# Focal persistence and phylodynamics of Heartland virus in Georgia

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## Abstract

Heartland virus (HRTV) is an emerging tick-bone virus associated with severe illness in the U.S. There are large gaps in knowledge of HRTV diversity, evolution, and transmission due to a paucity of HRTV-positive samples and genome sequences. We identified a focal site of HRTV-positive *Amblyomma americanum* ticks in central Georgia and developed a novel multiplex-amplicon sequencing assay to generate full HRTV genome sequences. By screening over 21,000 field-collected ticks from 2021-2023, we identified six positive pools. Five were collected from the site in central Georgia where our group first detected HRTV-positive ticks in 2019, and one from a site in western Georgia approximately 175 km away. The HRTV genome sequences from Georgia were highly related, even across this distance and over five years. Reference HRTV genome sequences from across the U.S. were also geographically clustered. Time-scaled phylogenetic analysis suggested recent spread of HRTV in the U.S., with all available sequences sharing a common ancestor within the last 300 years, and sequences from Georgia sharing a common ancestor within the last 40 years. Our observed spatial clustering of HRTV and the high degree of genetic conservation in our persistent focus suggest the importance of small spatial dynamics in HRTV transmission ecology.

## Author Summary

Heartland virus (HRTV) was first discovered in humans in 2009 and has since caused over 60 cases of severe and fatal disease in the United States. HRTV is transmitted by the lone star tick, *Amblyomma americanum*, across the Southeast, East coast, and Midwest. Little information is known about how this virus circulates and changes across time and space due to a lack of genetic data. Here, we created a new procedure to generate more genetic sequence data for HRTV and

collected over 21,000 ticks to screen for HRTV across three years in Georgia. We generated 6 new HRTV sequences and compared them to existing sequences from our group in Georgia, and across the country, finding evidence of regional clustering of HRTV and highly related HRTV across time in Georgia. Our analyses additionally found that this virus was likely introduced to the U.S. in the last 300 years. Our study provides new context and information in understanding the landscape and transmission of HRTV in the U.S.

## Introduction

In 2009, two farmers in northwestern Missouri presented with symptoms similar to ehrlichiosis and were found to have a novel pathogen, Heartland virus (HRTV).(1) Originally categorized as a *Phlebovirus*, HRTV was recategorized to the genus *Bandavirus* in the family *Phenuiviridae,* order *Bunyavirales* in 2020.(2) HRTV is vector-borne and transmitted by the lone star tick (*Amblyomma americanum*), whose range spans the majority of the eastern half of the United States.(3, 4) To date, HRTV has been associated with over sixty cases of human disease in fourteen U.S. states and has an observed case fatality rate of 5-10%.(5)

HRTV, like other bunyaviruses, has a segmented negative-sense single-stranded RNA genome with three segments.(1) The L segment encodes the RNA-dependent RNA polymerase (RdRp) and is 6.4 kb long. The M segment encodes two viral glycoproteins (Gn, Gc) and is 3.4 kb long. Lastly, the S segment employs an ambisense strategy to encode the nucleoprotein (Np) and nonstructural protein (NSs) in 1.7 kb. HRTV is most closely related to Dabie bandavirus (DBV) (previously known as Severe Fever and Thrombocytopenia Syndrome virus, SFTSV) which circulates in China, Japan, South Korea, Vietnam and Taiwan.(6) While different genotypes of

DBV have been documented across Asia(7) and have been associated with different pathogenicity in humans,(8) very little is known about the genotypic and phenotypic diversity of HRTV. This knowledge gap stems from limited surveillance, low HRTV prevalence in ticks, (9-11) and few detected human cases.(12)

HRTV has been identified in *A. americanum* populations in eight states across the U.S., spanning the Northeast, Southeast, and Midwest.(13) Prior surveillance studies for HRTV in ticks have been episodic, typically responding to newly discovered human cases, and continuing for one to two years.(9-11, 14) Few studies have continued surveillance beyond two years, and there is very little HRTV genome sequence data available, creating a gap in understanding the enzootic transmission and evolutionary dynamics of HRTV.

The objective of this study was to begin to fill this knowledge gap through long term surveillance at established HRTV-positive tick sites in central Georgia (GA), where our group initially detected HRTV in 2019.(15) In our prior study, we found a minimum infection rate of 0.46/1000 HRTV-positive *A. americanum* ticks at two locations where HRTV seropositivity was previously reported in deer populations,(16) and adjacent to the county where a human case was reported.(12) In the present study, we build upon this by performing extended HRTV surveillance in GA across multiple years. We use a novel multiplex amplicon sequencing approach to generate full HRTV genome sequences and analyze phylogenetic and phylogeographic relationships of HRTV in GA and across the U.S., gaining insight into potential mechanisms of virus transmission and evolution.

## Methods

Field collections

Ticks were collected from March-August 2021-2023, corresponding to the seasonality of *Amblyomma americanum* in Georgia.(17) Collection locations varied by year: in 2021, repeated sampling was conducted at the two sites where our group detected Heartland virus in 2019(15); in 2022, sampling was performed in forty-three locations across the state(18); and in 2023, five locations with the highest density of *A. americanum* from the previous years were repeatedly sampled to maximize potential HRTV detection. Free flagging was conducted at each site according to previously described protocols,(19) with the addition of transect sampling during 2022.(18) Ticks were transported live in plastic vials to the laboratory and microscopically identified with taxonomic keys.(20, 21)

Tick processing, RNA extraction, and qRT-PCR

Surface disinfection was conducted on ticks in field vials using a wash of 10% bleach for five minutes, 3% hydrogen peroxide for five minutes, and three rinses of distilled water for five minutes each. The ticks were pooled in groups of approximately five adults or twenty-five nymphs per species per site. Differing amounts of BA-1 diluent (1× medium 199 with Hanks balanced salt solution, 0.05 mole/L Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g sodium bicarbonate/L, 100 µg/L streptomycin, 1 µg/mL amphotericin B) were added to each pool based on the number of ticks in the pool (1 ml for >1 adult or >10 nymphs, 0.5 mL for 1 adult or <10 nymphs) to preserve concentrations of the ground ticks in media. Pools were ground thoroughly using a 7-mL glass TenBroeck grinder (Fisher Scientific) with alundum bedding material (Fisher Scientific) as an abrasive.

132 Tick homogenates underwent RNA extraction (QIAmp TNA Extraction Kit) following the
133 manufacturer's protocol (Supplemental Table S1). Extracted RNA was tested using quantitative
134 reverse-transcriptase PCR (qRT-PCR) with primer-probe set 1 for the S segment of HRTV
135 described by Savage et al.(10) The AgPath-ID One-Step RT-PCR kit (Thermo Fisher Scientific)
136 was used as per the manufacturer's instructions, with cycling conditions of 45°C for 10 minutes,
137 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 45 seconds. The reaction
138 mix and primer and probe sequences are available in Supplemental Table S2. Samples were
139 tested in duplicate with positive controls (extracted HRTV virus stock from Romer et al.(15))
140 and negative controls (water) on each plate. As a quality control check for RNA extraction, tick
141 actin RT-qPCR was run in parallel using the Applied Biosystems Power SYBR Green RNA-to-Ct
142 1-step PCR Kit (Fisher Scientific) with primers, probes, and reaction conditions listed in
143 Supplemental Table S3.
144
145 Infection rate estimation
146 The minimum infection rate (MIR) of HRTV was estimated by site and year by dividing the
147 number of positive pools at a location by the number of *A. americanum* of the same life stage
148 collected at the site and year and multiplying by 1,000.
149
150 HRTV multiplex amplicon sequencing primer design
151 Multiplex amplicon sequencing primers for HRTV were designed with primalscheme
152 (v1.4.1)(22) using an alignment of 4 (L) or 5 (M and S) complete reference HRTV
153 sequences.(23) Primers that had high annealing temperatures (65°C or greater) or had SNPs
154 expected to affect primer binding, as determined by comparison to additional partial genome

155 sequences, were redesigned in Geneious Prime (v2022.2). A set of 36 L, 18 M, and 10 S primer
156 pairs were created to cover the entire genome, with each primer pair amplifying an
157 approximately 250bp region (Supplemental Table S4). The HRTV-specific primer sequences
158 were concatenated with a universal Nextera adapter sequence to allow Nextera XT indexing and
159 amplification.(24, 25) Primers were synthesized by IDT, pooled, and tested using a previously-
160 sequenced HRTV-positive sample.(15) Primer concentrations were optimized to ensure
161 approximately equal coverage across the entire genome.
162
163 HRTV full genome multiplex amplicon sequencing
164 RNA samples that tested positive for HRTV via RT-qPCR underwent heat-labile dsDNase
165 treatment (ArticZymes), single-stranded cDNA synthesis (Fisher/Invitrogen) and multiplex PCR
166 using Q5 Hot Start High-Fidelity Polymerase (New England Biolabs) using the custom-designed
167 primers. The resulting amplicons were indexed using Nextera XT (Illumina) and quantified using
168 the KAPA Universal Complete Kit (Roche). Libraries were pooled to equimolar concentration
169 and sequenced with 300bp paired-end reads on an Illumina MiSeq. Reads were filtered for
170 quality, and adaptor sequences were removed, before undergoing reference-based assembly using
171 ViralRecon v. 2.6.0 with reference sequences L: NC_024495, M: NC_024494, and S:
172 NC_024496. A more complete protocol for this sequencing method can be found in
173 Supplemental Text S1.
174
175 Phylogenetic analysis
176 Twelve reference sequences of Heartland virus L, M, and S segments present in GenBank as of
177 March 1st, 2024 were downloaded for analysis. Selection criteria included: related to a field

178 collected case (tick or human), no identical isolates (if an isolate was sequenced more than once,
179 the earliest was selected), and >95% genome coverage. Newly-generated sequences and
180 reference sequences were aligned by segment in Geneious Prime (v2022.2.2) using MAFFT
181 (v7.450) with default parameters.(26, 27) Maximum-likelihood phylogenetic trees were
182 generated for each segment using PhyML 3.0,(28, 29) with best fit substitution models: GTR+G
183 for the L segment, HKY85+G for the M segment, and K80+I for the S segment. Trees were
184 subsequently visualized and annotated using iTOL(30). Reassortment was assessed through two
185 methods 1) by visually comparing segment relationships across different clades within the trees
186 and 2) by testing aligned concatenated coding regions with the recombination detection program,
187 RDP4.(31)
188
189 Bayesian time-scaled phylogenetic analysis
190 To construct time-scaled phylogenies, the temporal signal of each segment's coding region was
191 evaluated using TempEST(32), and one outlier was removed using a threshold of 0.03 with
192 unbalanced residuals on the residual histogram distribution (reference sequence OK480062.1,
193 Supplemental Figure S1). There was evidence for moderate temporal signal that varied by
194 segment, with root-to-tip correlation coefficients of 0.59 for the L segment, 0.55 for the M
195 segment, and 0.33 for the S segment. Time-scaled phylogenies were therefore constructed only
196 for the L and M segments, using BEAST 2.7.6 with a chain length of 600 million.(33) We used a
197 GTR+gamma nucleotide substitution model, a constant coalescent population, and a strict
198 molecular clock with a prior of 2E-4,(34-36) with a lognormal distribution. The log files were
199 examined with Tracer v1.7.2 and confirmed to have ESS values >200 for all parameters.(37)
200 Maximum clade credibility trees were generated with 10% burn-in using TreeAnnotator

201 v2.7.6.(33) More complex models, including a relaxed molecular clock and skyline models of
202 population growth, did not converge, likely as a result of the limited HRTV data available.
203
204 Geographic clustering analysis
205 The Mantel test(38) was used to assess the relationship between geographic distance and genetic
206 divergence. Because exact coordinates were not known for the reference sequences, the location
207 was estimated using the finest resolution information present. For samples with only a state
208 recorded for the source location in GenBank, the center point of the most central county in the
209 state was assigned, using USGS(39) and Latitude.to (v1.64-im). For samples with a county and a
210 state recorded for the source location in GenBank, the center of the county listed for that
211 sequence was assigned. For sequences associated with publications, the smallest resolution
212 estimate was made based on recorded location information.(9, 11, 40) Lastly, for the two initial
213 Missouri human case sequences, Andrews county and Nodaway county centers were used based
214 on Savage et al.(40) The Mantel test was run using the vegan package (v2.5-7) in R (v4.0.4)
215 using 1000 permutations.
216
217 Host bloodmeal analysis
218 Nucleic acid extracted from HRTV-positive *A. americanum* pools and matched HRTV-negative
219 *A. americanum* pools – containing a similar number of ticks, and the same life stage, collection
220 site, and collection date – were tested by multiplex qPCR targeting retrotransposons of potential
221 hosts, according to established protocols.(41, 42) Species included in the assay were: mice,
222 voles, rabbits, birds, shrews, squirrels/chipmunks, deer, skunks and opossums.
223

## Results

Six HRTV positive tick pools were detected across central and western Georgia, U.S., from 2021 to 2023

In total, 21,386 ticks were collected across the three year period: 7,687 in 2021, 3,444 in 2022, and 10,255 in 2023 (Table 1). Most (20,959, 98%) were *A. americanum* adults or nymphs. Six pools of *A. americanum* ticks tested positive for Heartland virus: 1 pool of 19 nymphs from 2021, 2 pools of 27 nymphs and 28 nymphs from 2022, and 3 pools of 25 nymphs, 5 adult females, and 25 nymphs in 2023 (Table 2). Five of the six HRTV-positive pools were collected at ST road site (ST) in central GA, and the sixth was collected at Chattahoochee Bend State Park (CB) in 2023 (Figure 1). The minimum infection rates (MIRs) calculated for each site and year were 0.33 per 1000 nymphs at ST in 2021, 1.97 per 1000 nymphs at ST in 2022, 0.59 per 1000 nymphs at ST in 2023, 2.56 per 1000 adults at ST in 2023 and 0.46 per 1000 nymphs at CB in 2023.

**Table 1. Species and life stage of ticks collected during field sampling from 2021-2023**

| Species | Stage | 2021 | 2022 | 2023 |
|---|---|---|---|---|
| *Amblyomma americanum* | Adult | 1984 | 600 | 1109 |
| *Amblyomma americanum* | Nymph | 5584 | 2708 | 8974 |
| *Dermacentor variabilis* | Adult | 100 | 54 | 19 |
| *Amblyomma maculatum* | Adult | 19 | 13 | 14 |
| *Ixodes scapularis* | Adult | 0 | 66 | 64 |
| *Ixodes* species | Nymph | 0 | 0 | 8 |
| *Haemaphysalis longicornis* | Nymph | 0 | 3 | 67 |
| **Total** | | 7687 | 3444 | 10255 |

**Table 2. Heartland positive pool characteristics**

| Year | Lab Code | Location | Date Collected | Number of Ticks | Life Stage |
|---|---|---|---|---|---|
| 2019* | 23 | KH | 4/28/19 | 5 | Adult (Female) |
| 2019* | 26 | KH | 4/28/19 | 25 | Nymph |
| 2019* | 504 | ST | 6/14/19 | 5 | Adult (Male) |

| | | | | | |
|---|---|---|---|---|---|
| 2021 | 404 | ST | 6/15/21 | 19 | Nymph |
| 2022 | 100 | ST | 5/12/22 | 27 | Nymph |
| 2022 | 187 | ST | 6/10/22 | 28 | Nymph |
| 2023 | 56F | ST | 5/12/23 | 25 | Nymph |
| 2023 | 432R | ST | 6/16/23 | 5 | Adult (Female) |
| 2023 | 642T | CB | 7/19/23 | 25 | Nymph |

All pools consist of *Amblyomma americanum*. Location abbreviations: KH = KH road site, ST = ST road site, CB = Chattahoochee Bend State Park. *2019 HRTV-positive pools were originally published in Romer et al.(15)

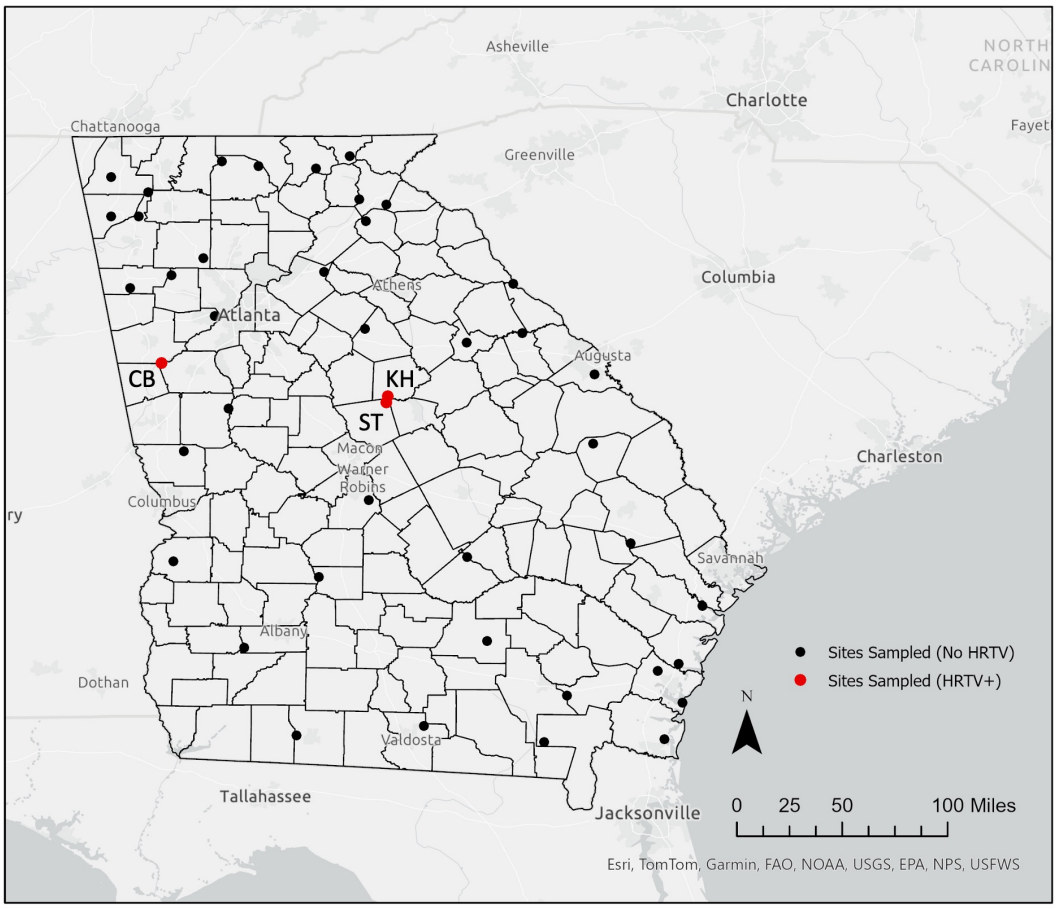



**Figure 1. Map of collection sites visited from 2021-2023**. State and county boundaries for Georgia are highlighted in black. Black dots represent locations sampled where HRTV positive pools of ticks were not found. Red dots represent locations where HRTV positive pools were found with each location labeled with its two letter site code (KH = KH road site, ST = ST road site, CB = Chattahoochee Bend State Park). Though no positive pools of HRTV were found from 2021-2023, KH is still highlighted due to presence of positive ticks from 2019 detected by our group as originally documented in Romer et al(15) and described in Table 2. Map created in ArcGIS Pro v3.1.0.

255 HRTV sequences from GA are highly related across space and time

256 Full HRTV genome sequences were generated using our novel HRTV-specific multiplex
257 amplicon protocol, with 97-100% coverage achieved across all three segments from the six
258 positive tick pools. Including the three HRTV positive tick pools collected and sequenced by our
259 group in 2019 from sites ST and KH,(15) the nine GA sequences were highly similar with 99.52-
260 99.99% nucleotide identity across the entire genome and similar identity relationships across the
261 individual segments (Table 3). The largest genetic differences were seen between the sample
262 collected at site CB in 2023 (Sample 642T) and other samples from GA, collected approximately
263 175 km away. Sample 642T differed from the other GA sequences by 44-51 single nucleotide
264 polymorphisms (SNPs), while the sequences from sites KH and ST differed from one another by
265 only 1-27 SNPs (Table 3). Few non-synonymous SNPs were found in sample 642T: 4 (of 24 total
266 SNPs) in the L segment, 1 (of 13) in the M segment, and none in the S segment. Interestingly,
267 while three of these non-synonymous SNPs were unique to sample 642T, two SNPs in the L
268 segment (T168A and R183K) were shared with the other twelve reference sequences obtained
269 from across the country.

270
271
272
273

**Table 3. Pairwise genetic distances between HRTV sequences in Georgia**

| Location | | Site KH | | Site ST | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HRTV-23_T_GA_KH_2019 | HRTV-26_T_GA_KH_2019 | HRTV-100_T_GA_ST_2022 | HRTV-404_T_GA_ST_2021 | HRTV-187_T_GA_ST_2022 | HRTV-504_T_GA_ST_2019 | HRTV-056F_T_GA_ST_2023 | HRTV-432R_T_GA_ST_2023 |
| Site KH | HRTV-26_T_GA_KH_2019 | 3 (99.97%) | | | | | | | |
| Site ST | HRTV-100_T_GA_ST_2022 | 10 (99.91%) | 7 (99.93%) | | | | | | |
| | HRTV-404_T_GA_ST_2021 | 8 (99.92%) | 7 (99.93%) | 2 (99.98%) | | | | | |
| | HRTV-187_T_GA_ST_2022 | 11 (99.9%) | 8 (99.92%) | 1 (99.99%) | 3 (99.97%) | | | | |
| | HRTV-504_T_GA_ST_2019 | 23 (99.78%) | 22 (99.79%) | 23 (99.78%) | 21 (99.8%) | 24 (99.77%) | | | |
| | HRTV-056F_T_GA_ST_2023 | 11 (99.9%) | 10 (99.91%) | 7 (99.93%) | 7 (99.93%) | 8 (99.92%) | 26 (99.75%) | | |
| | HRTV-432R_T_GA_ST_2023 | 12 (99.89%) | 11 (99.9%) | 8 (99.92%) | 10 (99.91%) | 9 (99.91%) | 27 (99.74%) | 7 (99.93%) | |
| Site CB | HRTV-642T_T_GA_CB_2023 | 47 (99.56%) | 44 (99.58%) | 46 (99.56%) | 46 (99.56%) | 47 (99.56%) | 51 (99.52%) | 47 (99.56%) | 46 (99.56%) |

Number of SNPs and percent nucleotide identity is displayed across the concatenated genome of the three segments excluding gaps. Background color gradient represents nucleotide identity with darker colors representing higher nucleotide identity. Sites are delineated by bolded lines.

When comparing all twenty-one HRTV sequences from the U.S., the nucleotide identity ranged from 92.76-99.99% across the three segments, equating to between 1 and 782 SNP differences (Supplemental Table S5). Across the HRTV genome, the GA sequences shared 70 SNPs (35 in L, 20 in M, 12 in S), 6 of which (1 in L, 2 in M, 3 in S) were non-synonymous. While the GA sequences were all derived from tick pools, the twelve available reference sequences from GenBank were from both tick pools (5) and human infections (7). There were no SNPs common to HRTV sequences from only ticks or only humans.

289 U.S. HRTV sequences demonstrate geographic clustering and potential reassortment

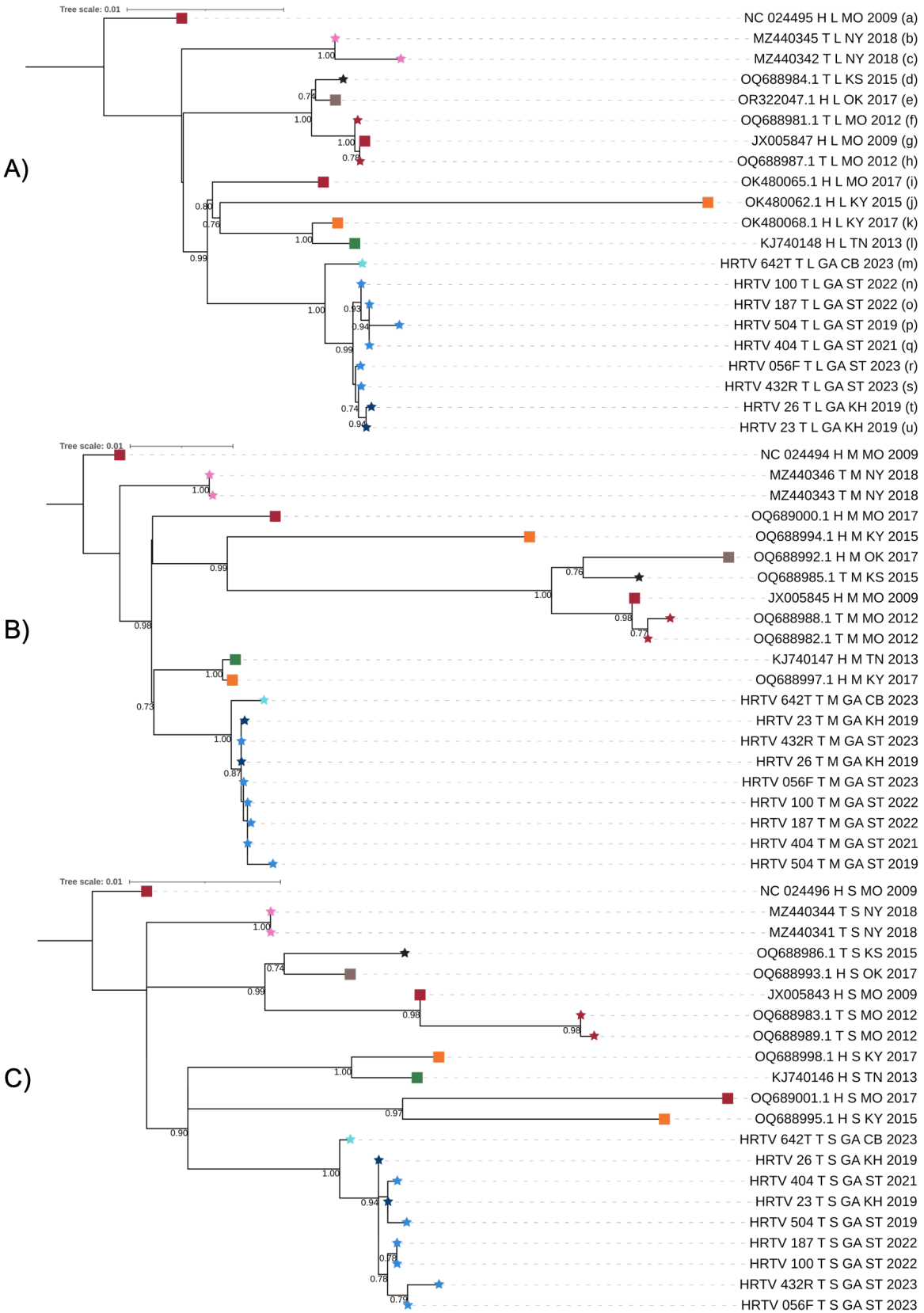
290 The 21 U.S. HRTV sequences generally clustered geographically on maximum-likelihood
291 phylogenetic trees for each segment (Figure 2). Across all trees, the GA sequences clustered
292 together and the sample collected at western site CB (HRTV 642T) was the most divergent. The
293 two sequences from NY ticks similarly clustered together across all segments. Furthermore,
294 samples from neighboring states generally clustered together (OK/KS/MO and TN/KY) in each
295 segment, regardless of date of sample collection or host. The Mantel test also supported
296 geographic clustering, with a correlation of 0.53 (p-value=0.001) between genetic distance and
297 spatial distance.

298

299 While the overall phylogenetic structure was similar across segments, we observed two examples
300 of potential reassortment between segments. First, sequences OQ688994-5, collected from a
301 human case in KY in 2015, clustered with the other KY and TN sequences in the L segment but
302 with the MO, OK, and KS clade in the M segment, and with a single MO sequence in the S
303 segment, all with moderate to high ultrafast bootstrap support (0.76, 0.99, 0.97 respectively). The
304 second example of potential reassortment is from our sample 432R, collected at site ST in 2023.
305 It clusters with the site KH sequences from 2019 (23 and 26) in the L segment, but with the other
306 site ST sequences (187, 100, 056F) in the S segment, with moderate ultrafast bootstrap support
307 (0.74, 0.79). These differences in tree topology may indicate reassortment, as has been reported
308 for the related Dabie bandavirus. RDP4 was also used to assess for reassortment events between
309 the HRTV segments and did not identify any events with high certainty.

310

311


A)
Tree scale: 0.01
NC 024495 H L MO 2009 (a)
MZ440345 T L NY 2018 (b)
MZ440342 T L NY 2018 (c)
OQ688984.1 T L KS 2015 (d)
OR322047.1 H L OK 2017 (e)
OQ688981.1 T L MO 2012 (f)
JX005847 H L MO 2009 (g)
OQ688987.1 T L MO 2012 (h)
OK480065.1 H L MO 2017 (i)
OK480062.1 H L KY 2015 (j)
OK480068.1 H L KY 2017 (k)
KJ740148 H L TN 2013 (l)
HRTV 642T T L GA CB 2023 (m)
HRTV 100 T L GA ST 2022 (n)
HRTV 187 T L GA ST 2022 (o)
HRTV 504 T L GA ST 2019 (p)
HRTV 404 T L GA ST 2021 (q)
HRTV 056F T L GA ST 2023 (r)
HRTV 432R T L GA ST 2023 (s)
HRTV 26 T L GA KH 2019 (t)
HRTV 23 T L GA KH 2019 (u)
B)
Tree scale: 0.01
NC 024494 H M MO 2009
MZ440346 T M NY 2018
MZ440343 T M NY 2018
OQ689000.1 H M MO 2017
OQ688994.1 H M KY 2015
OQ688992.1 H M OK 2017
OQ688985.1 T M KS 2015
JX005845 H M MO 2009
OQ688988.1 T M MO 2012
OQ688982.1 T M MO 2012
KJ740147 H M TN 2013
OQ688997.1 H M KY 2017
HRTV 642T T M GA CB 2023
HRTV 23 T M GA KH 2019
HRTV 432R T M GA ST 2023
HRTV 26 T M GA KH 2019
HRTV 056F T M GA ST 2023
HRTV 100 T M GA ST 2022
HRTV 187 T M GA ST 2022
HRTV 404 T M GA ST 2021
HRTV 504 T M GA ST 2019
C)
Tree scale: 0.01
NC 024496 H S MO 2009
MZ440344 T S NY 2018
MZ440341 T S NY 2018
OQ688986.1 T S KS 2015
OQ688993.1 H S OK 2017
JX005843 H S MO 2009
OQ688983.1 T S MO 2012
OQ688989.1 T S MO 2012
OQ688998.1 H S KY 2017
KJ740146 H S TN 2013
OQ689001.1 H S MO 2017
OQ688995.1 H S KY 2015
HRTV 642T T S GA CB 2023
HRTV 26 T S GA KH 2019
HRTV 404 T S GA ST 2021
HRTV 23 T S GA KH 2019
HRTV 504 T S GA ST 2019
HRTV 187 T S GA ST 2022
HRTV 100 T S GA ST 2022
HRTV 432R T S GA ST 2023
HRTV 056F T S GA ST 2023

312

D)

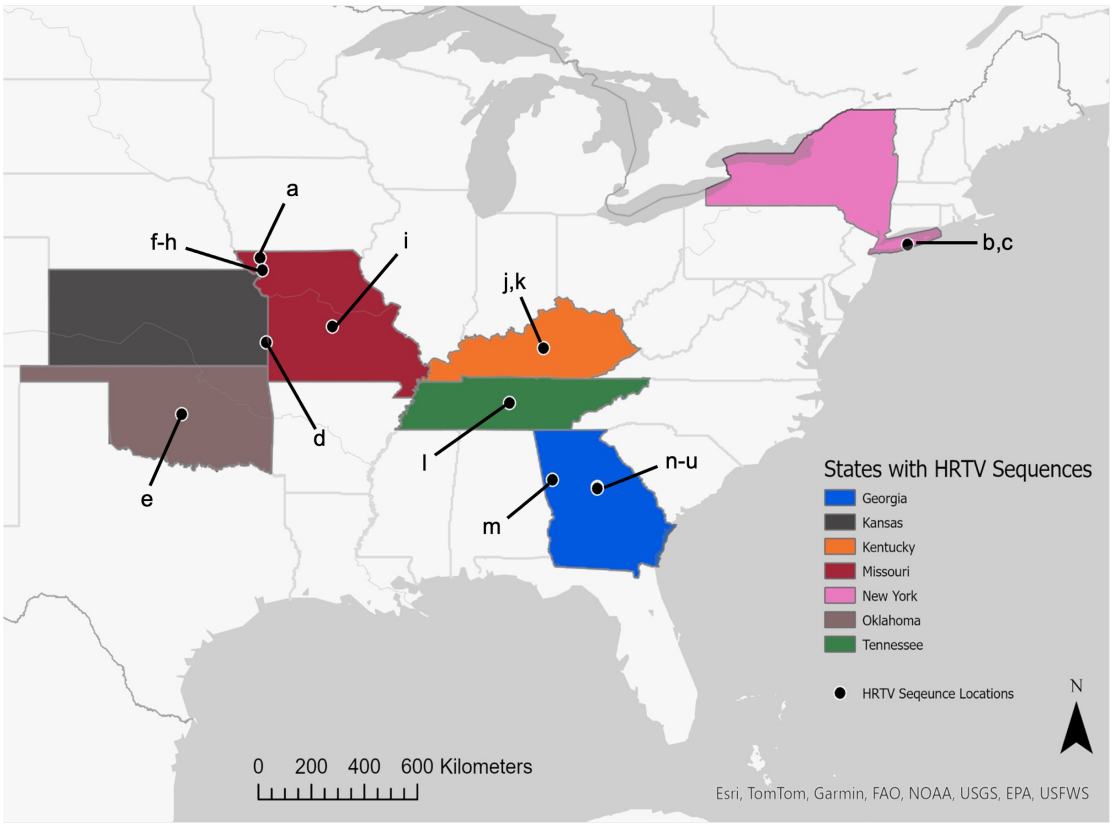



**Figure 2: Phylogenetic and geographic relationships of HRTV sequences in the U.S.** Segments were aligned separately, and maximum likelihood trees are displayed for the L segment (A), M segment (B), and S segment (C). Reference sequences from GenBank were named by the accession code followed by T (tick sample) or H (human sample), the segment, the state abbreviation for the collection location, and the year of collection. Sequences generated in this study were named by HRTV, sample number, T, GA, collection site, and year collected. All trees were rooted on the reference sequence from MO Patient 1. Ultrafast bootstrap values above 0.7 are displayed on the tree. Tips are labeled with a star for tick samples and square for human samples. The tip color indicates the location of collection. D) A map of the Eastern United States with states colored as in the phylogenetic trees (panels A-C). Locations for each sequence included are marked with a black dot. Dots are placed where either 1) sequences locations were documented in the literature(9, 11, 40) or 2) in the center of the state/county reported when finer resolution location data was unavailable. Dots are additionally marked with a-u and correspond to the sequences in the tree marked in panel A. Map created in ArcGIS Pro v3.1.0.

<u>Contemporary HRTV strains in the U.S. emerged within the last 300 years</u>

Time-scaled Bayesian phylogenetic analysis showed an evolutionary rate of 1.1 x$10^{-4}$ substitutions/site/year (s/s/y) in the L segment and 1.8 x$10^{-4}$ s/s/y in the M segment; this was not

333 performed for the S segment given the lower temporal signal observed. Based on the L segment,
334 the time to most recent common ancestor (tMRCA) for the GA clade was 23 years ago (95%
335 HPD 12-42); the tMRCA for the GA clade and its nearest neighbor, the MO/KY/TN clade, was
336 85 years ago (95% HPD 46-153); and the overall root of the tree was 105 years ago (95% HPD
337 57-187) (Figure 3). Based on the M segment, the tMRCA for the GA clade was 16 years ago
338 (95% HPD 8-29); the tMRCA for the GA clade and its nearest neighbor, the KY/TN clade, was
339 56 years ago (95% HPD 31-99); and the overall root of the tree was 150 years ago (95% HPD
340 78-286) (Figure 3).
341

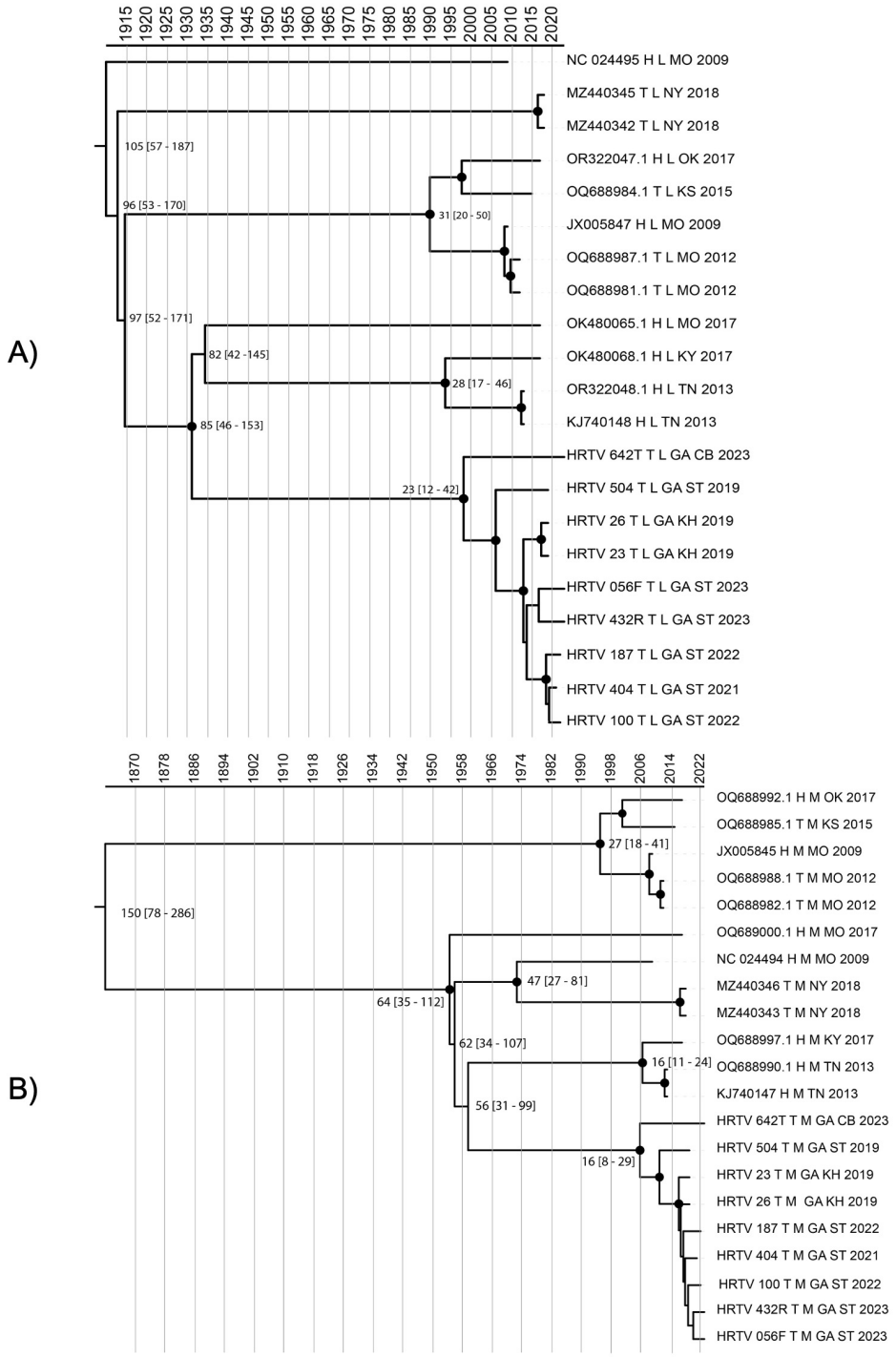



**Figure 3: Time-scaled Bayesian phylogenetic tree of HRTV segments L (A) and M (B).** Nodes are labeled with median time to recent common ancestor (tMRCA) with [95% HPD].

346 *Amblyomma americanum* ticks feed on a variety of wildlife in Georgia

347 To begin to evaluate potential reservoir species for HRTV, a total of 12 *A. americanum* pools
348 from GA underwent host bloodmeal analysis using retrotransposon qPCR. Each HRTV-positive
349 pool was matched with an HRTV-negative pool with ticks of the same life stage, collection site,
350 and collection date (Table 4) . The HRTV-positive pools of nymphs from ST (samples 404, 187,
351 100, and 56F) tested positive for birds, deer, rabbits, and squirrels/chipmunks. Their matched
352 controls from HRTV-negative pools had similar results, with two exceptions: HRTV-negative
353 pool 403 was positive for rabbit while its matched HRTV-positive pool 404 was not, and HRTV-
354 positive pool 56F was positive for squirrel/chipmunk, while its matched HRTV-negative pool
355 55E was not. Both pools of adult ticks from site ST tested negative for most hosts, other than
356 rabbit in the HRTV-negative pool. Finally, the nymph pools from CB both tested positive for
357 deer, and the HRTV-negative pool additionally tested positive for squirrels/chipmunks.

358

359 **Table 4. Host Bloodmeal PCR results**

| Sample Name | HRTV Status | Tick Stage (n) | Location, Year | Rabbit PCR | Bird PCR | Squirrel/ Chipmunk PCR | Deer PCR |
|---|---|---|---|---|---|---|---|
| 403 | - | Nymph (25) | ST, 2021 | + | + | - | + |
| 404 | + | Nymph (19) | ST, 2021 | - | + | - | + |
| 186 | - | Nymph (27) | ST, 2022 | - | - | - | + |
| 187 | + | Nymph (28) | ST, 2022 | - | - | - | + |
| 99 | - | Nymph (26) | ST, 2022 | + | + | + | + |
| 100 | + | Nymph (27) | ST, 2022 | + | + | + | + |
| 55E | - | Nymph (25) | ST, 2023 | + | + | - | + |
| 56F | + | Nymph (25) | ST, 2023 | + | + | + | + |
| 432R | + | Adult (5) | ST, 2023 | - | - | - | - |
| 433S | - | Adult (5) | ST, 2023 | + | - | - | - |
| 641S | - | Nymph (25) | CB, 2023 | - | - | + | + |
| 642T | + | Nymph (25) | CB, 2023 | - | - | - | + |

360 The following species with no positive results were not included in the table: mice, voles,
361 shrews, skunks, and opossums.

## Discussion

We identified a persistent focus of genetically conserved HRTV in central Georgia. We collected over 21,000 ticks across three years and found minimum infection rates (MIRs) ranging from 0.33 per 1000 nymphs in 2021 to 2.56 per 1000 adults in 2023, compatible with our prior work(15) and other studies.(40, 43) Using a novel multiplex amplicon sequencing assay, we generated six new HRTV genome sequences and examined phylogenetic relationships across multiple years and locations.

One key finding was the very high genetic conservation of HRTV from ticks collected at a focal site in central GA across five years. Studies of Powassan virus, an unrelated tick-borne virus also endemic to the U.S., have similarly reported foci of genetically conserved viruses that were stable across multiple years, but have also reported geographic dispersal of closely-related POWV strains.(44-46) We did not observe geographic dispersal of closely-related HRTV strains, instead we observed phylogenetic clustering by location throughout the U.S., though it is notable that sequence data are limited, with only 12 reference sequences available from elsewhere in the U.S. and our 9 from Georgia.

A second key finding was that time-scaled phylogenetic analysis indicated recent spread of HRTV in the U.S., with all 21 sequences sharing a common ancestor within the last 300 years, and sequences from Georgia sharing a common ancestor within the last 40 years. This timeline is compatible with historical patterns of *A. americanum* prevalence.(47) In the 18$^{th}$ century, dense forest supported high populations of deer and associated ticks, however deforestation and decimation of deer populations in the 19$^{th}$ century led to large-scale *A. americanum* range

386 retraction. The resulting fracturing of tick populations could have generated geographically
387 isolated HRTV lineages that diverged over the last several hundred years. Supporting this, there
388 is evidence for geographic population structure within *A. americanum* ticks between different
389 regions of the U.S.(48)
390
391 Overall, our phylodynamic results for HRTV mirror what has been reported for the closely
392 related Dabie bandavirus (DBV) in Asia. Our estimated evolutionary rate for HRTV, 1.1-1.8 $x10^{-}$
393 $^{4}$ s/s/y, is very similar to the DBV literature, where studies report rates ranging from 1.07 $x10^{-4}$ to
394 1.09 $x10^{-3}$ s/s/y across the three segments, with most estimates around 2 $x10^{-4}$.(34-36, 49, 50)
395 Similar to our results, many DBV studies have shown distinct genotypes clustering by
396 geographic region.(49, 51-53) Interestingly, however, several recent studies also report mixing of
397 DBV genotypes across different countries in Asia,(7, 36) which is thought to be due to migratory
398 bird movement carrying the DBV vector, *Haemaphysalis longicornis*, across longer
399 distances.(54)
400
401 Despite the small number of HRTV sequences, our phylogenetic analysis suggests the possibility
402 of reassortment between genome segments, a common mechanism of increasing genetic diversity
403 in bunyaviruses and other segmented viruses.(55) Reassortment has been frequently described
404 for DBV, allowing new genotypes to emerge after co-infection events.(7, 8, 56, 57) This is
405 important because reassorted viruses have the ability to overcome host barriers and have the
406 potential to generate strains of higher pathogenicity as seen in influenza virus.(55, 58) Because
407 reassortment requires multiple viruses to infect the same cell, it would be expected to occur less
408 frequently in viruses with low prevalence. Thus, our findings are surprising in light of the

409 reported low HRTV prevalences in our study and others, underscoring the need for expanded
410 surveillance and genetic studies of HRTV throughout the U.S.
411
412 Our observation of high HRTV genetic conservation at a focal site across multiple years suggests
413 several possible hypotheses about the enzootic cycle of HRTV, which remains largely
414 unknown.(3) One possibility is that closely related viruses may be sustained within the
415 population through transovarial transmission (also referred to as vertical transmission), as has
416 been documented in experimental studies of HRTV.(59) In this scenario, infected female *A.*
417 *americanum* would lay eggs at the site, passing on virus to their larvae, which would feed on
418 small-range hosts and remain within the site to molt and be captured as nymphs the following
419 year. Vertical transmission typically augments other transmission mechanisms and has not been
420 proposed as a sole mechanism for persistence of other tick-borne viruses.(60) Testing larval
421 populations for HRTV is an important next step in investigating this mechanism.
422
423 A second potential mechanism for HRTV focality involves propagation of virus through
424 cofeeding transmission, which has also been documented experimentally for HRTV(59) and is
425 important in other tick-borne viruses such as tick-borne encephalitis virus (TBEV).(61) In this
426 case, infected *A. americanum* feed next to uninfected ticks, leading to HRTV transmission
427 through infection of local skin and leukocytes. Co-feeding transmission provides a role for hosts
428 that are not competent for viremic infection themselves, as demonstrated for *Borrelia* species
429 that feed on hosts whose innate complement systems prevent systemic infection.(62) Cofeeding
430 on hosts with limited range could explain the persistence of highly related viruses across multiple
431 years.

432 Finally, HRTV could propagate through horizontal transmission by ticks feeding on infected
433 reservoir hosts. Serosurveys and experimental infections investigating potential HRTV reservoir
434 hosts have not yet identified any candidates.(63, 64) Evidence of seropositivity has been found in
435 multiple wildlife species with white-tailed deer, raccoons, horses, and dogs showing the highest
436 rates of neutralizing antibodies across studies.(3, 16, 65) However, experimental infection of
437 multiple wildlife hosts including white-tailed deer, raccoons, goats, chickens, rabbits, hamsters,
438 and C57BL/6 mice have failed to produce detectable viremia, except in an interferon receptor-
439 deficient mouse model.(63) Together, this information suggests that these animals may be
440 incidental hosts, and there is not yet evidence for a viremic reservoir host to support horizontal
441 transmission of HRTV. If there is a role for horizontal or cofeeding transmission in the HRTV
442 enzootic cycle, our HRTV phylogenetic analysis suggests the importance of geographically
443 restricted hosts. Our host bloodmeal analysis provides some possibilities, as *A. americanum* ticks
444 at our focal site in central Georgia fed on deer and rabbits (which do not support HRTV viremia
445 in experimental infection studies), as well as squirrels/chipmunks and birds (which have not been
446 evaluated in experimental infection studies). While we did not observe a clear difference
447 between HRTV-positive and negative samples, this analysis was limited by our analysis of tick
448 pools rather than individual ticks, and it is possible that yet-untested hosts are important.
449
450 In conclusion, through extensive field work and the use of a novel multiplex amplicon
451 sequencing assay, we have gained important insight into HRTV diversity and evolution. Key
452 findings are that HRTV sequences from a focal site in GA were highly related across time; that
453 there is strong geographic clustering of HRTV across the U.S. with this attribute being more
454 important than host or year; and that all available HRTV sequences share a common ancestor

within the recent past, suggesting that contemporary strains of HRTV emerged within the last 300 years. Our work offers potential insights into the enzootic cycle of HRTV and provides a foundation for further investigation.

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Acknowledgements

We thank undergraduate and graduate students from the Environmental Sciences Department at Emory University and Rollins School of Public Health for assistance with field work. We

651 additionally thank Yamila Romer, Kayla Adcock and Daniel Mead for their initial work on
652 Heartland virus detection and isolation from ticks in Georgia.
653

654 Author Contributions

655 SB, GVP, and AP conceived of the study. Field collections were conducted by SB, NS, AL, LA,
656 EF, TW, SC, IR, and MS. Lab processing, amplicon sequencing design and sequencing was
657 completed by SB, NS, EO, MS and HD. Phylogenetic analyses were conducted by SB, TF, and
658 AP. Host bloodmeal analysis was conducted by HKG and SRT. All authors read, edited, and
659 approved the final manuscript.
660

661 Competing Interests Statement

662 The authors declare they have no competing interests