Large-Scale Survey for Tickborne Bacteria, Khammouan Province, Laos

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We screened 768 tick pools containing 6,962 ticks from Khammouan Province, Laos, by using quantitative real-time PCR and identified Rickettsia spp., Ehrlichia spp., and Borrelia spp. Sequencing of Rickettsia spp.—positive and Borrelia spp.—positive pools provided evidence for distinct genotypes. Our results identified bacteria with human disease potential in ticks in Laos.

Rickettsia, Borrelia, Ehrlichia, Anaplasma, and Coxiella spp. are tick-associated bacteria and well-described human pathogens. All of these bacteria, except Coxiella spp., are primarily transmitted through tick bites and cause febrile disease with a wide spectrum of severity. Tickborne bacterial pathogens are believed to be an underrecognized cause of acute febrile illness in Southeast Asia (1).

In Laos, spotted fever group Rickettsia have been shown to cause undifferentiated fever in 2% of febrile hospitalized adult patients (2). However, data on bacteria in ticks in Laos are sparse. To date, 1 Rickettsia sp. has been identified in a Boophilus sp. tick from Luang Namtha Province; this species showed 99.8% similarity with the Rickettsia sp. FUJ98 ompA gene (3). No other tickborne bacteria have been reported from Laos. Therefore, we investigated Rickettsia, Borrelia, Ehrlichia, Anaplasma, and Coxiella spp. in ticks from Khammouan Province, Laos.

The Study
We collected ticks in Nakai District, Khammouan Province, during the dry seasons (December–April) during 2012–2014, as previously described (4) (online Technical Appendix Figures 1, 2, http://wwwnc.cdc.gov/EID/article/22/9/15-1969-Techapp1.pdf). A total of 6,692 ticks were pooled (n = 768 pools, 1–10 ticks/pool) according to genus, sex, developmental stage, collection period, and site. One Amblyomma testudinarium nymph that contained a blood meal was processed separately.

We extracted DNA by using the NucleoSpin 8 Virus Extraction Kit (Macherey-Nagel, Düren, Germany). Pools were screened by using single quantitative real-time PCRs specific for Rickettsia spp. (17-kDa gene), Borrelia spp. (23S rRNA gene), Anaplasma spp. (major surface protein 2 gene), Ehrlichia spp. (16S rRNA gene), and Coxiella spp. (IS1111) (5–8) (online Technical Appendix Table 1). Five microliters of diluted (1:10) template containing 1× Platinum Supermix-UDG (Invitrogen, Carlsbad, CA, USA) and bovine serum albumin (40 mg/mL) were used for each assay. Positive and nontemplate controls were included in each run. Screening by PCR was performed once per sample. In concordance with published guidelines, results were considered positive if they had a cycle quantitation (Cq) value <40 and likely positive if they had a Cq value 40–45 (9).

Sequencing was attempted for pools with Cq values <40 (online Technical Appendix Table 2) and performed by Macrogen (Seoul, South Korea). Consensus sequences were analyzed by using CLC Main Workbench 7 (http://www.clcbio.com/products/clc-main-workbench/) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank. Phylogenetic trees were constructed by using the Kimura 2-parameter model and the neighbor-joining method. Bootstrap values were determined by using 1,000 replications.

A total of 768 tick pools containing 6,692 ticks were screened. Pools contained 3 genera of ticks: 59.9% (460/768) Haemaphysalis spp., 36.3% (279/768) A. testudinarium, and 3.8% (29/768) Dermacentor auratus. Of the pools, 3% (23/768) contained adults, 36.5% (280/768) contained larvae, and 60.5% (465/768) contained nymphs (Table 1).

Rickettsia spp. were identified in 5.7% (44/768) of pools, and an additional 2.3% (18/768) of pools were likely positive for Rickettsia spp. Sequences consistent with 5 described Rickettsia species or genotypes were identified: R. tamurae, R. japonica, Rickettsia sp. ATT, Rickettsia sp. Kagoshima6, and Rickettsia sp. TwKM01 (Table 2; Figure 1).

Three novel genotypes (Table 2) were identified that might be new species. Candidatus Rickettsia laoensis (pool 447) was identified in 1 Haemaphysalis sp. pool. Phylogenetic analysis of 2845–2920-bp concatenated sequences of gltA, sca4, and ompB genes suggested that this bacteria

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belonged to the \textit{R. massiliae} group of rickettsiae (online Technical Appendix Figure 3). \textit{Candidatus} Rickettsia mahosotii (pools 81 and 372) was identified in \textit{Haemaphysalis} spp. and \textit{A. testudinarium} pools. Phylogenetic analysis of \textit{gltA}, \textit{sca4}, and \textit{ompB} genes suggested that this bacteria belonged to the \textit{R. rickettsii} group (online Technical Appendix Figure 3). \textit{Candidatus} Rickettsia khammouanensis was identified in 1 \textit{Haemaphysalis} sp. nymph pool (pool 120). Phylogenetic analysis of \textit{gltA}, 17-kDa, and \textit{ompB} genes suggested a relationship with the \textit{R. helvetica} group (online Technical Appendix Figure 4).

In addition, 15 \textit{A. testudinarium} pools showed dual peaks for 17-kDa gene sequences, which suggested the presence of \textit{R. tamurae} and \textit{Rickettsia} sp. ATT. Sequencing of \textit{sca4}, \textit{ompA}, and \textit{ompB} genes from 1 of these pools (pool 239) identified unique sequences (Table 2; online Technical Appendix Figure 4).

\textit{Borrelia} spp. were identified in 1.6\% (12/768) of pools (Table 1). Two unique sequences obtained from \textit{Haemaphysalis} spp. pools showed 99.3\% (298/300) (GenBank accession no. KR733069) and 98.7\% (296/300) (accession no. KR733068) identity with Shiretoko \textit{Haemaphysalis Borrelia} sp. (AB897888). Phylogenetic analysis confirmed that both bacteria were closely related to Shiretoko \textit{Haemaphysalis Borrelia} sp. (accession no. B897888) and belong to the relapsing fever group of \textit{Borrelia} (Figure 2).

Twelve (1.6\%) of 768 pools were positive for \textit{Ehrlichia} spp. (Table 1); an additional 6 pools (0.8\%) were likely positive. One short sequence from a \textit{Haemaphysalis} sp. nymph pool (pool 357) was obtained, and this sequence showed 100\% identity (116/116 bases) with the genus \textit{Ehrlichia}.

No pools were positive for \textit{Anaplasma} spp., but 2 were likely positive (Table 1). Although not all pools were tested for \textit{Coxiella} spp. (n = 511), 1 pool (0.2\%) was positive, and 4 pools were likely positive for \textit{C. burnetti}. No confirmatory sequences were obtained from these pools. The 1 tick that contained a blood meal (\textit{A. testudinarium nymph}) showed negative results by screening PCRs.

**Conclusions**

This study provides evidence that \textit{Rickettsia} spp., \textit{Borrelia} spp., and \textit{Ehrlichia} spp. are present in ticks in Laos. Several \textit{Rickettsia} spp. identified in this study are human pathogens. Infections with \textit{R. tamurae} (2) and \textit{R. japonica} are well described in Southeast Asia (10). However, the pathogenicity of \textit{Rickettsia} sp. TwkM01 (11), \textit{Rickettsia} sp. ATT (12), \textit{Rickettsia} sp. kagoshima6 genotypes (13) and potential novel \textit{Candidatus} Rickettsia laoensis, \textit{Candidatus} Rickettsia mahosotii, and \textit{Candidatus} Rickettsia khammouanensis is unknown. \textit{Candidatus} Rickettsia khammouanensis is phylogenetically related to \textit{R. helvetica}, for which there is serologic evidence for its role as a human pathogen in Laos (2). Unique \textit{ompA}, \textit{ompB}, and \textit{sca4} sequences identified in this study (Table 2) might indicate the presence of

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**Table 1. Tick pools tested for bacteria after screening by quantitative PCR, Khammouan Province, Laos**

| Bacteria and tick species | Total | Larvae | Nymphs | Adult males | Adult females |
|---------------------------|-------|--------|--------|-------------|---------------|
| \textit{Rickettsia} spp.  |       |        |        |             |               |
| All                       | 44/768 (5.7) | 6/280 (2.1) | 37/465 (8.2) | 0/12 (0) | 1/11 (9.1) |
| \textit{Amblyomma testudinarium} | 27/279 (10.0) | 0/61 (0) | 27/217 (12.9) | 0/1 (0) | 0/1 (0) |
| \textit{Haemaphysalis G1}  | 5/398 (1.3) | 8/194 (4.1) | 9/200 (4.5) | 0/3 (0) | 0/1 (0) |
| \textit{H. hystricis}     | 1/6 (16.7) | NS      | NS      | 0/3 (0) | 1/3 (33.3) |
| \textit{Dermacentor auratus} | 1/29 (3.4) | 0/0 (0) | 1/26 (3.8) | 0/2 (0) | 0/1 (0) |
| \textit{Ehrlichia} spp.   |       |        |        |             |               |
| All                       | 12/768 (1.6) | 4/280 (1.4) | 6/465 (1.3) | 1/12 (8.3) | 1/11 (9.1) |
| \textit{A. testudinarium} | 2/279 (0.7) | 0/61 (0) | 2/217 (0.9) | 0/1 (0) | 0/1 (0) |
| \textit{Haemaphysalis G1} | 8/398 (2.0) | 4/194 (2.1) | 4/200 (2.0) | 0/3 (0) | 0/1 (0) |
| \textit{H. aborensis}     | 2/6 (33.3) | NS      | NS      | 1/3 (33.3) | 1/3 (33.3) |
| \textit{Borrelia} spp.    |       |        |        |             |               |
| All                       | 12/768 (1.6) | 2/280 (0.7) | 8/465 (1.7) | 2/12 (16.7) | NS            |
| \textit{A. testudinarium} | 2/279 (0.7) | 1/61 (1.6) | 1/217 (0.5) | 0/1 (0) | 0/1 (0) |
| \textit{Haemaphysalis G1} | 6/398 (1.5) | 1/194 (0.5) | 5/200 (2.5) | 0/3 (0) | 0/1 (0) |
| \textit{Haemaphysalis G1.2} | 1/13 (7.7) | NS      | 1/13 (7.7) | NS | NS |
| \textit{H. aborensis}     | 2/6 (33.3) | NS      | NS      | 2/3 (66.7) | 0/3 (0) |
| \textit{D. auratus}       | 1/29 (3.4) | 0/0 (0) | 1/26 (3.8) | 0/2 (0) | 0/1 (0) |
| \textit{Coxiella} spp.    |       |        |        |             |               |
| All                       | 5/511 (1.0)† | 4/187 (2.1)† | 1/310 (0.3) | 0/8 (0) | 0/6 (0) |
| \textit{Haemaphysalis G1} | 5/279 (1.8)† | 4/162 (2.5)† | 1/117 (0.9) | NS | NS |
| \textit{Anaplasma} spp.   |       |        |        |             |               |
| All                       | 2/768 (0.3)† | 0/280 (0)† | 0/465 (0)† | 0/12 (0) | 0/11 (0) |
| \textit{A. testudinarium} | 1/279 (0.4)† | 0/61 (0) | 1/217 (0.5)† | 0/1 (0) | 0/1 (0) |
| \textit{Haemaphysalis G1} | 1/398 (0.3)† | 1/194 (0.5)† | 0/200 (0) | 0/3 (0) | 0/1 (0) |

*NS, no samples were available for screening.
†Includes samples with cycle quantitation values <40 and 40–45.
Table 2. Sequence data for Rickettsia species isolated from ticks, Khammouan Province, Laos*

| Tick pool | Tick species and stage | Rickettsia spp. gene, GenBank accession no., and % similarity (no. matching nucleotides/total) |
|-----------|------------------------|-----------------------------------------------------------------------------------------------|
| 110       | Amblyomma testudinarium nymph | Unclear sequence | Unclear sequence | KT753264, 100.0 (529/529) with Rickettsia sp.
|           |                        |                                                |                                                | TwKM01
| 177, 180, 216, 220 A. testudinarium nymph | KR733070, 100.0 (355/355) with R. tamurae AB114425 | KT753265, 99.8 (1,096/1,096) with R. tamurae AB812551 | KT753266, 99.7 (807/809) with R. tamurae DQ113911 | NS NS
| 315 A. testudinarium nymph | KT753267, 98.8 (407/412) with R. raoultii JX885457 | KT753268, 99.9 (1,036/1,037) with Rickettsia kagoshima6 JQ697956 | KT753269, 98.8 (795/821) with Rickettsia sp. AUS 118, KF666473 | Could not be amplified |
| 239 A. testudinarium nymph | KT753271, 99.7 (360/361) with Rickettsia sp. ATT AF483196 | KT753272, 99.7 (1,046/1,051) with R. tamurae (AB812551)/KT753273, 99.2 (367/370) with Rickettsia sp. hmn77 KC566999 | KT753274, 97.1 (759/782) with Rickettsia sp. AUS 118, KF666473 | KT753275, 87.2 (530/602) with R. raoultii JQ792137 |
| 76, 337, 450, 453 Haemaphysalis G1 nymphs (3), A. testudinarium nymph (1) | KT753277, 98.4 (417/423) with R. raoultii JX885457 | KT753278, 99.9 (1,037/1,038) with Rickettsia kagoshima6 JQ697956 | KT753279, 98.4 (794/807) with R. japonica AF155055 | Could not be amplified |
| 81, 372 Haemaphysalis G1 nymphs, A. testudinarium nymph (17 kDa only) | KT753283, 99.0 (408/412) with R. raoultii JX885457 | KT753284, 99.5 (1,090/1,096) with R. sibirica U59734 | KT753285, 98.5 (838/851) with R. japonica AF 155055 | Could not be amplified |
| 120 Haemaphysalis G1 nymph | KT753287, 96.1 (391/407) with R. helvetica GU827073 | KT753288, 97.1 (370/381) with Candidatus Rickettsia rara DQ365805 | Could not be amplified (x2) | Could not be amplified (x2) |
| 407 Haemaphysalis hysticas adult | KT733074, 100.0 (413/413), R. japonica AP011533 | KT753281, 100.0 (1,063/1,063), R. japonica AP011533 | NS NS | KT753282, 100.0 (1,191/1,191), R. japonica AP011533 |
| 447 Haemaphysalis G1 nymph | KT753291, 98.6 (407/413) with R. massilae CP000683 | KT753290, 99.6 (961/965) with R. raoultii JX885455 | KT753292, 97.5 (591/606) with Rickettsia sp. JL-02 AY093696 | KT753293, 97.5 (591/606) with Rickettsia sp. JL-02 AY093696 |
|           |                        |                                                |                                                | KT753294, 98.4 (1,137/1,156), with R. massilae CP003319 |

*New sequences were compared with reference sequences. NS, not sequenced.

Rickettsia sp. ATT (12), which was previously believed to be identical to R. tamurae (14), and suggests that it might be a distinct species. Further studies, including whole-genome sequencing, are required to identify and confirm these novel genotypes and understand their role in human disease.

Borrelia spp. sequences identified in Haemaphysalis spp. pools were shown to have high concordance with the Shiretoko Haemaphysalis Borrelia isolated from Haemaphysalis spp. ticks and deer in Japan (15). The species belongs to the relapsing fever group of Borrelia and is related to B. lonestari.

Sequence data for Ehrlichia spp. indicated the presence of these bacteria but were not sufficient to identify them to the species level. The Cq values were high (40–45) for Anaplasma spp., but no sequence data were obtained. Coxiella spp. were screened by using primers for IS1111, which are not specific for C. burnetii, and no confirmatory sequence data were obtained. Because of limited reagents, screening of all 768 pools for Coxiiella sp. was not completed. Further work is required to investigate the presence of these bacteria in Laos.

Our study had several limitations. First, pooling of ticks precludes an accurate assessment of prevalence of bacterial pathogens. Second, sequences obtained from some A. testudinarium pools had dual peaks, suggestive of multiple infections, and could therefore not be interpreted. Third, ticks were collected only from 1 area in Laos.
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(Khammouan Province); thus, extrapolating findings to the entire country must be done cautiously.

Our results highlight the frequency of tickborne bacterial infections in Laos. These findings emphasize the need for further research of tick-associated bacteria and their role in human disease.

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**Figure 1.** Phylogenetic analysis of *Rickettsia* spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (350 bp) of the 17-kDa gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.

**Figure 2.** Phylogenetic analysis of *Borrelia* spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (299–323 bp) of the flaB gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.
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Technical Appendix

Technical Appendix Table 1. Primers and probes used for screening quantitative PCR in large-scale survey for tickborne bacteria, Khammouan Province, Laos*

| Bacteria                  | Gene     | Primer or probe | Sequence, 5′→3′                   | Reference† |
|---------------------------|----------|-----------------|-----------------------------------|------------|
| Rickettsia spp            | 17 kDa   | R17K128F2       | F-GGGCGGATGAAAYAACAAAG            | (5)        |
| Spotted fever group       | 17 kDa   | R17K238R        | R-CCTACACCTACCTCCVACAAG           | (5)        |
| Spotted fever group       | 17 kDa   | R1K7202TACP     | P-CGAAATGGAAAACAGTAGGTC           | (5)        |
| Borrelia spp              | 23S rRNA | Bb23Sr          | R-TATTATGGCCAGCGGGGA             | (6)        |
| Borrelia spp              | 23S rRNA | Bb23Sp          | P-AGATGGTAGGGAGCCCAAGCAGGT        | (6)        |
| Coxliella spp             | IS1111   | IS1111l         | R-CAAGAACGGTACGGTGGC             | (7)        |
| Coxliella spp             | IS1111   | IS1111l         | R-CAAGAACGGTACGGTGGC             | (7)        |
| Coxliella spp             | IS1111   | IS1111probe     | P-CGAGGGAAACAGTAGGTC             | (7)        |
| Ehrlichsia spp            | 16S rRNA | EHR16S-17       | P-GCGGAGCAGCTACACAT              | (8)        |
| Ehrlichsia spp            | 16S rRNA | EHR16S-97       | R-CGAGGTCGACACTAAATTATT          | (8)        |
| Ehrlichsia spp            | 16S rRNA | EHR16S-38       | P-CGAGGTCGACACTAAATTATT          | (8)        |
| Anaplasma spp             | ms2      | ApMSP2f         | F-ATGGAAGGTGTTGTTGTTGTTGATG      | (6)        |
| Anaplasma spp             | ms2      | ApMSP2r         | R-TTGGTCTTGGAAGCGCTGTA           | (6)        |
| Anaplasma spp             | ms2      | ApMSP2p         | P-TTGGTCCGAGGGTCTGTTAGG          | (6)        |
*F, forward; IS, insertion sequence; ms2, major surface protein 2; P, probe; R, reverse.
†References are in the text of the article.

Technical Appendix Table 2. Primers used for sequencing in large-scale survey for tickborne bacteria, Khammouan Province, Laos*

| Bacteria                  | Gene     | Primer    | Sequence, 5′→3′                   | Size, bp   | Reference† |
|---------------------------|----------|-----------|-----------------------------------|------------|------------|
| Rickettsia spp            | 17 kDa   | R17KM61F† | F-.ACTTTACAAATTTCAAAACCATA       | 524        | (1)        |
| Spotted fever group       | 17 kDa   | R17K31F†  | R-CTTTGCGCTACTAAACATTACT         | 524        | (1)        |
| Spotted fever group       | 17 kDa   | R2609Rnewt†| R-CTTTGCGCTACTAAACATTACT         | 434        | (1)        |
| Spotted fever group       | gltA     | Rs1DF†    | R-ATGCAACTTGGAGAATAAA            | 1,237      | (2)        |
| Spotted fever group       | gltA     | Rs1SF†    | R-GGGGCGCTCGCTACGCGGG            | 382        | (2)        |
| Spotted fever group       | gltA     | Cs1DF†    | R-GGGGCGCTCGCTACGCGGG            | 382        | (2)        |
| Spotted fever group       | gltA     | Cs1SF†    | R-GGGGCGCTCGCTACGCGGG            | 382        | (2)        |
| Spotted fever group       | sca4     | RsD49F†   | R-GGGGCGCTCGCTACGCGGG            | 1,078      | (3)        |
| Spotted fever group       | sca4     | RsD928F†  | R-GGGGCGCTCGCTACGCGGG            | 1,078      | (3)        |
| Spotted fever group       | sca4     | RsD1826R† | R-GGGGCGCTCGCTACGCGGG            | 899        | (3)        |
| Spotted fever group       | ompA     | RompA50F†  | R-GGGGCGCTCGCTACGCGGG            | 692        | (4)        |
| Spotted fever group       | ompA     | RompA642R† | R-GGGGCGCTCGCTACGCGGG            | 692        | (4)        |
| Spotted fever group       | ompA     | RompA701R† | R-GGTGCTCGCTACGCGGG              | 692        | (4)        |
| Spotted fever group       | ompB     | RompB115F†  | R-GGTGCTCGCTACGCGGG              | 1,902      | (5)        |
| Spotted fever group       | ompB     | RompB607F†  | R-GGTGCTCGCTACGCGGG              | 1,902      | (5)        |
| Spotted fever group       | ompB     | RompB1902R† | R-GGTGCTCGCTACGCGGG              | 1,265      | (5)        |
| Borrelia spp              | fla B    | 280F†      | R-GGTGCTCGCTACGCGGG              | 1,452      | (5)        |
| Borrelia spp              | fla B    | 754R†      | R-GGTGCTCGCTACGCGGG              | 437        | (6)        |
| Borrelia spp              | fla B    | 301F†      | R-GGTGCTCGCTACGCGGG              | 437        | (6)        |
| Borrelia spp              | fla B    | 737R†      | R-GGTGCTCGCTACGCGGG              | 437        | (6)        |
| Ehrlichsia spp            | 16S rRNA | Ehr165F    | R-GGTGCTCGCTACGCGGG              | 437        | (7)        |
| Ehrlichsia spp            | 16S rRNA | Ehr16SR    | R-GGTGCTCGCTACGCGGG              | 437        | (7)        |
*F, forward; R, reverse.
†First reaction.
‡Second reaction.
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**Technical Appendix Figure 1.** Khammouan Province (red star), Laos, where ticks were collected in this study (see Technical Appendix Figure 2). Locations of previous studies investigating *Rickettsia* spp. in Laos are shown by the green star (8) and blue star (9).
Technical Appendix Figure 2. Location of tick collections sites (red stars) in Khammouan Province, Laos.
Technical Appendix Figure 3. Phylogenetic analysis of gltA, sca4, and ompB genes of candidate novel Rickettsia spp., Kammouan Province, Laos. The tree was constructed by using concatenated partial nucleotide sequences (2,845–2,920 bp) of gltA, sca4, and ompB genes; the Kimura-80 model; and the neighbor-joining method. Analyses were supported by using bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. Sample numbers identifying each tick pool from this study are shown in parentheses after the sequence name. Scale bar indicates nucleotide substitutions per site.

Technical Appendix Figure 4. Phylogenetic analysis of gltA, 17 kDa, and ompB genes of Rickettsia spp., Kammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (1,114–1,117 bp) of concatenated sequences of gltA, 17-kDa, and ompB genes; the Kimura-80 model; and the neighbor-joining method. Analyses were supported by using bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. Sample numbers identifying each tick pool from this study are shown in parentheses after the sequence name. Scale bar indicates nucleotide substitutions per site.