georgia	30
Chattanooga	Human	2000	Tennessee	30
Mitch	<i>O. virginianus</i>	1995–1996	Georgia	23
Dave	<i>O. virginianus</i>	1995–1996	Georgia	23

A. americanum = *Amblyomma americanum*.

Liberty, Osceola, Sapulpa, St. Vincent, Wakulla, and West Paces.

Molecular analyses. Standard PCR reactions were set up for six loci. Each PCR reaction consisted of 2 µL DNA template, 10 µL of Taq PCR Master Mix (QIAGEN, Valencia CA), and each primer at a final concentration of 1 µM in a final reaction volume of 20 µL. After a 5-minute denaturation at 95°C, each reaction underwent 40 cycles of a 30-second denaturation at 95°C, a 30-second annealing incubation (Table 2), and a 1-minute extension at 72°C for the *hemE* gene and intergenic regions ECH0217–0218 and ECH0033–ECH0035 (Table 2). This was followed by a final 10-minute extension at 72°C. PCR cycling conditions for the VLPT, 120 kDa, and 28 kDa protein genes were used as previously described.^{1,17,19} Amplicons were resolved in a 1% agarose gel and purified using the Promega Wizard SV gel extraction kit and PCR Clean-Up System (Promega, Madison WI) following the manufacturer's protocol.

Sequencing and phylogenetic analyses. One microliter of the purified PCR product was sequenced using a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3500 genetic analyzer. Each PCR amplicon was sequenced in both directions, and sequence reads were assembled using Geneious R11 (Biomatters Ltd.,

Auckland, New Zealand). MEGA7 was used to create DNA alignments for each locus using CLUSTAL W.²⁹ For the concatenated alignment, the loci were concatenated in the following order: ECH0033–ECH0035, ECH0217–ECH0218, *hemE*, VLPT, 28-kDa, and 120-kDa. MEGA7 was used to perform the phylogenetic analysis, a neighbor-joining tree was constructed using the maximum composite likelihood Jukes-Cantor model, and 1,000 bootstrap replicates were used to estimate the likelihood of the tree.

RESULTS

ECH0033–ECH0035. The primers for this locus amplified a 711-bp fragment of the intergenic region between two hypothetical proteins of unknown function (ECH0033–ECH0035). Five single nucleotide polymorphisms (SNPs) were identified within the amplicons generated by the ECH0033–ECH0035 primer pair (Figure 1), resulting in the identification of seven genotypes. The Lithonia isolate and tick DNA 2018NY-Aa140M are 100% identical to Arkansas^T (genotype I). When compared with genotype I, isolates V3, V8, V9, Chattanooga, Dave, Heartland, Sapulpa, and West Paces have a single SNP (C to T transition) at nucleotide 134 and comprise genotype II. Isolates V7, Liberty, and tick DNA samples CT17-121, and

TABLE 2
Primers used in this study

Target	Forward	Reverse	Amplicon size	Annealing temperature	Reference	Number of repeat units	PCR modifications
VLPT	AAGACTGAAACGTTATAGAG	GTGACATCTTAGTTTAAATAGAAC	643-901 bp	52°C	12	3-6 90-bp tandem repeat units (seven genotypes)	-
120 kDa	GAGAAATTGATTGTGGAGTTGG	ACATAACATTCCACTTTTCAAA	895 bp-1375 bp	50°C	12, 13	2-4 240-bp tandem repeat units (18 genotypes)	35 cycles, 50°C annealing temperature 40 cycles
28 kDa	ACTTCTACTATGTGAATTATTTATGTC AGTATCATTTTCCGACCCAGCA	GCTGTTGTGTAAGTGTAGACTGGT	832-867 bp	55°C	14	Five genotypes	-
hemE	GGTAG TGTCAGTTTCATATTTTACGGTG	AAGCAGGGCCCAAGCAAAAAG	920 bp	61°C	This study	Three genotypes	-
ECH0217- ECH0218	TTGT AGAGAGTCGTAATAGTGACGT	TCCCAAGAACACGTTGTCCA	561 bp	56°C	This study	Four genotypes	-
ECH0033- ECH0035	CCGCTTGGTCTAGCTCCATT	ACAGACTCACATAGAACCTGTACT	711 bp	61°C	This study	Seven genotypes	-

Tick DNA samples underwent 40 PCR cycles for VLPT, 120 kDa, and 28 kDa.

CT17-174 make up genotype III. Compared with strain Arkansas^T, these isolates contain a single SNP at nucleotide position 356 (C to T transition). Eight isolates (MO-3, MO-6, MO-17, MO-18, MO-19, V6, V10, and V19) are included in genotype IV. These isolates have two SNPs compared with genotype I, they are located at nucleotide positions 446 (T to C transition) and 476 (G to A transition). Genotype V contains isolates V4, V4C, Jax, Osceola, and Wakulla, and tick DNAs 2018NY-Aa95M and 2018NY-Aa171M. These strains are identical with strain Arkansas^T, except that they share the SNP at nucleotide 476 with genotype IV. The genotype VI isolates (V1, V5, 91HE17, MO-13, Mitch, and St. Vincent) share a single SNP with genotype IV at nucleotide position 446, whereas genotype VII, represented by tick DNAs CT17-107, CT17-126, and CT17-143, contains two SNPs at nucleotide positions 356 (shared with genotype III) and 659 (a unique A to G transition) when compared with genotype I.

ECH0217-ECH0218. These primers amplified a 561-bp region of the intergenic region between a putative methylthiotransferase (ECH0217) and a predicted thioredoxin (ECH0218). Three SNPs were detected in the ECH0217-ECH0218 intergenic region to that created four distinct genotypes (Figure 1). DNA from tick 2018NY-Aa171M, and isolates Lithonia, Mitch, V1, and V5 are 100% identical to Arkansas^T and comprise genotype I. Isolates V3, V8, V9, Chattanooga, Heartland, Sapulpa, St. Vincent, and West Paces, and DNA samples from ticks CT17-107, CT17-121, CT17-126, CT17-143, CT17-174, and 2018NY-Aa140M contain an SNP (G to A transition) at position 132 and form genotype II. Genotype III isolates (V7, 91HE17, MO-13, Dave, Osceola, and Wakulla) also have a single SNP located at nucleotide position 198 (T to C transition). The isolates that constitute genotype IV have two SNPs present at nucleotide positions 198 (T to C transition) and 458 (T to C transition) and include V4, V4C, V6, V10, V19, MO-3, MO-6, MO-17, MO-18, MO-19, Jax, and Liberty, and tick DNA 2018NY-Aa95M.

hemE protein gene. These primers amplified a 920-bp region of *hemE*, a uroporphyrinogen decarboxylase which is a component in heme biosynthesis.¹⁴ The *hemE* amplicons had nine SNPs producing a total of three distinct genotypes (Figure 1). Isolates V1, V5, Dave, Lithonia, Mitch, and Osceola, and tick DNA 2018NY-Aa95M were all identical to Arkansas^T and comprise genotype I. The Wakulla isolate had a unique genotype, with nine SNPs at nucleotide positions 11 (G to A transition), 28 (C to T transition), 62 (T to C transition), 173 (A to G transition), 203 (G to A transition), 306 (T to C transition), 797 (G to A transition), 884 (C to T transition), and 901 (C to A transversion). The remaining isolates and tick DNA samples were identical to each other and comprise genotype III. This genotype contains the SNPs found in genotype II at nucleotides 797, 884, and 901, but is identical to genotype I at all other positions.

28-kDa protein gene. The primers for this locus amplified an 832- to 867-bp (isolate dependent) region of the 28-kDa outer membrane protein, which is involved in disulfide-bond cross-linking of the outer membrane of *Ehrlichia*.¹ Two hundred two SNPs and 12 insertion/deletion (INDEL) events were identified in the 28-kDa amplicons, which created a total of five distinct genotypes (Figure 1). Isolates identical to Arkansas^T (genotype I) include V1, V5, Dave, Lithonia, Mitch, and Osceola. Genotype II isolates comprised V4, V4C, V7, MO-13, 91HE17, Liberty, and Jax. In comparison to Arkansas^T, genotype II

ECH0033-ECH0035						ECH0217-ECH0218				ECH075E												
Genotype	134	356	446	476	659	Genotype	132	198	458	Genotype	11	28	62	173	203	306	797	884	901			
I* (3)	C	C	T	T	G	A	I* (6)	G	T	T	I* (8)	G	C	T	A	G	T	G	C	C		
II (8)	T	C	T	T	G	A	II (14)	A	C	T	II (1)	A	T	C	G	A	C	A	T	A		
III (4)	C	T	T	T	G	A	III (6)	G	C	T	III (30)	G	C	T	A	G	T	A	T	A		
IV (8)	C	C	C	C	A	A	IV (13)	G	C	C	nt 920											
V (7)	C	C	T	A	A	nt 561																
VI (6)	C	C	C	G	A																	
VII (3)	C	T	T	G	G																	
nt 711																						
VLPT						28kDa																
Genotype	16-24	180	258-347	348-437	438-527	528-617	618-708	709-795	817	838	Genotype	No. of Repeats	No. of Repeat Types	No. SNPs	INDELS							
I* (12)	CATATAAAA	C	1	2	3	-	4	-	C	T	I* (7)	-	-	-	-							
II (9)	CATATAAAA	C	1	2	-	-	4	-	C	T	II (7)	-	-	111	10							
III (3)	CATATAAAA	C	1	2	3	3	4	-	C	T	III (9)	-	-	111	8							
IV (1)	CATATAAAA	C	1	2	3	-	4	-	C	C	IV (15)	-	-	111	8							
V (8)	-	T	1	2†	3	-	4‡	5*	C	T	V (1)	-	-	119	8							
VI (5)	-	T	1	2†	3	-	4	5	T	C	nt 832-867											
VII (1)	-	T	1	2†	3	3	4	5	T	C												
nt 643-901											120kDa											
Genotype	181-420	421-660	661-900	901-1140	226	240	399	401	405	412	419	466	503	558	566	652	743	798	806	892	1171	1186
I* (3)	1	6	6	-	A	G	A	T	A	T	C	G	A	G	G	C	A	G	G	C	A	A
II (3)	1	7	7	-	A	G	A	T	A	T	C	G	A	G	G	T	A	G	G	T	A	A
III (1)	1	8	-	-	A	G	A	T	A	T	C	A	A	A	A	C	A	G	G	C	G	A
IV (1)	1	4	6	6	A	G	A	T	A	T	C	G	A	A	A	T	A	G	G	C	A	A
V (2)	2	7	6	6	A	G	A	T	A	C	C	G	A	G	G	T	A	G	G	C	A	A
VI (1)	2	6	7	6	A	G	A	T	A	C	C	G	A	G	G	C	A	G	G	T	A	A
VII (1)	2	4	6	6	A	G	A	T	A	C	C	G	A	A	A	T	A	G	G	C	A	A
VIII (1)	2	7	10	6	A	G	A	T	A	C	C	G	A	G	G	T	G	G	G	T	A	A
IX (11)	2	7	6	-	A	G	A	T	A	C	C	G	A	G	G	T	A	G	G	C	A	A
X (3)	2	6	9	-	A	G	A	T	A	C	C	G	A	G	G	C	A	A	A	C	G	A
XI (1)	2	6	6	-	A	G	A	T	A	C	C	G	A	G	G	C	A	G	G	C	A	A
XII (1)	2	9	6	-	A	G	A	T	A	C	C	G	A	A	A	C	A	G	G	C	A	A
XIII (1)	2	10	6	-	A	G	A	T	A	C	C	G	G	G	G	T	A	G	G	C	A	A
XIV (1)	2	6	-	-	A	G	A	T	A	C	C	G	A	G	G	C	A	G	G	C	A	A
XV (1)	3	8	-	-	A	G	A	T	A	T	C	A	A	A	A	C	A	G	G	C	G	A
XVI (5)	4	4	4	-	G	A	A	T	A	T	C	G	A	A	A	T	A	A	A	T	A	A
XVII (1)	5	4	4	-	A	G	C	C	C	C	T	G	A	A	A	T	A	A	A	T	A	A
XVIII (1)	-	-	-	-	A	G	A	T	A	T	C	G	A	G	G	C	A	G	G	C	A	G
nt 406-1366																						

FIGURE 1. Genotypes of sequenced intergenic regions and outer membrane proteins. Each table shows individual genotypes identified for each locus. The numbers in parentheses indicate the number of isolates in each genotype. The total number of nucleotides in the consensus sequence of each locus is shown under the table, and the nucleotide positions indicated are relative to the aligned consensus sequence. * Type strain for this study is Arkansas. Note: VLPT sequence units are 90-bp and found in consensus nucleotides 258–347, 348–437, 438–527, 528–617, 618–708, and 709–795; sequence unit types are designated numbers 1–5. Note: 120 kDa sequence units are 240-bp and found in consensus nucleotides 181–420, 421–660, 661–900, and 901–1,140; sequence unit types are designated numbers 1–10. †single nucleotide polymorphisms at nucleotide position 420 (A to G transition). ‡18-bp deletion at consensus nucleotides 690–707. ^a71-bp deletion at consensus nucleotides 709–779. ^b16-bp deletion at consensus nucleotides 790–795.

isolates have 104 SNPs and 10 INDELS events. Genotype III isolates (V3, V8, V9, Chattanooga, Heartland, Sapulpa, St. Vincent, and West Paces, and tick DNA 2018NY-Aa140M) exhibit 116 SNPs and eight INDEL events. Genotype IV (isolates V6, V10, V19, MO-3, MO-6, MO-17, MO-18, MO-19, and Wakulla, and tick DNAs 2018NY-Aa171M, CT17-107, CT17-121, CT17-126, CT17-142, and CT17-174) and genotype V (tick DNA 2018NY-Aa95M) had 112 and 118 SNPs, respectively. Furthermore, genotype IV and V isolates share the same INDEL events at nucleotide positions 13, 85, 97, 110, 121–135, 223–228, 534–548 (genotype I Indel 534–545), 558–560 as genotype I.

120-kDa protein gene. The primers for this locus amplified a fragment of the 120-kDa antigen gene between 406 and 1,366 bp in length, depending on the isolate. This immunodominant protein is mainly expressed on dense-core factors of *E. chaffeensis*.¹⁷ Expression of this gene in *Escherichia coli* causes attachment and internalization by Vero cells, suggesting an important role for this protein in the attachment and invasion of host cells by *E. chaffeensis*.¹⁷ The amplicons produced by the 120-kDa primers contained two to four, 240-bp repeat units, as previously described.^{17,19} The 240-bp repeat units identified were found at consensus nucleotides 181–420, 421–660, 661–900, and 901–1,140 (Figure 1). In addition, a total of 18 SNPs were found among the 18 genotypes identified (Figure 1). Isolates V1 and Mitch are identical to Arkansas^T (Genotype I). These isolates have three repeat units with unique repeat unit types and sequence profile 1, 6, and 6 (Figure 1). Other genotypes that have three repeat units

with distinct sequence profiles include genotypes II (isolates MO-17, MO-18, and MO-19), IX (V3, V8, V9, Chattanooga, Heartland, Liberty, Sapulpa, and West Paces, and tick DNAs CT17-107, CT17-126, and CT17-174), X (isolates Osceola, and St. Vincent, and tick DNA 2018NY-Aa140M), XI (isolate MO-13), XII (isolate 91HE17), XIII (tick DNA CT17-143), XVI (isolates MO-3, MO-6, V6, V10, and V19), and XVII (tick DNA 2018 NY-171M) (Figure 1). They also share the same INDEL between nucleotide positions 901–1,140 (Figure 1). In comparison to Arkansas^T, the isolates that comprise genotypes IV (Wakulla), V (V4C, Jax), and VII (V7), and tick DNAs comprising genotypes VI (2018-NYAa95M) and VIII (CT17-121) share four repeat units, again, with their own distinct repeat unit types and profiles (Figure 1). Furthermore, isolates Lithonia (genotype III), V4 (genotype XIV), and V5 (genotype XV) have only two repeat units and share the same INDEL events at nucleotide positions 421–660 and 661–900 (Figure 1). In contrast to all other strains, Wakulla (genotype XVIII) was the only isolate that had no repeat units present (Figure 1). In conclusion, there was a total of 10 different 240-bp imperfect repeat unit types identified within the four 240-bp repeat regions which produced 18 genotypes (Figure 1).

Variable length PCR target protein gene. The primers for the locus amplified a 643- to 901-bp region of the VLPT protein. This gene has been shown to both be a sensitive and specific marker for *E. chaffeensis* and to allow for limited differentiation between strains of *E. chaffeensis*.⁴ Four SNPs, six INDELS, up to six imperfect repeat units (three to six repeat units, depending on the isolate), and five versions of the repeat

FIGURE 2. Phylogenetic associations of *Ehrlichia chaffeensis* tick DNAs and isolates. Maximum composite likelihood Jukes-Cantor model phylogenetic relationships of *E. chaffeensis* are based on the concatenated sequences six loci (ECH0033–ECH0035, ECH0217–ECH0218, *heme*, VLPT, 28-kDa, and 120-kDa). Numbers at the nodes are bootstrap values based on 1,000 bootstrap replicates. Only bootstrap values > 70% are shown.

sequence were detected in the VLPT amplicons that collectively created seven distinct genotypes (Figure 1). Four SNPs were detected at nucleotide positions 180 (C to T transition), 420 (A to G transition), 817 (C to T transition), and 838 (T to C transition). As previously described, VLPT amplicons also contained three to six repeat units at nucleotide positions 258–347, 348–437, 438–527, 528–617, 618–708, and 709–795, respectively.⁴ Isolates V1, V4, V4C, V5, Dave, Jax, Lithonia, Mitch, and Osceola, and tick DNA specimens 2018NY-Aa95_M and 2018NY-Aa171_M are 100% identical to Arkansas^T (Genotype I) and consist of four repeat units with repeat profiles 1, 2, 3, and 4 (Figure 1). Isolates V3, V8, V9, Heartland, Sapulpa, St. Vincent, 91HE17, and Chattanooga, and tick DNA 2018NY-Aa140_M, comprising genotype II, have a ninety-bp deletion at consensus nucleotides 438–527 when compared with Arkansas^T. The three repeat units have a repeat profile of 1, 2, and 4. Genotype III consists of the isolates MO-13, V7, and West Paces, and has five repeat units with the profiles 1, 2, 3, 3, and 4. A single SNP was identified at nucleotide position 838 (T to C transition) that differentiates isolate Liberty (Genotype IV) from strain Arkansas^T and genotype I. Isolates MO-3, MO-6, MO-17, MO-18, MO-19, V6, V10, V19, comprising genotype V, contain a unique 90-bp deletion between nucleotides 690–779 (in repeats four and five). This genotype also contains a 9-bp deletion of nucleotides 16–24. In addition, this genotype contains an SNP at nucleotide 180 (C to T transition) and an additional SNP in repeat two at nucleotide 420 (A to G transition). These isolates possess five repeat units with a repeat profile of 1, 2, 3, 4, and 5. Tick DNA samples CT17-107, CT17-121, CT17-126, CT17-143, and CT 17–174 comprise genotype VI. This genotype shares the deletion of nucleotides 16–24 and the SNP at position 180 with genotype V and contains full-length versions of repeats four and five. In addition, there is a SNP at position 817 (C to T transition), and this genotype shares the SNP at nucleotide 838 with genotype IV. The isolate Wakulla has a unique genotype (genotype VII). This isolate is identical to genotype VI, with the exception that it shares a second copy of repeat three with genotype III. Thus, this isolate contains six repeat units with the repeat pattern 1, 2, 3, 3, 4, and 5.

Phylogenetic analysis. Concatenation of the six loci separated the 30 isolates and nine tick DNA samples into 23 genotypes comprising six phylogenetic clades (Figure 2). MEGA7 was used to perform the phylogenetic analysis, a neighbor-joining tree was constructed using the maximum composite likelihood Jukes-Cantor model, and 1,000 bootstrap replicates were used to estimate the likelihood of the tree. Clades were established based on bootstrap values > 70. Any bootstrap value < 70 was considered statistically nonsignificant to designating a distinct clade. Clade I contained four closely related genotypes, comprising five isolates from Missouri, one isolate from Florida, a tick DNA sample from Suffolk County, New York, and three isolates from Tennessee/Kentucky. Clade II comprised all five tick DNA samples from Connecticut in four closely related genotypes. Clade III consists of nine isolates in two closely related genotypes. The first genotype from Clade III consists of an isolate from Florida and one tick DNA sample from Suffolk County, New York. The second genotype in Clade III consists of isolates from Nebraska, Georgia, Oklahoma, and Tennessee/Kentucky. Clade IV consists of a single tick DNA

sample from Suffolk County, New York. There are seven isolates in six closely related genotypes coming from geographic locations such as Arkansas, Missouri, Florida, and Tennessee/Kentucky in clade V. Finally, clade VI has seven isolates with six closely related genotypes from Florida, Tennessee, Arkansas, and Georgia.

DISCUSSION

In this study, a multi-locus typing system was developed that allows for the genetic differentiation of *E. chaffeensis*. To our knowledge, this represents the largest genotypic evaluation of this tick-borne pathogen and included 30 unique isolates and nine tick-associated DNA specimens from 11 U.S. states. The analysis identified a total of 515 individual mutations (234 SNPs and 21 INDELs) among these six targets that generated 23 different genotypes that could be grouped into six different phylogenetic clades (Figure 2). The 120-kDa locus presents the largest number of *E. chaffeensis* genotypes (18), whereas the VLPT locus exhibits seven genotypes among the isolates. Moreover, the 28-kDa locus shows the greatest number of SNPs and INDEL events between the isolates. None of the other loci (*hemE*, ECH0033–ECH0034, and ECH0217–ECH0218) revealed repeat sequences or INDELs. What differentiated the strains were the presence of 9, 5, and 3 SNPs at different nucleotide positions, respectively. The data in this study suggest that protein genes VLPT, 120-kDa, 28-kDa, and *hemE* can be used as dependable markers to distinguish among several isolates of *E. chaffeensis*, as well as the intergenic regions ECH0033–ECH0034 and ECH0217–ECH0218.

To our knowledge, this study represents the largest analysis of genetic diversity in *E. chaffeensis*, both in the number of isolates or DNA samples and in the number of genetic loci analyzed. Although our study generally agrees with the results of previous studies, it provides increased granularity into the genetic diversity of this pathogen and allows for additional separation of isolates and DNA samples. By using only the 28-kDa protein gene, Yu et al. were able to separate five isolates into three genetic groups.¹ Our analysis identified the same three groupings, but also identified an additional three clades. Also, using the 28-kDa gene, Cheng et al.³⁰ analyzed 10 isolates and found the same three groups. Our analysis characterized three additional clades and divided one of their groups (group 3) into two distinct clades (clade I and clade III). Finally, Sumner et al.⁴ reported 12 genotypes when analyzing the nucleotide sequences of the VLPT gene from nine isolates, seven positive patient DNA samples, and eight positive tick pool DNA samples. Our analysis nearly doubled the number of genotypes.

Our analyses did not identify specific associations between disease severity and various genotypes or clades. Among the four clades with representation by isolates of human origin, three (clades III, V, and VI) contained *E. chaffeensis* genotypes cultivated from patients with fatal disease (i.e., Jax, St. Vincent, Osceola, and Chattanooga)²⁶ or life-threatening infection (i.e., Sapulpa and 91HE17),^{16,25} as well as isolates from patients with varying degrees of disease severity who survived their illnesses (i.e., Arkansas and V1–V7).^{3,27} Similarly, clade I contained isolates from patients with disease of sufficient severity to require hospitalization (i.e., Wakulla), but who recovered

fully because of early diagnosis and appropriate antibiotic therapy.³¹ In this context, it is possible that most or possibly all genotypes of *E. chaffeensis* are capable of causing severe or fatal disease in a human host. Multiple other intrinsic and extrinsic factors, including the age and underlying immune status of the host, and the timeliness of diagnosis and treatment, are crucial considerations assessing patient outcomes following infection.^{27,31} With a few exceptions, no clear patterns emerged from our analysis that predicted geographical distribution based on genotype. Surprisingly, we found that multiple isolates and DNA specimens shared similar or identical genotypes, despite widely separated geographical origins. Also surprising was the identification of extensive genetic variability among samples of *E. chaffeensis* originating from relatively close geographical regions. For example, clade VI comprised seven isolates from Florida, Georgia, Tennessee or Kentucky, and Arkansas. These seven isolates have closely related genotypes, with three isolates (from Arkansas, Georgia, and Tennessee or Kentucky) sharing an identical genotype. Yet, the seven isolates from the adjacent states of Tennessee and Kentucky contain seven different genotypes that group into five separate clades, and the four isolates from Florida contain four genotypes grouped in four clades. Likewise, the five DNA samples from Connecticut have four closely related genotypes that group into a single clade (Clade II), whereas the three DNA samples from New York have three very different genotypes, each grouping into different clades (clade I, clade III, and clade IV).

The broad geographic distribution of *E. chaffeensis* strains that share similar genotypes may represent the introduction of a new lineage into a geographic area due to the migration of vertebrate hosts. *Amblyomma americanum* (lone star) ticks are the principal vectors of *E. chaffeensis*.² These ticks are the most abundant and aggressive human-biting tick in the southeastern United States, and are most commonly associated with white-tailed deer and wild turkeys.^{32,33} However, they feed on a wide range of mammals including cattle, horses, cats, coyotes, dogs, foxes, opossums, rabbits, raccoons, rodents, and squirrels as well as birds (i.e., gallinaceous and passerine birds).³² Most importantly, white-tailed deer, wild turkeys, and coyotes have played an important role as hosts for all life stages of the lone star ticks.²¹ The emergence of *Amblyomma*-associated ehrlichiosis has been influenced in part by the explosive growth of various wildlife species, especially white-tailed deer, wild turkeys, and coyotes.²¹ For instance, coyotes have expanded their range throughout North America from the Great Plains and western United States to the southeast.²¹ These carnivores exist in most habitats and have become established in suburban and urban locations which increase the potential for infected lone star ticks to be seeded into the peri-domestic environment.²¹ Last, as the wild turkey populations have increased throughout their historic geographic range (New York to the north and Kansas to the west), they are credited with reintroducing and increasing population densities of *A. americanum*.²¹ However, although increases in the populations of white-tailed deer, coyotes, and wild turkeys may explain local increases in *E. chaffeensis*-infected ticks, their relatively small home ranges (182–416 ha, 3,130–6,870 ha, and 1,295–3,514 ha, respectively) would not explain the existence of closely related

or identical genotypes of *E. chaffeensis* across large geographic distances, especially given the genetic diversity seen in localized areas.^{34–36}

The distribution of *E. chaffeensis* across large geographical distances by highly mobile species such as birds could explain the occurrence of identical genotypes in distant locations. Ehrlichial DNA, including that of *E. chaffeensis*, has been detected in the blood of various avian species including the northern crested caracara (*Caracara cheriway*), the American kestrel (*Falco sparverius*), and the black vulture (*Coragyps atratus*).^{37,38} In this context, birds could serve as amplifying hosts for the bacteria, providing a source of infection for naive ticks as well as the distribution of bacteria across broad geographic regions.^{38–40} Indeed, one study investigating the tick species found on birds in the state of Georgia reported that the predominate tick identified was *A. americanum*, the primary vector of *E. chaffeensis*.⁴¹ In addition, ehrlichial DNA has been detected in nymphal stage lone star ticks removed from multiple avian species, including the pale thrush (*Turdus pallidus*), the chaffinch (*Fringilla coelebs*), the common blackbird (*Turdus merula*), the brambling (*Fringilla montifringilla*), the Eurasian wren (*Troglodytes troglodytes*), and the willow warbler (*Phylloscopus trochilus*).^{42,43}

The absence of correlation among various genotypes and geographical origins is a notable aspect of this study. The only clade represented by a single source is clade II. These ticks were collected on a small island off the coast of Connecticut and could represent a geographically isolated population.²⁴ Although most of the isolates in this study originated from infections of humans, no genetic distinctions were identified between isolates of human origin and those directly from white-tailed deer or tick DNA. Future studies are needed to determine if any of the genetic differentiations correlate with differences in virulence.

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Disclaimer: The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the CDC.

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