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Beyond Rocky Mountain spotted fever: investigation of the presence and diversity of spotted fever *Rickettsia* species in ticks submitted from forestry workers

Vishvapali C. Kobbekaduwa*[,1], Jennifer G. Chandler[1], James T. Vogt[2], and Rebecca T. Trout Fryxell[1]

[1]Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN, USA
[2]USDA Forest Service, Southern Research Station, Athens, GA, USA

*Corresponding author. Department of Entomology and Plant Pathology, University of Tennessee, 370 Plant Biotechnology Building, 2505 E J Chapman Drive, Knoxville, TN 37996, USA (Email: vishvapali@gmail.com).

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Ticks present a significant risk to people in the southern United States, particularly those who spend time outdoors, as ticks can transmit agents that cause various diseases. This study evaluated the risk of exposure to ticks positive for spotted fever group (SFG) *Rickettsia* species among forestry workers. From 2017 to 2021, forestry workers passively collected ticks during field surveys for the USDA Forest Service's Forest Inventory and Analysis program. We screened 1395 ticks for SFG-*Rickettsia*, including *Amblyomma americanum* (51.5% positive, *N* = 1,279), *A. maculatum* (40% positive, *N* = 10), and *Dermacentor variabilis* (22.6%, *N* = 106). The agent of Rocky Mountain spotted fever, *R. rickettsii*, was not detected; however, 7 different SFG *Rickettsia* species were identified. *Rickettsia amblyommatis* was the most common, present in 95.7% of *A. americanum* ticks. The pathogenic *R. parkeri* was rare, detected in 2 *A. americanum* nymphs and 2 *A. maculatum* males only. Several *Rickettsia* species, such as *R. montanensis*, *R. monacensis*, *Candidatus* R. andeanae, and *R. tamurae* subsp. *buchneri*, were identified in *D. variabilis*. Some of these species are suspected to be pathogenic. *Rickettsia*-positive ticks were detected year-round, with the highest prevalence in Tennessee and Kentucky, possibly due to larger sample submissions, which may have increased detection rates. *Dermacentor variabilis* were less likely to be *Rickettsia*-positive compared to *A. americanum*. Male ticks were less likely to carry *Rickettsia* than females and nymphs. The presence of *Rickettsia*-positive ticks found in this study poses a risk to forestry workers, emphasizing the importance of ongoing surveillance and education to prevent tick-borne infections.

Keywords: spotted fever group rickettsiosis, ticks, southeastern United States, forestry workers

Introduction

The risk of encountering ticks and contracting tick-borne diseases has risen considerably in the United States due to factors such as increasing tick populations, climate change, land use changes, wildlife movements, and increased participation in outdoor recreation activities (Eisen et al. 2017, Rosenberg et al. 2018, Beard et al. 2021, Eisen and Paddock 2021). While Lyme disease remains the most frequently reported vector-borne disease in the United States, cases of other tick-borne diseases—including human granulocytic anaplasmosis (de la Fuente et al. 2023), babesiosis (Bloch et al. 2022, Ssentongo et al. 2024, Gray and Herwaldt 2019), ehrlichiosis (Fleshman et al. 2022), and Rocky Mountain spotted fever (RMSF)—have also increased in the United States (Dahlgren et al. 2012, Biggs et al. 2016). Ticks are estimated to transmit about 75% of vector-borne disease infections in the United States (Eisen et al. 2017, Beard et al. 2021, Eisen and Paddock 2021, Eisen 2022, de la Fuente et al. 2023). In the Southern United States, spotted fever group rickettsioses (SFGRs) are the most reported tick-borne disease (Fritzen et al. 2011, Biggs et al. 2016, Drexler et al. 2016, Rosenberg et al. 2018, Bishop et al. 2022, Zhang et al. 2023), with this region contributing to about 68% of the total SFGR cases in the United States (Adjemian et al. 2009, Drexler et al. 2016, Bishop et al. 2022). These cases are primarily reported from Arkansas, North Carolina, Oklahoma, Missouri, and Tennessee (Biggs et al. 2016, Drexler et al. 2016, Rosenberg et al. 2018, Bishop et al. 2022).

SFGRs are caused by intracellular bacteria in the genus *Rickettsia*; however, not all *Rickettsia* species are pathogenic. Typical SFGR symptoms include flu-like symptoms, with some cases presenting with a rash or eschar (Biggs et al. 2016). While many SFG *Rickettsia* species have been isolated from ticks, many are tick endosymbionts and do not cause human disease (Parola et al. 2013, Biggs et al. 2016, Fang et al. 2017, Zhang et al. 2023). Among human pathogens, RMSF, caused by *R. rickettsii*, is the most severe and can be fatal if not treated promptly

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(Dahlgren et al. 2012, Biggs et al. 2016, Drexler et al. 2016, Rosenberg et al. 2018, Jay and Armstrong 2020). *Rickettsia parkeri* is another known human pathogen, typically causing milder symptoms than RMSF (Paddock et al. 2004, Moo-Llanes et al. 2021, Silva-Ramos et al. 2021). *Rickettsia amblyommatis* is another SFG *Rickettsia* species, commonly found in ticks and identified as an endosymbiont (Stothard 1995, Karpathy et al. 2016), which may potentially cause mild human disease (Billeter et al. 2007, Snellgrove et al. 2021, Yen et al. 2021, Richardson et al. 2023), but further research is needed to determine its pathogenicity.

Distinguishing between different SFG *Rickettsia* species is challenging because diagnosis relies on amplifying gene fragments and DNA sequencing (Husin et al. 2021, Stewart and Stewart 2021). This often results in misdiagnosis, with less severe SFGRs sometimes being mistaken for RMSF, especially since they share similar clinical signs and treatments (La Scola and Raoult 1997, Husin et al. 2021, Stewart and Stewart 2021). More research is necessary to fill the discrepency between the number of human spotted fever rickettsiosis cases and the low detection rates of *R. rickettsii* and other pathogenic *Rickettsia* in field-collected ticks.

Individuals at higher risk of contracting tick-borne diseases often spend significant time in wooded areas where ticks are common (Piacentino and Schwartz 2002, Di Renzi et al. 2010, Donohoe et al. 2018, Magnavita et al. 2022, Roome et al. 2022, Schielein et al. 2022, Stufano et al. 2022). As an occupational hazard, forestry workers frequently report tick bites and related infections, with about half having experienced tick contact or contracted a tick-borne disease (Covert and Langley 2002, Cisak et al. 2012, Roome et al. 2022, Knecht et al. 2024, Douglas and Scott 2025). Although many studies have assessed tick-borne disease risks among forestry workers, most have been conducted in Europe, primarily due to the prevalence of tick-borne encephalitis and evaluations of vaccine effectiveness (Beauté et al. 2018, Riccò et al. 2020, Hofhuis et al. 2021, Miazga et al. 2023). In contrast, despite the relatively high incidence of RMSF in the Southern United States, few studies have examined the potential risk of tick-borne diseases for forestry workers in this region. The increasing tick populations across the United States have heightened the risk of exposure to tick-borne pathogens for forestry workers (Adjemian et al. 2012, Geissler et al. 2014, Wallace et al. 2016, Trout Fryxell and Vogt 2019, Schotthoefer et al. 2020, Roome et al. 2022).

Recognizing the significant occupational exposure of forestry workers, we partnered with the USDA Forest Service's Forest Inventory and Analysis (FIA) program (Bechtold and Patterson 2005, Trout Fryxell and Vogt 2019) to conduct a multi-year, geographically extensive passive surveillance of ticks found by forest crews routinely working in wooded environments. These crews spend much of their workday in forests, which increases their risk of encountering ticks and contracting SFGR compared to other jobs. Our study hypothesized that these crews encounter ticks infected with various spotted fever group *Rickettsia* species, consistent with the known epidemiology of SFGR in the Southern United States. Our goals included identifying common *Rickettsia* species, determining their infection prevalence, analyzing seasonal patterns and distribution of encounters, and exploring factors that contribute to *Rickettsia*-positive tick encounters, ultimately aiming to improve understanding of occupational SFGR risk in this high-incidence region.

Materials and Methods

Field Sampling

The FIA program conducts forest inventories at field sites that include fixed locations on various types of land ownership, such as previously forested land or land used for agriculture, residential purposes, or urban development. These sites are spread across the Southern United States (Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Texas, Virginia, and West Virginia). They are spaced roughly every 24 km^2 in a random pattern. To minimize sampling variance and reduce the likelihood of selecting non-forested land, sites are stratified using satellite-based Landsat Thematic Mapping (Bechtold and Patterson 2005). This sampling design was created to cover boundaries between different forest types and habitats; as a result, foresters conduct their forest sampling within each FIA site across 4 separate plots. Additional details on how foresters carried out their analyses can be found in Bechtold and Patterson (2005). The sampling effort spanned 7 yrs, from 2014 to 2021, during which 74 crews of 2 to 3 people visited a total of 45,244 sites across the South and Southeastern states (Trout Fryxell and Vogt 2019).

Tick Collections by Field Crews and Tick Identification

Before starting the passive tick collections, forest crews reviewed a comprehensive safety lecture focused on tick precautions. Held in spring 2014, the first year of the study, the lecture emphasized safety measures. Forest inventory and analysis crew members volunteered for passive tick collection. They received tick collection kits, which included an instructional guide on tick removal, vials filled with 80% ethanol, and forceps for handling ticks. Our tick surveillance was entirely passive, relying solely on ticks found on the crew members themselves. Crew members were instructed to collect only the ticks they personally encountered and found attached or unattached on themselves, explicitly not collecting any ticks from nearby vegetation. These ticks were then placed in the provided 80% ethanol vials (Trout Fryxell and Vogt 2019).

As previously reported in Trout Fryxell and Vogt (2019), crew members spent between 10 mins to about 6 hrs at each site, with the exact time durations spent on tick checks varying. Usually, during their surveys, crew members covered at least 400 meters from the center to the edge and around the perimeter of each site while taking forest health measurements. Ticks encountered could come from either location when crew members visited 2 sites in one day or during the walk to and from each site. However, each crew member was advised to perform thorough tick checks after completing forest inventories at each site to reduce errors in locating the ticks.

Ticks collected by each crew were then sent to the FIA headquarters for initial processing before being forwarded to the University of Tennessee Medical and Veterinary Entomology Laboratory for further identification and testing. Ticks were identified at the laboratory by species, life stage, and sex using published dichotomous keys (Cooley and Kohls 1945, Clifford et al. 1961, Keirans and Litwak 1989, Durden and Keirans 1996, Keirans and Durden 1998).

DNA Extraction and *Rickettsia* Species Detection

After identifying the ticks, each tick was cut lengthwise, with one half preserved as a voucher specimen and DNA extracted from the other half. Tissue was broken down using 3 mm

tungsten beads in the TissueLyser II (Qiagen, Hilden, Germany) for two 45-s cycles at 15 Hz, and total DNA was extracted using the QIAamp 96 DNA kit (Qiagen, Hilden, Germany), following the manufacturer's protocol on the QIAcube HT (Qiagen, Hilden, Germany). Due to challenges in distinguishing different species of SFG-*Rickettsia*, we used 3 separate PCRs to amplify 3 distinct gene regions (Husin et al. 2021). These regions included (i) a 510 bp segment of the *ompA* gene responsible for encoding the outer membrane protein A of *Rickettsia* species, followed by a restriction fragment length polymorphism (RFLP) assay on the amplified product; (ii) a 350 bp segment of the *23S-5S intergenic spacer region (IGS)* gene; and (iii) a 617 bp segment of the *gltA* gene, which encodes the citrate synthase enzyme. PCR reactions were performed using a Veriti 96-Well Thermocycler (Applied Biosystems, Foster City, California). PCR products were visualized using ethidium bromide-stained 1.5% agarose gels, which were run for 1.5 h at 100 V.

We performed conventional PCR targeting the *ompA* gene using previously published primers and reaction conditions (Eremeeva et al. 1994). The total 40 µl *ompA* PCR reaction included 20 µl of DreamTaq Hot Start PCR master mix, 0.25 µM of each primer, 16 µl of nuclease-free water, and 2 µl of extracted DNA. For the *23S-5S IGS* gene, a nested PCR assay was conducted with primers and conditions as previously described (Kakumanu et al. 2016). The 10 µl primary 23S-5S *IGS* PCR reactions consisted of 5 µl of DreamTaq Hot Start PCR master mix, 0.5 µM of each primer, 2 µl of nuclease-free water, and 2 µl of extracted DNA. The 30 µl nested PCR reactions for the *23S-5S IGS* gene used 15 µl of DreamTaq Hot Start PCR master mix, 0.22 µM of each primer, 12.66 µl of nuclease-free water, and 1 µl of primary PCR product. The conventional PCR targeting the *gltA* gene also used previously published primers and reaction conditions (Kollars and Kengluecha 2001). The 30 µl total *gltA* PCR reaction contained 15 µl of DreamTaq Hot Start PCR master mix, 0.33 µM of each primer, 11 µl of nuclease-free water, and 2 µl of extracted DNA.

The positive control for these PCRs was previously *Rickettsia*-positive tick DNA, while the negative control was a no-template control and previously *Rickettsia*-negative tick DNA. A sample was considered *Rickettsia*-positive by a gene if a distinct DNA band was seen on the agarose gel after electrophoresis at approximately 500 base pairs for the *ompA* gene, around 300-400 base pairs for the *23S-5S IGS* gene, and about 600 base pairs for the *gltA* gene, relative to the DNA ladder. Conversely, samples without any visible DNA bands were classified as *Rickettsia*-negative in all 3 gene target PCR assays. Positive samples for the *ompA* gene PCR were then subjected to RFLP analysis (Huang et al. 2022).

SFG *Rickettsia* Identification to Species

To verify the results and identify potential *Rickettsia* species, a subset of positive amplicons was bidirectionally sequenced using Sanger sequencing. The PCR products were purified with 1.5 µl ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts) per 5 µl of amplified PCR products. The cleaned PCR products were sent to Eurofins Genomics (Louisville, Kentucky, United States) for Sanger sequencing. The resulting forward and reverse DNA sequences for the *ompA*, *23S-5S IGS*, and *gltA* genes were aligned in BioEdit v7.0.9 to generate a consensus sequence for each gene fragment. These consensus sequences were then analyzed using BLAST with the NCBI GenBank repository to identify the *Rickettsia* species most closely related to each gene fragment. Only sequence fragments with clear, non-overlapping chromatogram peaks were used for BLAST analysis. Fragments with overlapping chromatogram peaks, which could indicate mixed infections, were excluded to reduce ambiguity. Sequences with conflicting species-level identifications from forward and reverse reads were also excluded. Sequences with more than 95% similarity to entries in GenBank are reported here and used for further analyses.

Data Analysis

The prevalence of *Rickettsia* infection in each tick species was calculated by dividing the number of *Rickettsia*-positive ticks by the total number of ticks screened using a specific molecular method. A tick was considered *Rickettsia*-positive if at least one of 3 targeted *Rickettsia*-specific genes produced a visible DNA band on agarose gel electrophoresis after PCR, consistent with previously validated methods. Ticks that did not show amplification for any gene target were classified as *Rickettsia*-negative. We further identified the *Rickettsia* species present within each tick species and life stage and analyzed the seasonality of *Rickettsia* infection prevalence. All statistical analyses and figures were generated in R (Version 2023.12.0), with error bars representing 95% binomial confidence intervals. To compare the infection prevalence of *Rickettsia* species among tick species and different life stages, we used a chi-square test and Fisher's exact test (for cases where $N < 5$ for any life stage or tick species). We also compared the prevalence of different *Rickettsia* species detected in the ticks using chi-square and Fisher's exact tests where appropriate. When conducting pairwise comparisons among tick species and life stages, a Bonferroni correction was applied to chi-square and Fisher's exact tests. Additionally, we examined the seasonality of *Rickettsia* species found in the ticks sampled during forest surveys. All analyses and data visualizations were performed using the packages rstatix (Kassambara 2019), fmsb (Nakazawa 2010), and ggplot2.

County-scale distribution maps of *Rickettsia*-positive ticks and maps of *Rickettsia* species diversity by county were created in ArcGIS Pro 3.0.0 (© 2022 Esri Inc.). To examine co-infections and co-occurrence patterns, we analyzed *Rickettsia* species among tick species collected within the same county, focusing the analyses on Kentucky and Tennessee due to the sampling distribution. Co-occurrence analyses among *Rickettsia*-positive tick species and individual *Rickettsia* species within sampling groups were performed when at least 3 groups per state submitted a combined minimum of 50 ticks. These analyses used the cooccur (Griffith et al. 2016), igraph (Csárdi et al. 2025), and EcoSimR (Gotelli et al. 2014) packages. Finally, we used a logistic regression model to determine whether tick species affected the overall *Rickettsia* species infection status, considering tick life stages, sampling year, and month of encounter.

Results

Overall, we identified 1965 ticks encountered and submitted by FIA crews, some of which were previously reported in Butler et al. (2023) and Butler et al. (2024). Of these samples, we screened 1,395 ticks from 9 different states across the Southern

United States (Table 1). The ticks included 3 species: *Amblyomma americanum* (1 larva, 694 nymphs, 322 females, and 262 males), *A. maculatum* (3 females, 6 males, and 1 nymph), and *Dermacentor variabilis* (62 females, 43 males, and 1 nymph). Although we encountered other tick species (eg *Ixodes scapularis*), we excluded them from this study due to their inability to transmit SFGR.

Infection Prevalence of *Rickettsia* Species

The overall infection prevalence of *Rickettsia* species in all sampled ticks was 49.2% (687 out of 1,395 ticks, Table 1). Infection rates varied among the tick species (Supplementary Fig. S1a): *Amblyomma americanum* (51.5%, 659/1,279), *A. maculatum* (40%, 4/10), and *D. variabilis* (22.6%, 24/106). The prevalence of *Rickettsia* species infection was significantly higher in *A. americanum* compared to *D. variabilis* (Fisher's test p-value < 0.001). No significant difference in infection prevalence was observed between the other tick species.

Amblyomma americanum was the only species with enough samples across all life stages (adults, nymphs, and larvae, Table 1) to compare among stages. In adult *A. americanum*, the overall prevalence of *Rickettsia* species infection was 56.2% (328/584 ticks). Adult female *A. americanum* had a significantly higher infection prevalence compared to adult males (χ^2 test p-value = 0.04, Supplementary Fig. S1b). Nymphal *A. americanum* showed an infection prevalence of 47.6% (330/694 ticks, Supplementary Fig. S1b). Pairwise tests revealed a significantly higher prevalence of *Rickettsia* species infection in adult female *A. americanum* compared to nymphs (χ^2 test p-value = 0.0003). No other pairwise comparisons between *A. americanum* life stages showed significant differences in infection prevalence. There was no significant difference in *Rickettsia* species infection rates between sexes of *A. maculatum* or D. variabilis.

Rickettsia Species Identified in Ticks

We examined the composition of *Rickettsia* species amplified from ticks using 3 gene fragments. Due to limited funding, we were only able to sequence a subset of the *Rickettsia*-positive ticks for each gene. Specifically, we sequenced 81 out of 545 *Rickettsia*-positive ticks for the *ompA* gene fragment, 59 out of 613 for the *IGS* gene fragment, and 64 out of 532 for the gltA gene fragment. We did not find any ticks positive for *R. rickettsii*, the agent of RMSF. However, we identified 10 different *Rickettsia* species based on our analysis of 3 gene sequences. The most common species was *R. amblyommatis*, found in 90.6% of positive ticks (529/583 sequences; Table 2, Fig. 1). The only confirmed pathogenic species identified was *R. parkeri* (0.7% of ticks, 4/583, Table 2, Fig. 1), which causes a milder form of SFGR. We also detected other *Rickettsia* species, including *Rickettsia* endosymbiont of *D. variabilis* (1.5%, 9/583, Table 2, Fig. 1), *Candidatus Rickettsia andeanae* (0.2%, 1/583, Table 2, Fig. 1), *R. montanensis* (0.2%, 1/583, Table 2, Fig. 1), and *R. rhipicephali* (0.2%, 1/583, Table 2, Fig. 1). Additionally, 38 sequences were classified as undefined because they tested positive for multiple *Rickettsia* species based on the gene targets used and/or the RFLP method. These undefined *Rickettsia* species included *R. amblyommatis*, *Rickettsia* endosymbiont of *D. variabilis*, *R. tamurae* subsp. *buchneri*, *R. montanensis*, *I. scapularis Rickettsia* endosymbiont, *R. rhipicephali*, and *R. monacensis*. Furthermore, a unique *ompA* gene fragment was detected from a *Rickettsia*-positive female *A. americanum* tick that did not match to any known *Rickettsia* species or *Rickettsia*ceae lineage in GenBank (Fig. 1, Table 2). We examined the prevalence of specific *Rickettsia* species within each tick species (Supplementary Table S1, Supplementary Fig. S2). In *A. americanum* ticks, *R. amblyommatis* was the most frequently detected *Rickettsia* species, accounting for approximately 98.9% of *Rickettsia*-positive samples (554/583, Table 2). Specifically, 62 out of 69 ticks tested positive for *R. amblyommatis* using the *ompA* gene, mostly matching GenBank MN947674 (99.77% to 100% identity); 10 out of 48 ticks were positive for *R. amblyommatis* based on the *IGS* gene, matching GenBank CP015012 (98.2% to 100% identity); and all 55 ticks tested positive for *R. amblyommatis* using the *gltA* gene, matching GenBank CP015012 (98.2% to 100%

Table 1. Total number of ticks assayed and *Rickettsia* positives by gene fragment

	Percent Rickettsia positive (No. Positive/No. Screened)			
Tick species and life stage	***ompA*-positive**	***23S-5S rRNA-IGS* positive**	***gltA* positive**	**Total[a]**
Amblyomma americanum				
Female	49.1% (158/322)	54.9% (174/317)	48.6% (154//317)	60.9% (196/322)
Male	36.5% (101/262)	44.7% (113/253)	38.3% (97/253)	50.4% (132/262)
Nymph	39.7% (265/694)	44.6% (309/693)	38.8% (269/693)	47.6% (330/694)
Larva	0% (0/1)	100% (1/1)	0% (0/1)	100% (1/1)
Total	40.1% (524/1,279)	47.2% (597/1,264)	41.1% (520/1,264)	49.2% (687/1,395)
Amblyomma maculatum				
Female	0% (0/3)	33.3% (1/3)	33.3% (1/3)	1/3 (33.3%)
Male	33.3% (2/6)	50.0% (3/6)	33.3% (3/6)	3/6 (50.0%)
Nymph	0% (0/1)	0% (0/1)	0% (0/1)	0/1 (0%)
Total	20.0% (2/10)	40.0% (4/10)	40.0% (4/10)	4/10 (40.0%)
Dermacentor variabilis				
Female	19.4% (12/62)	9.7% (6/62)	3/62 (4.8%)	15/62 (24.2%)
Male	16.3% (7/43)	14.3% (6/42)	5/42 (11.9%)	9/43 (20.9%)
Nymph	0% (0/1)	0% (0/1)	0/1 (0%)	0/1 (0.0%)
Total	19/106 (17.9%)	11.4% (12/105)	8/105 (7.6%)	24/106 (22.6%)
Total	39.1% (545/1,395)	44.4% (613/1,379)	38.6% (532/1,379)	49.2% (687/1,395)

[a]At least one positive *Rickettsia* species detected in a tick by at least one gene target.

Table 2. Total number of *Rickettsia* species detected in ticks encountered by foresters between 2014 and 2021

Tick species and life stage (number sequenced)	Rickettsia species detected based on the 3 gene targets and RFLP assay					
	R. amblyommatis	*R. parkeri*	*R. montanensis*	*R. andeanae*	*R. rhipicephali*	Undefined[a]
Amblyomma Americanum						
Female (*n*=170)	94.7% (161/170)	0	0	0	0	5.3% (9/170)
Male (*n*=111)	95.5% (105/111)	0	0	0	0	5.4% (6/111)
Nymph (*n*=279)	90.0% (251/279)	0	0	0	0	10.0% (28/279)
Total (*n*=560)	92.3% (517/560)	0	0	0	0	7.7% (43/560)
Amblyomma maculatum						
Male (*n*=2)	0	50% (1/2)	0	0	0	50% (1/2)
Total (*n*=2)	0	50% (1/2)	0	0	0	50% (1/2)
Dermacentor variabilis						
Female (*n*=13)	69.2% (9/13)	0	7.7% (1/13)	0	0	23.1% (3/13)
Male (*n*=8)	37.5% (3/8)	0	0	12.5% (1/8)	12.5% (1/8)	37.5% (3/8)
Total (*n*=21)	57.1% (12/21)	0	4.8% (1/21)	4.8% (1/21)	4.8% (1/21)	28.6% (6/21)
Total (*n*=583)	90.7% (529/583)	0.2% (1/583)	0.2% (1/583)	0.2% (1/583)	0.2% (1/583)	8.6% (50/583)

[a]Undefined: *Rickettsia* include endosymbionts, unique sequences not found in GenBank, sequences that were identified as multiple different species of *Rickettsia* based on the 3 different gene targets and RFLP.

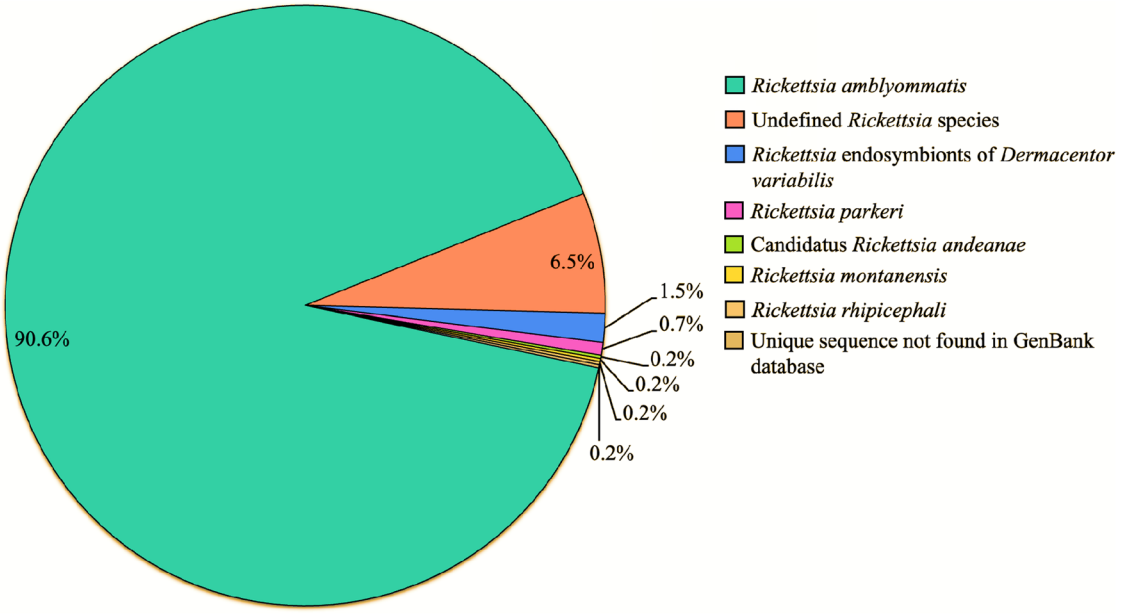

Fig. 1. The *Rickettsia* species composition in the ticks submitted from foresters in the Southeastern United States from 2014 to 2021. The values represent the relative proportions of each *Rickettsia* species in all tick species presented as a percentage. The category denoted by undefined are ticks that were presented with multiple *Rickettsia* species based on different gene targets. The unique sequence not found in GenBank did not match any species in the database; therefore, we kept it as a separate category, as grouping it with the undefined *Rickettsia* species would have been inaccurate

identity). We also detected *D. variabilis Rickettsia* endosymbionts (38/48, 99.4 to 100% *IGS* gene identity to GenBank KT374192), *I. scapularis Rickettsia* endosymbiont (3/69, 83.71% to 100% *ompA* gene identity to GenBank KP172259 and MN639216, Supplementary Table S1), *R. parkeri* (2/69, 96.35 to100% *ompA* gene identity to GenBank MT712872 and MT787018, Supplementary Table S1), and *Rickettsia tamurae* subsp. *buchneri* (1/62, 100% ompA gene identity to GenBank CP113531).

Dermacentor variabilis ticks showed the greatest diversity of *Rickettsia* species, although they had a relatively low prevalence compared to *R. amblyommatis*. Based on the *IGS* genes, we detected *R. montanensis* (4/9, 99.7% to 99.8% identity to GenBank CP003340, Supplementary Table S1), *R. tamurae* subsp. *buchneri* (2/9, 100% identity to GenBank CP113531, Supplementary Table S1), *Candidatus R. andeanae* (1/9, 100% identity to GenBank OK129300, Supplementary Table S1), *D. variabilis Rickettsia* endosymbionts (1/9, 99.4% to 100% identity to GenBank KT374192, Supplementary Table S1), and *R. amblyommatis* (1/9, 100% identity to GenBank CP015012, Supplementary Table S1). Using the *ompA* gene, we identified *R. amblyommatis* (5/10, 99.8% to 100% identity to GenBank EF689733, Supplementary Table S1), *R. montanensis* (3/10, 99.4% to 100% identity to GenBank MG834522, Supplementary Table S1), and *R. rhipicephali* (2/10, 95.8% to 100% identity to GenBank CP003342 and MN811615, Supplementary

Table S1). Based on the *gltA* gene sequences, we detected *R. montanensis* (4/7, 100% identity to GenBank CP003340, Supplementary Table S1), *R. monacensis* (2/7, 99.8% to 100% identity to GenBank OR399955, Supplementary Table S1), and *Candidatus Rickettsia andeanae* (1/7, 100% identity to GenBank KX576677, Supplementary Table S1). Some of these *Rickettsia* species were identified within the same tick using different target genes, which are explained in detail later.

We detected *R. parkeri* in 2 adult *A. maculatum* ticks. These 2 sequences, based on the *ompA* gene, showed 100% identity to GenBank accessions KP861344 and MW008551. Based on the *IGS* gene, these sequences had 99.4% to 100% identity to GenBank KT340608, and based on the *gltA* gene, the sequences showed a 100% identity to GenBank RPU59732 (Supplementary Table S1). Additionally, a *Rickettsia* endosymbiont was detected in one adult *A. maculatum* tick, showing 99.6% identity to GenBank HM5872528 based only on the *ompA* gene (Supplementary Table S1).

Mixed *Rickettsia* Species Sequences

Some of the previously mentioned sequences are ticks that we detected as harboring multiple *Rickettsia* species, based on the target genes used; we call these mixed infections. Mixed infections—where 2 or more *Rickettsia* species are found in the same tick based on several genes and/or RFLP analysis—were identified in 41 ticks. Of these, 35 were *Rickettsia*-positive *A. americanum* ticks (5 females, 4 males, 26 nymphs) with mixed infections. Thirty-one of these *A. americanum* ticks carried *D. variabilis Rickettsia* endosymbionts according to the *IGS* gene (Supplementary Table S1). However, based on *ompA* and *gltA* genes, 4 of these ticks also showed similarity to *R. amblyommatis* (Supplementary Table S1). Additionally, 2 of the 26 *A. americanum* nymphs positive for *D. variabilis Rickettsia* endosymbionts by the *IGS* gene (Supplementary Table S1) also tested positive for *I. scapularis Rickettsia* endosymbiont by the *ompA* gene and for *R. amblyommatis* by the *gltA* gene (Supplementary Table S1). Furthermore, 25 *A. americanum* ticks (3 females, 4 males, 18 nymphs) positive for *D. variabilis Rickettsia* endosymbionts by the *IGS* gene (Supplementary Table S1) also tested positive for *R. amblyommatis* by the *gltA* gene, with RFLP confirming *R. amblyommatis* in 20 of these (3 females, 18 nymphs) ticks. Two *A. americanum* nymphs, initially RFLP-positive for *R. amblyommatis*, had *ompA* gene sequences similar to *R. parkeri*. Lastly, one *A. maculatum* male, RFLP-positive for *R. amblyommatis*, displayed sequences matching *R. parkeri* in the *ompA* gene, *IGS* gene (Supplementary Table S1), and *gltA* gene (Supplementary Table S1).

Seasonality of Infection Prevalence of *Rickettsia* Species in Ticks

The presence of positive ticks throughout the year varied slightly, likely influenced by tick activity and FIA crew activity (Fig. 2a). Specifically, the overall prevalence of *Rickettsia* species from early spring to mid-summer (March to July) ranged from 47.3% to 56.5%, then decreased slightly from 36.4% in late summer to 10% in early fall (August to October). In November, no *Rickettsia*-positive ticks were found. Conversely, in February, all ticks collected by the crews tested positive for *Rickettsia*. However, due to the small sample size ($N = 3$), no definitive conclusions can be made from this finding.

We also examined the seasonal patterns of *R. amblyommatis* (Fig. 2b) and found no significant change from February to May; specifically, the infection prevalence of *R. amblyommatis* in ticks remained consistent during this period. As summer progressed (June to September), the prevalence of *R. amblyommatis* decreased by September. We saw a rise in prevalence by October; however, due to small sample sizes, we cannot draw definitive conclusions. Because the sample sizes for other *Rickettsia* species were too small (less than 5% of the data), we could not produce seasonal curves for those species; however, *R. parkeri* was detected in 3 different months (Fig. 2a, the red arrows), mainly from late spring to late summer (April, June, and August).

We reviewed the incident reports submitted by FIA crews. We differentiated reports involving tick bites from other work-related incidents, such as insect bites or vehicle accidents. Then, we compared patterns of tick activity with the times these tick-related incidents were reported by FIA crews (Fig. 2c). Tick-related incidents, first reported in February (N = 2 tick reports/41 incidents), increased throughout spring and summer, reflecting the activity of *Rickettsia*-positive ticks. The highest occurrence was between May (N = 7 tick reports/28 incidents) and June (N = 9 tick reports/36 incidents), with a secondary peak in September (N = 7 tick reports/26 incidents).

Distribution of *Rickettsia* Species

We surveyed ticks collected by crews working in 8 different states (Arkansas, Florida, Georgia, Kentucky, Louisiana, Oklahoma, South Carolina, and Tennessee, Supplementary Fig. S3) across 119 different counties (Arkansas 4/75 counties, Florida 11/67 counties, Georgia 2/159 counties, Kentucky 55/120 counties, Louisiana 2/64 counties, Oklahoma 3/77 counties, South Carolina 22/46 counties, and Tennessee 20/95 counties, Supplementary Fig. S3). *Rickettsia*-positive ticks were found in all 8 states, detected in approximately 75% (N = 89 total counties, Supplementary Fig. 3) of the counties where ticks were collected; specifically, Arkansas 1/4 counties, Florida 7/11 counties, Georgia 2/2 counties, Kentucky 46/55 counties, Louisiana 1/2 counties, Oklahoma 3/3 counties, South Carolina 16/22 counties, and Tennessee 13/20 counties (Fig. 3).

Rickettsia amblyommatis showed the broadest geographic distribution in our samples (Fig. 4a–c), being found in 7 of 8 states (all except Louisiana) and in 75 of 128 counties where ticks were tested. The county-level distribution included Arkansas (1/4 counties), Florida (6/11 counties), Georgia (2/2 counties), Kentucky (46/56 counties), Oklahoma (3/3 counties), South Carolina (12/22 counties), and Tennessee (12/24 counties). *Rickettsia parkeri*-positive ticks were identified in 4 counties across 3 states: Kentucky (Nelson and Hopkins counties), South Carolina (Richland County), and Tennessee (Van Buren County). These detection sites matched areas where a significant number of tick submissions were received.

We determined whether *Rickettsia*-positive ticks were randomly distributed across sampling regions or if there was evidence of positive or negative interactions among these ticks. A total of 24 teams contributed to our passive surveillance in the region. For this analysis, we selected states with at least 3 crews, including Florida (3 crews), Kentucky (9 crews), South Carolina (6 crews), and Tennessee (3 crews). When analyzing the forestry crew data, 19 of 24 forestry crews encountered and submitted at least one *Rickettsia*-positive tick, with 18 crews

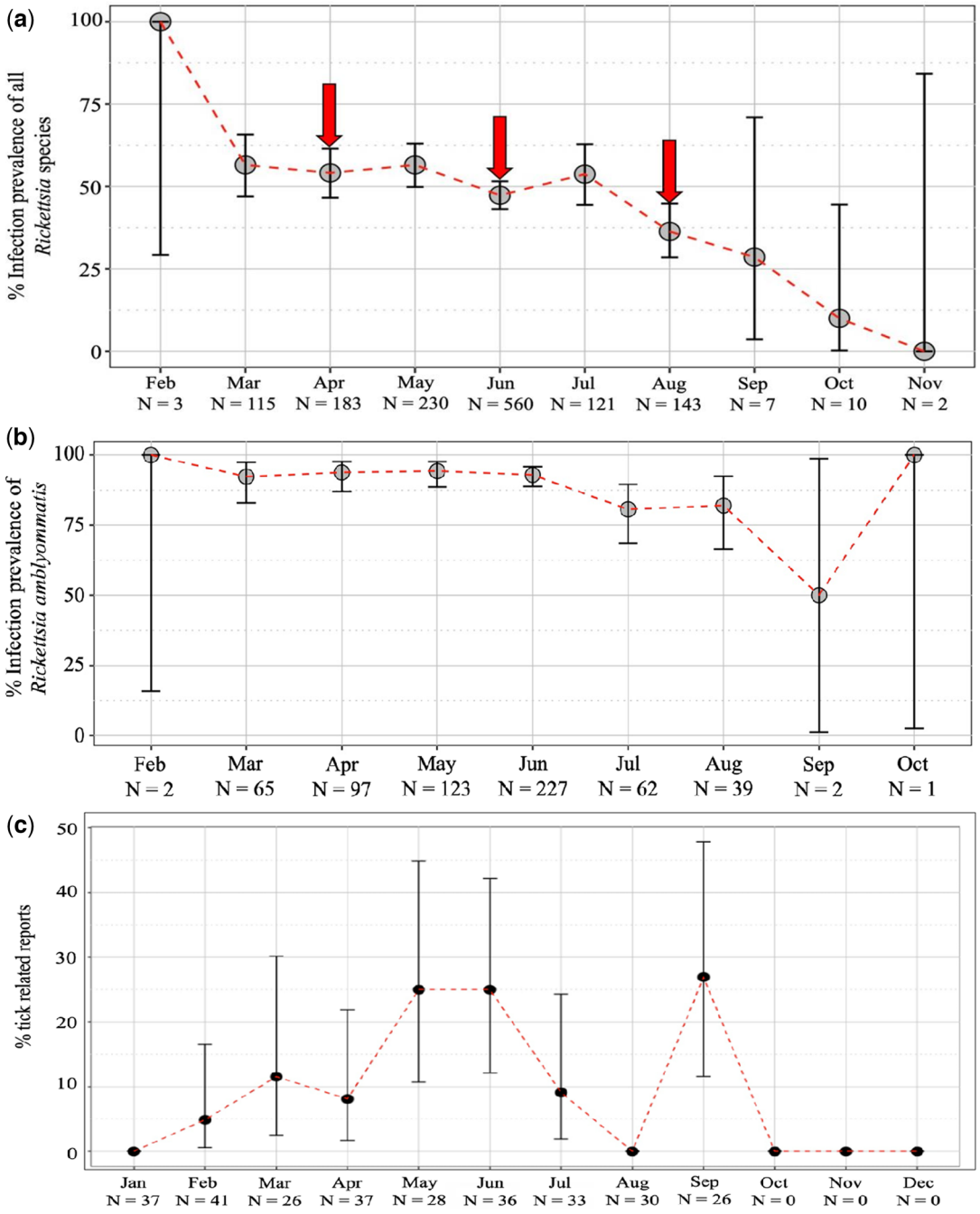

Fig. 2. Seasonality of *Rickettsia* species-positive ticks and tick activity a) Phenology of the infection prevalence of *Rickettsia* species-positive ticks submitted by forest crews from 2014 to 2021 in the Southeastern United States. The error bars depict the 95% binomial confidence intervals, and values underneath each month represent the total number of ticks that we screened each month. The 3 red arrows denote the months that *R. parkeri* was detected in ticks. b) Phenology of the infection prevalence of *Rickettsia amblyommatis* infecting ticks. c) Seasonality of tick-related incidence reports of the FIA forestry workers reported between April 2011 and May 2021. The percentage of tick-related reports is calculated as the total number of tick-related reports over the total number of work-incident reports submitted by the forestry workers. The numbers (N) underneath the months indicate the total number of work-incident reports that were submitted

collecting ticks positive for *R. amblyommatis*. Most of the *R. amblyommatis*-positive ticks were found in Kentucky (Fig. 4a–c).

In the co-occurrence analysis of *Rickettsia*-positive ticks, we found no positive or negative co-occurrence probabilities between any pairs of *Rickettsia*-positive tick species among the forestry teams that collected *Rickettsia*-positive ticks. Additionally, there were no positive or negative co-occurrence probabilities between any of the individual *Rickettsia* species identified in the ticks encountered by the teams.

Logistical Regression Model

In the logistic regression model, several variables were identified as statistically significant (Supplementary Table S2).

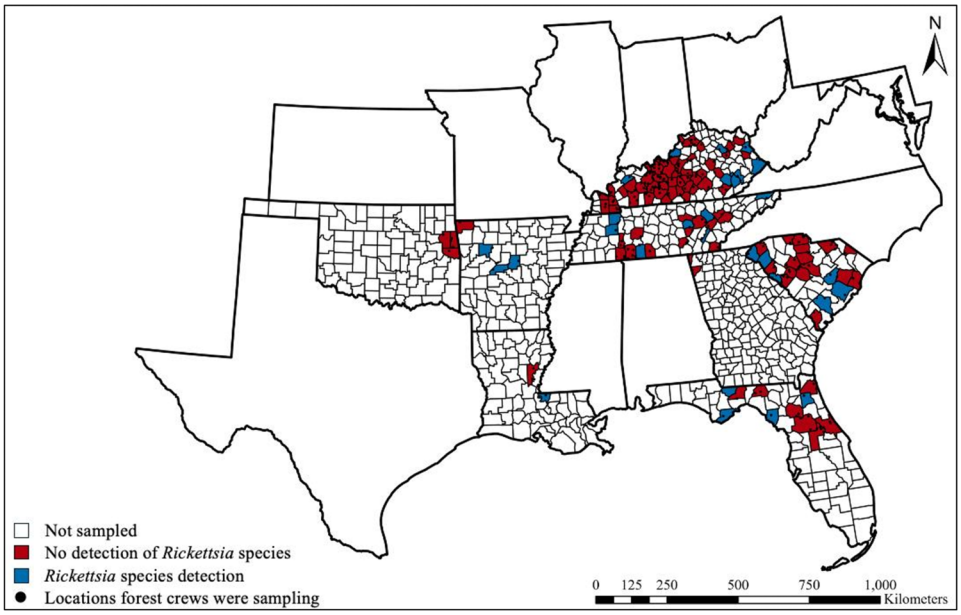

Fig. 3. Spatial distribution of *Rickettsia*-positive ticks in the Southeastern United States, 2014 to 2021. The map highlights counties where at least one *Rickettsia*-positive tick was encountered by FIA crews. Red counties indicate the presence of *Rickettsia*-positive ticks, while blue counties indicate no detections

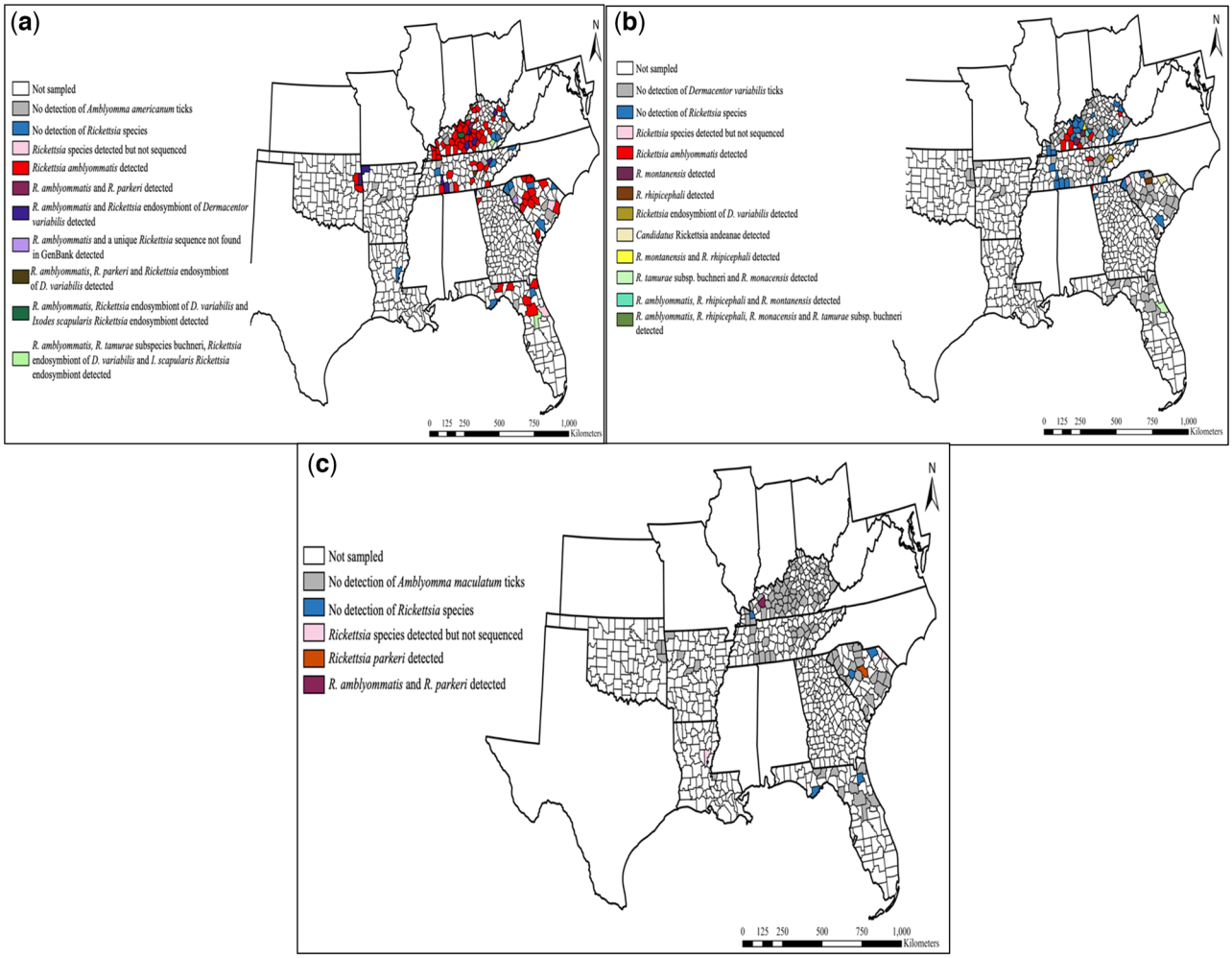

Fig. 4. The spatial distribution of the *Rickettsia* species detected in three tick species submitted by FIA foresters in the Southeastern United States, 2014 to 2021. a) *Rickettsia* species detected in *Amblyomma americanum* ticks. b) *Rickettsia* species detected in *A. maculatum* ticks. c) *Rickettsia* species detected in *Dermacentor variabilis* ticks.

Specifically, the year of tick collection (p-value =0.0018), the location (State) where the tick was collected (p-value = 9E–04), the tick species identified (p-value = 1.5E–09), and the tick's life stage (p-value = 0.0014) all were significantly associated with *Rickettsia* species infection.

To further investigate factors related to encountering *Rickettsia*-positive ticks, we analyzed data from tick collections conducted between 2017 and 2021. Results indicated significant variation year-to-year in the likelihood of finding *Rickettsia*-positive ticks. Compared to 2017, the odds of detecting infected ticks were significantly higher in 2018 (odds ratio [OR] = 2.57, p-value = 0.021, Table 3), 2019 (OR = 1.88, p-value = 0.0007, Table 3), and 2021 (OR = 6.40, p-value = 0.03, Table 3). Geographic location of the tick encounter was also correlated with whether a tick tested positive for *Rickettsia;* ticks collected in Tennessee had significantly lower odds of *Rickettsia*-positivity compared to those encountered in Kentucky (OR = 0.5, p-value = 2.77E–05). No other collection state had significant differences in *Rickettsia* species occurrence compared to Kentucky. Additionally, the species of tick was linked to the risk of *Rickettsia* positivity, with *D. variabilis* ticks having significantly lower odds of being *Rickettsia*-positive than *A. americanum* (OR = 0.70, p-value = 4.51E–10). The tick's life stage was also linkedto *Rickettsia* positivity; nymphal ticks had significantly lower odds of testing positive than female ticks (OR = 0.59, p-value = 0.0003). There was no significant difference in *Rickettsia*-positive status between male and female ticks (p-value = 016). Due to limited sample sizes, a reliable analysis of factors related to encountering *R. amblyommatis*-positive ticks was not possible.

Discussion

Forestry workers in the Southern United States face a high occupational risk of encountering SFGR. This concern is supported by documented tick-related workplace incidents among FIA forest crews and their routine outdoor activities in tick-endemic habitats (Trout Fryxell and Vogt 2019). Notably, out of 315 workplace incidents reported by FIA forest crews between April 2011 and May 2021, 34 (11%) involved ticks, including tick attachment, tick bites, and symptoms such as swollen lymph nodes, headaches, and nausea (unpublished FIA data).

Which *Rickettsia* Species Were Detected?

Among all tick species submitted by foresters, *A. americanum* was the most frequently encountered. This high frequency likely reflects upon the habitats where forest crews conducted their surveys, which are endemic areas for *A. americanum*, where the species occurs at relatively high densities (Butler et al. 2023, 2024). Notably, none of the ticks submitted by foresters were infected with *R. rickettsii*. Since RMSF is a severe and potentially fatal form of SFGR, this study suggests that the risk of foresters contracting RMSF is relatively low; however, it is important to note that the absence of *R. rickettsii*-infected ticks does not definitively mean that foresters and outdoor workers are not at risk of contracting RMSF in the Southern US. There remains a risk, as incidence reports indicate that most RMSF cases are primarily reported from several states in the southern United States (Biggs et al. 2016, Rosenberg et al. 2018, Silva-Ramos et al. 2021). Consistent with our findings, other studies have similarly reported either no detection or very low prevalence of *R. rickettsii* in ticks collected through active or passive surveillance, even in regions with relatively high numbers of reported human RMSF cases (Carmichael and Fuerst 2010, Moncayo et al. 2010, Berrada et al. 2011, Trout Fryxell et al. 2015, Ribeiro 2021).

Table 3. The coefficients and the odds ratio of the logistical regression model for the infection status of *Rickettsia* species among ticks submitted by forest crews from 2017 to 2021 in the Southeastern United States

Variables	Coefficients	Standard Error	z-value	*P*-value	Odds ratio (95% confidence intervals)
Intercept	0.62	0.39	1.59	0.11	1.86 (0.87–4.02)
Collection years compared to the first year 2017					
2018	0.94	0.41	2.30	0.021	2.57 (1.16–5.81)
2019	0.63	0.19	3.39	0.0007	1.88 (0.32–2.71)
2020	0.32	0.17	1.90	0.057	1.38 (0.99–1.91)
2021	1.86	0.85	2.17	0.03	6.40 (1.36–45.95)
Collection month compared to March					
April	–0.45	0.35	–1.29	0.2	0.64 (0.32–1.26)
May	–0.24	0.37	–0.67	0.51	0.78 (0.38–1.61)
June	–0.18	0.37	0.48	0.63	0.84 (0.4–1.72)
July	–0.28	0.37	–0.76	0.45	0.75 (0.36–1.56)
August	–0.59	0.4	–1.49	0.14	0.55 (0.25–1.12)
Collection state compared to Kentucky					
Tennessee	–0.73	0.17	–4.2	2.77e–05	0.48 (0.34–0.68)
Florida	–0.39	0.29	–1.34	0.18	0.68 (0.38–1.20)
Georgia	–0.06	0.46	–0.14	0.89	0.94 (0.38–2.31)
South Carolina	–0.36	0.47	–0.77	0.44	0.70 (0.26–1.76)
Tick species compared to *A. Americanum*					
A. maculatum	–1.44	0.85	–1.69	0.09	0.23 (0.03–1.11)
D. variabilis	–1.67	0.27	–6.23	4.51e–10	0.19 (0.11–0.31)
Life stage compared to all female adult ticks					
Male	–0.23	0.167	–1.40	0.16	0.79 (0.57–1.01)
Nymph	–0.53	0.15	–3.57	0.0003	0.59 (0.44–0.79)

Although *R. rickettsii* was not detected, we identified a *Rickettsia* species capable of causing SFG rickettsiosis, *R. parkeri*, which is commonly transmitted by *A. maculatum* ticks (Paddock et al. 2004, Wright et al. 2015, Suwanbongkot et al. 2019, Ramírez-Garofalo et al. 2022, Gual-Gonzalez et al. 2024). It causes a mild form of SFG rickettsiosis characterized by an inoculation skin eschar at the tick bite site (Biggs et al. 2016, Molaei et al. 2021, 2024, Moo-Llanes et al. 2021, Silva-Ramos et al. 2021). Although we received only a few *A. maculatum* specimens from foresters, approximately 33% tested positive for *R. parkeri*. While these occasional encounters with *A. maculatum* ticks suggest a relatively low risk for foresters, their extensive outdoor exposure in forests may increase their overall chances of contact with infected ticks compared to individuals in other occupations.

We also detected *R. parkeri* in nymphal *A. americanum* ticks at very low prevalence, consistent with other studies (Wright et al. 2015, Egizi et al. 2020, Ramírez-Garofalo et al. 2022, Gual-Gonzalez et al. 2024). Although no human transmission via *A. americanum* has been documented, guinea pig studies demonstrated that *A. americanum* can transmit *R. parkeri*, and cause mild symptoms (Goddard 2003, Wright et al. 2015). Detecting *R. parkeri* in *A. americanum* nymphs raises particular concern because the nymphs are active during peak outdoor recreational seasons and often go unnoticed due to their small size. In the southern United States, *A. americanum* is the main tick species that bites humans. Therefore, despite the low pathogen prevalence, frequent human–tick encounters may pose a moderate transmission risk, and the presence of *R. parkeri* in *A. americanum* will support the maintenance of *R. parkeri* in enzootic cycles.

Rickettsia amblyommatis was the most frequently detected *Rickettsia* species in our study, with all our sequences matching to those of *R. amblyommatis* sequences found in GenBank. It was primarily detected in *A. americanum* ticks. Several studies from regions where *A. americanum* ticks are endemic, such as Kentucky, Tennessee, and North Carolina, have also reported a high prevalence of *A. americanum* ticks carrying *R. amblyommatis* (Moncayo et al. 2010, Fritzen et al. 2011, Karpathy et al. 2016, Richardson et al. 2023, Gual-Gonzalez et al. 2024). Additionally, *R. amblyommatis* is also considered the most prevalent *Rickettsia* species in *D. variabilis* and *A. maculatum* ticks (Lee et al. 2014 , Karpathy et al. 2016, Richardson et al. 2023). Some studies suggest *R. amblyommatis* may cause mild flu-like symptoms in humans and have shown serological evidence of infection (Billeter et al. 2007, Apperson et al. 2008, Richardson et al. 2023). Furthermore, a xenodiagnostic study using guinea pigs and mice demonstrated that *A. americanum* ticks infected with *R. amblyommatis* could transmit the bacterium through bites, resulting in mild clinical symptoms in both animals (Snellgrove et al. 2021, Yen et al. 2021). With this evidence, *R. amblyommatis*-related spotted fever could be mistaken for other types of SFG-Rickettsioses. More research and better disease monitoring are needed to better understand whether some cases of SFG-Rickettsioses are potentially caused by *R. amblyommatis* (Mixson et al. 2006, Pornwiroon et al. 2015, Delisle et al. 2016).

As a result, foresters may be at risk of contracting mild SFGR from bites by *R. amblyommatis*-positive *A. americanum* ticks. This risk might be higher because *A. americanum* was the most common tick encountered by foresters. In some cases, the same crew encountered multiple *R. amblyommatis*-positive ticks on the same day (71 out of 204 sampling days). In our study, 18 of the 24 forestry teams encountered ticks infected with *R. amblyommatis*. The high prevalence of *R. amblyommatis* in *A. americanum*, along with the possibility that *R. amblyommatis* causes mild symptoms similar to SFGR, may suggest an increased risk among forestry workers. Our findings, together with those from other studies (Mixson et al. 2006, Pornwiroon et al. 2015, Delisle et al. 2016), emphasize the urgent need for further epidemiological research to determine whether some reported SFG rickettsiosis cases are caused by *R. amblyommatis* infections rather than other pathogenic SFG *Rickettsia* species.

Among the ticks we examined, several other *Rickettsia* species belonging to the SFGR group were identified. Most of these *Rickettsia* species are either not known to be pathogenic to humans or could potentially cause very mild clinical symptoms. Interestingly, *D. variabilis* ticks carried a greater diversity of *Rickettsia* species, although the prevalence was relatively low (Supplementary Fig. S2). This diversity could be due to several factors, including the variety of hosts fed on by larval and nymphal stages of *D. variabilis* ticks, potential transovarial transmission of some *Rickettsia* species, and the possibility that our methods only detect the most common *Rickettsia* in a tick (Carmichael and Fuerst 2010, Harris et al. 2017, Kakumanu et al. 2018, Hecht et al. 2019). We also identified several symbiotic SFG *Rickettsia* species, such as *R. tamurae* subsp. *buchneri*, which is commonly found on *I. scapularis* ticks (Kurtti et al. 2015, De et al. 2023), *D. variabilis Rickettsia* endosymbionts (Kakumanu et al. 2016), and *I. scapularis Rickettsia* endosymbionts (Lee et al. 2014). Previous studies have suggested that non-pathogenic SFG-*Rickettsia* species (eg *R. rhipicephali* or *R. montanensis*) infecting *D. variabilis* outcompete and interfere with the transovarial transmission of *R. rickettsii* (Macaluso et al. 2002). This phenomenon may help explain why we did not detect *R. rickettsii* in the submitted *D. variabilis* ticks from an area with high RMSF incidence.

Although *A. americanum* ticks are known to carry *R. rickettsii* and may serve as competent vectors (Stromdahl et al. 2008, Berrada et al. 2011, Breitschwerdt et al. 2011, Levin et al. 2017), we did not detect *R. rickettsii* in any of the *A. americanum* ticks we tested. This absence might be due to *R. amblyommatis* outcompeting *R. rickettsii in A. americanum* ticks, as supported by several studies showing high *R. amblyommatis* prevalence in field-collected ticks (Billeter et al. 2007, Blanton et al. 2014, Wright et al. 2015). In mixed infections, higher levels of *R. amblyommatis* DNA may outcompete *R. rickettsii* during PCR amplification. When *R. rickettsii* DNA levels are lower, its detection may be masked and go unnoticed. This should be considered when interpreting prevalence data, as it could lead to an underestimation of *R. rickettsii* in field ticks. Future studies using specific and sensitive quantitative PCR or next-generation sequencing approaches could improve detection and differentiation of co-infections.

Evidence for this competitive exclusion appears in both mammalian hosts and tick vectors. Blanton et al. (2014) demonstrated that guinea pigs previously inoculated with *R. amblyommatis* and then challenged with high doses of *R. rickettsii* did not fall ill, unlike those not previously inoculated. Similarly, Wright et al. (2015) documented that *A. americanum* nymphs infected with *R. amblyommatis* were less likely to

acquire *R. rickettsii*. Furthermore, Levin et al. (2018) found that the proliferation of *R. rickettsii* cells in *A. americanum* larvae already infected with *R. amblyommatis* was reduced. Later, guinea pigs infected through xenodiagnosis with those larvae showed only mild symptoms of spotted fever. Therefore, the higher prevalence of *R. amblyommatis* in ticks may limit *R. rickettsii* proliferation within the tick vector.

In our molecular diagnostics, we used 3 different gene targets and the RFLP method to identify *Rickettsia* species. We did not observe any mixed DNA sequences where the chromatograms presented with overlapping peaks at the same nucleotide positions from the same gene target, indicating that our methods did not detect the simultaneous presence of *R. rickettsii* and any other *Rickettsia* species. While many of our sequences achieved high percentage identities confirming species-level classification, we noted that some high-quality sequences were less than 98% similar to their closest GenBank counterparts. In such cases, we present the highest available match as an indicator of genetic relatedness; however, for formal species assignment, we applied the established phylogenetic and percent identity cutoff criteria for *Rickettsia* species (Fournier et al. 2003). One important observation was the presence of a distinct 427 bp *ompA* sequence from a female *A. americanum* tick sampled in Abbeville County, South Carolina (near Ware Shoals). The BLAST analysis optimized for highly similar sequences (MegaBLAST) against the GenBank database revealed no exact matches for this sequence, suggesting it may represent a previously undocumented *Rickettsia* genotype or a novel species circulating in this geographic area. The sequence produced clear peaks with no peak-over-peak overlap in both forward and reverse reads. A subsequent BLAST search using the "somewhat similar sequences" option (BLASTn) also failed to match any *Rickettsia* species or species within the *Rickettsia*ceae lineage. Future analyses using additional target genes, such as the *23S-5S IGS* and *gltA* genes, may help further define the identity of this sequence.

Seasonal Patterns of Infection Prevalence in Ticks

Rickettsia species were detected throughout the year in ticks submitted by foresters across all life stages of the identified tick species. There was no clear pattern in the seasonality of *Rickettsia* infection prevalence in the ticks. During spring and summer (March to July), the prevalence of *Rickettsia*-positive ticks fluctuated less than in fall and winter (Fig. 2a). This could be due to larger sample sizes of submitted ticks during spring and summer, when many tick species actively quest. In fall and winter, we saw a slight decrease in tick infection prevalence (Fig. 2a), likely because fewer ticks were submitted. Notably, most of the FIA work-related tick incident reports occurred during the same period when ticks were SFGR-positive—12% of reports in March, 8% in April, 25% in May, and 25% in June. We believe this seasonal pattern mainly reflects the activity patterns of the tick species.

We then examined the seasonality of *R. amblyommatis*-positive ticks (Fig. 2b). We observed that infection prevalence remained relatively high during the summer months (N = 428 *R. amblyommatis*-positive ticks), which could again be attributed to the activity patterns of the associated ticks. As tick activity declines toward the end of summer and into fall, we also noted a drop in *R. amblyommatis*-positive ticks. To put these findings into context, we compared the observed seasonality patterns of R. *amblyommatis* in ticks with the seasonality of human SFGR case incidence published by the Centers for Disease Control and Prevention (National Center for Emerging and Zoonotic Infectious Diseases, CDC). Using our tick data and CDC case data, we hypothesize that some of the previously reported disease incidences labeled as RMSF may potentially be due to *R. amblyommatis*, because historically, serology tests used to diagnose RMSF could not differentiate between *Rickettsia* species (La Scola and Raoult 1997, Biggs et al. 2016, Stewart and Stewart 2021), leading to many non-RMSF cases being categorized as RMSF. However, changes in case definitions regarding serology testing after 2020 resulted in a decline in RMSF incidence (Centers for Disease Control and Prevention 2019, 2021). Therefore, we suggest that some *R. amblyommatis*-positive ticks may contribute to the overall incidence of SFGR, highlighting the importance of accurate *Rickettsia* species identification of SFGR, especially when laboratory evidence is used to confirm RMSF in patients.

The Distribution of SFG *Rickettsia* Species in the Southeast

Unsurprisingly, most of our *R. amblyommatis* detections came from ticks submitted from Tennessee and Kentucky (Fig. 3). Our findings match those of other studies regarding the distribution of *R. amblyommatis*. *Rickettsia amblyommatis* is often found in *A. americanum* ticks, so its range mainly overlaps with areas where *A. americanum* is endemic (Mixson et al. 2006, Jiang et al. 2010, Ponnusamy et al. 2014). This correlation highlights the importance of understanding the geographic distribution of tick species and their associated pathogens for effective monitoring and control of tick-borne diseases.

Furthermore, we observed that in most counties across the states where ticks were submitted to us, ticks carried multiple *Rickettsia* species (Fig. 3). This suggests that in the Southern states, several additional *Rickettsia* species may be circulating and maintained enzootically. The transovarial transmission trait of some of these *Rickettsia* species further plays an important role in sustaining their enzootic cycles in nature (Socolovschi et al. 2009, Harris et al. 2017, Levin et al. 2018). These findings raise concerns because these species, along with *R. amblyommatis*—which may potentially cause a mild form of rickettsiosis in humans—could go undiagnosed or be misdiagnosed as RMSF due to non-specific diagnostic tools. A notable epidemiological paradox exists in the Southern United States: where despite a high incidence of human RMSF cases compared to other US regions (Dahlgren et al. 2012, Biggs et al. 2016, Rosenberg et al. 2018), the prevalence of *R. rickettsii*, the causative agent, remains consistently very low in key tick vectors such as *D. variabilis* (Dyer et al. 1931, Warner and Marsh 2002, Levin et al. 2020, Stanley et al. 2020), *D. andersoni* (Ricketts 1991), *Rhipicephalus sanguineus* (Burgdorfer et al. 1975), and *A. americanum* (Berrada et al. 2011, Breitschwerdt et al. 2011). This pattern strongly suggests that other circulating *Rickettsia* species might be causing infections currently misdiagnosed as RMSF, emphasizing an urgent need to develop and implement more accurate laboratory diagnostic tools to clarify this discrepancy.

While the CDC reports the highest incidence of RMSF in Arkansas, followed by Tennessee (Bishop et al. 2022), we did not detect any *R. rickettsii* in the ticks submitted by foresters using any of our 4 detection methods. The limited number of

ticks from Arkansas (N = 4, as previously mentioned) prevents definitive conclusions about *R. rickettsii* prevalence in that state. However, *R. rickettsii* was also not found in the larger sample of ticks from Tennessee. With further studies, there is potential to confirm more *Rickettsia* species as pathogenic, as is the case with *Rickettsia* sp. CA6269, which is described as a pathogenic *Rickettsia* from California (Probert et al. 2024).

Most *Rickettsia*-positive ticks were collected from central Kentucky and western Tennessee, especially around the Interior Plateau, with the majority being *R. amblyommatis*-positive (Fig. 4a–c). We also observed *R. amblyommatis*-positive tick clusters along the Central Appalachians in eastern Tennessee and northern Florida. These patterns generally matched suitable habitats for *A. americanum* and *D. variabilis* predicted by Butler et al. (2024). However, 31 of 57 ticks from north-central and northern coastal South Carolina tested positive for *Rickettsia*, despite these areas (Middle Atlantic Coastal Plains, Southeastern Plains, and Piedmont ecoregions) being predicted as unsuitable habitats for these tick species (Butler et al. 2023) . This discrepancy suggests that creating models specifically for *Rickettsia*-positive ticks could offer additional insights into regions of the southeastern United States with higher risks for SFR exposures and warrants further spatial-temporal analysis.

In the co-occurrence matrix, we did not observe any positive or negative co-occurrence probabilities among any of the tick species or *Rickettsia* species. All 3 tick species analyzed (*A. americanum*, *A. maculatum*, and *D. variabilis*) are generalist feeders and occupy similar ecological niches. We might not have detected any positive or negative co-occurrence probabilities due to the smaller sample sizes of *A. maculatum* and *D. variabilis* compared to *A. americanum*, as well as the limited samples of *Rickettsia* species other than *R. amblyommatis*.

Factors Affecting the Detection of *Rickettsia* Species in Ticks

We examined several factors related to ticks and collection methods that were connected to detecting *Rickettsia* species in ticks. This included tick-related factors such as the tick species, life stage, and sex, as well as collection-related factors like seasonality, the years, and the locations where samples were collected. Our model indicated a lower likelihood of *Rickettsia* positivity in Tennessee ticks compared to Kentucky ticks, potentially due to a larger sample size and broader spatial sampling in Kentucky. Additionally, a significant increase in *Rickettsia*-positive ticks was observed in subsequent years compared to 2017, suggesting a possible rise in tick densities or increased tick surveillance by foresters with program experience. Furthermore, tick species and life stages were significant predictors, as each may have different tendencies for *Rickettsia* acquisition and host preferences. The notable difference in *Rickettsia* species infection status between *A. americanum* and *D. variabilis* ticks is likely due to the higher prevalence of *Rickettsia* species, especially *R. amblyommatis* in *A. americanum* ticks. With a larger sample size of *A. americanum* ticks, the chance of detecting *Rickettsia* infections is higher, particularly since most of these ticks were infected with *R. amblyommatis*. Conversely, *D. variabilis* ticks had a lower prevalence of *Rickettsia* species infections. The limited sample sizes of *A. maculatum* may have contributed to the inability to detect significant differences in their *Rickettsia* species infection status compared to *A. americanum*.

When comparing *Rickettsia*-positive status, we found that nymphal ticks were less likely to be infected than adult female ticks. Conversely, adult females had a higher likelihood of *Rickettsia*-positive status than nymphs. No significant difference in *Rickettsia*-positive status was observed between male and female ticks. This pattern of higher infection prevalence in adult ticks, especially females, aligns with observations for other tick-borne pathogens like *Borrelia burgdorferi* (Feldman et al. 2015, Ramírez-Garofalo et al. 2022, Price et al. 2024). This is mainly due to the cumulative feeding opportunities: an adult tick, which has already taken 2 blood meals (as a larva and a nymph), has had twice the chance to acquire a bacteria from an infected host compared to a nymph, which has only taken one blood meal (as a larva). Therefore, adult ticks inherently have a higher probability of being *Rickettsia*-positive than nymphs. Further research into the mechanisms behind these differences in infection susceptibility among tick life stages would provide valuable insights into tick-borne disease dynamics. Although we had a substantial overall sample size of *R. amblyommatis*-positive ticks, we did not perform a logistic regression model because the distribution of these ticks was not uniform across our model variables, resulting in limited statistical power for robust comparisons.

Conclusion

The occurrence of SFGR cases in humans is increasing, especially in the Southern United States. Occupations that involve extended outdoor activity are associated with a higher risk of SFGR transmission. Collaborative passive surveillance provides a cost-effective way to monitor the spread of SFGR among tick species. Our study used this approach to examine the prevalence of *Rickettsia* species in ticks, focusing on the Southern region, and to evaluate the risk of SFGR transmission to humans. Forest characteristics influence overall tick abundance, which may also impact encounters with *Rickettsia*-positive ticks (Butler et al. 2023). Understanding these environmental factors is essential for developing strategies—such as guidelines for forestry workers and public awareness efforts—to reduce exposure to infected ticks and lower SFGR cases. Further research is needed to establish direct connections between environmental factors and encounters with *Rickettsia*-positive ticks in this context.

Because many of the clinical symptoms of SFGR are nonspecific, outdoor workers may not immediately recognize them as signs of SFGR. Therefore, it is essential to raise awareness among outdoor workers, especially about SFGR, and to highlight the importance of preventing tick encounters and bites, as well as monitoring themselves for symptoms during high-risk periods. This awareness is essential for the prevention and early detection of SFGR in outdoor workers.

Since our main goal was to identify common *Rickettsia* species in ticks collected by forestry workers, we did not analyze the genetic lineages of the *Rickettsia* sequences obtained. Future research should include detailed genetic analyses to better understand the transmission patterns and genetic diversity of *Rickettsia* species circulating in ticks across the southern United States. Often, SFGR case diagnoses do not specify which *Rickettsia* species cause clinical symptoms (Biggs et al. 2016). However, advances in case diagnostics show promise for improving our understanding of *Rickettsia* transmission. With better

diagnostics and surveillance programs, hopefully our knowledge of SFGR spread will expand, leading to more effective control and prevention strategies to reduce disease cases, especially RMSF.

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Author Contributions

Vishvapali Kobbekaduwa (Data curation [equal], Formal analysis [lead], Investigation [equal], Methodology [equal], Software [lead], Validation [lead], Visualization [lead], Writing—original draft [lead]), Jennifer G. Chandler (Data curation [equal], Formal analysis [supporting], Investigation [equal], Methodology [equal], Supervision [equal], Validation [supporting], Writing—review & editing [supporting]), Rebecca Trout Fryxell (Conceptualization [lead], Data curation [supporting], Formal analysis [supporting], Funding acquisition [equal], Investigation [equal], Methodology [equal], Project administration [lead], Resources [lead], Supervision [lead], Validation [supporting], Visualization [supporting], Writing—review & editing [lead]), and James Vogt (Funding acquisition [lead], Investigation [equal], Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [supporting])

Supplementary Material

Supplementary material is available at *Journal of Medical Entomology* online.

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Conflicts of Interest

None declared.

Data Availability

Data from this study are available at datadryad.org: 10.5061/dryad.0p2ngf2f8. Kobbekaduwa et al. (2025).

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