

16S rRNA Gene Sequence of *Neorickettsia helminthoeca* and Its Phylogenetic Alignment with Members of the Genus *Ehrlichia*

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Neorickettsia helminthoeca (tribe *Ehrlichieae*, family *Rickettsiaceae*) is the agent of salmon poisoning disease, which affects members of the family Canidae. This bacterium is unusual in that it is the only known obligately intracellular bacterium that is transmitted via a helminth vector. The nucleotide sequence of the *N. helminthoeca* 16S rRNA gene was determined and compared with the sequences of intracellular bacteria belonging to the alpha subgroup of the *Proteobacteria*. The *N. helminthoeca* sequence was most similar to the sequences of two *Ehrlichia* species, *Ehrlichia risticii* and *Ehrlichia sennetsu* (levels of sequence similarity, >95%). All other members of the tribe *Ehrlichieae*, including members of the other *Ehrlichia* species, and the related species *Cowdria ruminantium* and *Anaplasma marginale*, were only distantly related phylogenetically (levels of sequence similarity, 84 to 86%). Our results corroborate the results of previous ultrastructural and Western blot (immunoblot) comparisons of *N. helminthoeca* with other ehrlichial species. The genus *Ehrlichia* is phylogenetically incoherent and can be separated into three identifiable clusters of species. Each cluster is closely associated with a species classified in another non-*Ehrlichia* bacterial genus. The close relationships among *N. helminthoeca*, *E. risticii*, and *E. sennetsu* and the striking differences between these organisms and other members of the tribe *Ehrlichieae* suggest that in the future, these organisms should be treated as members of a new bacterial genus separate from the genus *Ehrlichia*.

Neorickettsia helminthoeca is the first and only obligately helminth-borne pathogenic bacterium that has been recognized (11). This obligately intracellular bacterium infects the fluke *Nanophyetus salmincola*, which in turn infects salmonid fish. *N. helminthoeca* can cause a severe disease in dogs or wild members of the family Canidae, referred to as salmon poisoning disease, if an animal ingests salmonid tissue that is infested with the infected trematode (7, 12). The disease is indigenous around rivers of the Pacific coast of the United States from northern California to southwestern Washington (3). Adult trematodes can live in the intestinal lumen of a canine host and shed infected eggs into the environment via the feces of the animal. The trematode requires another specific intermediate host, the snail *Oxytrema silicula*, for completion of its life cycle. *N. helminthoeca* is maintained by transovarial passage in the helminth and is found throughout the life cycle of the fluke, including in free-swimming cercariae (9, 10). Salmon poisoning disease is a pathological consequence of infection by *N. helminthoeca*, not the fluke or other salmon properties. The disease is acute and is characterized by fever, depression, anorexia, dehydration, diarrhea, and severe lymphadenopathy. The fatality rate in untreated infected dogs approaches 90% (3, 12). *N. helminthoeca* has been successfully isolated in a dog continuous cell line (17). This allows workers to cultivate and obtain the organism in sufficient quantities for antigenic and genetic analyses.

On the basis of the results of morphological comparisons (17) and, more recently, antigenic cross-reactivity data (14), it has been suggested that *N. helminthoeca* may be very closely related to some organisms currently placed in the genus *Ehrlichia* (tribe *Ehrlichieae*, family *Rickettsiaceae*) (15). Morpho-

logically, *N. helminthoeca* and two *Ehrlichia* species, *Ehrlichia risticii* (the agent of Potomac horse fever) and *Ehrlichia sennetsu* (the agent of human Sennetsu fever), are very similar (17). As determined by antigenic cross-reactivity between *N. helminthoeca* and various *Ehrlichia* species, *N. helminthoeca* is antigenically more closely related to *Ehrlichia sennetsu* and *Ehrlichia risticii* than either it or the last two species are related to *Ehrlichia canis* (14). Previously, live *N. helminthoeca* cells were not maintained in any laboratory, and cultivation of large numbers of organisms was not possible. Consequently, phylogenetic comparisons of *N. helminthoeca* with other members of the *Rickettsiales* in which nucleic acid sequences were used were not possible previously. In this paper we report the results of a comparative study of the 16S rRNA gene sequences of *N. helminthoeca*, related members of the genus *Ehrlichia*, and other intracellular bacteria belonging to the alpha subgroup of the *Proteobacteria*.

MATERIALS AND METHODS

Culture of *N. helminthoeca*. *N. helminthoeca* which was previously isolated and cultured in dog macrophage cell line DH82 (17) was propagated in Dulbecco's modified minimal essential medium (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum and 2 mM L-glutamine in 5% CO₂-95% air (17). Cells were harvested when the infection rate was 90%, as determined by microscopic examination of cytocentrifuged cells stained with Dif-Quik (Baxter Scientific Products, Oletz, Ohio).

Infected cell suspensions were pooled and centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was discarded. The pellet was suspended in Dulbecco's modified minimal essential medium at a concentration of 5 × 10⁶ cells per ml, and the suspension was sonically disrupted at 20 kHz for 5 min. Unbroken cells and nuclei were pelleted by centrifugation and resuspended in Dulbecco's modified minimal essential medium. The sonic lysis procedure was repeated twice, and the supernatants were reserved, pooled, and kept at 4°C.

DNA extraction. *N. helminthoeca* was purified by passing supernatants from sonic lysates through Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) low-pressure liquid chromatography columns as previously described (13). Purified organisms were washed and pelleted from sterile phosphate-buffered saline (9 mM Na₂HPO₄, 6 mM NaH₂PO₄, 150 mM NaCl; pH 7.4). After suspension in TE buffer (40 mM Tris, 1 mM EDTA; pH 8.0) containing 1% sodium dodecyl sulfate and 20 µg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml, the

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TABLE 1. Levels of similarity and evolutionary distances between 16S rRNA gene sequences

Organism	Level of similarity or evolutionary distance ^a									
	<i>N. helminthoeca</i>	<i>Ehrlichia risticii</i>	<i>Ehrlichia sennetsu</i>	<i>Ehrlichia canis</i>	<i>C. ruminantium</i>	<i>A. marginale</i>	<i>Ehrlichia equi</i>	<i>W. pipientis</i>	ISYM- <i>Nasonia</i> ^b	<i>Rickettsia rickettsii</i>
<i>N. helminthoeca</i>		0.0409	0.0412	0.1552	0.1522	0.1588	0.1561	0.1664	0.1696	0.1769
<i>Ehrlichia risticii</i>	96.05		0.0065	0.1696	0.1659	0.1625	0.1592	0.1689	0.1748	0.1847
<i>Ehrlichia sennetsu</i>	96.03	99.36		0.1657	0.1619	0.1603	0.1572	0.1657	0.1718	0.1827
<i>Ehrlichia canis</i>	86.17	85.09	85.38		0.0241	0.0748	0.0770	0.1330	0.1357	0.1802
<i>C. ruminantium</i>	86.43	85.36	85.67	97.64		0.0722	0.0722	0.1263	0.1331	0.1681
<i>A. marginale</i>	85.93	85.63	85.81	92.98	93.22		0.0264	0.1367	0.1401	0.1731
<i>Ehrlichia equi</i>	86.12	85.89	86.03	92.78	93.21	87.42		0.1295	0.1315	0.1760
<i>W. pipientis</i>	85.33	85.18	85.42	88.07	88.62	87.76	88.31		0.0086	0.1747
ISYM- <i>Nasonia</i>	85.08	84.73	84.94	87.84	88.17	87.48	88.14	99.15		0.1835
<i>Rickettsia rickettsii</i>	84.40	83.83	83.98	84.21	85.12	84.76	84.52	84.66	84.02	

^a The values on the upper right are the average numbers of substitutions per sequence position (evolutionary distances), adjusted as described by the Kimura two-parameter model for multiple substitutions at individual positions and calculated for 1,446 positions which could be aligned unambiguously. The values on the lower left are the levels of fractional nucleotide identity between sequences.

^b ISYM-*Nasonia*, incompatibility endosymbiont of *Nasonia* sp.

lysate was incubated at 65°C for 2 h. After extraction with phenol-chloroform and chloroform, DNA was precipitated from the aqueous phase by adding 3 volumes of cold 95% ethanol in the presence of 0.3 M sodium acetate (pH 5.2), and the mixture was kept at -70°C for 20 min. The precipitated DNA was collected by centrifugation, and the pellet was washed once with 70% ethanol, dried, and redissolved in sterile distilled water. The DNA was examined spectrophotometrically at A_{260} and A_{280} and stored as 50- μ g aliquots at -20°C until it was used.

PCR amplification of the 16S rRNA gene. PCR was used to amplify the 16S rRNA gene from DNA extracts of *N. helminthoeca*. To do this, we used two primers that included *Bgl*II and *Sal*I restriction sites to facilitate cloning into compatible vectors. We used the following two primers, which flanked the coding region of the 16S rRNA gene and originally were designed to amplify the entire 16S rRNA gene in *Rickettsia* spp. (20): RA-17 (5'-GGCTGCAGTCGACGTTTGATCCTGGCTCAG-3') and R3-17 (5'-CCAGATCTGAGCTCAAGGAGGTGATCCAGCC-3'). PCR was performed for 35 cycles (each cycle consisted of 1.5 min at 94°C, 3 min at 40°C, and 3 min of extension at 70°C) with an Ericomp thermocycler (Ericomp, San Diego, Calif.). Each reaction mixture contained 1 μ g of template DNA, each primer at a concentration of 50 pM, each deoxynucleoside triphosphate at a concentration of 5 mM, and 2.5 U of *Taq* polymerase (U.S. Biochemicals, Cleveland, Ohio) in buffer supplied by the manufacturer. The reaction mixtures were overlaid with 100- μ l portions of sterile mineral oil (Sigma). The mineral oil overlay was removed by chloroform extraction, and 0.1 volume of the reaction mixture was examined visually on 1.5% agarose gels stained with ethidium bromide. A single amplified product that was approximately 1.5 kb long was observed. The reaction products were extracted with phenol-chloroform and chloroform and precipitated in 70% ethanol at -70°C for 30 min. The precipitates were collected by centrifugation, washed once with 70% ethanol, and dissolved in a minimum volume of distilled water. The PCR product was purified by using GeneClean (BIO 101 Inc., La Jolla, Calif.), and the resulting product was suspended in TE buffer.

Cloning and sequencing the 16S rRNA gene. The PCR product was digested with restriction enzymes *Bgl*II and *Sal*I according to the instructions of the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The digests were extracted with phenol-chloroform and precipitated with ethanol. Bacteriophage vector M13 (mp18 and mp19) was digested with *Bam*HI and *Sal*I, extracted with phenol-chloroform, and precipitated with ethanol. The digested vector and PCR product were resuspended in water and ligated with T4 ligase (Boehringer Mannheim). Since *Bgl*II and *Bam*HI produce compatible ends after digestion, the PCR products were cloned into phage M13 in known orientations. After ligation at room temperature for 4 h, *Escherichia coli* JM101 cells were transfected with a plasmid (2). Single-stranded DNA from the bacteriophage (obtained from representative colorless plaques) was precipitated with polyethylene glycol, extracted with phenol-chloroform, and precipitated with ethanol. After suspension in distilled water, the single-stranded DNA was used as a template for DNA dideoxy chain termination sequencing in which Sequenase II (U.S. Biochemicals) and ³⁵S-labeled dATP were used. The sequencing primers used to determine the sequence of the *N. helminthoeca* 16S rRNA gene included the universal M13 primers (Sequenase II kit; U.S. Biochemicals) and a set of internal 16S rRNA primers which we designed (20). These primers are similar to those used by other workers (21). The gene was sequenced in both orientations by using the following primers: RA-17 (located at position 8 in *Escherichia coli*), 16S-400 (located at position 371 in *Escherichia coli*; 5'-CAATGGCGAAAGC

CTGATCC-3'), 5003-18 (located at position 519 in *Escherichia coli*; 5'-GTAT TACCGCGGCTGCTG-3') 5004-20 (located at position 907 in *Escherichia coli*; 5'-CCGTC AATTCCTTTGAGTTT-3'), 2-20 (located at position 1181 in *Escherichia coli*; 5'-GACTTGACGTCATCCCCACC-3'), and R3-17 (located at position 1522 on the standard *Escherichia coli* 16S rRNA sequence).

Phylogenetic comparison. We performed phylogenetic analyses by using programs in the PHYLIP phylogenetic package (version 3.5) (5). A matrix of pairwise corrected nucleotide divergence values was constructed by using DNA-DIST and the Kimura two-parameter correction. This matrix was then used to produce a phylogenetic tree by the neighbor-joining method implemented in NEIGHBOR. The other sequences used to construct phylogenetic trees included the sequences of *Ehrlichia sennetsu*, *Ehrlichia risticii*, *Ehrlichia phagocytophila*, *Ehrlichia equi*, *Ehrlichia bovis*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Anaplasma marginale*, *Cowdria ruminantium*, *Wolbachia pipientis*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rickettsia bellii*, an intracellular endosymbiont of *Nasonia vitripennis*, and the intracellular endosymbiont of *Rhinocyllus conicus* (20a).

Nucleotide sequence accession numbers. The GenBank accession number for the nucleotide sequence of the *Escherichia coli* 16S rRNA gene is J01695. The sequence of the 16S rRNA gene of *N. helminthoeca* has been deposited in the GenBank data library under accession number U12457. The accession numbers for the other sequences used to construct phylogenetic trees are as follows: *Ehrlichia sennetsu*, M73225; *Ehrlichia risticii*, M21290; *Ehrlichia phagocytophila*, M73220; *Ehrlichia equi*, M73223; *Ehrlichia bovis*, U03775; *Ehrlichia canis*, M73221; *Ehrlichia chaffeensis*, M73222; *Ehrlichia ewingii*, M73227; *Ehrlichia muris*, U15527; *A. marginale*, M60313; *C. ruminantium*, X61659; *W. pipientis*, X61768; *Rickettsia prowazekii*, M21789; *Rickettsia rickettsii*, U11021; *Rickettsia bellii*, U11014; an intracellular endosymbiont of *Nasonia vitripennis*, M84687; and the intracellular endosymbiont of *Rhinocyllus conicus*, M85267.

RESULTS

We obtained a 1,453-base sequence which corresponds approximately to bases 27 to 1478 in the complete rRNA gene sequence of *Ehrlichia risticii* (22). The sequence of the *N. helminthoeca* 16S rRNA gene was aligned with the 16S rRNA sequences of other members of the genus *Ehrlichia*, of members of the genus *Rickettsia*, and of representatives of several groups of insect-borne intracellular bacteria which have been placed in the alpha subgroup of the *Proteobacteria*. In agreement with the results of previous immunological studies, the 16S rRNA gene sequence of *N. helminthoeca* is most similar to the sequences of *Ehrlichia risticii* and *Ehrlichia sennetsu* (levels of complete structural similarity [including insertion or deletion sites], 95.7 and 96.0%, respectively). The results of a comparison performed with other representative intracellular bacteria belonging to the alpha subgroup of the *Proteobacteria*

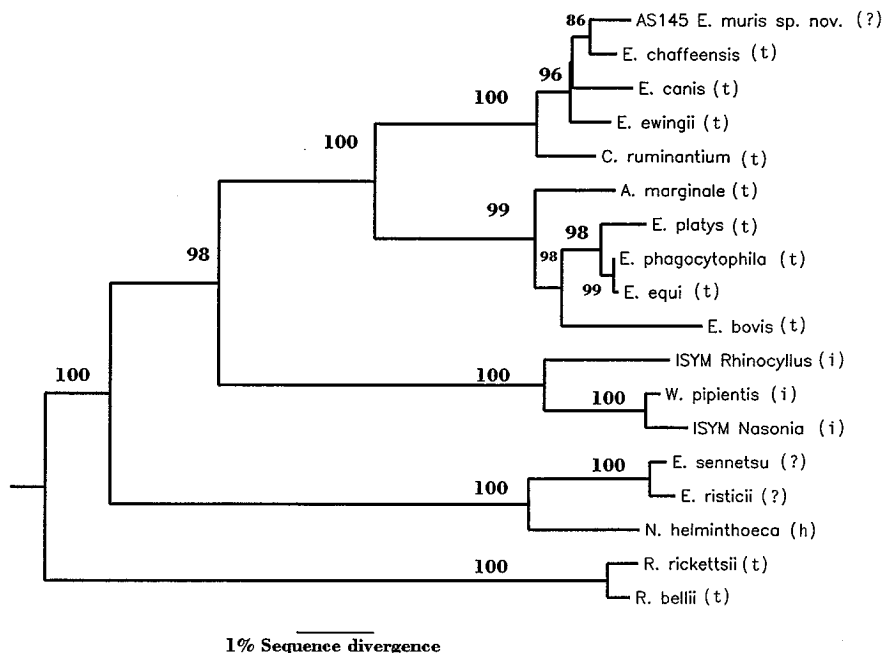


FIG. 1. Phylogenetic relationships between *N. helminthoeca* and various intracellular bacteria belonging to the alpha subgroup of the *Proteobacteria*. The tree was constructed by using a neighbor-joining method (NEIGHBOR in PHYLIP) from a matrix of corrected nucleotide divergence values. The root of the tree was determined by using the sequences of *Rochalimaea quintana* and *Agrobacterium tumefaciens* as outliers. The evolutionary distance (percentage of nucleotide divergence) between a pair of species was calculated by adding the distances along the horizontal axis from the last common ancestor of the terminal taxa. The numbers on the branches are the bootstrap values calculated in PHYLIP; only bootstrap values greater than 50% are shown. Modes of transmission are indicated in parentheses, as follows: (h), helminth; (i), insect; (t), tick; (?), unknown. ISYM *Rhinocyllus*, incompatibility intracellular symbiont of *Rhinocyllus* sp.; ISYM *Nasonia*, incompatibility intracellular symbiont of *Nasonia* sp.

are shown in Table 1. Our phylogenetic analysis of 16S rRNA sequences included more taxa, including all available sequences for members of the genus *Ehrlichia* and the insect intracellular endosymbionts which are phylogenetically related to the genus *Ehrlichia*. The data in Table 1 include data for only a representative sample of these taxa. The most closely related *Ehrlichia* species other than members of the *N. helminthoeca*-*Ehrlichia risticii*-*Ehrlichia sennetsu* group was *Ehrlichia equi*, which exhibited only 86.4% 16S rRNA sequence similarity to *N. helminthoeca*.

Figure 1 shows the phylogenetic relationships among various taxa, as determined by the neighbor-joining method. The genus *Ehrlichia* is divided into three well-differentiated groups. The cluster which includes *N. helminthoeca*, *Ehrlichia risticii*, and *Ehrlichia sennetsu* is a highly distinct clade, and the average level of corrected sequence difference from all other species belonging to the genus *Ehrlichia* is 16 to 17%. This clade is only slightly more closely related to the other *Ehrlichia* clusters than it is to the members of the genus *Rickettsia* (average distance, 19.3%). It represents the deepest branch within the tribe *Ehrlichieae*.

Each of the three clades of the genus *Ehrlichia* is characterized by being associated with a bacterium that is not classified as an *Ehrlichia* species. In addition to the *N. helminthoeca* triad, *C. ruminantium* clusters with three *Ehrlichia* species, including *Ehrlichia canis*, while in the third cluster *A. marginale* clusters with four members of the genus *Ehrlichia*. Thus, the three *Ehrlichia* clusters are separated from each other by at least two noncongeneric named forms. The average level of intercluster sequence divergence between members of the *Cowdria*- and *Anaplasma*-associated clusters is about 7.5%. In addition, there appears to be some similarity in the invertebrate vectors for the *Ehrlichia* species which cluster with mem-

bers of the genera *Cowdria* and *Anaplasma*. In all cases in which a vector is known, the species are associated with ticks. The vectors for *Ehrlichia risticii* and *Ehrlichia sennetsu* have not been identified, despite searches for an arthropod vector.

An additional sister clade of the tick-borne *Ehrlichia* clade is shown in Fig. 1. There is a significant cluster of insect-borne intracellular endosymbionts, which includes *W. pipientis*. The members of this clade are more similar to the tick-borne members of the genus *Ehrlichia* than either they or the tick-borne ehrlichial species are to the members of the *N. helminthoeca* cluster. The average level of sequence divergence between the insect endosymbionts and the tick-borne members of the genus *Ehrlichia* is 14.2%, while the average level of divergence between the insect endosymbionts and members of the *N. helminthoeca* cluster is 17.4%. The inclusion of the insect-borne intracellular endosymbionts with the tick-borne members of the genus *Ehrlichia* creates a distinct group of sister clades which are significantly differentiated both from the helminth-borne *Neorickettsia* species and the associated *Ehrlichia* species and from the tick-borne *Rickettsia* species. We propose that the *N. helminthoeca* cluster should be referred to as the "neoehrlichia" cluster.

DISCUSSION

The causative agent of salmon poisoning disease (10), *N. helminthoeca*, was described by Philip in 1953. On the basis of disease and morphological characteristics, early investigators were not able to place the organism in a previously described genus, and the genus *Neorickettsia* is still classified as an independent group within the tribe *Ehrlichieae* (15, 18). The species *N. helminthoeca* is unique in its association with a helminth vector. Our data suggest that there is a strong and unique

phylogenetic association among three intracellular bacterial species, *N. helminthoeca*, *Ehrlichia risticii*, and *Ehrlichia sennetsu*. The results of a phylogenetic analysis indicate that these organisms have been differentiated from other previously studied intracellular members of the alpha subgroup of the *Proteobacteria* for a very long time. *Ehrlichia risticii* is the agent of Potomac horse fever, an acute diarrheic disease of equids whose nonmammalian vector and mode of transmission remain unknown (19). *Ehrlichia sennetsu*, the causative agent of Sennetsu fever in Japan, is anecdotally associated with the consumption of a particular raw fish (6).

Ultrastructurally, *N. helminthoeca* is similar to other ehrlichial species. All of these organisms are intracellular bacteria with two unit membranes, ribosomes, and fine DNA strands. However, *N. helminthoeca* organisms are tightly enveloped by the host membrane, like *Ehrlichia sennetsu* and *Ehrlichia risticii* but unlike *Ehrlichia canis*, which produces morula inclusions containing large numbers of organisms (16). *N. helminthoeca*, *Ehrlichia risticii*, and *Ehrlichia sennetsu* infect monocytes and macrophages rather than granulocytes. However, during infection none of these species produces large dense morulae, in contrast to other members of the genus *Ehrlichia*, such as *Ehrlichia canis*. The antigenic cross-reactivity between *N. helminthoeca* and several members of the genus *Ehrlichia* has been investigated previously (14). The results of indirect fluorescent labeling and Western blot (immunoblot) experiments make it clear that *Ehrlichia sennetsu* and *Ehrlichia risticii* are antigenically more similar to *N. helminthoeca* than to *Ehrlichia canis*, *Ehrlichia equi* (14), or ehrlichial strain AS145, which was isolated from a wild mouse in Japan (8).

The results of a comparison of the sequences of the 16S rRNA genes of various intracellular bacteria, including members of the genus *Rickettsia* and other members of the genus *Ehrlichia*, confirmed that *N. helminthoeca* shares a close common ancestry with *Ehrlichia risticii* and *Ehrlichia sennetsu*. In addition, since the results of a phylogenetic analysis indicated that this trio of species is not closely related to any of the arthropod-borne *Rickettsia* or *Ehrlichia* species or to the *W. pipientis*-like insect endosymbionts, it is not unreasonable to hypothesize that the three species may be transmitted by a different vector, such as the fluke vector which is known to be involved in the *N. helminthoeca* life cycle.

The possibility that *N. helminthoeca*, *Ehrlichia risticii*, and *Ehrlichia sennetsu* are all helminth-associated bacteria can now be examined by using the 16S rRNA gene as a molecular probe. The evidence that *N. helminthoeca* is present in various stages of the life cycle of its trematode vector is based only on the results of transmission experiments performed with dogs. Our data suggest that the 16S rRNA sequence could be used as a probe to determine the infection rates of each stage of the trematode life cycle and to diagnose salmon poisoning disease in dogs. Furthermore, the search for vectors of Potomac horse fever and Sennetsu fever should be broadened to include possible infection of fish or snail populations in geographic foci of infection by trematodes which could act as the vectors of ehrlichial infection.

Using the phylogenetic evidence presented above, we suggest that the close clustering of the three species *N. helminthoeca*, *Ehrlichia risticii*, and *Ehrlichia sennetsu* should eventually result in careful reconsideration of nomenclature of the genus *Ehrlichia*. The inclusion of many intracellular bacteria, including members of the genera *Cowdria* and *Anaplasma* and a number of *Wolbachia* sp. strains (data not shown), in the broad cluster represented by the named *Ehrlichia* species weakens the coherence of the taxon *Ehrlichia* as a genus. The genetic differences between the *Neorickettsia* cluster and the two other

clusters of *Ehrlichia* species are almost equivalent to the genetic differences between any of the *Ehrlichia* or *Wolbachia* species and *Rickettsia* species. Thus, data from the phylogenetic analysis, together with other biological characteristics, suggest that *Ehrlichia risticii* and *Ehrlichia sennetsu* should eventually be removed from the genus *Ehrlichia* and reclassified together with *N. helminthoeca*. In addition, the results of our analysis strongly suggest that consideration should be given to reclassification of *A. marginale* and *C. ruminantium* together with associated members of the genus *Ehrlichia*.

Finally, it is worth noting that the diversity of forms in the phylogenetic cluster which contains *N. helminthoeca* is likely to be greater in the future. Additional studies of the mode of transmission and the phylogenetic placement of the two other members of the genus *Neorickettsia*, the elusive organism *Neorickettsia elokominica* (4) and the SF agent (6), in relation to *N. helminthoeca* and to other *Ehrlichia* species are under way.

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