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Single Genotype of *Anaplasma phagocytophilum* identified from Ticks, Camargue, France

To the Editor: Granulocytic anaplasmosis is a tickborne zoonosis caused by *Anaplasma phagocytophilum* bacteria, which are emerging in Europe. Besides infecting humans, *A. phagocytophilum* infect a wide range of wild and domestic mammals (*1*). In Europe, the *Ixodes ricinus* tick is the main vector for the bacteria, but *A. phagocytophilum* has also been detected in association with *Rhipicephalus* and *Dermacentor* spp. ticks (*2*). The climate and biotopes of the Mediterranean region are particularly favorable for several species of ticks and, therefore, for tickborne diseases.

Although *I. ricinus* ticks are rare or absent in the Mediterranean Basin, serosurveys performed on equine populations in Camargue, southern France, indicated an *A. phagocytophilum* seroprevalence of ≈10% (*3*). To investigate the prevalence and diversity of *A. phagocytophilum* bacteria in ticks in Camargue, we collected questing ticks from horse pastures and feeding ticks from horses.

Ticks feeding on horses were collected in randomly selected stables during 2007 (84 stables), 2008 (72 stable), and 2010 (19 stables). The stables were chosen among those where evidence of *A. phagocytophilum* seroconversion in horses had been previously found (*3*). In 2008 and 2010, questing ticks were collected by the dragging method in 19 pastures, around bushes, and in areas where horses spent the most time. Surveys were conducted in the spring, which represents the peak activity time of *Ixodes* ticks.

A total of 406 adult ticks were collected, representing 6 species: *Rhipicephalus bursa, R. sanguineus*, *R. turanicus*, *R. pusillus*, *Dermacentor marginatus*, and *Hyalomma marginatum*. Tick species were identified by morphologic criteria and molecular analyses based on mitochondrial 12S rDNA sequences (*4*). Total DNA was extracted from the ticks by using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) (*5*). *A. phagocytophilum* was detected by nested PCR targeting the 16S rDNA (online Technical Appendix 1, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp1.pdf).

Of the 406 ticks, 40 were infected with *A. phagocytophilum*. The infected group included ticks from all 6 collected species except *R. pusillus*. Infection rates among the species ranged from 0 to 22% (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp2.pdf). The prevalence of *A. phagocytophilum* infection did not differ significantly between species (logistic regression model, $p = 0.76$) but was higher among questing ticks than feeding ticks ($p<0.001$; odds ratio 1.15).

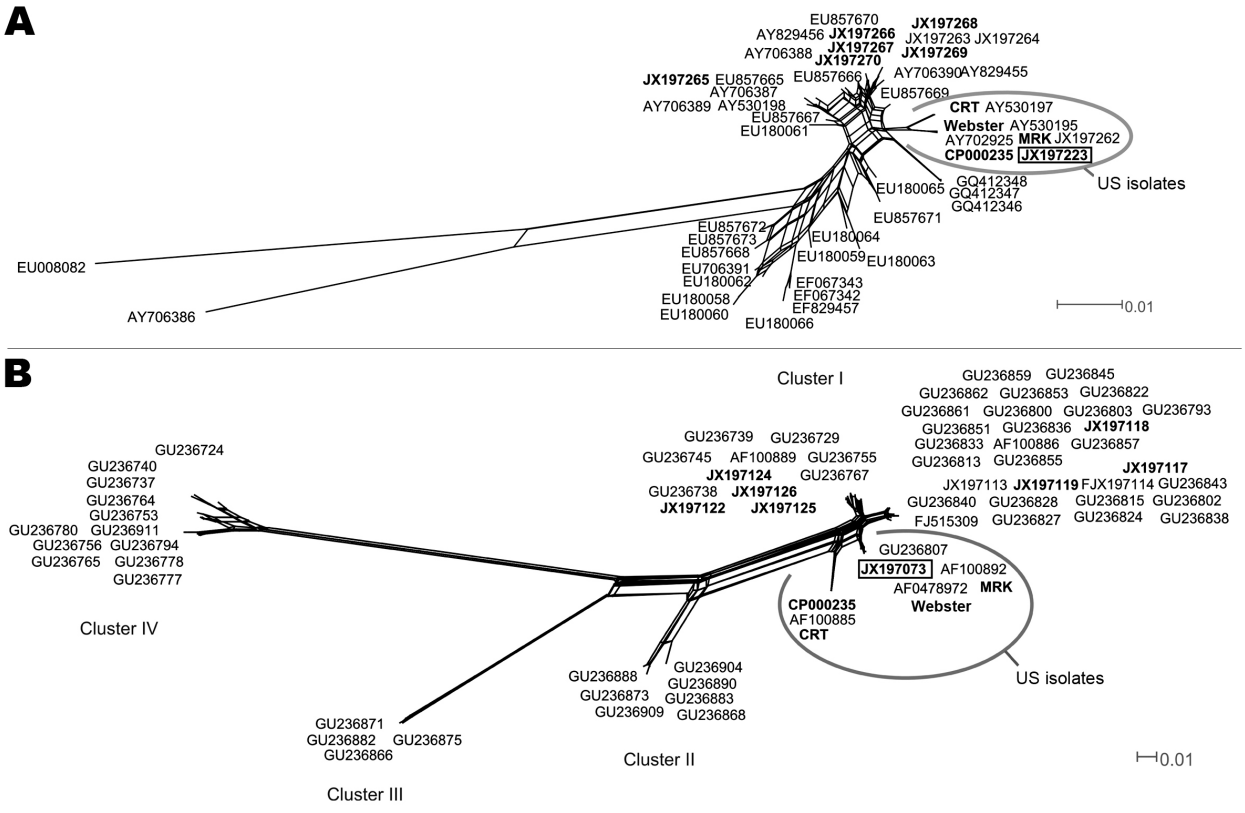

Figure. Phylogenetic networks of *Anaplasma phagocytophilum* based on *msp4* (A) and *ankA* (B) genes and built with SplitsTree4 (version 4.11.3; http://splitstree.org/) by the Neighbor-Net method. The sequences of the genotype described in Camargue, France, is framed. Sequences found in the 2 networks are in **boldface**: *A. phagocytophilum* amplified from ticks collected in Combrailles, Auvergne region, France (JX197116–JX197126 and JX197265–JX197270), a human isolate (strain Webster, EU857674 and GU236811]), an American roe deer isolate (strain CRT, JX197261 and JX197113), and *an American horse isolate (*strain MRK, AY530196 and AF153716). Scale bars indicate number of nucleotide substitutions per site.

We amplified 6 loci by nested PCR (online Technical Appendix 1) to characterize *A. phagocytophilum* genetic diversity in positive samples: *ankA*, *msp4*, *pleD*, t*ypA*, and intergenic regions *hemE–APH_0021* and *APH_1099–APH_1100* (National Center for Biotechnology Information annotation). The GenBank accession numbers for the nucleotide sequences are JX197073–JX197368. No polymorphism was found among the 6 loci tested in the 40 *A. phagocytophilum*–positive ticks. The genotype identified was 100% identical to the reference sequence (NC_007797) for loci *msp4*, *pleD*, and *typA* and for intergenic regions *hemE–APH_0021* and *APH_1099–APH_1100*. The *ankA* sequence was 96% similar (487 nt) to the reference sequence. The relevance of these loci as markers of diversity was verified (online Technical Appendix 3, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp3.pdf).

To study the phylogenetic relationships between cognate sequences, we included in our analysis all sequences available in GenBank for genes *ankA* and *msp4*. To account for recombination events that affect *ankA* and *msp4* (data not shown) in phylogenetic analyses, we used Neighbor-Net networks (Figure). Phylogenetic analysis of *msp4* (Figure, panel A) indicated that the genotype of *A. phagocytophilum* from ticks in Camargue was included in a clade that also includes genotypes that infect humans and horses in the United States.

The diversity of *ankA* sequences has been described as 4 phylogenetic clusters (*6*). All sequences obtained in our study were included in cluster I, particularly in a branch composed exclusively of sequences of *A. phagocytophilum* isolated from humans in the United States (Figure, panel B).

Previous studies investigating *A. phagocytophilum* have revealed a genetic diversity that is thought to have been caused by sympatric epidemiologic cycles involving different vectors and reservoir hosts (*1*,*6*,*7*). In 5 species of ticks (40 ticks total) that we collected from a 250-km^2 area in southern France, we found only 1 genotype of *A. phagocytophilum*, which we determined to be phylogenetically close to genotypes found in the United States. Sequences phylogenetically related to bacteria in the United States were also observed in Sardinia (*8*) and Sicily (*9*).

The low diversity we found could be explained by a recent introduction of the bacteria into the area [although *A. phagocytophilum*–seropositive horses have been found in the area since 2001 (*3*)] or by a selective sweep linked to the particular ticks and host reservoir in Camargue. The 5 species of ticks that we found positive for *A. phagocytophilum* have been described as potential vectors of *A. phagocytophilum* in the Mediterranean Basin (*2*,*10*). Among the tick species in our investigation, *R. bursa* and *R. sanguineus* ticks are the 2 main carriers of *A. phagocytophilum*, and these ticks are likely to feed on humans and, thus, pose a risk of infection to the local population. Further studies are needed to address the potential effect of *A. phagocytophilum*–infected ticks on human health in this area and, more specifically, the relationship between genotype and pathogenicity.

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West Nile Virus Lineage 2 Strain in Greece, 2012

To the Editor: West Nile virus (WNV) has been in Europe at least since the 1960s (*1*). Before 2010, WNV epidemics in Europe were caused mainly by lineage 1 strains. However, in 2010, a major WNV epidemic in Central Macedonia, Greece, was caused by a lineage 2 strain (Nea Santa-Greece-2010) (*2*).

This strain also circulated during 2011 (*3*), causing a second epidemic among humans throughout the country (*4*). Although the virus was closely related to the goshawk-Hungary-2004 strain circulating in Hungary, Austria, and Italy (*5*–*7*), severe epidemics occurred only in Greece; 273 cases of West Nile neuroinvasive disease (WNND) in humans were reported during the 2 seasons (*2*,*4*).

A third epidemic occurred in 2012, and 109 WNND cases were reported (*8*). Until mid-August, most cases were in central (Attica; 29 cases) and northeastern Greece (East Macedonia and Thrace; 10 cases). In contrast, during the same period, only 3 cases were confirmed at the location of the 2010 epidemic epicenter, in Central Macedonia (*9*). This situation led to the question of whether the Nea Santa-Greece-2010 strain was responsible for the third epidemic in Greece.

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Single Genotype of *Anaplasma phagocytophilum* Identified from Ticks, Camargue, France

Technical Appendix 1

Primers used for sequencing *Anaplasma phagocytophilum*. The primers were defined with the Primer-BLAST software.

Primer		Sequences (5′–3′)	Annealing temperature	Fragment length (bp)
16S_ external *	*ge3a*	CACATGCAAGTCGAACGGATTATTC	58°C	932
	ge10r	TTCCGTTAAGAAGGATCTAATCTCC		
16S_ internal *	*ge9f*	ACGGATTATTCTTTATAGCTTGCT	58°C	544
	ge2r	GCAGTATTAAAAGCAGCTCCAGG		
ankA - external	forward	TGAGCCTCACCCGCAGCATG	72°C	540
	reverse	CTCTGCGTTGCTGGAGCCCC		
ankA - internal	forward	CTCACCCGCAGCATGTTG	66°C	534
	reverse	GTTGCTGGAGCCCCTTTATCC		
msp 4 - external	forward	TCGCTGCAATACGATTCCGA	66°C	1300
	reverse	GAGTCTTCCACACCATCGGTT		
msp 4 - internal†	forward	TTAATTGAAAGCAAATCTTGCTCCTATG	66°C	849
	reverse	ATGAATTACAGAGAATTGCTTGTAGG		
pleD - external	forward	ACAAGTGGCCCTGAAGCAAT	66°C	1101
	reverse	TGCGTCGTAGCCTGTCTGCA		
pleD - internal	forward	TGCACTTTGCCGGAGATGGGT	69°C	576
	reverse	same of *pleD*-external reverse		
typA - external	forward	CCTGGACATGCTGACTTCGG	66°C	1455
	reverse	CGGCGGAACTAACCTCACAG		
typA - internal	forward	TGCCTCTGAGGGCCCTATGCC	71°C	550
	reverse	AGCCCTTTCCAGCCCTGCAAC		
hemE-aph0021 - external	forward	GCGATCCTGCCAAGGGTATT	66°C	1070
	reverse	AGCCCTAATTTCCGACCTTGC		
hemE-aph0021 - internal	forward	AGCGCTGTGTGCTTCTTCTGGT	66°C	537
	reverse	AGAGACGCGCTTTCCAGCGA		
aph1099-aph1100 - external	forward	ACAGTGCCCAACCTAGACGA	66°C	1453
	reverse	TGGAAGAACACGGTGGTTGC		
aph1099-aph1100 - internal	forward	GTTGCACATCCTGCTGGGGTGT	69°C	574
	reverse	GCCCCTCTGCAGACAAAGAAGC		

*Published by Massung RF, Slater K, Owens JH, Nicholson WL, Mather TN, Solberg VB, et al. Nested PCR assay for detection of granulocytic ehrlichiae. Journal of Clinical Microbiology. 1998;36:1090-5.
†Published by de La Fuente J, Massung RF, Wong SJ, Chu FK, Lutz H, Meli M, et al. Sequence analysis of the msp4 gene of *Anaplasma phagocytophilum* strains. Journal of Clinical Microbiology. 2005;43:1309-17.

All fragments were amplified by nested PCR. For the first PCR, each reaction contained 40 ng of total DNA in a solution of 25 µL with 1 U of Taq polymerase (Qiagen), 2 µL of each primer at 10 µM, 2 µL of dNTP at 25 mM, 5 µL of Q solution (Qiagen) and 1 µL of $MgCl_2$ at 25 mM. Tests were performed to choose optimal annealing temperatures. The PCR program began by an initial denaturation step of 3 min at 95°C, then 40 cycles consisted of a denaturing step of

30 s at 94°C, an annealing step of 30 s at the temperature corresponding to the target gene (see Table 2) and an extension step of 90 s at 72°C, and finally an extension step of 10 min at 72°C. The nested PCR was performed with 5 µLof the first PCR product in a total volume of 50 µL containing 2 U of Taq polymerase, 4 µL of each primer at 10 µM and 4 µL of dNTP at 25 mM. Nested cycling conditions were as described for the primary amplification.

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Technical Appendix 2

Number (%) of collected ticks positive for *Anaplasma phagocytophilum*, Camargue, France, 2007–2010

	No. positive/no. analyzed (%positive)		
Genus spp.	Ticks questing in pastures	Ticks feeding on horses	Total
Rhipicephalus bursa	3/12 (25)	19/247 (7.7)	22/259 (8.5)
Rhipicephalus sanguineus	11/53 (20.7)	2/58 (3.4)	13/111 (11.7)
Rhipicephalus turanicus	2/4 (50)	0/5 (0)	2/9 (22.2)
Rhipicephalus pusillus	0/9 (0)	0/1 (0)	0/10 (0)
Dermacentor marginatus	1/3 (33.3)	1/9 (11.1)	2/12 (16.7)
Hyalomma marginatum	0/0 (0)	1/5 (20)	1/5 (20)

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Single Genotype of *Anaplasma phagocytophilum* Identified from Ticks, Camargue, France

Technical Appendix 3

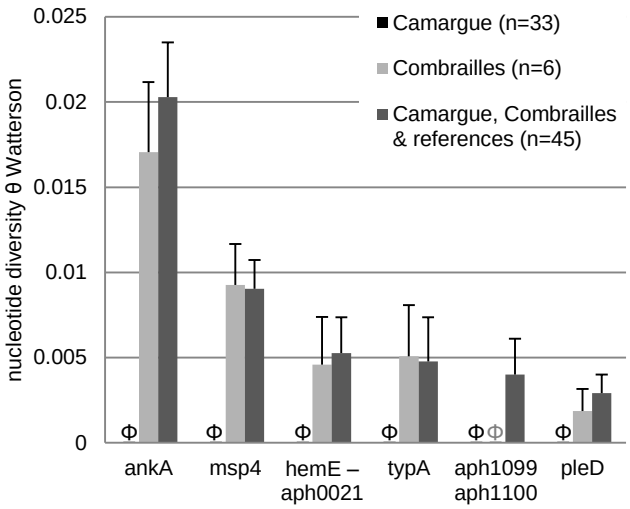
Verification of the nucleotide diversity of *Anaplasma phagocytophilum*, as calculated by use of the Watterson estimator

To verify the relevance of our loci as markers of *A. phagocytophilum* diversity, we compared the nucleotide diversity of *A. phagocytophilum* found in questing ticks collected in Camargue, France, with that found in 6 questing *Ixodes ricinus* ticks collected in the Combrailles (Auvergne, Central France, HALOS 2010 [*1*]); and with the totality of studied sequences that include 2 cognate sequence obtained in diseased horses from France, 2 American human strains (HGE and Webster), and 1 American roe deer strain (CRT).

The nucleotide diversity was calculated with the Watterson estimator:

$$\theta = \mathrm{K} \quad \sum_{i=1}^{n-1} \frac{1}{i}$$

with K the proportion of single nucleotide polymorphisms in the sequences. Diversity in Combrailles ticks showed that the markers used in this study were appropriated to describe significant level of diversity (see graph on following page).



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Nucleotide diversity of *Anaplasma phagocytophilum* calculated with the Watterson estimator. n: number of sequences analyzed and Φ indicates the zero values. Error bars indicate upper limit of the standard deviation of the Watterson estimator. Length of the sequences analyzed: 462 pb for ankA, 709 pb for msp4, 457 pb for aph1099-aph1100, 477 pb for hemE-aph0021, 308 pb for typA and 471 pb for pleD.

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