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High Diversity of *ankA* Sequences of *Anaplasma phagocytophilum* among *Ixodes ricinus* Ticks in Germany

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In Germany humans with acute granulocytic ehrlichiosis have not yet been described. Here, we characterized three different genes of *Anaplasma phagocytophilum* strains infecting German *Ixodes ricinus* ticks in order to test whether they differ from strains in other European countries and the United States. A total of 1,022 *I. ricinus* ticks were investigated for infection with *A. phagocytophilum* by nested PCR and sequence analysis. Forty-two (4.1%) ticks were infected. For all positive ticks, parts of the 16S rRNA and *groESL* genes were sequenced. The complete coding sequence of the *ankA* gene could be determined in 24 samples. The 16S rRNA and *groESL* gene sequences were as much as 100% identical to known sequences. Fifteen *ankA* sequences were ≥99.37% identical to sequences derived from humans with granulocytic ehrlichiosis in Europe and from a horse with granulocytic ehrlichiosis in Germany. Thus, German *I. ricinus* ticks most likely harbor *A. phagocytophilum* strains that can cause disease in humans. Nine additional sequences were clearly different from known *ankA* sequences. Because these newly described sequences have never been obtained from diseased humans or animals, their biological significance is currently unknown. Based on this unexpected sequence heterogeneity, we propose to use the *ankA* gene for further phylogenetic analyses of *A. phagocytophilum* and to investigate the biology and pathogenicity of strains that differ in the *ankA* gene.

Anaplasma phagocytophilum is a gram-negative, obligately intracellular bacterium that replicates within neutrophils. Recently, the organisms formerly known as *Ehrlichia phagocytophila*, the causative agent of tick-borne fever in cattle and sheep, *Ehrlichia equi*, the causative agent of equine granulocytic ehrlichiosis (EGE), and the agent of human granulocytic ehrlichiosis (HGE) have been assigned to the single species *A. phagocytophilum* based on 16S rRNA and *groESL* gene analyses, the presence of shared antigens, and common biological characteristics (15).

A. phagocytophilum is tick transmitted and causes febrile diseases in humans and animals. Clinically, HGE is characterized by fever, headache, myalgia, and arthralgia. Typical laboratory findings are leukopenia, thrombopenia, and elevated liver enzyme levels (5). HGE was first described in the United States in 1994 (12), and more than 600 cases have been reported since then in the United States (5). However, in Europe, HGE has been diagnosed only in a small number of patients living in Slovenia (4, 30, 37), The Netherlands (52), Sweden (7, 25), Norway (26), and Poland (51).

The presence of *A. phagocytophilum* in *Ixodes ricinus* ticks in Europe is well documented (1, 3, 13, 14, 20, 24, 28, 33–35, 38, 40, 43, 54). In German *I. ricinus* ticks, the reported infection rate ranges from 1.6 to 2.3% (6, 17, 21). Despite the presence of *A. phagocytophilum* in German ticks and seropositivity rates as high as 2.6% in the normal population and 14% in certain risk groups (16, 23), human patients with granulocytic ehrlichiosis have not yet been described in Germany.

We therefore asked whether *A. phagocytophilum* strains infecting German ticks differ from strains known to be pathogenic in other European countries or in the United States. A total of 1,022 *I. ricinus* ticks were investigated for the presence of *A. phagocytophilum* by means of PCR, and the infecting strains were characterized by sequence analysis of three different genes (the 16S rRNA gene, *groESL*, and *ankA*).

MATERIALS AND METHODS

Tick collection. A total of 1,022 *I. ricinus* ticks were collected between 1998 and 2001. Of these, 1,019 ticks were from different regions of Germany (733 from Bavaria, 269 from Baden-Württemberg, 8 from Nordrhein-Westfalen, 8 from Lower Saxony, and 1 from Rheinland-Pfalz) and 3 were from Austria. Two hundred eighty-seven ticks had been part of a previous study (6). Ticks were removed from the vegetation or from the fur or skin of dogs, cats, deer, or humans. Six hundred ninety-seven ticks were adult females, 223 were adult males, 73 were nymphs, and 2 were larvae. For 12 adults the sex was not determined, and for 15 ticks the instar was unknown. Ticks were stored at −20°C until analysis.

PCR analyses. DNA was extracted using the QIAamp tissue kit (Qiagen, Hilden, Germany). PCR amplification of the tick mitochondrial 16S rRNA gene was carried out as a quality control for the prepared DNA (8). Nested PCRs for detection of *A. phagocytophilum* that amplify a 546-bp product of the 16S rRNA gene were performed as described previously (6). In samples that were positive for *A. phagocytophilum* DNA, 1,297 bp of the *groESL* heat shock operon was amplified (30, 48). Amplification of the *ankA* gene of *A. phagocytophilum* (also termed *ank*) with published primers (32) was possible for only 15 out of 42 positive samples. When PCRs were carried out at low stringency, parts of the *ankA* genes of eight additional samples could be amplified. These PCR products were sequenced, and primers for the amplification of the complete open reading frame could be obtained (Table 1). In 1 of 42 samples, only the first half of the *ankA* gene was amplified with previously published primers (32). By the strategy described above, the second half of the gene could be amplified. Primers used for

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TABLE 1. Primers used for amplification and sequencing of the *ankA* gene

No. of ticks	First PCR		Nested PCR		Additional primers used for sequencing[a]
	Primers used	Reference or source	Primers used	Reference or source	
15 of 42	U7 + 1R1	32	U8 + 1R7	32	FvL ankU3
			U5 + 1R4	32	FvL ankU1
			U3 + 1R2	32	FvL ankU2
15 of 42	1F + 4R1	32	1F + 1R	32	FvL ank1
			2F1 + 2R1	32	FvL ank2
			3F + 3R	32	FvL ank3
			4F1 + 4R1	32	
15 of 42	4F2 + D2	32	4F3 + D1	32	
8 of 42	U8 + 1Rmod	32; this study	U8 + 1R7mod	32; this study	
			U5mod + 1R4mod	This study	
			U3mod + 1R2mod	This study	U3 Seq1
					U3 Seq2
					U3 Seq3
					U3 Seq4
8 of 42	1F + 4R1mod	32; this study	1Fmod2 + 1Rmod	This study	
			2F1 mod + 2R1mod	This study	
8 of 42	1Fmod2 + D1	This study; 32	3Fmod + D1	32; this study	3Fmod Seq1
					3Fmod Seq2
					3Fmod Seq3
					3Fmod Seq4
					3Fmod Seq5
					3Fmod Seq6
8 of 42	4F2 + D2	32	4F3 + D1	32	
1 of 42	U7 + 1R1	32	U8 + 1R7	32	FvL ankU3
			U5 + 1R4	32	FvL ankU1
			U3 + 1R2	32	FvL ankU2
1 of 42	1F + 4R1mod	32; this study	1F + 1R	32	
			2F1neu + 4Rneu	This study	2F1neu Seq1
					2F1neu Seq2
					2F1neu Seq3
					2F1neu Seq4
					4F1neu Seq1
					4F1neu Seq2
					4F1neu Seq3
					4F1neu Seq4
					4F1neu Seq5
1 of 42	4F2neu + D1	32; this study			

[a] Primers were designed for this study and are listed in Table 2.

PCR and sequencing of the *ankA* gene are summarized in Table 1; the respective nucleotide sequences are shown in Table 2.

PCRs for amplification of the *ankA* gene were carried out as follows: 2 to 5 μl of DNA was used as a template in a 50-μl reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 μM each primer, and 0.2 μl (1 U) of *Taq* DNA polymerase (Gibco BRL, Karlsruhe, Germany). The PCRs were performed in a Gene Amp PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, Calif.) under the following cycling conditions: denaturation for 3 min at 94°C followed by 40 cycles at 94°C for 30 s, the predicted melting temperature of the primers minus 4°C for 30 s, and 72°C for 30 s. Final extension of the product was carried out at 72°C for 10 min. Nested amplifications used 1 μl of the primary PCR product as the template. DNA from *A. phagocytophilum* (*E. equi* MRK) was used as a positive control (kindly provided by J. Stephen Dumler, The Johns Hopkins Medical Institutions, Baltimore, Md.), and sterile H_2O served as a negative control. To avoid contamination, the extraction of DNA, the preparation of the master mixes, and the PCR were performed in three separate rooms.

DNA sequencing and data analysis. All PCR products were bidirectionally sequenced without prior subcloning by using the ABI Prism 310 genetic analyzer and the Big Dye Cycle Sequencing Kit (Applied Biosystems). In the case of the 546-bp product of the 16S rRNA gene, sequencing was performed with the primers used for the PCR as well as with two other primers (342 f [CTA CGG GAG GCA GCA GT] and 356 r [TGC TGC CTC CCG TAG GA]). The *groESL* sequence was determined as described previously (11, 30, 48) together with primer FvL groESL (GGG AGA TGG AAC TAC TAC AT). For sequence analysis of the *ankA* gene, the same primers as those for the PCR were used (32), but complementation was achieved by use of additional sets of primers (Table 1). Sequences were edited and assembled with the SeqMan program (Lasergene package; DNAstar, Madison, Wis.). Alignments were performed using the PileUp program of the Wisconsin Package (version 10.2; Genetics Computer

TABLE 2. Nucleotide sequences of primers used for amplification and sequencing of the *ankA* gene

Primer	Nucleotide sequence
Amplification	
U5mod	GCA AGC ACG TGA GGC AGC G
U3mod	GCG TAT GGA TCA CGA GAA AA
1Fmod2	GTA TTC TGC AGT CTG CTC TTA G
1Rmod	CTT AGT GCT TCA GCG GTC AG
1R2mod	GCG TAG GAA ACT TAG AAC TA
1R4mod	CAT ACT GCA CTG CAC TCA TCC
1R7mod	ATA TCT GCT CCA TAC CAC TG
2F1mod	CAC TGA AGT GGT TGC TGT AA
2R1mod	GAA TAT AAA GCC TCA TGT AAC G
2F1neu	CAA GAG ACA ACT CTG GCT TTT
3Fmod	GCT GCA AGC CGC TCC ATT CT
4F2neu	CAG AAG GAT TAC AGG GAG CA
4R1mod	CTT TGA GGA GCT TCT GGT TG
4Rneu	GGA TCC TGC TTT GCC TTT TC
Sequencing	
FvL ankU1	CAC TCC GCT TCA TAT TGC TA
FvL ankU2	ACT CCT GAG TCT GTT GTA AA
FvL ankU3	TAA CAC ATC CTC TTT CAA CC
FvL ank1	GAA GAA ATT ACA ACT CCT GAA
FvL ank2	GCA GCT GCT AAT GGT GAC GG
FvL ank3	TAT CAT CCT TGG GTA GTG G
U3 Seq1	GCT CAG GTT CCC CAG AAG TA
U3 Seq2	AAT TTT GGC TAT CAT TAG AAG AT
U3 Seq3	GAA CAC CCT CAA TCA ACA TC
U3 Seq4	CAT CTC CAG TGA CAG GTG TA
2F1neu Seq1	GGA TCT ATG GAT GAA CAA GG
2F1neu Seq2	ATT AAC AAG TGC CAG GTG TAA
2F1neu Seq3	CAG TAA TGA TTC TCC GCT TG
2F1neu Seq4	GTA TCA CCA GAA TGT TTC CT
3Fmod Seq1	CCA TTT GCT CTT TGC TGA GAA
3Fmod Seq2	CTG GGG CAA CAG CAT CAG T
3Fmod Seq3	CAC TGG AGA ATA AAG AAG TAG G
3Fmod Seq4	ATG CAA CAG TGA AAA AGG GTC
3Fmod Seq5	CTT TTT GAG AAG TAT CAC TC
3Fmod Seq6	GCT GAA GGT CTA GGA ACG CC
4F1neu Seq1	GTC AGC ATA GAT AGA TTC AGG
4F1neu Seq2	AGA AGA AAT AGA TTC CCA AGT A
4F1neu Seq3	GCC TAT CTA CGA GGA CAT TA
4F1neu Seq4	TAG AAG AGC GTG TCC AAG TA
4F1neu Seq5	TCT TTC TTC TTA ACC ACA GAT A

Group [GCG], Madison, Wis.). Percent identity and percent similarity were determined by the OldDistances program of the GCG package by using the length of the shorter sequence without gaps as the denominator. For amino acid sequence comparison, a BLOSUM62 matrix was applied. The GrowTree program of the GCG package was used for phylogenetic analyses, and phylograms were generated by the unweighted pair-group method using arithmetic averages (UPGMA).

Nucleotide sequence accession numbers. The 16S rRNA gene sequence of sample D14 matches the previously reported sequence of the *Ehrlichia* sp. "Frankonia 1" (GenBank accession no. AF136713). The 16S rRNA gene sequences of samples G35 and G22 are identical to the previously described sequences of the *Ehrlichia* sp. "Frankonia 2" (GenBank accession no. AF136712) and the *Ehrlichia* sp. "Baden" (GenBank accession no. AF136714), respectively. The remaining 16S rRNA gene sequences received GenBank accession no. AY281771 to AY281809. GenBank accession numbers for the *groESL* and *ankA* sequences are AY281810 to AY281851 and AY282368 to AY282391, respectively.

RESULTS

In 46 of 1,022 *I. ricinus* ticks, the 16S rRNA gene-based nested PCR for detection of *A. phagocytophilum* yielded products of the correct size. Sequence analysis revealed that 42 (4.1%) ticks were infected with *A. phagocytophilum.* The infection rate was 4.3% (30 of 697) in adult females, 4.5% (10 of 223) in adult males, and 2.7% (2 of 73) in nymphs. The prevalences of infected ticks in Bavaria (4.1%; 30 of 733) and Baden-Württemberg (3.7%; 10 of 269) were comparable. One of 111 ticks removed from humans was infected (sample P80). Four hundred ninety-six base pairs (without primer sequences) of three samples were 99.80 to 100% identical to the 16S rRNA gene of *Wolbachia pipientis* (GenBank accession no. AJ306308). The sequenced 331 bp of another PCR product was 100% identical to the 16S rRNA gene of the *Ehrlichia*-like sp. "Schotti variant" (GenBank accession no. AF104680). The negative-control samples included in each PCR run did not yield amplification products.

16S rRNA gene sequences. Sequence analysis of 497 bp (without primer sequences) of the 16S rRNA gene revealed seven sequence types of *A. phagocytophilum* infecting German ticks. The sequences were 99.20 to 100% identical to each other and 99.40 to 100% identical (one to three nucleotide exchanges) to the prototype sequence of *A. phagocytophilum*, which was originally derived from a patient with HGE (GenBank accession no. U02521). Sixteen sequences (samples G35, G55, I68, I121, I200, K71, K84, S16, W34, W37, W63, W98, W140, W229, W284, and X7) were 100% identical to a sequence previously obtained from German *I. ricinus* ticks (GenBank accession no. AF136712). Ten sequences (samples D12, D13, D14, D20, D21, D22a, I63, I145, I158, and X17) showed 100% identity to a sequence which was previously derived from *I. ricinus* ticks in Sweden (GenBank accession no. AJ242784) and Germany (GenBank accession no. AF136713) and from roe deer in Switzerland (GenBank accession no. AF384212) and Slovenia (GenBank accession no. AF481850). In six sequences (samples G22, G24, G26, I20, J1, and SG3), 100% identity to another sequence from *I. ricinus* ticks in Sweden (GenBank accession no. AJ242783) and in Germany (GenBank accession no. AF136714) was found. An *A. phagocytophilum* strain with the latter sequence has been implicated in a case of granulocytic ehrlichiosis in a roe deer in Norway (46). One sequence (sample W271) was 100% identical to the prototype sequence of *A. phagocytophilum* (GenBank accession no. U02521). Another sequence (sample N6) showed 100% identity to a sequence from red deer in Slovenia (GenBank accession no. AF481852) and from *I. ricinus* ticks in the United Kingdom (GenBank accession no. AY149637).

Three sequences (samples I80, P80, and W298) had nucleotide exchanges at two to three positions compared to the prototype sequence of *A. phagocytophilum* (GenBank accession no. U02521) and were also different from any other known *A. phagocytophilum* sequence. Two of them were identical to each other. The analysis of five sequences yielded one double peak in the respective chromatograms. These peaks occurred mainly at positions known for nucleotide exchanges, suggesting that one individual tick might harbor more than one *A. phagocytophilum* variant. Apart from these double peaks, two sequences (samples I94 and I149) were identical to the prototype sequence of *A. phagocytophilum* (GenBank accession no. U02521), and three (samples D16, D17, and G34) matched the sequence from *I. ricinus* ticks in Sweden and Germany and from roe deer in Switzerland and Slovenia described above (GenBank accession no. AJ242784, AF136713, AF384212, and

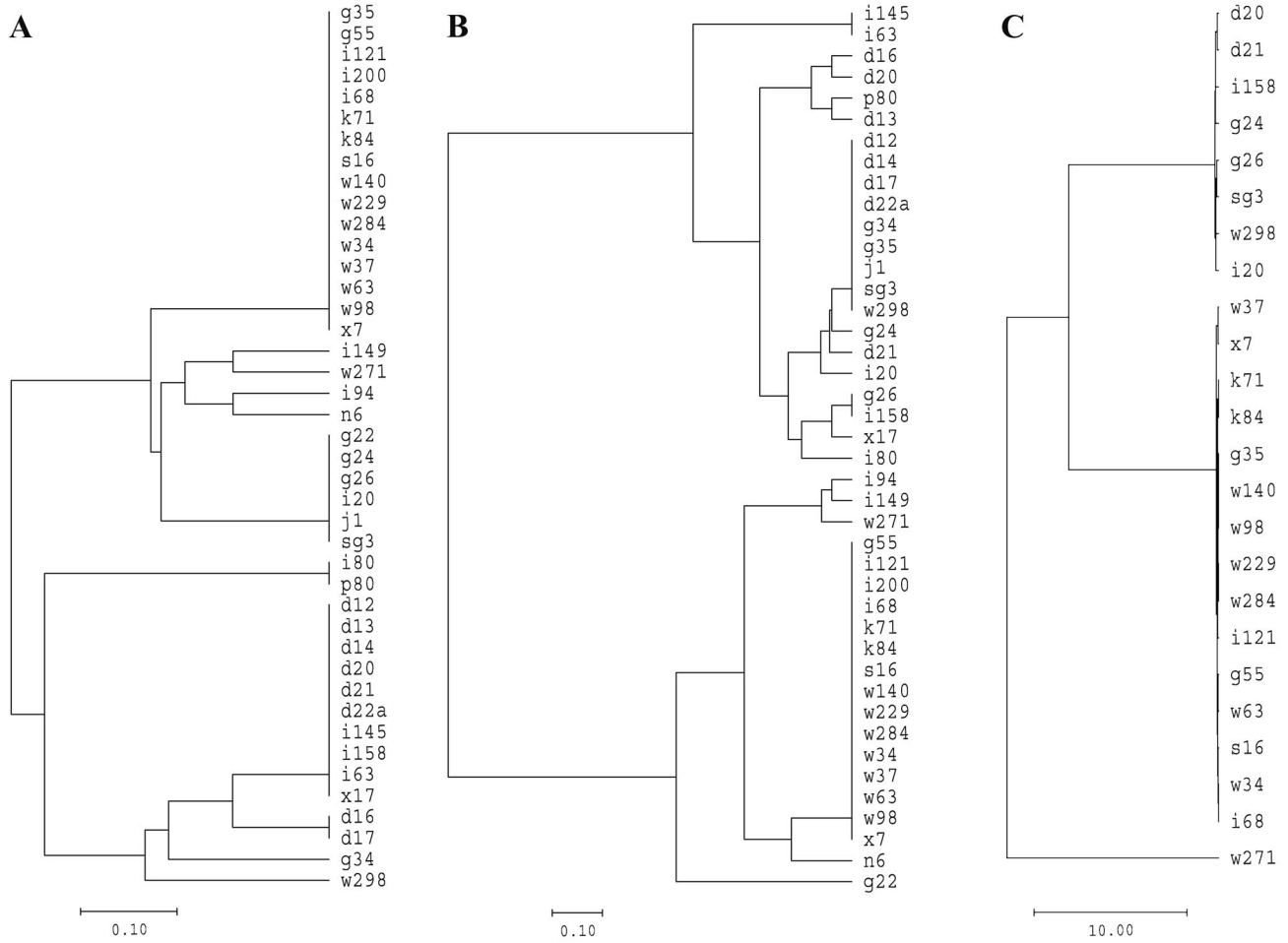

FIG. 1. Phylograms showing the relationships of the 16S rRNA gene (A), *groESL* (B), and *ankA* (C) nucleotide sequences. Scale bars indicate substitutions per 100 residues.

AF481850). The phylogram presented in Fig. 1A shows the relationships of the 16S rRNA gene sequences obtained.

***groESL* sequences.** A total of 1,256 bp (without primer sequences) of the *groESL* heat shock operon was analyzed for the 42 ticks that were infected with *A. phagocytophilum*. The sequences were 98.01 to 100% identical to each other. Although the majority of these sequences showed the highest degree of identity to sequences derived from roe deer, red deer, and *I. ricinus* ticks in Europe, 98.41 to 100% identities to sequences from HGE patients and horses with EGE in North America and Europe were also found.

The majority of the *groESL* sequences segregated into two main sequence types. The first type, represented by 15 sequences (samples G55, I68, I121, I200, K71, K84, S16, W34, W37, W63, W98, W140, W229, W284, and X7), showed 99.92% identity to sequences from red deer (GenBank accession no. AF478562) and roe deer (GenBank accession no. AF478558) in Slovenia. The second type, consisting of nine sequences (samples D12, D14, D17, D22a, G34, G35, J1, SG3, and W298), was 100% identical to a sequence from roe deer in Switzerland (GenBank accession no. AF383226) and Slovenia (GenBank accession no. AF478551). The remaining 18 sequences showed 99.68 to 100% identities to various *groESL* sequences deposited in GenBank. One of them (sample N6) was 100% identical to a sequence from a Slovenian HGE patient (GenBank accession no. AF033101) and a German horse with EGE (GenBank accession no. AF482760), but in this sample a 16S rRNA gene sequence variant was found. The sample with the 16S rRNA gene prototype sequence (sample W271) showed 100% identity to the *groESL* sequence from a Scottish goat (GenBank accession no. U96729), an animal known to be susceptible to *A. phagocytophilum*. In six sequences, one to two double peaks were detected in each of the chromatograms. They occurred mainly at positions where nucleotide exchanges were observed and in samples that also had double peaks in their 16S rRNA gene sequences. Double peaks were restricted to the third position of the codons and did not lead to amino acid exchanges. The phylogram in Fig. 1B shows the relationships of the *groESL* nucleotide sequences obtained.

The nested PCR we used targets the spacer region between the *groES* and *groEL* genes and the first part of the *groEL* gene. Hence, the first 402 amino acids of the GroEL sequences were analyzed. Only one amino acid exchange was observed (at position 242); all other nucleotide changes were silent. Twenty-two sequences had an alanine at that position, and 20 sequences had a serine.

TABLE 3. Identities of *ankA* nucleotide sequences and similarities of deduced amino acid sequences

Sample	% Identity of *ankA* nucleotide sequences (roman) and % similarity of deduced amino acid sequences (italics) for the indicated samples																			
	D20	D21	G24	G26	SG3	I20	W298	I158	W37	X7	K71[a]	G35[b]	W229[c]	G55	W63	S16	W34	I121	I68	W271
D20		99.81	99.78	99.70	99.54	99.43	99.43	99.78	82.14	82.14	81.54	81.60	81.57	81.57	81.57	81.52	81.49	82.18	82.18	74.42
D21	*99.75*		99.73	99.73	99.57	99.51	99.57	99.70	82.06	82.06	81.46	81.52	81.49	81.49	81.49	81.43	81.41	82.10	82.10	74.34
G24	*99.42*	*99.34*		99.86	99.73	99.62	99.62	99.84	82.17	82.17	81.57	81.62	81.60	81.60	81.60	81.54	81.52	82.21	82.21	74.42
G26	*99.59*	*99.67*	*99.59*		99.81	99.70	99.76	99.78	82.11	82.11	81.52	81.57	81.54	81.54	81.54	81.49	81.46	82.15	82.15	74.32
SG3	*99.51*	*99.59*	*99.59*	*99.67*		99.68	99.62	99.62	82.06	82.06	81.46	81.52	81.49	81.49	81.49	81.43	81.41	82.10	82.10	74.26
I20	*99.18*	*99.42*	*99.26*	*99.34*	*99.51*		99.68	99.57	81.82	81.82	81.22	81.28	81.25	81.25	81.25	81.20	81.17	81.88	81.88	74.03
W298	*99.18*	*99.42*	*99.26*	*99.51*	*99.34*	*99.51*		99.70	81.87	81.87	81.27	81.33	81.30	81.30	81.30	81.24	81.22	81.91	81.91	74.15
I158	*99.50*	*99.42*	*99.42*	*99.50*	*99.34*	*99.26*	*99.50*		82.13	82.13	81.54	81.59	81.56	81.56	81.56	81.51	81.48	81.91	81.91	74.37
W37	*78.37*	*78.12*	*78.04*	*78.12*	*78.21*	*77.80*	*77.88*	*78.22*		99.97	97.73	97.67	97.70	97.70	97.70	97.73	97.70	99.67	99.70	81.43
X7	*78.37*	*78.12*	*78.04*	*78.12*	*78.21*	*77.80*	*77.88*	*78.22*	*99.92*		97.70	97.70	97.67	97.73	97.73	97.70	97.67	99.65	99.67	81.46
K71[a]	*77.14*	*76.89*	*76.81*	*76.89*	*77.14*	*76.56*	*76.64*	*76.98*	*97.32*	*97.24*		99.87	99.89	99.81	99.81	99.87	99.84	99.89	99.84	81.01
G35[b]	*77.22*	*76.97*	*76.89*	*76.97*	*77.06*	*76.64*	*76.73*	*77.06*	*97.16*	*97.24*	*99.59*		99.97	99.89	99.84	99.79	99.76	99.89	99.78	81.01
W229[c]	*77.22*	*76.97*	*76.89*	*76.97*	*77.06*	*76.64*	*76.73*	*77.06*	*97.24*	*97.16*	*99.68*	*99.92*		99.87	99.81	99.81	99.79	99.92	99.81	80.98
G55	*77.22*	*76.97*	*76.89*	*76.97*	*77.06*	*76.64*	*76.73*	*77.06*	*97.24*	*97.32*	*99.43*	*99.68*	*99.59*		99.95	99.84	99.87	99.84	99.89	81.06
W63	*77.30*	*77.06*	*76.97*	*77.06*	*77.06*	*76.73*	*76.81*	*77.15*	*97.24*	*97.32*	*99.43*	*99.51*	*99.43*	*99.84*		99.84	99.92	99.78	99.95	81.06
S16	*77.22*	*76.97*	*76.89*	*76.97*	*77.06*	*76.64*	*76.73*	*77.06*	*97.40*	*97.32*	*99.68*	*99.43*	*99.51*	*99.59*	*99.59*		99.92	99.81	99.92	81.09
W34	*77.22*	*76.97*	*76.89*	*76.97*	*77.06*	*76.64*	*76.73*	*77.06*	*97.32*	*97.24*	*99.59*	*99.35*	*99.43*	*99.68*	*99.84*	*99.76*		99.78	100	81.06
I121	*77.78*	*77.53*	*77.45*	*77.53*	*77.61*	*77.20*	*77.28*	*77.36*	*99.25*	*99.25*	*99.75*	*99.83*	*99.83*	*99.67*	*99.50*	*99.59*	*99.50*		99.78	82.67
I68	*77.86*	*77.61*	*77.53*	*77.61*	*77.69*	*77.28*	*77.36*	*77.45*	*99.34*	*99.25*	*99.59*	*99.42*	*99.50*	*99.75*	*99.92*	*99.75*	*100*	*99.50*		82.72
W271	*70.64*	*70.48*	*70.31*	*70.39*	*70.48*	*70.23*	*70.39*	*70.54*	*79.10*	*78.61*	*78.07*	*78.07*	*78.07*	*78.15*	*78.15*	*78.23*	*78.15*	*79.68*	*79.77*	

[a] K71 also represents K84.
[b] G35 also represents W140 and W98.
[c] W229 also represents W284.

***ankA* sequences.** In 24 of 42 samples of DNA from infected ticks, the *ankA* gene could be amplified by PCR as described in Material and Methods. Sequencing of the total open reading frames revealed three distinct sequence types. The first sequence type comprised 15 sequences (samples G35, G55, I68, I121, K71, K84, S16, W34, W37, W63, W98, W140, W229, W284, and X7) that were closely related to known *ankA* sequences and were 97.67 to 100% identical to each other at the nucleotide level. At the amino acid level, the percentage of similarity was 97.16 to 100% (Table 3). Two of these sequences (samples I68 and I121) showed 99.45 and 99.40% identities, respectively, to a sequence from a Swedish HGE patient (GenBank accession no. AF100888) and from a German horse with EGE (GenBank accession no. AF482759). In 11 sequences (samples G35, G55, K71, K84, W34, W63, W98, W140, W229, W284, and S16), an as yet undescribed 75-bp (25-amino-acid) direct repeat, starting at nucleotide position 2145, was found. Except for that repeat, these sequences were again 99.37 to 99.48% identical to the sequence from the Swedish HGE patient and the German horse with EGE. In two sequences (samples X7 and W37), another novel, 96-bp (32-amino-acid) direct repeat structure beginning at nucleotide position 2775 was detected. It spans the same region as a previously described 81-bp direct repeat (32). Except for the direct repeat structure, these two sequences were 99.49 and 99.52% identical to sequences from two Slovenian HGE patients (GenBank accession no. AF100886 and AF100887, respectively). All 15 samples representing the first *ankA* sequence type showed the same 16S rRNA gene sequence variant which was previously obtained from German *I. ricinus* ticks (GenBank accession no. AF136712). The same was true with respect to the *groESL* sequences, since all, except sample G35, harbored the same *groESL* sequence, which was 99.92% identical to sequences from Slovenian red deer and roe deer (GenBank accession no. AF478562 and AF478558, respectively).

The second *ankA* sequence type was detected in eight samples (D20, D21, G24, G26, I20, I158, SG3, and W298) and did not match with known *ankA* sequences over the entire open reading frame. They were 99.43 to 99.86% identical to each other on the nucleotide level and 99.18 to 99.75% similar to each other on the amino acid level (Table 3). The identity to sequences of the first sequence type was 81.17 to 82.21% on the nucleotide level but was limited to 76.56 to 78.37% on the amino acid level (Table 3). One sequence (sample I158) showed an unique 12-bp deletion at nucleotide position 1102. In three sequences (samples G24, G26, and I158) that showed ambiguous nucleotides in their *groESL* sequences, as many as six double peaks in the *ankA* chromatograms were detected. Four of 12 of these ambiguous nucleotides would lead to an amino acid exchange. This indicates that one tick might harbor more than one *A. phagocytophilum* variant, since BLAST searches in the *A. phagocytophilum* genome (http://www.tigr.org/tdb/mdb/mdbinprogress.html) revealed only one copy of each of these genes. The infection of ticks (31, 43), rodents (28), and sheep (47) with more than one *A. phagocytophilum* variant has also been noted by others.

The third sequence type was displayed only by one sequence (sample W271). The first (nucleotides 1 to 1667) and last (nucleotides 3543 to 3764) parts of this sequence were 99.28 and 91.89% identical, respectively, to sequences from HGE patients in Europe and from a German horse with EGE (GenBank accession no. AF100886, AF100887, AF100888, and AF482759), whereas in the middle part the identity to known sequences was limited to about 60%. At the nucleotide level, the third sequence type was 80.98 to 82.72% and 74.03 to 74.42% identical to the first and the second *ankA* sequence

types described above, respectively. At the amino acid level, similarities of 78.07 to 79.77% to the first *ankA* sequence type and 70.23 to 70.64% to the second *ankA* sequence type were found (Table 3). Although sample W271 showed the unique *ankA* sequence described, its 16S rRNA gene and *groESL* sequences were 100% identical to the prototype sequence of *A. phagocytophilum* (GenBank accession no. U02521) and to a sequence from a Scottish goat (GenBank accession no. U96729), respectively.

The phylogram presented in Fig. 1C shows the relationships of the *ankA* nucleotide sequences obtained. The same clusters (except for sample G35) were achieved by using *groESL* sequences for the construction of the phylograms (Fig. 1B). The origin of the tick (Bavaria or Baden-Württemberg) had no obvious influence on the position of the respective sequence in the phylograms.

DISCUSSION

Using a large collection of *I. ricinus* ticks, the present study not only extends previous reports on the infection rate of ticks with *A. phagocytophilum* in Germany (6, 17, 21) but also provides a detailed phylogenetic analysis of *A. phagocytophilum* in Europe. Most importantly, the data demonstrate an unexpected and marked nucleotide polymorphism of the *ankA* gene. Compared to the infection rates observed in other European countries (about 1 to 30%), the prevalence of *A. phagocytophilum* infection in German ticks lies in the middle of the range of reported values (1, 3, 13, 14, 20, 24, 28, 33–35, 38, 40, 43, 54). The amplification of the 16S rRNA genes of *W. pipientis* and of the *Ehrlichia*-like sp. Schotti variant with primers thought to be specific for *A. phagocytophilum* underlines the necessity to confirm the specificity of the PCR products by sequence analysis. Amplification of related species has also been reported for other primer combinations (24, 27).

The number of proven cases of acute HGE in Europe is low compared to that in the United States. Nevertheless, in several European countries including Germany, significant rates of infection of *I. ricinus* ticks with *A. phagocytophilum* have been reported, but no cases of HGE have been diagnosed to date (1, 3, 13, 14, 28, 33, 35, 40). It is unlikely that ticks infected with *A. phagocytophilum* do not bite humans, because the same species of ticks most successfully transmits *Borrelia burgdorferi* in these countries, and ticks coinfected with *A. phagocytophilum* and *B. burgdorferi* have been detected repeatedly (6, 13, 14, 17, 20, 28, 43). Furthermore, in the present study, one tick that was removed from a human was found to be infected with *A. phagocytophilum* (sample P80).

Differences between European and North American strains of *A. phagocytophilum* have been claimed, because in European HGE patients the clinical course appears to be less severe and morulae in peripheral blood neutrophils are uncommon (9). However, at the moment the number of cases in Europe is still too small for a definite conclusion. We therefore characterized three different genes (the 16S rRNA gene, *groESL*, and *ankA*) of *A. phagocytophilum* strains infecting German *I. ricinus* ticks to test whether these strains differ from strains known to be pathogenic in other European countries or in the United States. The results clearly show that almost half of the analyzed *A. phagocytophilum* strains in German *I. ricinus* ticks are closely related by sequence identity to strains that caused granulocytic ehrlichiosis in humans or animals in other European countries and in the United States as well as to a strain that was found in a horse with EGE in Germany (53). Therefore, German *I. ricinus* ticks most likely harbor *A. phagocytophilum* strains that are able to elicit granulocytic ehrlichiosis in humans also.

Sequence analysis of the 16S rRNA genes of 42 samples revealed that only 1 sequence was 100% identical to the prototype sequence of *A. phagocytophilum* (GenBank accession no. U02521). All other sequences, although highly related to the prototype sequence, showed one to three nucleotide exchanges. The impact of such *A. phagocytophilum* 16S rRNA gene variants has been a matter of debate. Apart from two HGE patients in California, who were infected with strains that had the same 16S rRNA gene sequences as isolates from sick horses (19), all other cases of HGE were, to our knowledge, caused by strains with the 16S rRNA gene prototype sequence. Otherwise, *A. phagocytophilum* 16S rRNA gene variants have been shown to elicit diseases in horses (11), in roe deer (46), and in sheep (47).

In our positive samples we also sequenced the *groESL* gene, since the 16S rRNA gene is too conserved to allow for the analysis of genetic heterogeneity. The highest degree of identity found was to *A. phagocytophilum* sequences from roe deer, red deer, and *I. ricinus* ticks in Europe. Roe and red deer have been suggested to be one of the reservoirs for *A. phagocytophilum* in Europe (2, 29, 36), although it is not clear whether the variants infecting these animals can cause disease in humans. Nevertheless, the *groESL* sequences found in our study showed 98.41 to 100% identities to sequences from HGE patients and horses with EGE in North America and Europe. Two main types of *groESL* sequences were detected. The latter result is in agreement with the findings of Petrovec et al., who reported two genetic lineages of *groESL* sequences, one infecting mainly red deer and one infecting roe deer (36).

To further characterize *A. phagocytophilum* variants infecting German ticks, we chose to analyze the *ankA* gene, since it is known to be immunodominant (10, 45) and its expression may be subject to immunoselection. The function of AnkA remains unknown to date, but a role in protein-protein interactions and a possible impact on host cell gene transcription have been suggested (10, 32, 45). In a previous pioneering sequence analysis, the identities of the *ankA* genes of 14 *A. phagocytophilum* specimens from America and Europe were generally very high, but three different clades of sequences could be clearly identified (32). In our study we observed a high and so far unrecognized diversity of the *ankA* gene sequences. Except for sample G35, the *groESL* and *ankA* sequences clustered in the same way, although the separation into three sequence types was much more distinct in the case of the *ankA* sequences. It appears that the *ankA* gene is a valuable tool for describing genetic heterogeneity in *A. phagocytophilum*. It is possible that the strains that carry the newly described *ankA* sequences are nonpathogenic, since these sequences have never been detected in cases of HGE or granulocytic ehrlichiosis of animals. Alternatively, these new *ankA* sequence variants might simply reflect the still-limited sequence information on the *ankA* gene. Another explanation is the existence of *A. phagocytophilum* strains that are adapted to

a certain animal reservoir and do not cause disease in all hosts to the same extent. This hypothesis is in line with the limited efficacy of some of the cross-infection experiments with *A. phagocytophilum* strains of human, equine, bovine, and ovine origins (18, 22, 39, 41, 42, 44). Differences in antigenicity and virulence have already been suggested between *A. phagocytophilum* strains (47, 49, 50).

In addition to the *A. phagocytophilum* strains carrying the newly described *ankA* sequences, we also found strains with identities as high as 99.52% to *ankA* sequences from European patients with HGE and from a German horse with EGE. All these strains carried the same 16S rRNA gene variant. Thus, a defined 16S rRNA gene variant did not allow one to predict a certain *ankA* sequence different from known sequences. Conversely, 100% identity in the 16S rRNA or *groESL* gene to sequences from patients with HGE was not associated with an *ankA* sequence with high identity to known *ankA* sequences. Therefore, the sequence variability of one gene is not sufficient to determine the genetic diversity of a certain strain. In the future, it will be necessary to cultivate some of the *A. phagocytophilum* strains and to compare them in animal models in order to unravel the biological significance of the observed genetic diversities.

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