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Evolution

Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences

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ABSTRACT **Ticks are parasitiform mites that are obligate hematophagous ectoparasites of amphibians, reptiles, birds, and mammals. A phylogeny for tick families, subfamilies, and genera has been described based on morphological characters, life histories, and host associations. To test the existing phylogeny, we sequenced ≈460 bp from the 3′ end of the mitochondrial 16S rRNA gene (rDNA) in 36 hard- and soft-tick species; a mesostigmatid mite, *Dermanyssus gallinae*, was used as an outgroup. Phylogenies derived using distance, maximum-parsimony, or maximum-likelihood methods were congruent. The existing phylogeny was largely supported with four exceptions. In hard ticks (Ixodidae), members of Haemaphysalinae were monophyletic with the primitive Amblyomminae and members of Hyalomminae grouped within the Rhipicephalinae. In soft ticks (Argasidae), the derived phylogeny failed to support a monophyletic relationship among members of Ornithodorinae and supported placement of Argasinae as basal to the Ixodidae, suggesting that hard ticks may have originated from an *Argas*-like ancestor. Because most *Argas* species are obligate bird ectoparasites, this result supports earlier suggestions that hard ticks did not evolve until the late Cretaceous.**

Ticks are classified in the suborder Ixodida of the order Parasitiformes, one of the two orders of mites (Acari) (1). They are unique among Acari in possessing a large body size (2–30 mm) and specialized mouthparts. All ticks are hematophagous, obligate ectoparasites of terrestrial vertebrates including amphibians, reptiles, birds, and mammals. The group is relatively small, consisting of about 850 species divided into two major families: the Argasidae ("soft" ticks) and the Ixodidae ("hard" ticks) (2, 3). The third family, Nuttalliellidae, contains only a single species, which shares characters of both Argasidae and Ixodidae in addition to having many derived features (4).

The family Ixodidae is divided into the Prostriata and Metastriata. The Prostriata (subfamily Ixodinae) comprise about 240 species in a single genus, *Ixodes*. The Metastriata are divided into four subfamilies (5): the Amblyomminae (125 species in two genera), Haemaphysalinae (147 species), Hyalomminae (22 species), and Rhipicephalinae (119 species in eight genera). The family Argasidae contains about 170 species divided into two subfamilies, Argasinae (56 species) and Ornithodorinae (114 species in three genera).

The most commonly cited phylogeny among tick families, subfamilies, and genera is that of Hoogstraal and Aeschlimann (6) (Fig. 1). Hoogstraal's conception of the long-term evolution of ticks combined a scenario of broad cospeciation on specific hosts with an assumption that ticks are a group of ancient derivation (5–8). Hoogstraal and earlier workers suggested that various structural modifications of the mouthparts and coxae were associated with specialization for particular hosts. They noted that changes in these characters in different instars appeared to be correlated with the host species parasitized by each instar and concluded that adaptation to hosts played a major role in tick evolution. This adaptation was assumed to lead to host specificity and eventually to parallel evolution (cospeciation) between ticks and their hosts. Hoogstraal suggested that the ancestral ticks, resembling the present-day Argasidae, arose in the late Paleozoic or early Mesozoic associated with "slow-moving, smooth-skinned reptiles" (5). The Prostriata was among the earliest line to differentiate from those ancestral forms. Within the Metastriata, Hoogstraal proposed that the Amblyomminae originated on reptiles in the late Permian and radiated on those hosts during the Triassic and Jurassic. The Haemaphysalinae appeared on reptiles later in the Triassic, whereas the Hyalomminae evolved on early mammals late in the Cretaceous. The Rhipicephalinae did not appear until the Tertiary and, like the Hyalomminae, evolved primarily on mammals.

The fossil record provides few clues to tick evolution. The first mite fossils date from the Devonian and closely resemble extant taxa. However, all representatives of that fauna belong to the suborder Acariformes. Fossils of the Parasitiformes, and in particular of ticks, are quite rare and much more recent. For example, hard ticks appear only in amber from the Eocene and Oligocene (9–11). Direct evidence regarding the time of origin of ticks is therefore absent, and scenarios which vary markedly from Hoogstraal's have been proposed. Oliver (2) suggested an even earlier age for the evolution of ticks, by assuming an origin close to that of the Parasitiformes. He established the age of the latter group by comparison with the known age of its sister group, the Acariformes. He suggested a possible origin on amphibians. Alternatively, Russian workers have rejected amphibians or reptiles as ancestral hosts and suggested a much more recent origin of at least the Prostriata. The Russian school proposed that the Ixodidae arose in the Cretaceous because nearly all extant Ixodidae occur on mammals or birds and "primitive" Ixodidae occur on primitive mammals (marsupials and monotremes) (12, 13).

Hoogstraal and Aeschlimann's phylogeny for hard- and soft-tick genera has never been tested with a formal cladistic analysis. The purpose of this study was to use sequence variation from the 3′ end of the mitochondrial 16S rRNA gene ("16S" rDNA)§ to derive a molecular phylogeny for hard- and soft-tick families, subfamilies, and genera. Molecular characters provide an objective means to test the existing phylogeny and, in particular, afford a neutral background against which to examine the morphological characters and cospeciation mechanisms used by Hoogstraal. Objective phylogenies also permit examination of alternative modes of speciation not considered by Hoogstraal, including habitat

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§The sequences reported in this paper have been deposited in the GenBank database (accession nos. L34292–L34330).

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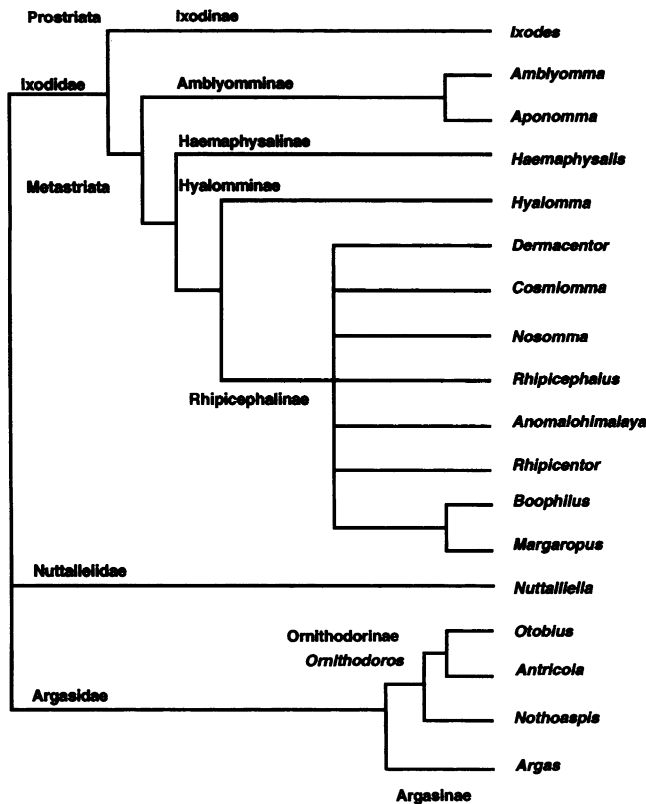

FIG. 1. Phylogeny of families, subfamilies, and genera of soft and hard ticks proposed by Hoogstraal and Aeschlimann (6) and based on morphology, life history, and host associations.

adaptation and vicariant speciation following biogeographical separation.

MATERIALS AND METHODS

PCR Amplification. DNA was isolated by freezing and crushing individual ticks (14) in 60 μl of homogenization buffer (15). Amplification was initially accomplished with two primers, 16S+1 (5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′) and 16S−1 (5′-CCGGTCTGAACTCAGATCAAGT-3′). The predicted product size is ≈460 bp. Full-length amplification was unsuccessful in over half of the specimens and we instead amplified overlapping halves by using the 16S+1 primer in combination with 16S−2 (5′-TTACGCTGTTATCCCTAGAG-3′) to amplify the first half and 16S−1 with 16S+2 (5′-TTGGGCAAGAAGACCCTATGAA-3′) to amplify the second half. The 16S+2 and 16S−2 primers were designed from conserved regions determined during initial sequencing of distant taxa.

PCRs were done in 50 μl of reaction buffer (50 mM KCl/10 mM Tris·HCl, pH 9.0/1.5 mM $MgCl_2$/0.01% gelatin/0.1% Triton X-100/200 μM dNTPs/1 μM each primer) in 500-μl microcentrifuge tubes with ≈25 μl of mineral oil layered on top. These tubes were exposed at a distance of 5 cm to ultraviolet light (260 nm) for 10 min to destroy contaminating template DNA. Tick template DNA (1.5 μl) was then added through the oil. The tubes were placed in a PTC-100 thermal cycler (MJ Research, Watertown, MA) and heated at 95°C for 5 min; the temperature was then reduced to 80°C and 1 unit of *Taq* DNA polymerase (Promega) was added. Amplification was completed with a program consisting of 10 cycles of 1 min at 92°C, 1 min at 48°C, and 1.5 min at 72°C. This was followed by 32 cycles of 1 min at 92°C, 35 sec at 54°C, and 1.5 min at 72°C. A final extension reaction was carried out for 7 min at 72°C and the reaction mixture was stored overnight at 4°C. Negative controls (no template) were always run simultaneously and reaction mixtures were discarded when any DNA appeared in the negative control.

DNA Sequencing. Amplified DNA was sequenced directly in all taxa. The amplified DNA was purified by Magic PCR Preps (Promega) according to manufacturer protocols and resuspended in 20 μl of 10 mM Tris·HCl/1 mM EDTA, pH 8.0. Double-stranded DNA sequence was determined by cycle sequencing (*fmol* system; Promega).

Six primers were used for sequencing. The original PCR primers were used for sequencing from the ends. In addition, two primers overlapping and complementary to the 16S−2 and 16S+2 primers were designed: 16S+3 (5′-ATACTCTAGGGATAACAGCGT-3′) and 16S−3 (5′-AAATTCATAGGGTCTTCTTGTC-3′). When the entire 460-bp fragment was amplified, six separate sequencing reactions were used to sequence over the entire length on both strands. When the region was amplified in two overlapping halves, four primers were used to sequence over each half on both strands so that eight separate sequencing reactions were run on one taxa. The 16S+1/16S−2 half was sequenced with these primers in addition to the 16S+2 and 16S−3 primers, whereas the 16S−1/16S+2 half was sequenced with the PCR primers in addition to the 16S−2 and 16S+3 primers.

Sequence Alignments and Phylogenetic Inferences. Sequences were read manually into a computer from autoradiographs using SEQAID II 3.6 (16). These were initially aligned using CLUSTALV (17). Nucleotides that were obviously misaligned were manually shifted. Distance, maximum-parsimony, and maximum-likelihood methods were used in phylogeny reconstruction. For distance analysis, a neighbor-joining tree (18) was generated from a Kimura two-parameter distance matrix (19) using MEGA (20) or PHYLIP 3.5C (21) with NEIGHBOR and DNADIST. Maximum-parsimony analysis was performed with PHYLIP 3.5C using DNAPARS. Support for derived phylogenies was examined with PHYLIP 3.5C using bootstrapping over 1000 replications. Maximum-likelihood analysis (22) was performed with PHYLIP 3.5C using DNAML.

RESULTS

Sequence Data. The average length of the amplified 16S region in the 38 tick taxa was 460 bp [standard deviation (SD) = 8.2]. The average length in the Ornithodorinae alone was slightly longer, 476 bp (SD = 3.0). The amplified sequence corresponds with the *Drosophila yakuba* 16S rDNA between positions 12,866 and 13,367 (23). With gaps added for alignment, 506 sites were used in all analyses. Of these, 202 sites were constant, 49 sites were phylogenetically uninformative, and 255 sites were informative. The alignment is available upon request from W.C.B.

The frequencies of adenine, cytosine, guanine, and thymine were 0.373, 0.094, 0.161, and 0.372, respectively. The average rate of gaps (Table 1) in the alignment was 0.045 per nucleotide. The average substitution rate was 0.215 per site, of which the average transition rate was 0.053 and the average transversion rate was 0.117. Transitions between adenine and guanine were predominant (67.8%). Most of transversions were between adenine and thymine (82.7%) whereas adenine/cytosine, guanine/cytosine, and guanine/thymine transversions accounted for only 6.3%, 0.7%, and 10.3% of the remainder, respectively.

The average number of substitutions per site among different taxa are listed in Table 1. In the hard ticks, substitutions within the subfamily Amblyomminae were almost as great as for the entire Ixodidae. In the soft ticks, substitutions within the subfamily Ornithodorinae were large and equal to those for the entire Ixodidae.

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Table 1. Substitution, transition, transversion, and gap rates per nucleotide site among and within taxonomic groups

Taxonomic group	Substitution	Transition	Transversion	Gap
Ixodidae	0.175	0.042	0.100	0.033
Prostriata	0.102	0.033	0.054	0.016
Ixodes scapularis vs. *dammini*	0.004	0.002	0.002	0.000
Subgenus *Ixodes*	0.091	0.028	0.048	0.015
Metastriata	0.152	0.038	0.092	0.023
Amblyomminae	0.136	0.037	0.076	0.023
Amblyomma spp.	0.140	0.040	0.078	0.022
Amblyomma (neotropical spp.)	0.146	0.040	0.080	0.025
Amblyomma (African spp.)	0.085	0.036	0.042	0.008
*Amblyomma americanum**	0.004	0.000	0.004	0.000
Apponomma spp.	0.125	0.022	0.081	0.022
Haemaphysalinae	0.103	0.028	0.059	0.016
Rhipicephalinae	0.123	0.032	0.073	0.018
Hyalomminae	0.059	0.018	0.034	0.008
Rhipicephalinae (*Hyalomma* removed)	0.115	0.029	0.068	0.019
Rhipicephalus spp.	0.081	0.027	0.037	0.017
Boophilus spp.	0.018	0.008	0.004	0.006
Dermacentor spp.	0.051	0.010	0.034	0.008
Argasidae	0.201	0.059	0.102	0.039
Argasinae	0.115	0.045	0.051	0.018
Ornithodorinae	0.176	0.061	0.087	0.028
Ornithodoros spp.	0.136	0.056	0.055	0.025
Ornithodoros (moubata complex)	0.061	0.030	0.014	0.017
Antricola	0.103	0.047	0.042	0.014
Over all taxa	0.215	0.053	0.117	0.045

*Two populations.

Secondary Structure Analysis. The *Drosophila yakuba* 16S rRNA secondary structure (24) was used to predict the secondary structure of the *Haemaphysalis cretica* sequence (Fig. 2). We assumed that the secondary structure would be conserved because the locations of stems and loops are conserved between human, mouse, and *Drosophila* (24). The *H. cretica* sequence was chosen in order to facilitate analysis because it had the highest sequence similarity to the *D. yakuba* 16S gene. All nucleotides were easily identified as belonging in either a stem or a loop. This secondary structure is representative of the other taxa (data not shown) with the exception that all members of the Ornithodorinae had 17–19 more nucleotides in the stem–loop region between positions 200 and 255. The presumptive secondary structure for this region in *Ornithodoros moubata* is also shown in Fig. 2.

Phylogenies. Neighbor-joining and maximum-parsimony trees had virtually equivalent topologies (analyses not shown). The results of bootstrap analysis with distance and maximum-parsimony analyses are shown in Fig. 3 (branch lengths are proportional to the average percent divergence among taxa). There were two regions of the molecule in which alignments were ambiguous. These correspond with nucleotides 202–254 and 291–311 (Fig. 2). These regions were removed and the entire bootstrap analysis was repeated. The support for branches with and without removal of nucleotides is indicated.

There was 85–92% support for monophyly of the hard ticks, 97–100% support for monophyly of metastriate ticks, and 81–98% support for monophyly of prostriate ticks. Members of the subfamily Argasinae form a monophyletic group with 97–100% support and form a monophyletic group with the hard ticks with 71–90% support. There was only weak support (up to 68%) for members of the subfamily ornithodorinae forming a monophyletic group.

Within the metastriate ticks, members of the subfamilies Rhipicephalinae and Hyalomminae form a monophyletic group with 85–100% support. There was weak support (53%) for grouping the *Haemaphysalis* on a common branch with some of the *Amblyomma* species. Prostriate ticks are divided into subgenera (25). All of the *Ixodes* examined in this study, with the exception of *I. hexagonus*, are in the subgenus *Ixodes*. There was 77–98% support for monophyly of taxa in the subgenus *Ixodes*.

DISCUSSION

The phylogeny for hard and soft ticks derived from variation in the mitochondrial 16S rDNA nucleotide sequence largely supports the phylogeny derived by Hoogstraal and Aeschlimann (6), with four important exceptions.

(*i*) Members of Amblyomminae and Haemaphysalinae occurred on a common branch. While this is not well supported with the 16S-based phylogeny, analysis of the combined 16S and 12S mitochondrial rDNA datasets (D. Norris, personal communication) provides strong support for this result. The grouping of Amblyomminae and Haemaphysalinae was not proposed by Hoogstraal and Aeschlimann (6). Hoogstraal preferred to group the Haemaphysalinae in a lineage with the Hyalomminae and Rhipicephalinae (Fig. 1), based on the shared presence of hair-hooking devices (spines and hooks on the legs and mouthparts) and modified palps. The Amblyomminae retain the long, leg-like palps found in Argasidae, but most *Haemaphysalis* are characterized by shortened palps with the palp femur projecting beyond the lateral margin of the capitulum. However, the "primitive" *Haemaphysalis* have poorly developed hair-hooking devices and retain relatively long, leg-like palps. It is also unclear whether the different hair-hooking devices are homologous among various taxa. Members of Amblyomminae did not form a monophyletic group in our analysis. However, reanalysis of these same taxa with combined 16S and 12S datasets (D. Norris, personal communication) indicates a well-supported monophyletic relationship among Amblyomminae. Branch lengths are deep in this combined analysis as well.

(*ii*) Members of Hyalomminae occurred on a common branch with members of the Rhipicephalinae. A primary reason that these were treated by Hoogstraal and Aeschlimann (6) as a separate subfamily, more primitive and basal to the Rhipicephalinae, was based on the morphology of the

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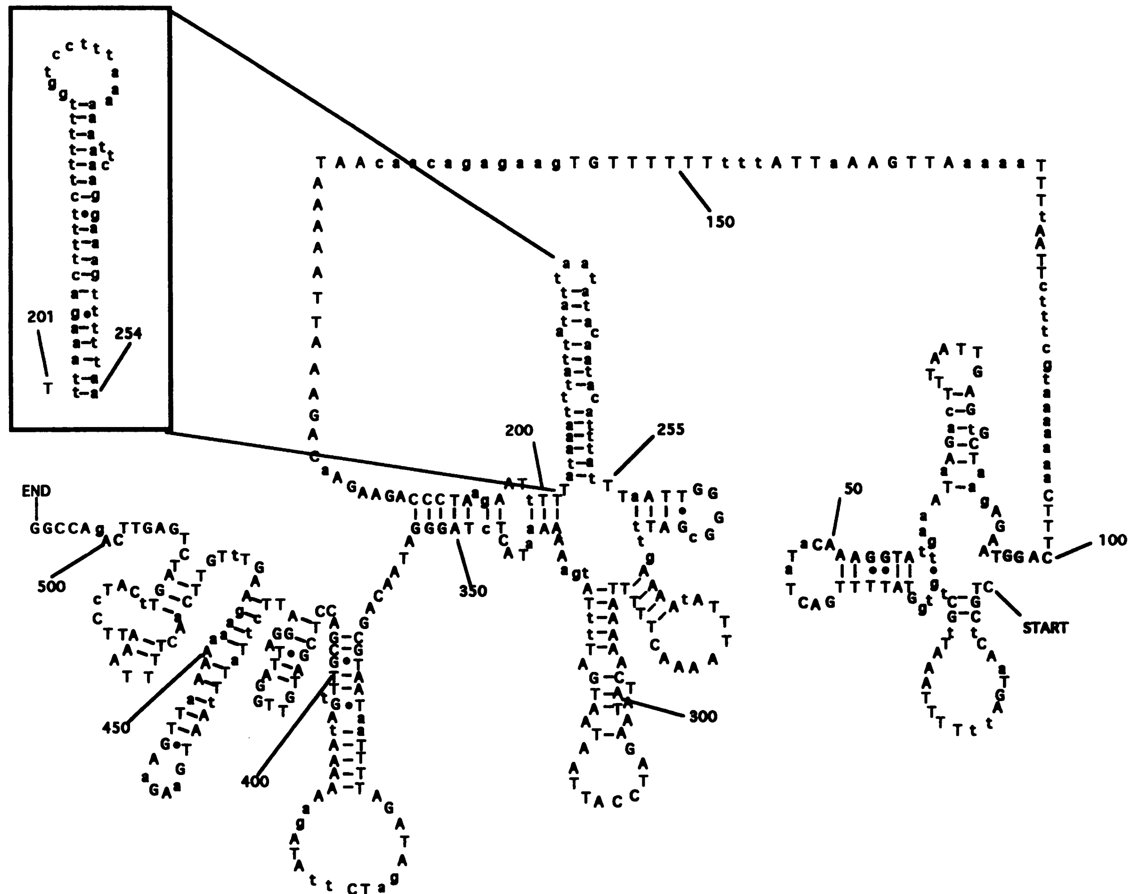

FIG. 2. Secondary structure of 460 nucleotides in the 3′ end of the mitochondrial 16S rRNA in *Haemaphysalis cretica*. This sequence had the greatest similarity with *Drosophila yakuba*. Sequences that were conserved with *D. yakuba* appear in uppercase letters. The box contains the stem–loop structure in *Ornithodoros moubata* located between positions 200 and 255. Members of Ornithodorinae contained 17–19 more nucleotides at this site than the other taxa. Sequences arranged vertically between positions 100 and 150 and between 150 and 200 correspond with stem regions in *D. yakuba*. The horizontal line of nucleotides in this region corresponds to single-stranded RNA in the 16S molecule.

mouthparts. Hoogstraal believed that larger and longer mouthparts were primitive characters and that there was tendency for mouthparts to become shorter in more recently derived lineages. The 22 species of Hyalomminae have elongated hypostomes and palps. They are distributed primarily on mammals, but one species is specific for tortoises,

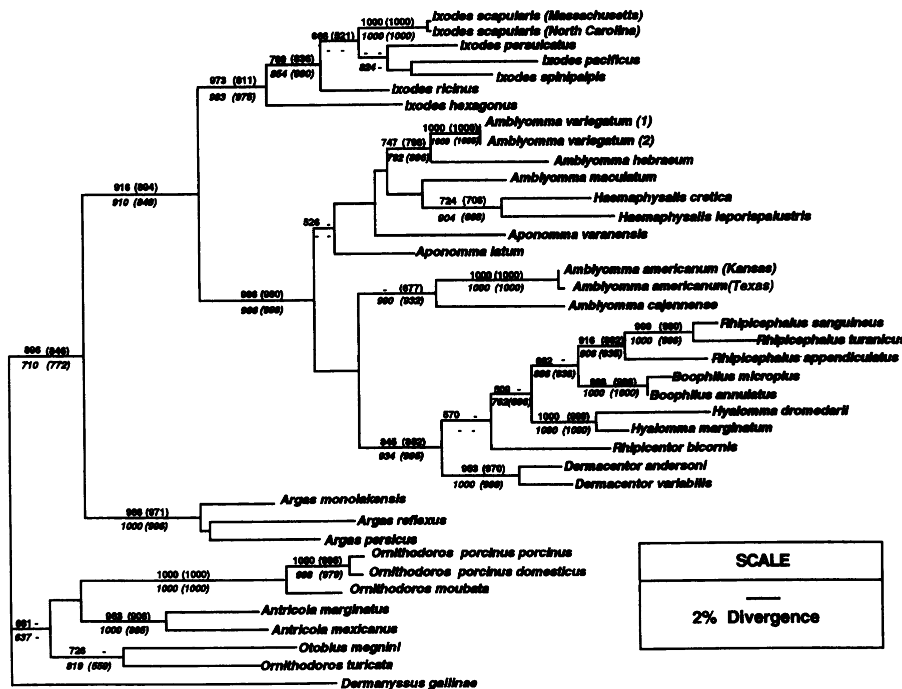

FIG. 3. Maximum-likelihood tree derived by the method of Felsenstein (22) with PHYLIP 3.5C. *Dermanyssus gallinae* was treated as an outgroup. The transition/transversion ratio was 0.453. The natural logarithm of the likelihood ratio was −6441. Branch length is proportional to percent divergence. To test the support for the derived branches, 1000 bootstrap replications (SEQBOOT) were performed with PHYLIP 3.5C. The number of replications supporting each branch when parsimony analysis was performed (DNAPARS) appears above each branch. The frequency with which each branch was supported when Kimura's two-parameter distance (DNADIST) and neighbor-joining (NEIGHBOR) were used appears below each branch. Numbers in parentheses indicate the level of support when nucleotides with ambiguous alignments (positions 202–254 and 291–311) were removed from the analysis. Branches that occurred <50% of the time in all analyses are not numbered.

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and immature individuals may feed on birds. They are believed to have originated in semidesert or steppe lowlands in Central Asia. Our data strongly suggest that *Hyalomma* species share a common ancestor with the Rhipicephalinae and should not be placed in a separate subfamily. The characters which Hoogstraal and Aeschlimann (6) considered primitive in placing Hyalomminae at the base of Rhipicephalinae may in fact be secondarily derived.

(*iii*) There was only weak support for monophyly of the members of the Ornithodorinae. However, there were a large number of substitutions among the taxa examined, suggesting that the time of the earliest divergences within this taxon is ancient. It is possible that examination of additional sequences will give stronger support for this clade. Certain consistent topologies were resolved within Ornithodorinae. Members of the *moubata* complex: *O. moubata, O. porcinus porcinus*, and *O. porcinus domesticus* (26) fell on a common branch with 100% support. The two *Antricola* [reclassified as *Carios* (27)] species formed a common branch with 90–100% support.

(*iv*) There was support for members of *Argas* forming a monophyletic and basal group with the hard ticks. This result is quite unexpected. Although a few derived characters are shared between most *Argas* and some hard ticks (mostly derived *Ixodes*), it is very difficult to derive this relationship from morphological characters alone. Examination of sequences from members of the other subgenera of *Argas* will determine whether this topology is due to exemplar effects. Examination of other DNA sequences will indicate whether this pattern is unique to the 16S rDNA. However, if, after examination of other genes and other taxa this arrangement is confirmed, it would have some interesting consequences for our view on the time of origin of the Ixodidae. With *Argas* restricted to birds, it would lend support to an origin of the hard ticks no earlier than the late Jurassic (140 million years ago), when primitive bird fossils first appeared, and probably no later than the rapid radiation of bird taxa during the late Cretaceous or early Tertiary (50–100 million years ago). This is much more recent than a late Permian (245 million years ago) origin on reptiles as originally conceived by Hoogstraal and Aeschlimann (6) but supports Filippova's (12) theory of an origin of the Ixodidae somewhere in the Cretaceous based on host associations.

We were able to resolve a consistent topology for prostriate ticks within the subgenera *Ixodes* and *Pholeoixodes* (25). Furthermore, the *Ixodes* subgenera that we have examined are all vectors of Lyme disease. Our data therefore not only support the current subgeneric classification but strengthen the argument of Filippova (28) that the main vectors of Lyme disease are monophyletic. However, we have examined only a few of the taxa which Filippova (28) considered. Further work in this group will be required to test her hypothesis that the primary vectors are Palearctic in origin.

I. dammini has been reduced to a junior synonym of *I. scapularis* (29). Recent analysis of sequence variation in the internal spacer regions of the rRNA genes among and within populations of *I. scapularis* and the former *I. dammini* showed that the populations continually overlapped, suggesting continual gene flow (30). Our study of the *I. scapularis* populations from North Carolina and Massachusetts (formerly *I. dammini*) is not a careful examination of population breeding structure; however, the number of substitutions between the individuals sampled from two populations are equal to those found intraspecifically in *Amblyomma americanum* or *A. variegatum*. Additional data on the 16S and 12S rRNA genes collected from *I. scapularis* populations throughout the geographic range of the species indicate a greater number of intraspecific substitutions than reported here but continue to support a monophyletic relationship among populations (D. Norris, personal communication).

In general these results indicate that examination of the mitochondrial 16S rDNA will be useful in examining the phylogenetics of hard- and soft-tick taxa at or below the family level. However, all of the trends that we have observed and all of the discrepancies with the phylogeny envisaged by Hoogstraal and Aeschlimann (6) need to be tested by examination of other DNA sequences.

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