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Citrate Synthase Gene Sequence: a New Tool for Phylogenetic Analysis and Identification of *Ehrlichia*

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The sequence of the citrate synthase gene (*gltA*) of 13 ehrlichial species (*Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia muris*, an *Ehrlichia* species recently detected from *Ixodes ovatus*, *Cowdria ruminantium*, *Ehrlichia phagocytophila*, *Ehrlichia equi*, the human granulocytic ehrlichiosis [HGE] agent, *Anaplasma marginale*, *Anaplasma centrale*, *Ehrlichia sennetsu*, *Ehrlichia risticii*, and *Neorickettsia helminthoeca*) have been determined by degenerate PCR and the Genome Walker method. The ehrlichial *gltA* genes are 1,197 bp (*E. sennetsu* and *E. risticii*) to 1,254 bp (*A. marginale* and *A. centrale*) long, and GC contents of the gene vary from 30.5% (*Ehrlichia* sp. detected from *I. ovatus*) to 51.0% (*A. centrale*). The percent identities of the *gltA* nucleotide sequences among ehrlichial species were 49.7% (*E. risticii* versus *A. centrale*) to 99.8% (HGE agent versus *E. equi*). The percent identities of deduced amino acid sequences were 44.4% (*E. sennetsu* versus *E. muris*) to 99.5% (HGE agent versus *E. equi*), whereas the homology range of 16S rRNA genes was 83.5% (*E. risticii* versus the *Ehrlichia* sp. detected from *I. ovatus*) to 99.9% (HGE agent, *E. equi*, and *E. phagocytophila*). The architecture of the phylogenetic trees constructed by *gltA* nucleotide sequences or amino acid sequences was similar to that derived from the 16S rRNA gene sequences but showed more-significant bootstrap values. Based upon the alignment analysis of the ehrlichial *gltA* sequences, two sets of primers were designed to amplify tick-borne *Ehrlichia* and *Neorickettsia* genogroup *Ehrlichia* (*N. helminthoeca*, *E. sennetsu*, and *E. risticii*), respectively. Tick-borne *Ehrlichia* species were specifically identified by restriction fragment length polymorphism (RFLP) patterns of *Acs*I and *Xho*I with the exception of *E. muris* and the very closely related ehrlichia derived from *I. ovatus* for which sequence analysis of the PCR product is needed. Similarly, *Neorickettsia* genogroup *Ehrlichia* species were specifically identified by RFLP patterns of *Rca*I digestion. If confirmed this technique will be useful in rapidly identifying *Ehrlichia* spp.

Ehrlichiae were previously known mainly as important agents of veterinary disease (13). For example, *Ehrlichia canis*, *Ehrlichia equi*, *Ehrlichia phagocytophila*, *Ehrlichia platys*, *Ehrlichia risticii*, *Cowdria ruminantium*, *Anaplasma marginale*, and *Neorickettsia helminthoeca* have been known as veterinary pathogens. However, over the last decade, several new *Ehrlichia* species or strains have been isolated and characterized from human patients and are known as major emerging tick-borne pathogens. The human granulocytic ehrlichiosis (HGE) agent, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* are now included among emerging ehrlichial agents of humans. Diagnostic methods of emerging ehrlichial infection include isolation, serology, and molecular techniques. Isolation is the "gold standard" for diagnosis; however, this method is time-consuming and expensive. Although serology is the most frequently used method for diagnosis, serological cross-reactions occur between closely related ehrlichiae, leading to misinterpretation and misdiagnosis (3, 17). With the recent development of molecular biology methods, specific and sensitive assays such as PCR and sequencing are now used for detection of ehrlichiae.

The 16S rRNA encoding gene sequence is most often used for the identification of *Ehrlichia*. Using the 16S rRNA, the genus *Ehrlichia* was found to belong to the alpha-subgroup of *Proteobacteria* closely related to the genus *Rickettsia* (5). The *Ehrlichia* clade also includes the genera *Neorickettsia*, *Cowdria*, and *Anaplasma* and the species *Wolbachia pipientis* (27). Polyphasic taxonomy had been advocated in order to ensure well-balanced determinations of taxonomic relationships (26), but few genes are available for investigating the genetics of ehrlichiae. A phylogenetic tree derived from nucleotide sequences of the heat shock protein gene (*groESL*) was the only alternative tree, and it supported the relationships among *Ehrlichia* species previously determined by comparison of 16S rRNA gene sequence (24). Other determined ehrlichial sequences, i.e., those of the quinolinate synthetase gene (31) and the *ankA* gene (4, 12, 28), have provided useful information for phylogenetic study of ehrlichiae although a limited number of strains or isolates have been tested. Consequently, studies of additional genes are required to improve the classification, identification, and diagnosis of ehrlichiae and ehrlichial diseases.

The citrate synthase gene (*gltA*) encodes the first enzyme of the tricarboxylic acid cycle, which is a key regulator of intracellular ATP production in nearly all living cells (29). Sequences of *gltA* contribute to the phylogenetic analysis and identification of *Rickettsia* (19) and *Bartonella* species (2, 9) and exhibit higher variation than the 16S rRNA gene, therefore allowing better discrimination among closely related species. *gltA* analysis is currently one of the best tools for this

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TABLE 1. Ehrlichial strains studied

Organism	Strain	Source
HGE agent	Webster	J. S. Dumler, The Johns Hopkins Medical Institutions, Baltimore, Md.
E. equi	MRK	J. E. Madigan, University of California, Davis
E. phagocytophila	1602	A. Garcia-Perez, Fundation Hospital Alcoron
A. marginale	South Idaho	G. Palmer, Washington State University
A. marginale	Florida	G. Palmer, Washington State University
A. centrale	Aomori	Y. Terada, National Institute of Animal Health
E. canis	Oklahoma	J. Dawson, Centers for Disease Control and Prevention, Atlanta, Ga.
E. chaffeensis	Arkansas	J. Dawson, Centers for Disease Control and Prevention, Atlanta, Ga.
E. muris		M. Kawahara, Nagoya City Public Health Research Institute
Ehrlichia detected from *I. ovatus*	Yamaguchi	H. Inokuma
C. ruminantium		C. E. Yunker, University of Pretoria, Pretoria, Republic of South Africa
E. risticii	ATCC	
E. sennetsu	Miyayama	G. Dasch, Naval Medical Research Center, Bethesda, Md.
N. helminthoeca		Y. Rikihisa, Ohio State University, Columbus

purpose and for phylogenetic analysis of these two closely related genera (2, 19).

We determined the *gltA* sequences of 13 ehrlichial species by combining consensus degenerate PCR and Genome Walker approaches. *gltA*-based phylogenetic analyses were performed, and consensus primers were developed to amplify partial sequences of *gltA* of ehrlichial species within a group. A preliminary PCR-restriction fragment length polymorphism (RFLP) assay was developed to allow identification of ehrlichial species.

MATERIALS AND METHODS

Ehrlichia strains and DNA preparation. All ehrlichial strains included in this study are listed in Table 1. The HGE agent and *E. equi* were cultured in HL-60 cells, and *E. canis*, *E. chaffeensis*, *Cowdria ruminantium*, *E. risticii*, *E. sennetsu*, and *N. helminthoeca* were cocultured with DH82 cells. *E. phagocytophila*-infected sheep blood was provided by A. Garcia-Perez, Foundation Hospital Alcoron, Derio, Spain. *Anaplasma centrale*-infected bovine blood was provided by Y. Terada, National Institute of Animal Health, Tsukuba, Japan (8). Genomic DNA was extracted from these infected cells by using the QIAamp blood kit (Qiagen GmbH, Hilden, Germany) and stored in 200 μl of Tris-EDTA (TE) buffer at −20°C until use. Genomic DNA of a recently discovered *Ehrlichia* species originally isolated from *Ixodes ovatus* (22) was extracted from an *I. ovatus* tick collected from a bear in Yamaguchi Prefecture, Japan (Inokuma et al., unpublished data). This isolate was a strain variant of the newly described *Ehrlichia* species previously isolated from *I. ovatus*. DNA extracted from *A. marginale*, strains South Idaho and Florida, and *Ehrlichia muris* were kindly provided by G. Palmer, Washington State University, Pullman, and M. Kawahara, Nagoya City Public Health Research Institute, Nagoya, Japan, respectively.

PCR amplification of *gltA* of HGE agent. The strategy for determining *gltA* sequences of HGE is summarized in Fig. 1. A partial sequence of the HGE agent *gltA* was first determined by using degenerated primers F3 and R1b designed after the alignment of the conserved regions of *gltA* among *Rickettsia prowazekii*, *Bartonella henselae*, and *Escherichia coli* (Fig. 1; Table 2). For the amplification, the reaction mixture contained 50 pmol of each primer, 1.5 U of *Taq* DNA polymerase (GibcoBRL, Gaithersburg, Md.), a 20 mM concentration of each deoxynucleoside triphosphate, 10 mM Tris-HCl, 50 mM KCl, 1.6 mM $MgCl_2$, and 5 μl of template DNA in a final volume of 50 μl. The amplifications were performed in a Peltier model PTC-200 thermal cycler (MJ Research, Inc., San Francisco, Calif.) with the following program: initial 5-min denaturation step at 95°C; 35 cycles of denaturation (95°C for 30 s), annealing (50°C for 30s), and extension (72°C for 90 s); and a final 5-min extension step at 72°C. Distilled water and DNA of *B. henselae* were included as negative and positive controls in each PCR. The amplification products were visualized on a 1% agarose gel after electrophoretic migration. The PCR products were purified for DNA sequencing using the QIAquick PCR purification kit (Qiagen) and sequenced using PCR primers when a single clear band was observed on the ethidium bromide-stained agarose gel. When multiple bands including bands of the expected size were obtained in PCR, the Qiagen gel extraction kit was used to purify the expected bands from the gel. After determination of the partial sequence, the unknown sequences of the 3′- and 5′-ends of the gene were amplified using the Universal Genome Walker Kit (Clontech Laboratories, Palo Alto, Calif.). Briefly, genomic DNA was digested with *Eco*RV, *Dra*I, *Pvu*II, *Stu*I, and *Sca*I. DNA fragments were ligated with a Genome Walker adapter, which had one blunt end and one end with a 5′ overhang. The ligation mixture of the adapter and ehrlichial genomic DNA fragments was used as template for PCR. This PCR was performed using an adapter primer supplied by the manufacturer and ehrlichial *gltA*-specific primers to walk downstream on the DNA sequence (Table 3). For the amplification, 1.5 U of ELONGASE (GibcoBRL) was mixed with 10 pmol of each primer, a 20 mM concentration of each deoxynucleoside triphosphate, 10 mM Tris-HCl, 50 mM KCl, 1.6 mM $MgCl_2$, and 5 μl of template DNA in a final volume of 50 μl. Distilled water and genomic DNA extracted from uninfected host cells (HL-60) was included as a negative control in each PCR. The following program was used for the amplification: an initial 2-min denaturation step at 94°C; 44 cycles of denaturation (94°C for 30 s), annealing (53°C for 60s), and extension (68°C for 60 s); and a final 3-min extension step at 68°C.

Determination of other ehrlichial *gltA* sequences. Additional primers were designed based upon the alignment of the complete *gltA* sequences of the HGE agent, *R. prowazekii*, and *B. henselae* (Table 2). Primers F4b and R1b were used for the amplification of *E. equi*, *E. phagocytophila*, *E. chaffeensis*, *E. muris*, *C. ruminantium*, and *A. marginale* strains South Idaho and Florida; primers F4e and R1b were used for the amplification of *N. helminthoeca*; and primers F1 and R1b were used for the amplification of *E. sennetsu.* The optimal annealing temperature (48 to 55° C) was determined for each species by empirical testing. After determination of the sequences of these short fragments (230 to 730 bp), the *gltA* sequences of *E. chaffeensis*, *E. muris*, *C. ruminantium*, *A. marginale* strain South Idaho, *N. helminthoeca*, and *E. sennetsu* were completed by using the Genome Walker method as described above for the HGE agent. Based upon the complete sequences of ehrlichial *gltA* described above, new primer sets were designed to amplify partial *gltA* sequences of *E. canis*, *A. centrale*, and *E. risticii* (Table 2). After sequencing of these partial *gltA* fragments, the Genome Walker method was used to determine the 3′ and 5′ ends of these three species. As the material of the *Ehrlichia* species detected from *I. ovatus* was not abundant enough to perform the Genome Walker method, two primer pairs, CAN-M61F–R1b and F1b-MUR1251R, were used to obtain a partial *gltA* sequence of the species (Table 4).

DNA sequencing. The fluorescence-labeled dideoxynucleotide technology was used for DNA sequencing reactions (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). The sequencing fragments were separated using an Applied Biosystems model ABI 310 automated DNA sequencer (Perkin-Elmer), and data were collected with an ABI PRISM 310 Genetic Analyzer package (Perkin-Elmer). The collected sequences were assembled and edited with the AutoAssembler (version 1.4; Perkin-Elmer).

Confirmation of the ehrlichial *gltA* sequence. In order to avoid the editorial error of the Genome Walker method, obtained sequences of the citrate synthase coding region, including the open reading frame at the 5′ end and the stop codon at the 3′ end, from each ehrlichial species except for the *Ehrlichia* sp. detected from *I. ovatus* were confirmed by PCR with the primers shown in Table 4 and also were sequenced.

Data analysis. The sequences of ehrlichial *gltA* and the registered *gltA* sequence of *R. prowazekii* and *B. henselae* deposited in GenBank were analyzed for GC content, level of similarity, and phylogenetic relationships. Pairwise percent

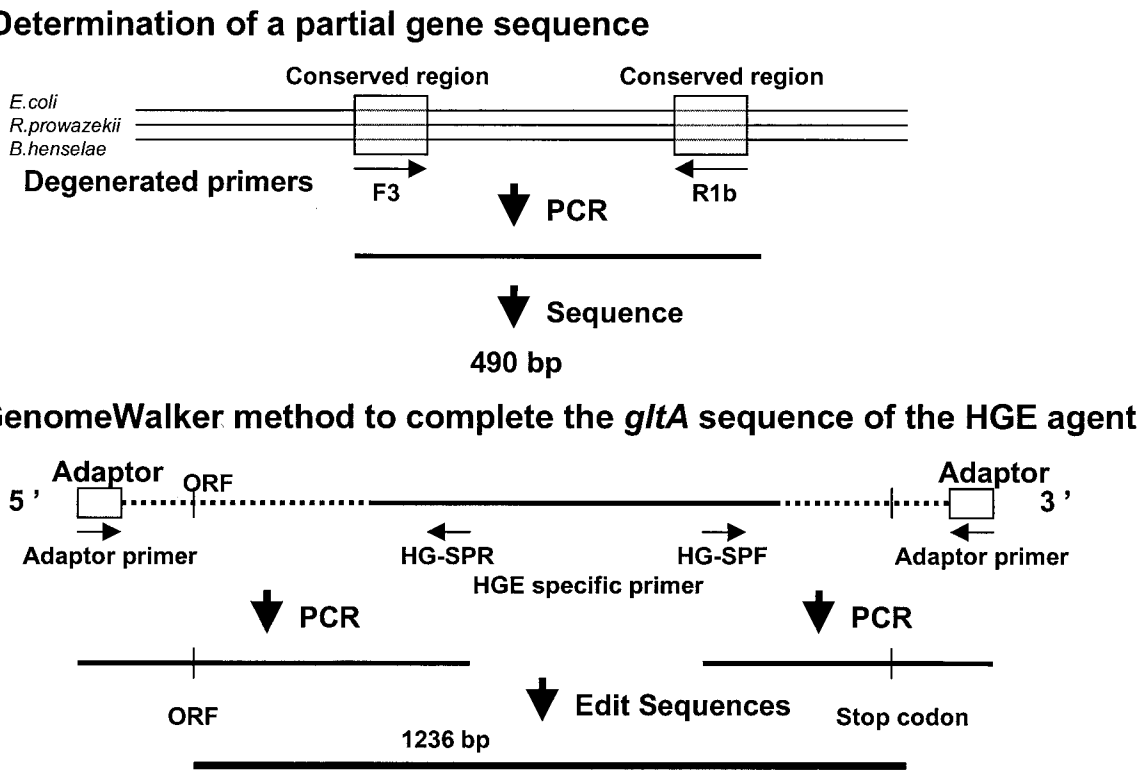

FIG. 1. Strategy for determination of the sequence of the citrate synthase gene (*gltA*) of the HGE agent. Primers F3 and R1b were determined after alignment of the *gltA* of *E. coli*, *R. prowazekii*, and *B. henselae*. After determination of the partial sequence, the unknown sequences of both the 3′ and 5′ ends of the gene were amplified by PCR using an adapter primer provided in the Universal Genome Walker kit and the HGE agent-specific primers based on the partial sequence. Assembly of these sequences determines the complete *gltA* sequence of HGE. ORF, open reading frame.

identities of the sequences with all gaps omitted were calculated by a program designed by H. Ogata, IGS, CNRS-UMR, France. Multiple alignment analysis, distance matrix calculation, and construction of a phylogenetic tree were performed with the ClustalW program (25), version 1.8 (available from the DNA Data Bank of Japan, Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). The distance matrices for the aligned sequences with all gaps ignored were calculated using the Kimura two-parameter method (10), and the neighbor-joining method was used for constructing a phylogenetic tree (21). The stability of the tree obtained was estimated by bootstrap analysis for 1,000 replications using the same program. Tree figures were generated using the TreeView program, version 1.61 (15). The same analysis of similarity and phylogenetic relationships was also performed for the deduced amino acid sequences of *gltA* and the 16S rRNA gene sequences.

Consensus PCR and PCR-RFLP analysis. Based upon the alignment analysis of the ehrlichial *gltA* sequences, a pair of primers, EHR-CS136F (5′-TTY-ATG-TCY-ACT-GCT-GCK-TG-3′) and EHR-CS778R (5′-GCN-CCM-CCA-TGM-GCT-GG-3′), was designed in order to specifically amplify partial sequences of the *gltA* gene of the following tick-borne *Ehrlichia* species: *E. chaffeensis*, *E. canis*, *E. muris*, the *Ehrlichia* sp. detected from *I. ovatus*, *C. ruminantium*, the HGE agent, *E. equi*, *E. phagocytophila*, *A. marginale*, and *A. centrale*. Another pair of

TABLE 2. Oligonucleotide primers used for PCR to amplify partial citrate synthase gene

Primer	Sequence (5′-3′)[a]	Organisms in which primer is used
Forward		
F3	TCT-TCT-CAT-CCT-ATG-GC	HGE agent
F4b	CCG-GGT-TTT-ATG-TCT-ACT-GC	HGE agent, *E. equi, E. phagocytophila, A. marginale* South Idaho, *A. marginale* Florida, *E. chaffeensis, E. muris, C. ruminantium*
F4e	ACT-GCT-TCK-TGT-SAR-TC	*N. helminthoeca*
F1	CAT-GAR-CAR-AAT-GCT-TC	*E. sennetsu*
Chaf-F	GGA-TTA-TGG-TWR-AAR-AAG-C	*E. canis*
EH130F	GGW-TTY-ATG-TCY-ACT-GCT-GC	*A. centrale*
SEN330F	AAA-TAT-CCG-TTC-TTT-CCC-AAC-G	*E. risticii*
Reverse		
R1b	CGA-TGA-CCA-AAA-CCC-AT	HGE agent, *E. equi, E. phagocytophila, A. marginale* South Idaho, *A. marginale* Florida, *E. chaffeensis, E. muris, C. ruminantium, N. helminthoeca, E. sennetsu*
Chaf-R	TAY-AAC-TGR-CGT-GGR-CG	*E. canis*
HG1085R	ACT-ATA-CCK-GAG-TAA-AAG-TC	*A. centrale*
SEN890R	GCT-TTA-ATA-TGG-CTG-CAC-GAG	*E. risticii*

[a] S, G or C; R, A or G; W, A or T; Y, C or T; K, G or T.

TABLE 3. Oligonucleotide primers and restriction genome libraries used for genome walking of the ehrlichial citrate synthase gene

Target species	Primer	Sequence (5′-3′)[b]	Restriction genomic library(ies)
HGE agent	HG-SPF	CGG-ATC-AAT-GCG-ACT-TGC-G	*Pvu*II
	HG-SPR	TTG-CTG-AGA-TTG-GTT-CAC-C	*Pvu*II
A. marginale South Idaho	MAR-SPR	GCC-ATT-GGG-TGG-GCA-TCA	*Dra*I
	MAR-SPF1	ATG-CTT-GAG-GAA-ATT-GGT-CGC-C	*Dra*I
	MAR-SPF2[a]	CCA-GTG-AAC-ATG-TTT-ACC-GCG	*Dra*I
A. centrale	CENT-SPR1	GAT-GAG-CAA-CGC-TGC-AGA-ACC	*Sca*I, *Dra*I
	CENT-SPR2[a]	CCA-CCT-GTC-GTG-ACA-AGA-TC	*Sca*I, *Dra*I
	CENT-SPF1	TTG-AGT-GCA-GGT-GCT-GCA-AC	*Pvu*II
	CENT-SPF2[a]	TTT-GAG-CTT-GAA-AGA-GTG-GCC	*Pvu*II
E. chaffeensis	F3R	CAT-AGG-ATG-TGA-ATC-T	*Dra*II
	CHAF-SPR[a]	ATT-TTG-CAA-ATC-CTC-AAG-ACC	*Dra*II
	F1	CAT-GAR-CAR-AAT-GCT-TC	*Eco*RV
	CHAF-SPF[a]	TGT-GAT-TAA-TAT-GTT-AAT-GGC	*Eco*RV
E. canis	CAN-SPR1	TAA-CTT-TAT-TTC-CAT-TAG-TAT-CAC	*Eco*RV, *Dra*I
	CAN-SPR2[a]	CGG-TAA-TTT-CAC-TTT-TTG-ACC	*Eco*RV, *Dra*I
	CAN-SPF	GTG-GTA-TGA-GAT-GGT-GGC-AGA-TAA-G	*Dra*I
E. muris	MUR-SPR1	TAA-ATC-TAC-TAT-GTT-ATG-TCC	*Eco*RV
	MUR-SPR2[a]	TCA-TAA-GTT-AAA-ACT-CCT-GTG-TC	*Eco*RV
	MUR-SPF1	AAT-GAC-AAT-TGA-AAA-ACC-AAG	*Dra*I
	ER-R1F[a]	ATG-GGT-TTT-GGT-CAT-AGA-G	*Dra*I
C. ruminantium	R1b	CGA-TGA-CCA-AAA-CCC-AT	*Dra*I
	CR-SPR[a]	TAA-ATT-CCT-GAA-GTT-GCT-CAG-CAT	*Dra*I
	F1b	GAT-CAT-GAR-CAR-AAT-GCT-TC	*Pvu*II
	CR-SPF[a]	GGA-TTC-CAG-TTA-AAA-TGT-TTA-CG	*Pvu*II
E. sennetsu	R1b	CGA-TGA-CCA-AAA-CCC-AT	*Dra*II, *Stu*I
	SEN-SPR1[a]	CTG-TAG-CCG-CAG-AAC-ATG-CC	*Dra*II, *Stu*I
	SEN-SPR2	TTG-CAT-GGA-GCA-GTT-TTT-GC	*Eco*RV, *Sca*I
	SEN-SPR3[a]	GCA-TAC-CCT-GGA-TCA-TAA-AAG	*Eco*RV, *Sca*I
	SEN-SPF1	GAG-AGG-TAT-AGT-TGA-AAG-CG	*Eco*RV
	SEN-SPF2[a]	AAG-GGA-AGA-GTA-CTT-TCT-GAG-TC	*Eco*RV
	SEN-SPF4	GAG-TGA-CAG-TGA-ACA-AAA-AC	*Sca*I, *Stu*I
	SEN-SPF5[a]	GCA-GAC-CTA-GAC-AAA-TTT-ACG	*Sca*I, *Stu*I
E. risticii	RIS-SPR	CGA-GCG-AAG-CAA-GTG-CAC-A	*Eco*RV, *Sca*I, *Dra*I, *Pvu*II
	RIS-SPF	GAG-AGG-TAT-CAT-TGA-AAG-CGG	*Eco*RV, *Sca*I
N. helmintoeca	NEO-SPR1	CCA-TGG-GAT-GTG-CAT-C	*Eco*RV
	NEO-SPR2[a]	TTT-TGC-GCG-GAC-AGG-GTC	*Eco*RV
	F1	CAT-GAR-CAR-AAT-GCT-TC	*Eco*RV
	NEO-SPF[a]	GAG-AAA-GTC-CTG-CAC-ATG-CTG	*Eco*RV

[a] Used in the nested PCR.
[b] R, A or G.

primers, NEO-CS142F (5′-ATY-ACY-TTC-RTA-GAY-GGT-GA-3′) and NEO-CS730R (5′-CGT-GCA-GTG-GWC-CCC-ATA-A-3′), was designed to specifically amplify *N. helminthoeca*, *E. risticii*, and *E. sennetsu*. The conditions for these two PCRs were the same as those described above with an annealing temperature at 55°C. Amplified products were digested with *Acs* I (Roche, Mannheim, Germany) and *Xho*I (Roche) for tick-borne *Ehrlichia* species and *Rca*I (Roche) for *Neorickettsia* genogroup ehrlichial species. Briefly, 7 μl of each PCR product was incubated with 2 μl of each enzyme and 1 μl of 10× buffer supplied by the manufacturer, and this was followed by incubation at 50°C for 1 h. Digestion products were separated by 1.5% agarose gel electrophoresis.

Nucleotide sequence accession numbers. The herein-determined *gltA* sequences of the following organisms have been deposited in the GenBank database under the indicated accession numbers: HGE agent, AF304136; *E. equi*, AF304137; *E. phagocytophila*, AF304138; *A. marginale* strain South Idaho, AF304139; *A. marginale* strain Florida, AF304140; *A. centrale*, AF304141; *E. chaffeensis*, AF304142; *E. canis*, AF304143; *E. muris*, AF304144; *Ehrlichia* sp. detected from *I. ovatus*, AF304145; *C. ruminantium*, AF304146; *E. risticii*, AF304147; *E. sennetsu*, AF304148; and *N. helminthoeca*, AF304149. The GenBank accession numbers of the *gltA* sequences of *R. prowazekii*, *B. henselae*, and *E. coli* used in this study were M17149, L38987, and J01619, respectively. The GenBank accession numbers of the following 16S rRNA gene sequences used to calculate percent identities and construct phylogenetic trees are as indicated: HGE agent, U02521; *E. equi*, M73223; *E. phagocytophila*, M73224; *A. marginale*, M60313; *A. centrale*, AF283007; *E. chaffeensis*, M73222; *E. canis*, M73221; *E. muris*, U15527; *Ehrlichia* sp. detected from *I. ovatus*, AF260591; *C. ruminantium*, AF069758; *W. pipientis*, AF179630; *E. risticii*, M21290; *E. sennetsu*, M73225; *N. helminthoeca*, U12457; *R. prowazekii*, M21789; and *B. henselae*, AJ223779. The GenBank accession numbers of the following heat shock protein-coding genes for most *Ehrlichia* species, except for *A. centrale* and *N. helminthoeca* or for the glutathione synthetase gene for *A. centrale*, that were used to compare the GC contents with those of *gltA* are as indicated: HGE agent, AF172163; *E. equi*, AF173988; *E. phagocytophila*, U96735; *A. marginale*, AF165812; *A. centrale*, M80425; *E. chaffeensis*, L10917; *E. canis*, U96731; *E. muris*, AF210459; *Ehrlichia* sp. detected from *Ixodes ovatus*, AB032712; *C. ruminantium*, U13638; *E. risticii*, AF206299; *E. sennetsu*, AF060197; *R. prowazekii*, Y15783; and *B. henselae*, U78514.

TABLE 4. Oligonucleotide primers used for PCR amplification and sequencing to determine the *gltA* sequences of various species and to confirm sequences of the *gltA* coding region in other ehrlichial strains studied[a]

Target species	Primer	Sequence (5′-3′)[c]
HGE agent, *E. equi, E. phagocytophila*	HG-M28F	GTA-ATA-AAT-TGT-ATT-ATC-AGA-G
	HG1257R	AAT-ACG-TGA-GTT-TGA-AAC-CA
	HG602F[b]	TGG-ATG-ATG-CAC-ATC-GTG
	HG700R[a]	TAC-GCA-CAG-TGG-AAG-TAG
A. marginale Florida and South Idaho	MAR-M35F	GTC-TGG-TGA-GTT-TGT-TGT-CC
	MAR-1287R	GCT-TGC-ACA-TCG-CTC-AAT-AA
A. centrale	CENT-M32F	GTG-TCC-AGT-AAA-CTT-GTT-GTC-GG
	CENT1534R	AAA-GCA-TGG-TGC-GAG-CAT-A
Ehrlichia detected from *I. ovatus*	CAN-M61F	TTA-TCT-GTT-TAT-GTT-ATA-TAA-GC
	R1b	CGA-TGA-CCA-AAA-CCC-AT
	F1b	GAT-CAT-GAR-CAR-AAT-GCT-TC
	MUR1251R	CTA-GAT-TTT-TGT-AAT-ATG-GCC-AG
E. chaffeennsis	CAN-M61F	TTA-TCT-GTT-TAT-GTT-ATA-TAA-GC
	CHAFF1285R	AAA-CAA-TAA-GCA-ATG-ATA-ATT-CAA
E. muris	CAN-M61F	TTA-TCT-GTT-TAT-GTT-ATA-TAA-GC
	MUR1278R	AAT-TTG-ATA-ACA-ATA-GCA-TAA-AAA-C
E. canis	CAN-M61F	TTA-TCT-GTT-TAT-GTT-ATA-TAA-GC
	CAN1317R	CAG-TAC-CTA-TGC-ATA-TCA-ATC-C
C. ruminantium	CR-M31F	ACG-CTT-TGT-TGT-TAT-TGT-ATT-AG
	CR1278R	CTA-CAA-AAG-GAA-ATA-CCT-TCA-C
E. sennetsu, E. risticii	SEN-M26F	CTC-AAC-TGG-AGA-ATA-TTT-AAA-GAA-G
	SEN–RIS-1218R	GCG-KAG-AAC-CAC-TAA-MAG-CTG
N. helminthoeca	NH-M91F	CAT-AGA-TTA-ACT-GTG-CTA-C
	NH1239R	GAT-GAA-ATT-CCA-TCC-TCG-TGT-G

[a] Specific or degenerated primers shown in Table 3 were also used as supplementary sequence primers if necessary.
[b] Sequence primers.
[c] R, A or G; M, A or C.

RESULTS

Determination of ehrlichial *gltA* sequences. After determination of the 482-bp partial sequence of the *gltA* of HGE agent, a 1,236-bp open reading frame extending from the ATG start codon down to the TAA stop codon was determined using the Genome Walker PCR method.

Complete *gltA* nucleotide sequences of *E. chaffeensis*, *E. muris*, *C. ruminantium*, *A. marginale* strain South Idaho, *N. helminthoeca*, and *E. sennetsu* have been determined, with lengths of 1,251, 1,251, 1,248, 1,254, 1,212, and 1,197 bp, respectively. As the 730-bp partial *gltA* sequence of *E. equi* and *E. phagocytophila* exhibited more than 99.0% similarity with that of HGE, primers HG-M28F and HG1257R, which could amplify the complete *gltA* sequence of the HGE agent, were used for determining the 1,236-bp *gltA* sequences of *E. equi* and *E. phagocytophila* (Table 4). As the 730-bp partial sequence of *A. marginale* strain Florida was identical to that of strain South Idaho, primers MAR-M35F and HG1287R, which could amplify the complete *gltA* sequence of *A. marginale* strain South Idaho, were used to amplify the 1,254-bp sequences of the *gltA* of *A. marginale* strain Florida (Table 4).

The complete *gltA* nucleotide sequences of *E. canis*, *A. centrale*, and *E. risticii* have been determined in the third step of the strategy. The *gltA* genes of these organisms have lengths of 1,251, 1,254, and 1,197 bp, respectively. Two primer sets, CAN-M61F–R1b and F1b-MUR1251R, were used to obtain a partial *gltA* sequence of the *Ehrlichia* sp. detected from *I. ovatus* (Table 2) and resulted in a 1,228-bp open reading frame near the 3′ end.

Comparison of *gltA* sequences. The GC content of the ehrlichial *gltA* genes varied from 30.5% for the *Ehrlichia* sp. detected from *I. ovatus* to 51.0% for *A. centrale* (Table 5).The multiple alignment analysis by the ClustalW program demonstrated several gaps in the alignment (data not shown). The percentages of similarity varied from 49.7% (*E. risticii* versus *A. centrale*) to 99.8% (the HGE agent versus *E. equi*) for the nucleotide sequence and from 44.4% (*E. sennetsu* versus *E. muris*) to 99.5% (the HGE agent versus *E. equi*) for the deduced amino acid sequence. The percent identities of the *gltA* nucleotide sequences between species in the *Neorickettsia* group (*N. helminthoeca*, *E. sennetsu*, and *E. risticii*) and other ehrlichial species varied from 49.7 to 55.3%; these were lower than those between other ehrlichial species and *R. prowazekii* (53.8 to 62.2%) or *B. henselae* (52.7 to 58.5%). The *gltA* nucleotide and deduced amino acid sequences of the HGE agent, *E. equi*, and *E. phagocytophila* were very similar: there were 3 nucleotide and 2 amino acid differences between the HGE agent and *E. equi* sequences, 9 nucleotide and 4 amino acid

TABLE 5. The length and GC contents of ehrlichial citrate synthase gene

Organism	Citrate synthase gene		%GC contents of other gene registered in GenBank (gene, accession number)
	Length (bp)[a]	GC content (%)	
HGE agent	1,236	38.3	42.4 (heat shock protein gene, AF172163)
E. equi	1,236	38.3	42.1 (heat shock protein gene, AF173988)
E. phagocytophila	1,236	38.2	42.1 (heat shock protein gene, U96735)
A. marginale	1,254	50.5	48.8 (heat shock protein gene, AF165812)
A. centrale	1,254	51.0	50.4 (glutathione synthetase gene, M80425)
E. canis	1,251	32.1	33.2 (heat shock protein gene, U96731)
E. chaffeensis	1,251	31.3	33.2 (heat shock protein gene, L10917)
E. muris	1,251	31.2	34.0 (heat shock protein gene, AF210459)
Ehrlichia detected from *I. ovatus*	1,228[b]	30.5	33.7 (heat shock protein gene, AB032712)
C. ruminantium	1,248	32.6	33.0 (heat shock protein gene, U13638)
E. risticii	1,197	43.7	41.8 (heat shock protein gene, AF206299)
E. sennetsu	1,197	43.9	42.4 (heat shock protein gene, AF060197)
N. helminthoeca	1,212	44.2	50.5 (16S rRNA gene, U12457)
R. prowazekii	1,311	33.6	33.8 (heat shock protein gene, Y15783)
B. henselae	1,296	38.6	44.3 (heat shock protein gene, U78514)

[a] From ATG start codon to stop codon.
[b] Partial sequence; sequencing of the 3′ end has not been completed.

differences between the HGE agent and *E. phagocytophila*, and 10 nucleotide and 4 amino acid differences between *E. equi* and *E. phagocytophila*.

Phylogenetic analyses. GltA-based phylogenetic reconstruction was compared with 16S rRNA-based analysis (Fig. 2). The phylogenetic tree based on the deduced amino acid sequences of ehrlichial species, *R. prowazekii*, and *B. henselae* was similar to the *gltA* gene tree (data not shown)

The topologies of the *gltA*-based phylogenetic trees were almost the same as those derived from the 16S rRNA gene sequence analyses. However, the trees constructed by *gltA* nucleotide sequences or amino acid sequences showed better bootstrap values than the 16S rRNA-based tree. Higher bootstrap values were obtained in the nucleotides of *gltA*-based trees for the relationships between *E. muris* and the *Ehrlichia* species detected from *I. ovatus* (bootstrap value: 1,000) and between *E. chaffeensis* and these two species (bootstrap value: 950). However, the bootstrap value between the HGE agent and *E. equi*

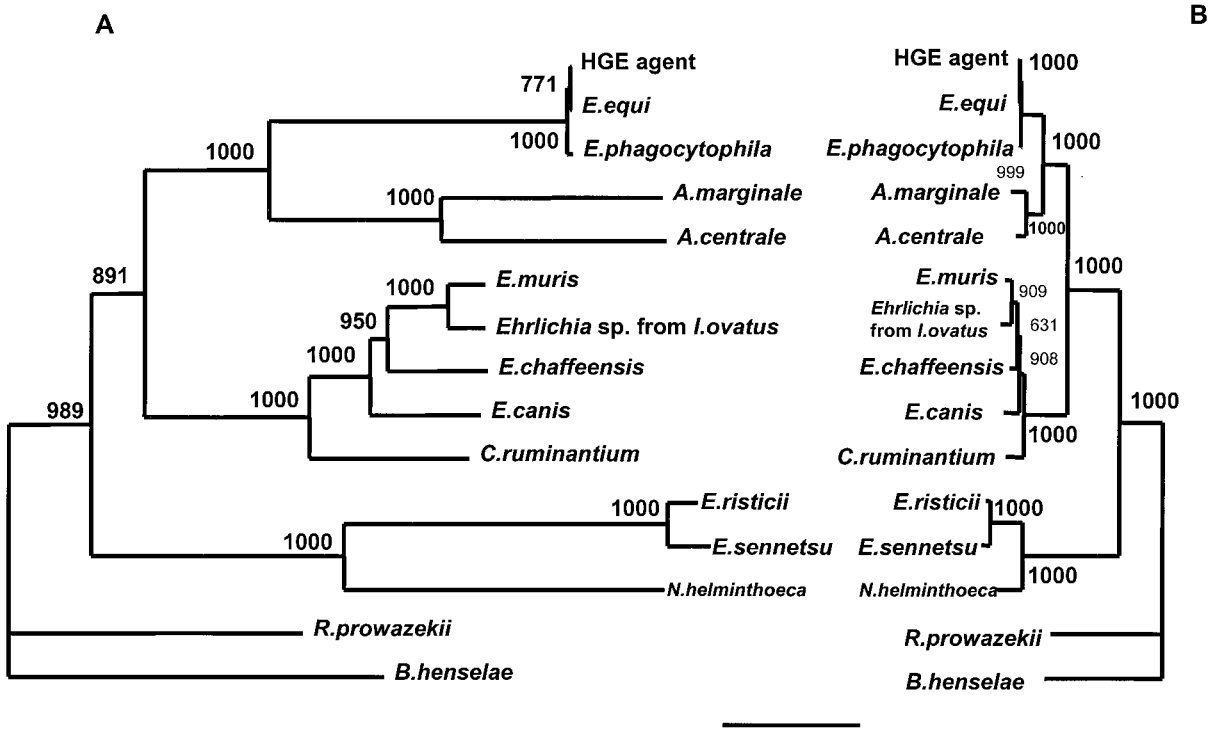

FIG. 2. Phylogenetic relationship of various *Ehrlichia* spp. based on the nucleotide sequences of citrate synthase gene (A) and 16S rRNA gene (B). The neighbor-joining method was used to construct the phylogenetic tree by using the ClustalW program. The scale bar represents 1% divergence. The numbers at nodes are the proportions of 1,000 bootstrap resamplings that support the topology shown.

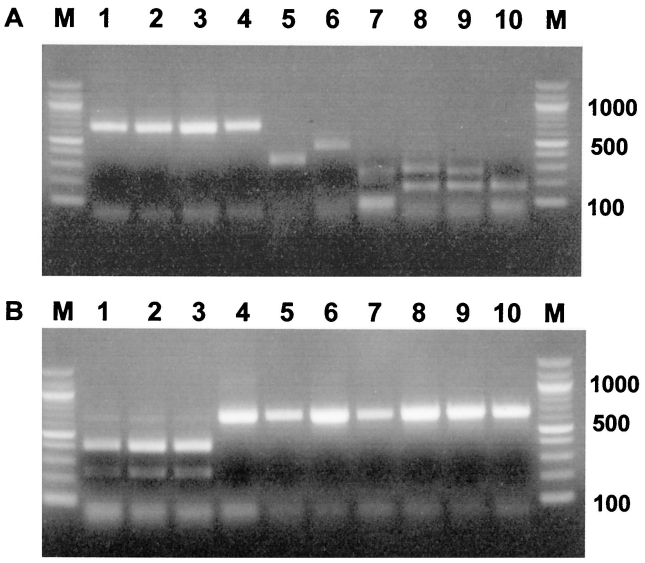

FIG. 3. Restriction profiles obtained after *Acs*I (A) and *Xho*I (B) digestion of a portion of the citrate synthase gene amplified from 10 tick-borne ehrlichial species by PCR using consensus primers EHR-CS136F–EHR-CS778R. Lanes: M, molecular weight markers (in thousands); 1, HGE agent; 2, *E. equi*; 3, *E. phagocytophila*; 4, *A. marginale*; 5, *A. centrale*; 6, *E. canis*; 7, *E. chaffeensis*; 8, *E. muris*; 9, *Ehrlichia* sp. detected in *I. ovatus*; 10, *C. ruminantium*.

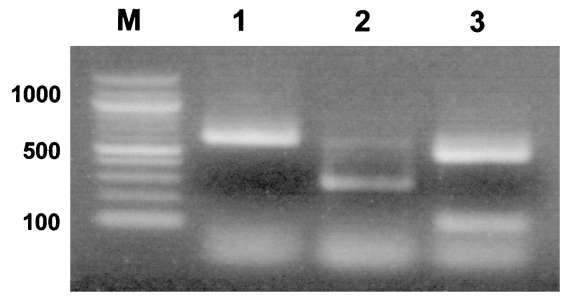

FIG. 4. Restriction profiles obtained after *Rca*I digestion of a portion of the citrate synthase gene amplified from three species of the *Neorickettsia* genogroup by PCR using consensus primer NEO-CS142F–NEO-CS730R. Lanes: M, molecular weight markers (in thousands); 1, *E. sennetsu*; 2, *E. risticii*; 3, *N. helminthoeca*.

was comparatively low (771 and 497 for nucleotide- and amino acid sequence-based trees, respectively).

Consensus PCR and PCR-RFLP analysis. A consensus primer pair, EHR-CS136F and EHR-CS778R, amplified a 643-bp partial sequence of *gltA* in 10 tick-borne *Ehrlichia* species. Predicted *Acs*I RFLP patterns were a single band (no digestion) for the HGE agent, *E. equi*, *E. phagocytophila*, and *A. marginale*; two bands of 312 and 331 bp for *A. centrale*; three bands of 37, 162, and 44 bp for *E. canis*; five bands of 14, 84, 87, 199, and 259 bp for *E. chaffeensis*; four bands of 38, 162, 171, and 272 bp for *E. muris*; five bands of 14, 38, 162, 171, and 258 bp for the *Ehrlichia* sp. detected from *I. ovatus*; and five bands of 37, 75, 167, 171, and 192 bp for *C. ruminantium* (Fig. 3A). The predicted patterns for *Xho*I were two bands of 242 and 401 bp for the HGE agent, *E. equi*, and *E. phagocytophila* and a unique band for the seven other species (Fig. 3B). Experimental results differed from those predicted, because bands that have molecular size of <100 bp were not easily detected and two or three bands which have similar molecular sizes could not be distinguished in agarose gels. The combination of both *Acs*I and *Xho*I digestion identified tick-borne ehrlichial species except those of the *E. phagocytophila* genogroup (HGE, *E. equi*, and *E. phagocytophila*), and *E. muris* and the *Ehrlichia* sp. detected from *I. ovatus* that showed the same RFLP patterns for both *Acs*I and *Xho*I digestion.

A consensus primer pair, NEO-CS142F and NEO-CS730R, amplified a 596-bp partial sequence in *E. sennetsu*, *E. risticii*, and *N. helminthoeca*. Predicted *Rca*I RFLP patterns included a unique band for *E. sennetsu*, two bands of 285 and 304 bp for *E. risticii*, and two bands of 109 and 487 bp for *N. helminthoeca*. The result of the RFLP is shown in Fig. 4. Although two bands of 285 and 304 bp for *E. risticii* were not distinguished in agarose gel, the RFLP profiles of these three species were apparently different from one to another.

DISCUSSION

To determine the complete *gltA* sequence of most ehrlichial species, a combination of consensus PCR amplification, sequencing, and the Genome Walker method was used in this study. To determine the RNA polymerase beta subunit (*rpoB*) gene of *Leptospira biflexa*, this strategy was recently evaluated as a convenient means for amplifying unknown sequences on the 3′ and 5′ ends (18). Semipurified genomic DNA of ehrlichial bacteria, including host cell genomic DNA, was used in this study, although the Genome Walker method is, as recommended by the manufacturer, usually performed with purified DNA in order to avoid nonspecific amplification. By using DNA from noninfected host cells as a negative control, nonspecific amplification in the Genome Walker PCR can be identified and controlled. Avoiding the purification steps saves material and time, which are critical issues when dealing with fastidious intracellular organisms such as ehrlichiae.

The length of the *gltA* sequences varied in ehrlichial species from 1,197 bp (*E. sennetsu* and *E. risticii*) to 1,254 bp (*A. marginale* and *A. centrale*), encoding proteins with deduced sequences of 398 to 421 amino acid residues. The length of ehrlichial *gltA* was shorter than that of *gltA* from closely related genera of *Rickettsia* (*R. prowazekii*, 1,311 bp) and *Bartonella* (*B. henselae*, 1,296 bp) (14, 30). The level of similarity among ehrlichial *gltA* was much lower than that of 16S rRNA gene sequences in the same species. The percent identities of the *gltA* nucleotide and deduced amino acid sequences vary from 49.7 to 99.8% and 44.4 to 99.5%, respectively. In contrast, those of the 16S rRNA gene vary from 83.5 to 99.9%. Percent identities were also found to be lower than those reported for *groESL* sequences (23), although the differences are small. These findings suggest that ehrlichial *gltA* sequencing may offer a tool with increasing discriminatory power for both phylogenetic and identification studies because of the greater variation in *gltA* than in any other gene currently determined for these species. Interestingly, the level of similarity between species in the *Neorickettsia* genogroup (*N. helminthoeca*, *E. sennetsu*, and *E. risticii*) and other ehrlichial species was lower than that between these species and *R. prowazekii* or *B. henselae*. The

gltA sequence analysis confirmed that this group of ehrlichiae forms a clade distinct from other tick-borne ehrlichial agents.

GC contents of the *gltA* gene also shows greater variation from 30.5 to 51.0%. The *C. ruminantium* genogroup (*E. canis*, *E. chaffeensis*, *E. muris*, *Ehrlichia* sp. detected from *I. ovatus*, and *C. ruminantium*) has lower GC content (30.5 to 32.6%) than the *E phagocytophila* genogroup (GC: 38.2 to 38.3%) and the *Neorickettsia* genogroup (GC: 43.7 to 44.2%). *A. marginale* and *A. centrale* show the highest percentages (50.5 and 51.0%, respectively). The GC content of other genes, mainly heat shock protein, shows values similar to those of *gltA*; 30.5 to 34.0% for the heat shock protein gene of *C. ruminantium* genogroup species, 48.8% for the heat shock protein gene of *A. marginale*, and 50.4% for the glutathione synthetase gene of *A. centrale*. The architecture of *gltA*-based phylogenetic trees was almost the same as the that of the tree derived from the 16S rRNA gene sequences. However, the trees constructed from *gltA* show more divergence than that from the 16S rRNA gene. The relationships of *E. muris*, *E. chaffeensis*, and the recently detected *Ehrlichia* species originally isolated from *I. ovatus* were well defined, with higher bootstrap values in the *gltA*-based tree than for those of the 16S rRNA-based tree. The bootstrap values for all of the nodes were greater than 85% in both nucleotide and deduced amino acid analyses. The only exception was the branching of the HGE agent and *E. equi*, due to the high sequence homology. These findings suggest that the *gltA*-based phylogeny of ehrlichial agents can be an additional phylogenetic tool and support the 16S rRNA-based phylogeny.

Although *A. marginale*, *A. centrale*, *E. phagocytophila*, *E. equi*, and the HGE agent are all tick-borne agents and most often detected in the cells in the peripheral blood that derive from bone marrow precursors in vivo, both *A. marginale* and *A. centrale* show biological differences from *E. phagocytophila*, *E. equi*, and the HGE agent. *Anaplasma* species infect predominantly erythrocytes in the ruminant host, while *E. phagocytophila* genogroup ehrlichiae are most often detected in granulocytes of various mammalian hosts, including humans. In vitro, *E. equi* and the HGE agent grew in the HL-60 human promyelocytic cell line (6, 7, 11), whereas no mammalian cell system allowed active replication of *Anaplasma* species. In the present study, both *A. marginale* and *A. centrale* show low levels of similarity with the *E. phagocytophila* genogroup (63.8 to 64.0%). The GC contents of both *A. marginale* and *A. centrale* are 50.5 and 51.0%, respectively, while those of the *E. phagocytophila* genogroup are of 38.2 or 38.3%. These two groups were distant in the phylogenetic tree. These data regarding the level of similarity between the *gltA* nucleotide sequences, GC content, and the *gltA*-based trees suggest that the *Anaplasma* group (*A. marginale* and *A. centrale*) forms a clade independent from the *E. phagocytophila* genogroup.

W. pipientis occupies a position intermediate between tick-borne *Ehrlichia* and the *Neorickettsia* clade as shown by 16S rRNA gene analysis (5). The *gltA* sequence of this species was not been analyzed in the present study, but its sequencing is under way in our laboratory.

The *gltA* nucleotide sequences of 13 ehrlichial species used in this study demonstrate both very conserved regions that allowed us to amplify DNA fragments by using both *B. henselae*- and *R. prowazekii*-derived degenerate primers and highly variable regions that allowed better definition of *Ehrlichia* species. Consequently, the design of *Ehrlichia* genus-specific primers has not been successful. However, tick-borne-*Ehrlichia*-specific or *Neorickettsia* genogroup-specific primer sets that amplify partial *gltA* genes of several ehrlichial agents were designed. In the present study, two sets of primers, EHR-CS136F–EHR-CS778R and NEO-CS142F–NEO-CS730R, amplified 10 tick-borne *Ehrlichia* species and three species among the *Neorickettsia* genogroup, respectively. This *gltA*-based group-specific PCR may be useful for epidemiological studies of ehrlichiosis, as previously demonstrated for *Rickettsia* (1) (2, 20). Although a unique isolate of each species has been tested herein, the conservation of these primer pairs among the different species argues for their conservation within a species suggesting their usefulness. Indeed, two new *Ehrlichia* genotypes have been recently found in African ticks in our laboratory, and partial *gltA* sequences of these species were determined by using a consensus primer set to characterize the phylogenetic position in ehrlichiae (16).

The results of RFLP analysis revealed that the combination of consensus PCR and RFLP can identify ehrlichiae at the species level with the exception of the *E. phagocytophila* genogroup and *E. muris* or *Ehrlichia* sp. detected in *I. ovatus*. It has been suggested that *E. phagocytophila*, *E. equi*, and the HGE agent are different strains of the same species and are not convincingly distinguishable by 16S rRNA analysis (4). Moreover, little is known about the newly described ehrlichia isolated from *I. ovatus* or its phylogenetic relationship with *E. muris*. RFLP analysis of *gltA* PCR products offers an effective new tool for identification of ehrlichial species among tick-borne *Ehrlichia* and *Neorickettsia* genogroup *Ehrlichia*.

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