

ANALYSIS OF THE SYSTEMATIC RELATIONSHIPS AMONG TICKS OF THE GENERA *RHIPICEPHALUS* AND *BOOPHILUS* (ACARI: IXODIDAE) BASED ON MITOCHONDRIAL 12S RIBOSOMAL DNA GENE SEQUENCES AND MORPHOLOGICAL CHARACTERS

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ABSTRACT: A portion of mitochondrial 12S rDNA sequences (337–355 base pairs) and 63 morphological characters of 36 hard-tick species belonging to 7 genera were analyzed to determine the phylogenetic relationships among groups and species of *Rhipicephalus* and between the genera *Rhipicephalus* and *Boophilus*. Molecular and morphological data sets were first examined separately. The molecular data were analyzed by maximum parsimony (MP), maximum likelihood, and neighbor-joining distance methods; the morphological data were analyzed by MP. After their level of congruence was evaluated by a partition homogeneity test, all characters were combined and analyzed by MP. The branches of the tree obtained by combining the data sets were better resolved than those of the trees inferred from the separate analyses. *Boophilus* is monophyletic and arose within *Rhipicephalus*. *Boophilus* species clustered with species of the *Rhipicephalus evertsi* group. Most of the clustering within *Rhipicephalus* was, however, consistent with previous classifications based on morphological data. Morphological characters were traced on the molecular reconstruction in order to identify characters diagnostic for monophyletic clades. Within the *Rhipicephalus sanguineus* complex, the sequences of specimens morphologically identified as *Rhipicephalus turanicus* were characterized by a high level of variability, indicating that *R. turanicus*-like morphology may cover a spectrum of distinct species.

Hard ticks are parasitiform mites, obligate hematophagous ectoparasites of terrestrial vertebrates. On the basis of morphological characters, the various hypotheses about taxonomic and phylogenetic relationships among hard-tick taxa have evolved considerably since the mid-19th century (Koch, 1844; Neumann, 1904; Warburton, 1912; Camicas and Morel, 1977; Hoogstraal and Aeschlimann, 1982). According to widely recognized concepts based on morphology, hard ticks are subdivided into the Prostriata (Ixodinae; *Ixodes*) and Metastricata. The Metastricata group comprises 4 subfamilies that are, in order of divergence from the original stem, Amblyomminae (*Amblyomma* and *Aponomma*), Haemaphysalinae (*Haemaphysalis*), and the sister clades Hyalomminae (*Hyalomma*) and Rhipicephalinae. The subfamily Rhipicephalinae encompasses 9 genera, among them *Rhipicephalus* (~75 species), *Dermacentor* (~30 species), *Margaropus* (3 species), and *Boophilus* (5 species) (Hoogstraal and Aeschlimann, 1982). These 4 genera contain more than 90% of the Rhipicephalinae species (Keirans, 1992). A study, based on the structure of the ventral skeleton of male ixodid ticks, suggested that Hyalomminae be included in the Rhipicephalinae (Filippova, 1993). A recent comprehensive re-evaluation of the relationships within the Metastricata, based on structural and developmental characters (Klompen et al., 1996, 1997), confirmed to some extent the classic morphological phylogeny (Hoogstraal and Aeschlimann, 1982). However, in agreement with the phylogeny proposed by Filippova (1993), *Hyalomma* species were included in the Rhipicephalinae.

Phylogenetic studies based on the variation of DNA sequences (Black et al., 1997) have also challenged the original classification of Rhipicephalinae, the main discrepancies being found in the relationships between Hyalomminae and Rhipicephalinae. Analyses based on nuclear and mitochondrial ribo-

somal genes sequences (Black and Piesman, 1994; Black et al., 1997; Mangold et al., 1998a, 1998b) confirm the fact that *Hyalomma* species are found within the Rhipicephalinae monophyletic clade, whereas *Dermacentor* are placed basal rather than within that clade.

The genus *Rhipicephalus* has been subdivided in groups and complexes according to morphological characters that were so variable that the composition of those groups kept changing over the years (Zumpt, 1941, 1942a, 1942b, 1943, 1944; Pegram and Walker, 1988). The latest morphological classifications of *Rhipicephalus* groups are based on the morphology of the immature stages (Walker, 1961; Walker et al., 2000). The number and names of species belonging to the genus *Boophilus* also varied considerably, although it is now accepted that this genus is represented by 5 species only (Curtice, 1891; Minning, 1934; Feldman-Muhsam and Shechter, 1970; Keirans, 1992). A global reassessment of the relative phylogenetic positions of *Rhipicephalus* groups and *Boophilus* species, based on molecular data, still needs to be undertaken; in the latest studies on global tick phylogeny, these 2 genera were represented by a very limited number of species (Black et al., 1997; Mangold et al., 1998a, 1998b; Murrell et al., 1999; Norris et al., 1999). Analyses of data sets, containing a relatively larger sample of *Rhipicephalus* and *Boophilus* species, not only could contribute to an understanding of how these taxa are related to each other but also may lead to a better understanding of the global structure of the Rhipicephalinae clades.

The 12S ribosomal DNA (rDNA) tick phylogenies have already proven to resolve relatively recent speciation events better than earlier ones (Murrell et al., 1999; Norris et al., 1999). This gene, therefore, appeared to be promising as a tool for examining relationships among recently diverged branches of hard-tick phylogenies. Morphological data were included to determine whether a total evidence redundant analysis would provide us with more consistent reconstructions than would the examination of molecular data alone. Furthermore, morphological characters were traced onto the phylogenetic tree inferred from molecular data, in order to establish which of them are unambiguously associated with a monophyletic group of species.

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MATERIALS AND METHODS

Tick specimens

Tick specimens were either collected by the authors and stored frozen at -80°C or obtained from the U.S. National Tick Collection (USNTC), where they had been stored for varying lengths of time in 70% ethanol. Table I lists the taxa of ticks we analyzed and their source. Each of the selected genera and each *Rhipicephalus* group (*sanguineus*, *simus*, *praeus*, *evertsi*, *appendiculatus*) were represented by at least 2 taxa to avoid as much as possible artifacts due to sampling bias. In our sample, we included broad geographical representation for certain species (*Ixodes ricinus*, *Amblyomma hebraeum*, *R. appendiculatus*, *R. sanguineus*, and *Rhipicephalus turanicus*) to evaluate the expected level of intraspecific variability of the selected 12S rDNA fragment. In our sample, the Metastriata were represented by 23 Rhipicephalinae (14 *Rhipicephalus*, 4 *Boophilus*, and 5 *Dermacentor* species), 4 *Hyalomma*, 5 *Amblyomma*, and 2 *Haemaphysalis* species. Two *Ixodes* species, representing the Prostriata, were used as outgroups. Although 12S rDNA sequences of other tick species are available in GenBank, we did not add them to our data sets because they only partially overlapped the DNA fragment we amplify with our primers (Murrell et al., 1999; Norris et al., 1999).

DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted from each specimen by using the Qiagen tissue kit (Qiagen, Chatsworth, California). Before DNA extraction, frozen ticks were thawed, whereas ethanol-preserved specimens were vacuum dried for 2 hr. Depending on the size of the tick, each specimen was triturated into variable volumes of a solution containing 90% of ATL lysis buffer (Qiagen) and 10% proteinase K (14 mg/ml) (Boehringer Mannheim, Indianapolis, Indiana). Unengorged *Ixodes* or *Rhipicephalus* ticks were triturated into a total volume of $\sim 200\ \mu\text{l}$, whereas engorged *Amblyomma* were resuspended in $\sim 600\text{--}1,000\ \mu\text{l}$ of lysis solution. The lysis was carried out overnight in a water bath at 55°C . Subsequently, if the cuticle of the tick did not appear to be clean, the same amount of proteinase K was added and the lysis carried out for 2–4 hr more. Tick debris was eliminated after a centrifugation at low speed. Except for the elution step that was performed by 2 subsequent additions of 25 μl (50–100 μl for bigger ticks) of hot (72°C) deionized water on the columns, the remaining extraction steps were performed according to the manufacturer's instructions. DNA was extracted simultaneously from Vero cells as an extraction control. The eluates were stored at $+4^{\circ}\text{C}$.

Two conserved primers (T1B = 5'-AACTAGGATTAGATACCCT-3', and T2A = 5'-AATGAGAGCGACGGGCGATGT-3') were chosen by aligning mitochondrial 12S rDNA GenBank sequences from widely divergent arthropods, i.e., *Drosophila yakuba*, *Anopheles gambiae*, *Daphnia pulex*, and *Apis mellifera* (GenBank accession numbers: NC 001322, NC 002084.1, Z15015, and L06178, respectively). The predicted product size is approximately 360 base pairs (bp) and corresponds to the fragment between position 14,232 and 14,592 of the complete mitochondrial genome sequence of *D. yakuba* (NC 001322.1).

For PCR, 2.5 μl of tick DNA were amplified in a 25- μl reaction mixture containing 2.5 pmol of each primer, 2.5 μl of *Taq* buffer (10 \times), 200 μM each of dATP, dTTP, dGTP, and dCTP, 0.1 μl of AmpliTaq polymerase (5 U/ μl), and 1.75 μl of MgCl_2 (25 mM) (PE Applied Biosystems, Foster City, California). PCR conditions were as follows: 5 min of DNA denaturation at 94°C , 5 cycles of denaturation at 94°C for 15 sec, annealing at 51°C for 30 sec, and elongation at 68°C for 30 sec, followed by 25 cycles of denaturation at 94°C for 15 sec, annealing at 53°C for 30 sec, and elongation at 70°C for 30 sec. The elongation was completed by a further 5-min step at 70°C , and the amplicons were then kept refrigerated at 4°C . Two negative controls, distilled H_2O and Vero cell DNA, were run simultaneously to detect possible contaminations during both the extraction and the amplification steps. Prior to sequencing, the PCR products were purified by using the Quiaquick PCR purification Kit (Qiagen) according to the manufacturer's protocols and resuspended in 25 μl deionized water. Each strand of the amplified fragment was directly sequenced by using primers T1B and T2A and the PRISM dRhodamine dye-terminator kit (PE Applied Biosystems). Samples were purified by using Centrisep spin columns (Princeton Separations, Adelphia, New Jersey), and sequences were resolved in an ABI 377 automated sequencer (PE Applied Biosystems).

The 2 strands of DNA were assembled using GAP4 (Wisconsin Sequence Analysis Package, Genetics Computer Group, version 8.1, Madison, Wisconsin). When the assembly of some base pairs was ambiguous, the fragments were reamplified and resequenced. Multiple alignments were done by using Clustal X, version 1.64b (Thompson et al., 1997). The GenBank accession numbers of the sequences are listed in Table I. Sequences were aligned progressively according to the guide tree with gap and gap-extension penalties set to 10. Obviously misaligned characters were manually shifted by using SEQLAB (Wisconsin Sequence Analysis Package, Genetics Computer Group, version 8.1, Madison, Wisconsin) and according to secondary structure criteria (Gutell et al., 1994; Hickson et al., 1996).

Morphological data

Most of the morphological characters were collected from available taxonomic literature (Robinson, 1926; Cooley and Kohls, 1944, 1945; Pomerantsev, 1950; Feldman-Muhsam, 1953, 1956; Theiler and Robinson, 1953; Hoogstraal, 1956; Theiler and Salisbury, 1959; Hoogstraal and Kaiser, 1960; Morel and Vassiliades, 1962; Kaiser and Hoogstraal, 1963, 1964; Feldman-Muhsam and Shechter, 1970; Nosek and Sixl, 1982; Yunker et al., 1986; Matthyse and Colbo, 1987; Pegram, Clifford et al., 1987; Pegram, Keirans et al., 1987; Pegram, Walker et al., 1987; Cordas et al., 1993). The characters of *Rhipicephalus* immature stages were derived from drawings and electron micrographs contained in a comprehensive work dedicated to *Rhipicephalus* (Walker et al., 2000). When descriptions of species or stages were not available, the data set was completed by direct observation of specimens of the USNTC collection. The number of hosts for each tick species was the only non-morphological character in our data set. One assumption of characters utilized in phylogenetic analyses is that all characters are independent. Little is known about relationships between morphological characters in ticks; it is, however, obvious that multistate characters cannot be totally independent. Character states are listed in Table II, and the character data matrix is presented in Table III.

Phylogenetic analysis

Because of the relatively small number of characters in our data sets, we first verified that data matrices contained phylogenetic signal. Ten thousand trees were randomly generated and the g_1 value for their tree-length distribution was evaluated by using the random-trees option in PAUP*, beta version 4.0b2* (Swofford, 1998), for both the molecular and the morphological data sets. The statistical significance of the obtained g_1 values were evaluated (Rohlf and Sokal, 1981; Hillis and Huelsenbeck, 1992). Phylogenetic reconstructions for the molecular data were inferred by using maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining distance (NJ) methods (PAUP*). All MP analyses were performed by heuristic search procedures, with tree bisection-reconnection branch swapping, MULTREES option, and random addition of taxa (10 replicates). Gaps were treated either as missing or a fifth character state for the analysis of molecular data and as missing characters for the analysis of the combined data set. Branch supports (MP and NJ) were calculated by bootstrap analyses (1,000 replicates for molecular and combined data sets). Branches with bootstrap values of $\geq 70\%$ were considered resolved (Hillis and Bull, 1993). Decay indices were calculated by using TreeRot, version 2 (Sorenson, 1999), combined with PAUP*. The shortest MP tree with the best ML score was used as the starting tree for the ML search, after transition/transversion ratios, the base substitution frequencies, and the alpha parameter of gamma distribution (Yang, 1996) were estimated. NJ distances were evaluated by ML. One thousand bootstrap replicates were generated to establish the support of NJ reconstructed branches.

Morphological data were analyzed by MP (100 bootstrap replicates, with MAXTREE set to 10,000 in PAUP). Gaps were treated as missing characters. A partition homogeneity test (100 replicates, with MAXTREE set to 100) was run with the molecular and the morphological data sets to establish whether the 2 partitions of characters are heterogeneous (significance threshold value is $P > 0.05$) (Bull et al., 1993). A combined phylogeny was inferred by simultaneously analyzing all characters in a single data set by MP (1,000 bootstrap replicates, random taxa addition). MacClade, version 3.05 (Maddison and Maddison, 1992), and the commands "describe tree" and "reconstruct tree" in PAUP were used to trace changes in morphological characters on the

TABLE I. List of taxa, their classification, geographical origin, USNTC accession numbers, and the GenBank accession numbers of the corresponding sequences.

Species	Rhipicephalus group	Classification (sensu Hoogstraal, 1982)	Geographical origin	Date	Collected from	Accession no. (USNTC)	Accession no. GenBank
<i>Ixodes ricinus</i>	—	Prostriata; Ixodinae	Switzerland	1993	Vegetation		AF150029
<i>I. ricinus</i>	—	Prostriata; Ixodinae	Portugal	1994	Vegetation		ND*
<i>I. ricinus</i>	—	Prostriata; Ixodinae	France	1993	Vegetation		ND
<i>Ixodes scapularis</i>	—	Prostriata; Ixodinae	Cell line IDE8, U.S.A.	na	na		AF150030
<i>Haemaphysalis leachi</i>	—	Metastrata; Haemaphysalinae	Zimbabwe	1992	<i>Canis familiaris</i>		AF150035
<i>Haemaphysalis punctata</i>	—	Metastrata; Haemaphysalinae	Switzerland	1991	Vegetation		AF150032
<i>Amblyomma hebraeum</i>	—	Metastrata; Amblyomminae	Zimbabwe	1992	<i>Bos taurus</i>		AF150049
<i>Amblyomma sparsum</i>	—	Metastrata; Amblyomminae	Zimbabwe	1992	<i>Diceros bicornis</i>	122782	AF150047
<i>Amblyomma variegatum</i>	—	Metastrata; Amblyomminae	Ivory Coast	1993	<i>Bos taurus</i>		AF150046
<i>Amblyomma americanum</i>	—	Metastrata; Amblyomminae	Colony, U.S.A.	1997	na		AF150050
<i>Amblyomma rhinocerotis</i>	—	Metastrata; Amblyomminae	Zimbabwe	1992	<i>D. bicornis</i>	122782	AF150048
<i>Hyalomma m. marginatum</i>	—	Metastrata; Hyalomminae	Morocco	1992	<i>B. taurus</i>		AF150034
<i>Hyalomma m. rufipes</i>	—	Metastrata; Hyalomminae	Zimbabwe	1992	<i>B. taurus</i>		AF150033
<i>Hyalomma dromedari</i>	—	Metastrata; Hyalomminae	Morocco	1992	<i>Camelus dromedarius</i>		AF150036
<i>Hyalomma truncatum</i>	—	Metastrata; Hyalomminae	Zimbabwe	1992	<i>B. taurus</i>		AF150031
<i>Dermacentor marginatus</i>	—	Metastrata; Rhipicephalinae	Switzerland	1991	Vegetation		AF150039
<i>Dermacentor reticulatus</i>	—	Metastrata; Rhipicephalinae	France	1995	Vegetation		AF150038
<i>Dermacentor variabilis</i>	—	Metastrata; Rhipicephalinae	Colony, U.S.A.	1997	na		AF150037
<i>Dermacentor andersoni</i>	—	Metastrata; Rhipicephalinae	Colorado	1998	Vegetation		AF150040
<i>Dermacentor albipictus</i>	—	Metastrata; Rhipicephalinae	Cell line, DAL, U.S.A.	na	na		AF150041
<i>R. sanguineus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>C. familiaris</i>	ND	ND
<i>R. sanguineus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Spain	1994	<i>C. familiaris</i>	ND	ND
<i>R. sanguineus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	France	1994	<i>C. familiaris</i>	AF150020	AF150020
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Greece	?	?	ND	ND
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Israel	?	?	AF150015	AF150015
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Israel	?	?	AF150014	AF150014
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Israel	?	?	AF150013	AF150013
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>Capra hircus</i>	AF150017	AF150017
<i>R. turanicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	France	1990	<i>Equus caballus</i>	AF150018	AF150018
<i>Rhipicephalus pusillus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	France	1995	Vegetation	AF150022	AF150022
<i>Rhipicephalus rossicus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Russia	1995	?	AF150021	AF150021
<i>Rhipicephalus pumilio</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Russia–Astrakhan	1994	<i>C. familiaris</i>	AF150023	AF150023
<i>Rhipicephalus bursa</i>	<i>R. evertsi</i>	Metastrata; Rhipicephalinae	Colony, Spain	1994	na	AF150053	AF150053
<i>Rhipicephalus e. evertsi</i>	<i>R. evertsi</i>	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>B. taurus</i>	AF150052	AF150052
<i>Rhipicephalus pulchellus</i>	<i>R. appendiculatus</i>	Metastrata; Rhipicephalinae	Tanzania	1956	<i>B. taurus</i>	96690	AF150024
<i>Rhipicephalus maculatus</i>	<i>R. appendiculatus</i>	Metastrata; Rhipicephalinae	South Africa	1995	Vet. Faculty Pretoria	AF150026	AF150026
<i>Rhipicephalus appendiculatus</i>	<i>R. appendiculatus</i>	Metastrata; Rhipicephalinae	Uganda	1955	<i>B. taurus</i>	121879	AF150028
<i>R. appendiculatus</i>	<i>R. appendiculatus</i>	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>B. taurus</i>	AF150027	AF150027
<i>Rhipicephalus kochi</i>	<i>R. pravus</i>	Metastrata; Rhipicephalinae	Tanzania	1975	<i>Nesotragus moschatus</i>	65551	AF150051
<i>Rhipicephalus praeus</i>	<i>R. pravus</i>	Metastrata; Rhipicephalinae	Tanzania	1975	<i>B. taurus</i>	65690	AF150025
<i>Rhipicephalus zampti</i>	<i>R. simus</i>	Metastrata; Rhipicephalinae	South Africa	1995	Vegetation	122657	AF150016
<i>Rhipicephalus simus</i>	<i>R. simus</i>	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>C. familiaris</i>	AF150019	AF150019
<i>Boophilus kohlsi</i>	—	Metastrata; Rhipicephalinae	Jordan	1959	<i>Ovis aries</i>	91415	AF150043
<i>Boophilus decoloratus</i>	—	Metastrata; Rhipicephalinae	Zimbabwe	1992	<i>B. taurus</i>	AF150044	AF150044
<i>Boophilus annulatus</i>	—	Metastrata; Rhipicephalinae	Yemen	?	<i>B. taurus</i>	90072	AF150045
<i>Boophilus microplus</i>	—	Metastrata; Rhipicephalinae	Nepal	1969	<i>B. taurus</i>	54465	AF150042

TABLE I. Continued.

Species	<i>Rhipicephalus</i> group	Classification (sensu Hoogstraal, 1982)	Geographical origin	Date	Collected from	Accession no. (USNTC)	Accession no. GenBank
<i>Rhipicephalus longus</i>	<i>R. capensis</i>	Metastrata; Rhipicephalinae	Belgian Congo	1912	<i>Syncerus caffer</i>	105252	ND
<i>Rhipicephalus senegalensis</i>	<i>R. simus</i>	Metastrata; Rhipicephalinae	Belgian Congo	1951	<i>Dendrocygna viduator</i>	36707	ND
<i>Rhipicephalus praetextatus</i>	<i>R. simus</i>	Metastrata; Rhipicephalinae	Tanzania	1974	<i>Taurotragus oryx</i>	122337	ND
<i>Rhipicephalus punctatus</i>	<i>R. pravus</i>	Metastrata; Rhipicephalinae	South Africa	?	?	117916	ND
<i>Rhipicephalus distinctus</i>	<i>R. simus</i>	Metastrata; Rhipicephalinae	Sudan	?	?	84982	ND
<i>Rhipicephalus zambesensis</i>	<i>R. appendiculatus</i>	Metastrata; Rhipicephalinae	Zimbabwe	1977	<i>B. taurus</i>	105751	ND
<i>Rhipicephalus sulcatus</i>	<i>R. sanguineus</i>	Metastrata; Rhipicephalinae	Ivory Coast	1967	<i>C. familiaris</i>	54376	ND
<i>Boophilus geigy</i>	—	Metastrata; Rhipicephalinae	Ivory Coast	1961	?	HH11788	ND
<i>B. geigy</i>	—	Metastrata; Rhipicephalinae	Nigeria	1967	<i>B. taurus</i>	HH12279	ND
<i>Margaropus reidi</i>	—	Metastrata; Rhipicephalinae	Sudan	1954	<i>Giraffa camelopardalis</i>	121281	ND
<i>Margaropus winthemi</i>	—	Metastrata; Rhipicephalinae	South Africa	1955	?	79681	ND

*ND = not done.

MP strict consensus tree inferred from molecular data. Tree View was used to prepare illustrations of trees (Page, 1996).

RESULTS

DNA extraction

DNA was easily obtained from all frozen specimens, whereas the yield of DNA from alcohol-preserved specimens, particularly from specimens collected at the beginning of the century, was highly variable. Unsuccessful DNA extractions are listed in Table I. Our actual sample was therefore reduced to 36 species.

Sequence data and alignment

Accession numbers of the sequences submitted to GenBank are listed in Table I. The length of the obtained sequences varied from 337 to 355 bp.

The alignment obtained with Clustal X was exported in GCG and obviously misaligned characters were detected and shifted by eye. Conserved regions alternating with highly variable regions are characteristic features of 12S rRNA gene sequences. When aligned to sequences with fully resolved secondary structure, the conserved regions correspond to the helices of the 12S rRNA secondary structures, whereas the highly variable regions corresponded to the unpaired loop regions that are often difficult to align in an unambiguous way (Hickson et al., 1996). Some of the stem regions of our sequences were also difficult to detect, particularly the sequence corresponding to the fragment between the end of stem 45 and the beginning of stem 33 (Hickson et al., 1996). In this part of the alignment, there were, however, recognizable patterns shared by taxa belonging to single groups of ticks. Therefore, we decided to align those regions by eye, rather than eliminate them. Only indels created by a single species were excised. The resulting alignment was 351 bp long (available upon request from L.B.). The general secondary structure of the sequence of *R. sanguineus* is shown in Figure 1. Within the 351 aligned sites, 162 were constant, 31 were parsimony-uninformative, and 158 were parsimony-informative (Table IV). Estimated mean frequencies of the 4 nucleotides were as follows: adenine 42.5%, cytosine 8.3%, thymidine 36.3%, and guanine 12.9%. Global transitions/transversions ratio was estimated at 0.78.

Morphological data

All morphological characters were treated as unordered, 59 were parsimony-informative, and none were constant.

Phylogenetic Analysis

The level of intraspecific variability of sequences within *I. ricinus* (from France, Switzerland, and Portugal), *R. appendiculatus* (from Zimbabwe and Uganda), *R. sanguineus* (from Spain, Portugal, France, and Zimbabwe), and *A. hebraeum* (2 different localities in Zimbabwe) appeared to be extremely low. The sequences of *I. ricinus* collected in Portugal differed by a single base pair from sequences from French and Swiss specimens. All *R. sanguineus* and all *A. hebraeum* sequences were identical. The 342-bp-long sequences of *R. appendiculatus* from Uganda and Zimbabwe differed by 5 bp (1.5%). However, the sequences of specimens of *R. turanicus* (from Greece, Zimbab-

TABLE II. List of the morphological characters (character numbers, name of character, character states).*

1. Eyes—1: (0) present, (1) absent.
2. Eyes—2: (0) bulging or orbited, (1) flat.
3. Anal groove—1: (0) present, (1) absent.
4. Anal groove—2: (0) posterior to anus, (1) anterior to anus.
5. Ornamentation on scutum: (0) present, (1) absent.
6. (A) festoons—1: (0) present, (1) absent.
7. (A) festoons—2: (0) with enameling, (1) not so.
8. (A) if festoons enameled: (0) black, (1) ivory, (2) dappled.
9. (A) number of festoons: (0) 11, (1) sometimes fused.
10. (L) festoons: (0) present, (1) absent.
11. (L) number of festoons: (0) 7, (1) 9, (2) 11.
12. Parma: (0) present, (1) absent.
13. (M) Permanent caudal process: (0) present, (1) absent.
14. (M) After feeding caudal process—1: (0) present, (1) absent.
15. (M) After feeding caudal process—2: (0) elongated, (1) short.
16. (A) basis capituli—1: (0) square-shaped, (1) trapezoid, (2) rectangular, (3) hexagonal, (4) triangular.
17. (A) basis capituli—2: (0) with cornua, (1) without cornua.
18. (A) palps—1: (0) about as wide as long, (1) palp II with lateral protuberance, (2) longer than wide.
19. (A) dorsal spur on palp II: (0) present, (1) absent.
20. (A) palps—2: (0) with ventrolateral ridges, (1) not so.
21. (N) basis capituli: (0) hexagonal, (1) trapezoid, (2) quadrangular, (3) triangular.
22. (N) If basis capituli hexagonal: (0) lateral angles pointed, (1) lateral angles blunt.
23. (N) lateral angles of basis capituli: (0) in anterior third of basis capituli, (1) at midlength, (2) in posterior third.
24. (N) palps—1: (0) broad and blunt, (1) tapering, (2) elongated and blunt.
25. (N) palps—2: (0) constricted proximally, (1) not so.
26. (L) basis capituli: (0) hexagonal, (1) quadrangular.
27. (L) lateral angles of basis capituli: (0) pointed, (1) blunt.
28. (L) palps—1: (0) broad and blunt, (1) tapering, (2) elongated and blunt.
29. (L) palps—2: (0) constricted proximally, (1) not so.
30. (AF) hypostomal dentition: (0) 3:3, (1) 4:4, (2) variable.
31. (AF) areae porosae: (0) round, (1) oval with long axes parallel and longitudinal, (2) oval with long axes V-shaped, (3) triangular or piriform.
32. (M) ventral shields: (0) present, (1) absent.
33. (M) adanal plates—1: (0) present, (1) absent.
34. (M) accessory plates: (0) present, (1) absent.
35. (M) subadanal plates—1: (0) present, (1) absent.
36. (M) subadanal plates—2: (0) in the axis of adanal plates, (1) exterior to the axis of adanal plates.
37. (M) adanal plates—2: (0) with internal tooth, (1) with posterior spur, (2) with 2 spurs, (3) not so.
38. (M) adanal plates—3: (0) $>2\times$ longer than broad, (1) $<2\times$ longer than broad.
39. (M) adanal plates—4: (0) sickle-shaped, (1) not so.
40. (M) marginal lines—1: (0) present, (1) absent.
41. (M) marginal lines—2: (0) limiting all festoons, (1) not so.
42. (M) posteromedian groove: (0) present, (1) absent.
43. (M) posterolateral grooves: (0) present, (1) absent.
44. (L) sensilla sagittiformia: (0) present, (1) absent.
45. (L) number of marginal dorsal setae anterior to wax gland: (0) 2, (1) 4, (2) 3, (3) 5 setae.
46. Number of hosts: (0) 1-host species, (1) 2-host species, (2) 3-host species.
47. (M) legs IV: (0) markedly bigger than other, (1) not so.
48. Trochanter I: (0) with dorsal spur, (1) not so.

TABLE II. Continued.

49. Ornamentation on legs—1: (0) present, (1) absent.
50. Ornamentation on legs—2: (0) ring-shaped, (1) dorsal longitudinal.
51. (M) coxa I: (0) with large dorsal projection, (1) not so.
52. (M) coxa I: (0) with 2 spurs, (1) with 1 spur.
53. (M) if coxa I with 2 spurs—1: (0) internal spur longer than external, (1) external spur longer than internal, (2) spurs of similar length.
54. (M) if spurs on coxa I of similar length: (0) similar and short, (1) similar and long.
55. (M) if coxa I with two spurs—2: (0) internal broader than external, (1) external broader than internal.
56. (F) genitalia: (0) U-shaped, (1) V-shaped, (2) shields, (3) oval and divided.
57. Spiracular plates: (0) round, (1) with dorsal projection.
58. Circumspiracular setae: (0) present, (1) absent.
59. Dorsal projection of spiracular plates (0) elongated, (1) short.
60. Goblet cells: (0) numerous and small, (1) medium size, (2) big.
61. Cervical fields: (0) present, (1) absent.
62. Internal cervical groove: (0) present, (1) absent.
63. External cervical groove: (0) present, (1) absent.

*(M) = male; (F) = female; (A) = adult; (N) = nymph; (L) = larva.

we, Israel, and France) were characterized by higher levels of variability (from 5 to 27 bp differences, 1.5–7.7%). Interestingly, the sequence of the French *R. turanicus* differed only by 8 bp (2.4%) from the sequence of *R. sanguineus* (Table V).

The frequency distribution of lengths of 10,000 randomly generated trees is left skewed for both molecular and morphological data, with g_1 values of -0.539673 ($P < 0.01$) and -0.490167 ($P < 0.01$), respectively, indicating that both data matrices contain significant phylogenetic information and are not burdened by excessive random noise.

Molecular data: The set of taxa was first analyzed by MP. The strict consensus of the 2 most parsimonious trees (length 720; CI = 0.425; RI = 0.628; RC = 0.267), inferred by treating gaps as missing characters, is shown in Figure 2a. When gaps were treated as a fifth character state, the topology of the tree was identical. Bootstrap values supporting some of the nodes were however different and are indicated in Figure 2a by * when the difference exceeded 5% of the value obtained by treating gaps as missing characters. Among the Metastriata, *Amblyomma* and *Haemaphysalis* constitute the first diverging lineages. The taxa of these 2 genera belong to two monophyletic sister groups. The single New World *Amblyomma* species, *A. americanum*, is basal to the remaining *Amblyomma* clade. The Rhipicephalinae and Hyalomminae (sensu Hoogstraal) are included in a monophyletic clade. Basal to this clade, the branching order of the *Hyalomma* and *Dermacentor* lineages, both monophyletic, is not clearly determined. Among *Dermacentor* taxa, New and Old World taxa appear to belong to 2 different, well-resolved clusters. Within the *Hyalomma* clade, relationships are also well resolved. *Boophilus* and *Rhipicephalus* taxa appear to be clustered in a fairly well-supported monophyletic group. Within this clade, *Boophilus* species and *Rhipicephalus evertsi-pravus* taxa seem to be more closely related to each other, than to the other *Rhipicephalus* taxa. Although not strongly supported, the phylogenetic position of this clade appears to be basal to the remaining part of the *Rhipicephalus* clade. In the *R. sanguineus* group of species, *R. tur-*

anicus (from Israel, Greece, and Zimbabwe) belongs to a clade distinct from that of *R. sanguineus* and *R. turanicus* (from France). The pairs of taxa, *R. simus*–*R. zumpti*, *R. rossicus*–*R. pumilio*, and *R. maculatus*–*R. pulchellus*, belong to well-supported lineages, which is in agreement with the morphological classification. The *R. simus* group is included in the *R. sanguineus* group. *Rhipicephalus appendiculatus* does not appear to cluster with the 2 other members of the group, *R. maculatus* and *R. pulchellus*. The respective order of branching within the *Rhipicephalus* lineages is not fully resolved.

ML (data not shown) and MP reconstructions show similar topologies, whereas the NJ tree differs in a single point, *Boophilus decoloratus*, that does not clearly cluster with the other *Boophilus* taxa in the ML and MP trees but appears to be included in the *Boophilus* clade (data not shown). By removing *B. decoloratus* from the sample and reanalyzing the data set, the ML, MP, and NJ topologies are consistent with each other, and the bootstrap values of 2 branches increase significantly. The cluster that includes all remaining *Boophilus* and the *R. evertsi*–*pravus* group of taxa (not resolved by MP and ML) is now supported by 78% bootstrap value, and the monophyletic *Boophilus*–*Rhipicephalus* clade is supported in 81%, rather than 75%, of the 1,000 replicates.

Morphological characters: The MP heuristic search resulted in 540 equally most parsimonious trees of length 199 (CI = 0.427; RI = 0.762; RC = 0.326). The strict consensus tree is presented in Figure 2b. The tree in Figure 2b only indicates that *Metastrata* are monophyletic. *Hyalomma* species are not included in the monophyletic *Rhipicephalinae* clade, where the *Dermacentor* lineage constitutes the basal branches.

Combined data sets: The partition homogeneity test results reject the null hypothesis of data set homogeneity ($P = 0.01$). Therefore, the results of analyses including all characters may have to be considered with caution. However, total evidence may provide better resolution than separate, not completely resolved analyses (Wheeler et al., 1993; Jackman et al., 1997). The combined data sets were analyzed by MP; all characters were equally weighted. The heuristic search resulted in a single tree, with a length of 914 (CI = 0.421; RI = 0.643; RC = 0.271) that is presented in Figure 3. The resolution of this reconstruction is better than that provided by 2 separate data sets. Nonetheless, among the monophyletic *Metastrata*, the relative position of the monophyletic *Haemaphysalis* and *Amblyomma* clades are not resolved when gaps are treated as missing character states. When treated as a fifth character, they would appear to be sister groups. *Hyalomma*, *Dermacentor*, and *Rhipicephalus*–*Boophilus* all constitute monophyletic clades. The order in which those clades diverged from the original stem appears to be better resolved than in the previous analyses. *Hyalomma* were found in a basal position, followed by *Dermacentor*. All species belonging to the *R. sanguineus* group were clustered in a single monophyletic lineage that contains, however, the *R. simus* taxa. Similarly, *Boophilus*, the *R. evertsi*, and the *R. pravus* groups were found in a fairly well-supported clade. Although the species belonging to the *R. appendiculatus* group appeared to be clustered together, their relationships to each other and to the other *Rhipicephalus* were not resolved.

Mapping of morphological characters: Character states identified by MacClade and PAUP to be ancestral to particular lineages are represented by an open circle on the molecular MP

strict consensus tree in Figure 3. Black circles represent characters that identify a clade but that have been lost by some members of that clade. Two character states identify *Metastrata* as a monophyletic clade, absence of ventral shields, 32 (1), and presence of sensilla sagittiformia, 44 (0). Three other characters support this branch, posterior position of the anal groove, 4 (0), presence of festoons in adults, 6 (0), number of festoons in adults, 9 (0). However, this implies that character states 4 (0), 6 (0), and 9 (0) have subsequently been lost by all *Boophilus*, 4 (–), 6 (1), and 9 (–), or reacquired by *Rhipicephalus* species in an independent way. Similarly, morphological character states that traditionally identified *Boophilus* species as a different genus, 3 (1), 6 (1), 20 (0), 45 (3), 54 (0), would have appeared independently in *Boophilus* species. States of character 11, number of larval festoons, are synapomorphic for the clade that includes *Amblyomma* and *Haemaphysalis*, 11 (2), for the *Hyalomma* clade, 11 (0), and for the clade that includes *Dermacentor* and *Rhipicephalus* taxa, 11 (1). Again, we would have to assume that this character state was lost by all *Boophilus* taxa, 11 (–). The number of spurs on coxa I, 52 (1), and the shape of palps, 18 (1), identify *Haemaphysalis* taxa. Character state 41 (0), marginal lines limiting all festoons, is shared by all *Amblyomma* taxa with the exception of *Amblyomma rhinocerotis*. A variable hypostomal dentition, 30 (2), identifies the cluster that includes *A. hebraeum*, *Amblyomma variegatum*, and *Amblyomma sparsum*. Festoons sometimes fused, 9 (1), square-shaped basis capituli, 16 (0), and presence of subadanal plates, 35 (0), are synapomorphic for the *Hyalomma* clade. The position of the subadanal plates, character 36, further identifies 2 groups of *Hyalomma*. The *Dermacentor* clade is supported by character state 45 (2), number of dorsal setae anterior to wax gland. Character state 16 (3), hexagonal basis capituli, is synapomorphic for the *Rhipicephalus*–*Boophilus* clade. The clustering of *Dermacentor*, *Rhipicephalus*, and *Boophilus* taxa is supported by character state 18 (0), palps about as wide as long. A short after-feeding caudal process, 15 (1), is synapomorphic for all ticks of the *R. sanguineus* complex, other than *R. sinus* where this character converges to state (1), which is found in some *Rhipicephalus* species that belong to other groups. Lateral angles of nymphal basis capituli pointed, 22 (0), identifies the *Rhipicephalus* and *Boophilus* clade and changes to (1) in the *R. bursa*–*R. evertsi* clade that is also characterized by its number of hosts, 46 (1).

DISCUSSION

Warburton (1912) observed that *Rhipicephalus* species were extremely difficult to classify because of their high level of morphological intrageneric uniformity and intraspecific variability. Moreover, most of the characters useful for classification are found in adult males only or in immature stages. Females are sometimes simply impossible to identify. It was, therefore, interesting to investigate whether the historical groupings of *Rhipicephalus*, based on morphological information only, would be substantiated by phylogenetic analysis of molecular data.

The poor phylogenetic resolution of the tree inferred by MP analysis of morphological data is not surprising. The reduced number of characters in itself constitutes the main cause of weakly supported phylogenies and low bootstrap values. Al-

TABLE III. Morphological character states for each taxon.*

Species	Character no.																																				
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	3	3		
<i>I. ricinus</i>	1	-	0	1	1	1	1	-	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
<i>I. scapularis</i>	1	-	0	1	1	1	1	-	-	1	1	1	1	1	1/4	1	2	1	1	1	1	1	2	-	-	2	0	1	-	0	0	1	1	0	1	3	0
<i>H. leachi</i>	1	-	0	0	1	0	1	-	0	0	2	1	1	1	-	2	1	1	0	1	2	-	-	-	0	1	1	-	1	0	1	1	1	1	1	1	1
<i>H. punctata</i>	1	-	0	0	1	0	1	-	0	0	2	1	1	1	-	2	1	1	1	1	2	-	-	-	0	1	1	-	0	1	1	0	1	0	1	1	1
<i>A. hebraeum</i>	0	1	0	0	0	0	0	1	0	0	2	1	1	1	-	2	1	2	1	1	2	-	-	-	2	0	0	0	2	0	2	0	2	0	2	0	1
<i>A. sparsum</i>	0	1	0	0	0	0	0	1	0	0	2	1	1	1	-	2	1	2	1	1	0	1	1	1	2	0	0	0	2	0	2	0	2	0	2	0/1	1
<i>A. variegatum</i>	0	0	0	0	0	0	0	0	0	0	2	1	1	1	-	2	1	2	1	1	2	-	-	-	2	0	1	-	2	1	2	1	2	0/1	1	1	1
<i>A. americanum</i>	0	1	0	0	0	0	0	2	0	0	2	1	1	1	-	2	1	2	1	1	2	-	-	-	2	0	1	-	2	1	2	1	2	0/1	1	1	1
<i>A. rhinocerotis</i>	0	1	0	0	0	0	0	2	0	0	2	1	1	1	-	2	1	2	1	1	2	-	-	-	2	0	1	-	2	1	0	0	1	0	0	1	1
<i>H. m. marginatum</i>	0	0	0	0	0	0	0	1	1	1	2	1	1	1	-	2	1	2	1	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>H. m. rufipes</i>	0	0	0	0	1	0	1	-	1	0	0	1	1	1	-	0	1	2	0	1	3	-	1	2	1	1	1	-	2	0	0	0	0	0	0	0	1
<i>H. dromedari</i>	0	0	0	0	1	0	1	-	1	0	0	1	1	1	-	0	1	2	0	1	3	-	1	2	1	1	1	-	2	0	0	1	1	1	1	1	1
<i>H. truncatum</i>	0	0	0	0	1	0	1	-	1	0	0	1	1	1	-	0	1	2	0	1	3	-	1	2	1	1	1	-	2	0	0	2	1	1	1	1	1
<i>D. marginatus</i>	0	1	0	0	0	0	0/1	2	0	0	1	1	1	1	-	2	1	0	1	1	3	-	1	2	1	1	0	0	0	0	0	0	1	1	1	1	1
<i>D. reticulatus</i>	0	1	0	0	0	0	0/1	2	0	0	1	1	1	1	-	2	1	0	0	1	3	-	1	1	1	1	0	0	0	0	1	0	0	1	0	0	1
<i>D. variegatum</i>	0	1	0	0	0	0	0/1	2	0	0	1	1	1	1	-	2	1	0	1	1	3	-	2	1	1	1	1	0	0	1	1	0	1	1	0	2	1
<i>D. andersoni</i>	0	1	0	0	0	0	0	2	0	0	1	1	1	1	-	2	1	0	1	1	3	-	1	1	1	1	0	0	1	1	0	1	1	0	2	1	1
<i>D. albipictus</i>	0	1	0	0	0	0	0	2	0	0	1	1	1	1	-	2	1	0	1	1	2	-	-	0	1	1	0	1	0	0	0	0	0	0	0	0	1
<i>R. sanguineus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	1	3	1	0	0	1	0	0	1	1	1	1	0	0	1	1	0	0	1	1	1	1
<i>R. turanicus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	1	3	1	0	0	1	0	0	1	1	1	1	0	0	1	1	0	0	1	0	0	1
<i>R. pusillus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	1	3	1	0	0	1	0	0	1	1	1	1	0	0	1	1	0	0	1	0	0	1
<i>R. rossicus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	1	3	1	0	1	1	0	0	1	1	1	1	0	0	1	1	0	0	1	0	0	1
<i>R. pumilio</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	1	3	1	0	1	1	0	0	1	1	1	1	0	0	1	1	0	0	1	0	0	1
<i>R. bursa</i>	0	0	0	0	1	0	1	-	0	0	1	1	1	1	-	3	1	0	1	1	0	1	0	1	1	0	1	1	-	0	1	0	1	1	1	1	1
<i>R. e. evertsi</i>	0	0	0	0	1	0	1	-	0	0	1	1	1	1	0	3	1	0	1	1	0	1	0	1	1	0	1	1	-	0	1	0	1	1	1	1	1
<i>R. pulchellus</i>	0	1	0	0	0	0	0	1	0	0	1	1	1	1	0	3	1	0	1	1	1	0	1	0	0	0	0	1	0	0	1	0	0	1	0	1	1
<i>R. maculatus</i>	0	1	0	0	0	0	0	1	-	0	0	1	1	1	-	3	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1
<i>R. appendiculatus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	-	3	1	0	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
<i>R. kochi</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	3	1	0	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
<i>R. pravus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	3	1	0	1	1	1	0	0	2	1	1	0	1	-	0	0	0	1	1	1	1	1
<i>R. sinus</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	3	1	0	1	1	1	3	0	2	1	1	0	0	1	0	0	1	0	0	0	1	1
<i>R. zumpti</i>	0	1	0	0	1	0	1	-	0	0	1	1	1	1	0	3	1	0	1	1	1	3	0	2	1	1	0	0	1	0	0	1	0	0	0	1	1
<i>B. kohlsi</i>	0	1	1	-	1	1	1	-	-	0	1	-	0	1	-	0/3	1	0	1	0	0	0	0	1	0	0	0	1	0	0	?	?	?	?	?	?	?
<i>B. decoloratus</i>	0	1	1	-	1	1	1	-	-	0	1	-	0	1	-	0/3	1	0	1	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1	0	3	1
<i>B. annulatus</i>	0	1	1	-	1	1	1	-	-	1	1	-	0	1	-	3	1	0	1	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1	1	1	1
<i>B. microplus</i>	0	1	1	-	1	1	1	-	-	1	1	-	0	1	-	3	1	0	1	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1	1	1	0

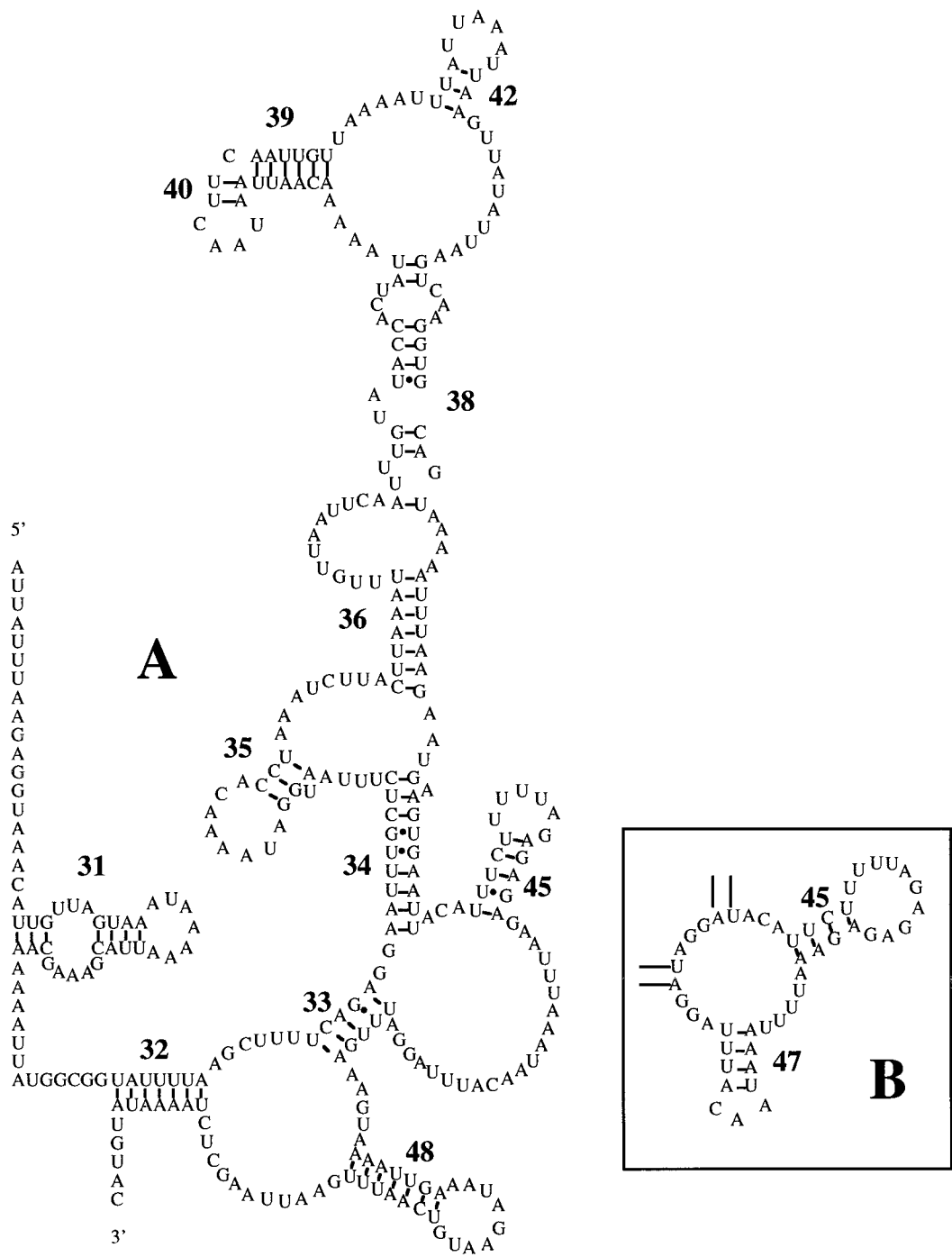


FIGURE 1. General secondary structure of 12S mitochondrial rRNA of *Rhipicephalus sanguineus*. The portion of the gene we sequenced corresponds to part of the II and to the III domain in the models proposed by Gutell et al. (1994) and Hickson et al. (1996). Two alternative solutions (A and B) are presented for the region between stem 45 and 33, where the presence of stem 47 is more or less evident depending on the analyzed taxon.

though our reconstruction is not in total agreement with previously published data (Klompfen et al., 1997), this discrepancy may be explained by different weighting procedures, different number of characters, and the different taxon representation. In our case, we tried to select characters available for all our hard-tick species and, at the same time, sensitive enough to differentiate the Rhipicephalinae taxa. This effort proved to be an

arduous exercise because, as previously mentioned, the morphology of *Rhipicephalus* species does not provide us with a wide array of discriminant characters.

The 12S rDNA phylogeny is largely consistent with previously published studies based on analyses of the same gene fragment (Murrell et al., 1999; Norris et al., 1999). However, with our sample of taxa (which unfortunately does not include

soft ticks, Australian, Asian, and South American *Amblyomma* and *Aponomma* species), *Amblyomma* and *Haemaphysalis* clades are both monophyletic. Tick phylogenies based on other regions of the genome, such as mitochondrial 16S rDNA and nuclear 18S rDNA sequences (Black and Piesman, 1994; Black et al., 1997), have shown that the *Hyalomma* lineage is placed within, rather than basal to the Rhipicephalinae. With our data set, *Hyalomma* species appear to be basal to the Rhipicephalinae that would be consistent with other tick phylogenies based on 12S rDNA sequences only (Murrell et al., 1999; Norris et al., 1999). However, the support of the branch clustering *Dermacentor* species with *Rhipicephalus* species is weak (61%). Different gap treatments do not appear to modify the overall tree structure. However, the support of the clade that includes *Amblyomma* and *Haemaphysalis* species significantly increases (from 64% to 89%), whereas the support of the branch that places *Dermacentor* taxa basal to the *Rhipicephalus*–*Boophilus* clade significantly decreases (from 61% to <50%), when gaps are treated as a fifth character state. This indicates that the number of indels in the 12S rDNA gene sequences have an important role in determining relationships between these clades. The monophyletic *Dermacentor* clade is clearly separated in 2 lineages, an Old World and a New World cluster. *Hyalomma* species are also monophyletic.

It has been argued that a significance threshold value of $P > 0.05$ for partition homogeneity tests may be too conservative, and that when P is > 0.01 the combined analysis still improves or at least does not reduce phylogenetic accuracy (Cunningham, 1997). Molecular data sets supposedly provide more objective information than morphological data sets, particularly because it is sometimes difficult to establish whether morphological features really constitute homologous characters. Combining the 2 data sets would, therefore, constitute a step back toward less informative reconstructions. In our case, however, this set of morphological characters collected from adult and immature stages of *Rhipicephalus* species has never been used as it is for classification purposes. For instance, despite its weak overall support, the reconstruction in Figure 2b indicates that *Boophilus* taxa are clustered with *R. pravus* and *R. evertsi* taxa, and that the *R. sanguineus* complex clade includes taxa of the *R. simus* group. With a single exception (Walker, 1961), these findings that are in agreement with the molecular reconstruction were not recognized by previous classifications based on phenotypic characters alone. Furthermore, the fact that the MP heuristic search of combined data finds a single most parsimonious tree and that the support for nodes is increased indicates that the 2 data sets may be complementary in resolving phylogenetic relationships unresolved when analyzed separately. It is plausible to believe that each of our 2 data sets is able to resolve a different section of the phylogenetic tree. When compared to results obtained with the molecular data set alone, both early diverging lineages, as well as more recent speciation events, are more fully resolved. The *Hyalomma* monophyletic group constitutes the lineage basal to the Rhipicephalinae and is followed by the *Dermacentor* clade. As mentioned for the analysis of molecular data, the support for the cluster that includes *Dermacentor* and *Rhipicephalus* taxa decreases significantly (from 74% to 50%) when gaps are treated as fifth characters. This indicates that the relationship between the *Hyalomma* and the *Dermacentor* clades is very dependent on the analysis methods

for indel positions, which may explain why 12S rRNA sequence analyses (Murrell et al., 1999; Norris et al., 1999) are in disagreement with reconstructions inferred by analyzing other genes. In the *Rhipicephalus*–*Boophilus* clade, the combined data set analysis recovered a tree that differs from that of molecular data alone only by a few points. *Boophilus* taxa are not placed basal to the clade. The clustering of *Boophilus*, *R. evertsi*, and *R. pravus* groups of taxa is confirmed. The *R. simus* group is consistently found within the *R. sanguineus* group. Finally, the position of the *R. appendiculatus* group of species (*R. maculatus*, *R. pulchellus*, and *R. appendiculatus*) is not resolved in any reconstruction.

When compared with morphological classification, our data provide confirmation for the existence of an *R. sanguineus* group of taxa. The fact that *R. simus* and *R. zumpti* are found within this clade is not surprising, because, on the basis of characters of the immature stages, this group of taxa has previously been included in the *R. sanguineus* complex (Walker, 1961). *Rhipicephalus maculatus* and *R. pulchellus* consistently cluster together; however, their relationship with *R. appendiculatus*, although slightly reinforced by adding morphological data, does not appear to be very robust. Their classification in a single group is based essentially on morphological characters of the immature stages (Walker, 1961) that constitute an important portion of our morphological data set. The morphology of *R. appendiculatus* adults is quite distinct from that of *R. pulchellus* and *R. maculatus*, the latter tick species being larger and ornate (Theiler and Robinson, 1953). *Rhipicephalus bursa* and *R. evertsi* were clustered in all our analyses. Analyses of the molecular data set provided support for the *R. pravus* group of taxa.

In early taxonomic studies, *B. annulatus* was named *Rhipicephalus annulatus*. (Neumann, 1901). Thereafter, other *Boophilus* species were described and they were all classified in a separate genus (Minning, 1934; Feldman-Muhsam and Schechter, 1970; Curtice, 1981; Keirans, 1992). More recently, *Boophilus* taxa have been found to be most closely related to the *R. evertsi* group (Mangold et al., 1998b). Our results not only substantiate that the *R. evertsi* group is more closely related to *Boophilus* species than to other *Rhipicephalus* clades, but that this is also the case for the members of the *R. pravus* group. Within *Boophilus*, *B. annulatus* and *B. microplus* appear to be more closely related to each other than to other *Boophilus* species. *Boophilus decoloratus* seems to be less closely related to the other *Boophilus* taxa than are ticks of the *R. evertsi* and *R. pravus* groups. Only morphological data succeeded in clustering *B. decoloratus* with other *Boophilus* species. Indeed, by eliminating the sequence of *B. decoloratus* from the molecular data set, the resolution of the clade that includes *Boophilus* and *R. pravus*–*evertsi* taxa is significantly increased. Analyses based on more discriminant molecular tools may help in resolving the relationships of this particular group of ticks and whether *Boophilus* should be a synonym of *Rhipicephalus*.

We believe that once more discriminant molecular data will succeed in resolving relationships within hard-tick species, the addition of relatively few morphological characters to global analyses may not prove to be as important as it is in our case. In order to establish whether morphological characters are synapomorphic or not, we should map them on a fully resolved molecular reconstruction. Although our molecular tree is not

TABLE V. Percentage of base-pair differences between 12S rDNA gene sequences of *R. sanguineus* and *R. turanicus*.

	<i>R. sanguineus</i>	<i>R. turanicus</i> (France)	<i>R. turanicus</i> (Israel 1)	<i>R. turanicus</i> (Israel 35)	<i>R. turanicus</i> (Greece)	<i>R. turanicus</i> (Israel 63)	<i>R. turanicus</i> (Zimbabwe)
<i>R. sanguineus</i>	0	2.4	5.9	6.5	6.5	5.9	8.3
<i>R. turanicus</i> (France)		0	4.7	5.3	5.3	5.6	7.7
<i>R. turanicus</i> (Israel 1)			0	1.5	1.5	3.5	6.2
<i>R. turanicus</i> (Israel 35)				0	0	4.1	5.9
<i>R. turanicus</i> (Greece)					0	4.1	5.9
<i>R. turanicus</i> (Israel 63)						0	5.3
<i>R. turanicus</i> (Zimbabwe)							0

fully resolved, it is, however, interesting to map morphological characters onto the molecular tree and try to establish which morphological character is diagnostic for, at least, the well-resolved clades. When character changes are mapped on the MP strict consensus tree, relatively few character states appear to identify monophyletic groups. Some characters, very useful for taxonomic purposes, are of a homoplastic nature. For instance,

bulging eyes, 2 (0), appear independently in 1 *Amblyomma*, in all *Hyalomma*, and in 2 *Rhipicephalus* species. Similarly, scutal ornamentation is found in *Amblyomma*, *Dermacentor*, and 2 *Rhipicephalus* taxa. This is the case for other characters easily identifiable in Table III. It is evident that it is difficult to determine the phylogenetic validity of morphological characters when the molecular reconstruction is not fully resolved. The

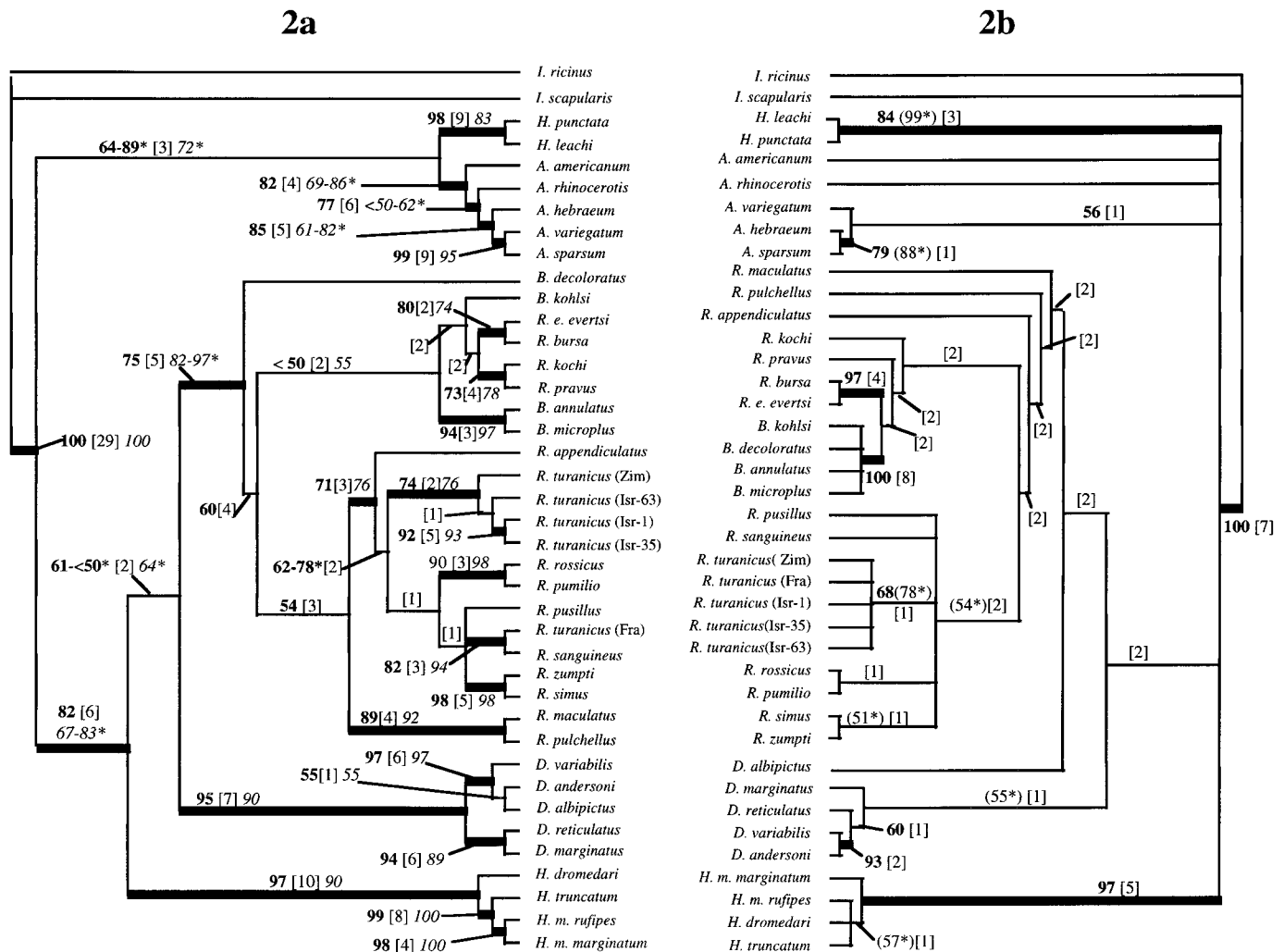


FIGURE 2. MP strict consensus trees inferred from the molecular (a) and from the morphological (b) data sets. MP bootstrap values are in bold; NJ in italics. Bootstrap values followed by an * indicate values obtained when treating gaps as a fifth character state. Decay indices are in square brackets. Only bootstraps >50% are indicated. Branches in bold are supported by bootstrap values >70%.

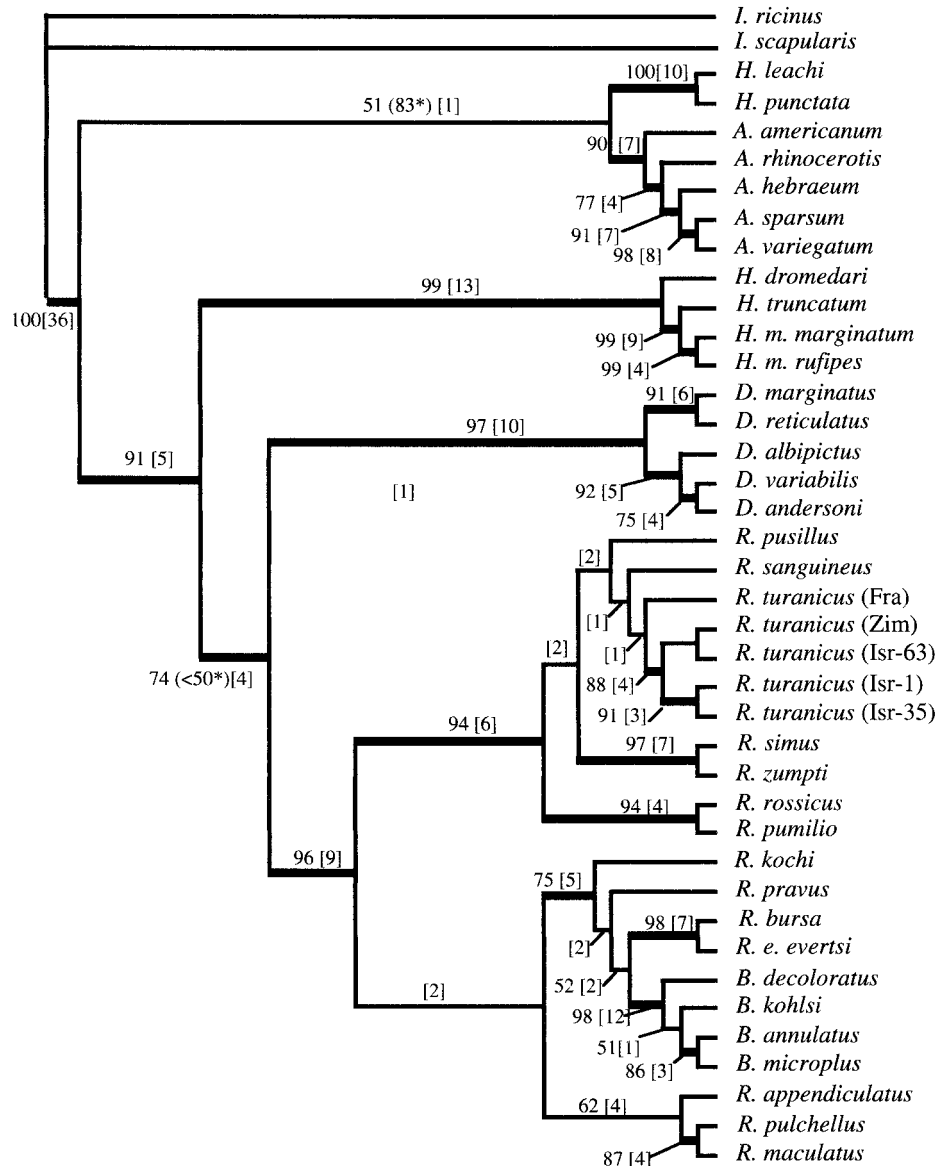


FIGURE 3. MP single tree inferred from the combined data set. Only bootstrap values >50% are indicated. Branches in bold are supported in >70% of the 1,000 bootstrap replicates.

peculiar situation of character states strictly associated with *Boophilus* taxa illustrates this problem. If the topology of the molecular MP strict consensus tree is correct, *Boophilus* taxa are basal to the *Rhipicephalus* clade and are not monophyletic. Among Metastriata species, we find however at least 7 morphological character states strictly associated with *Boophilus* taxa. It is not parsimonious to suggest that all these characters are homoplastic and that they either appeared independently in all *Boophilus* species or were present at the root of the *Rhipicephalus-Boophilus* clade and then disappeared in all *Rhipicephalus* clades. Similarly, whereas character states 11 (1), number of larval festoons, and 18 (0), shape of palps, support the clustering of *Dermacentor* with the *Rhipicephalus-Boophilus* clade, character states 45 (1), number of larval marginal dorsal setae anterior to wax gland, 33 (0), the presence of adanal plates, 16 (2), the shape of the basis capituli, and 34 (0), the

presence of accessory plates rather support the placement of *Hyalomma* species basal to the *Rhipicephalus-Boophilus* clade. We will not be able to dismiss morphological data as noninformative or homoplastic characters until the branching order of each clade, particularly the *Boophilus* clade, is clearly determined by analyzing more discriminant molecular data sets.

Although the main aim of our study was to provide a phylogenetic tree as the basis for further comparative studies, rather than a taxonomic review of this group of arthropods, the peculiar findings associated with the sequences within the *R. sanguineus* group deserve a few comments. On the basis of ITS2 sequences, Zahler et al. (1997) suggested that *R. sanguineus* (Azerbaijan and Burkina Faso) and *R. turanicus* (Turkmenistan) were a single species. This taxonomy was also suggested by other authors, who studied 12S rDNA phylogenies of Spanish specimens (Mangold et al., 1998b). These results were not in

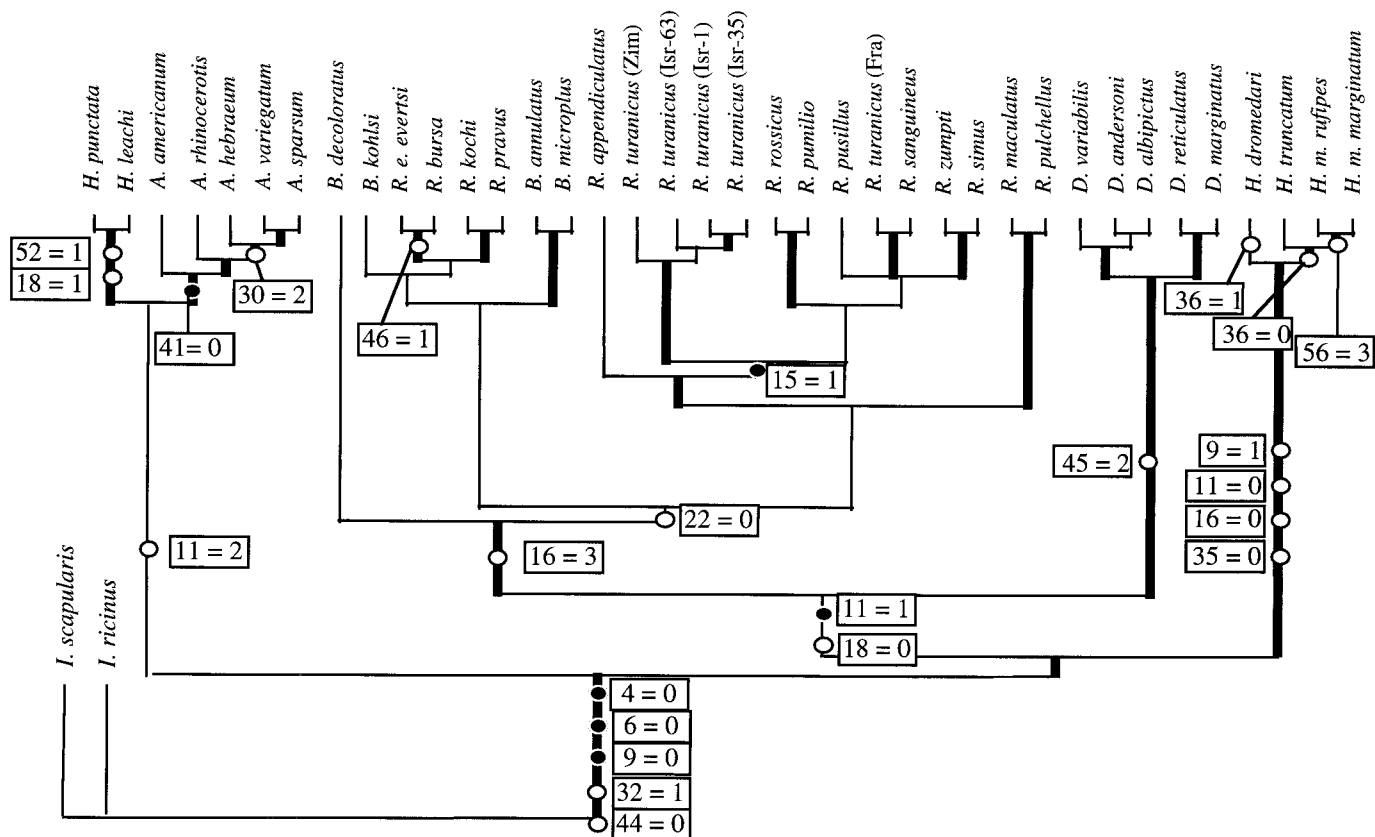


FIGURE 4. Character states that identify a particular lineage are represented by an open circle. Black circles indicate character states that are shared by a monophyletic group of taxa, but have been lost by some members of the clade.

agreement with previously published data based on morphology, biology, and molecular sequences of *R. turanicus* collected in Israel (Black and Piesman, 1994; Ioffe-Uspensky et al., 1997; Norris et al., 1999). If we combine that information with our results (difference of 2.4%), *R. turanicus* and *R. sanguineus* appear to be a single species, or at least more closely related, along the northwestern Mediterranean coast and Turkmenistan, although it is unclear how the Turkmen and western European strains are connected to each other. The situation changes, however, in the eastern Mediterranean area and in southern Africa, where *R. turanicus* differs more markedly from *R. sanguineus* (5.9–8.3%). The differences between *R. turanicus* from France and the Greek/Israeli strains (4.7–5.6%) is as great as the difference between the Israeli strains (that are also different from each other) and the Zimbabwean strain (5.3–6.2%), and the difference between the French and the Zimbabwean sequences is 7.7%. For comparison, the sequence of 2 tick species clearly distinct from *R. sanguineus*, *R. simus*, and *R. pumilio* differ from the sequence of *R. sanguineus* by 6.1% and 7.0%. These observations suggest that many cryptic species (including *R. sanguineus*) may possess a rather uniform *R. turanicus*-like morphology. The extent of the genetic and morphological variability of this relatively recently evolving lineage deserves to be studied further. Similarly, and in agreement with previously published data (Zahler et al., 1997), base pair differences between *R. rossicus* and *R. pumilio* are compatible with their placement in a single species (5/339 bp; 1.5%). This is also the

case with the 2 subspecies of *Hyalomma marginatum*, *H. m. marginatum*, and *H. m. rufipes* (6/341 bp differences; 1.8%). This is consistent with these morphologically slightly different subspecies belonging to a single species.

Our results, particularly those obtained by combining the 2 data sets, have confirmed some of the existing morphological and molecular hypotheses about Rhipicephalinae phylogeny. Furthermore, our analysis has provided information about the relationships among taxa that had not previously been included in a phylogenetic study.

Several species belonging to the Rhipicephalinae subfamily are vectors of microorganisms of medical and veterinary interest. Thus, the study of their phylogenetic relationships not only provides us with a fundamental knowledge of their evolutionary history but also may lead to a better understanding of their association with pathogens.

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