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Detection of New *Francisella*-Like Tick Endosymbionts in *Hyalomma* spp. and *Rhipicephalus* spp. (Acari: Ixodidae) from Bulgaria▽

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We report on the identification of two new *Francisella*-like endosymbionts (FLEs) found in three different tick species from Bulgaria. The FLEs were characterized by 16S rRNA and *tul4* gene sequencing and seem to lack the molecular marker RD1. These two new taxa seem to be facultative secondary endosymbionts of ticks.

Francisella is an expanding genus of closely related Gram-negative coccobacilli. In the past 2 years, at least three new taxa that are pathogens either in fish or humans have been described (9). Yet the classification of many so-called *Francisella*-like endosymbiotic (FLE) bacteria found in both hard and soft ticks remains unresolved (13, 16, 18).

FLEs seem to replicate intracellularly, they are transmitted transovarially, and to date, there is no evidence of horizontal transmission through tick bites. FLEs have been found mainly in the female's reproductive tissues (16), but recently a *Dermacentor variabilis* endosymbiont (DVF) was detected in the hemolymph, potentially suggesting colonization of the salivary glands (7). The pathogenic potential of FLEs remains unknown, although sequences homologous to *iglC* and *mglA* genes of *Francisella tularensis* implicated in pathogenicity have been detected (4, 12).

Studies involving FLEs are hampered by their inability to grow on cell-free media. Hence, most of the molecular studies have been performed with total DNA extracts from ticks or tissues rather than on FLE cultures. This together with the fact that FLEs have never been detected outside ticks suggested that they represent secondary endosymbionts. FLEs seem to be widely distributed, and during the last decade, a number of diverse FLEs have been reported in various tick genera on at least four continents (12, 14, 16–18). To date, the only FLE ever reported from Europe has been isolated from *Dermacentor reticulatus* in Hungary (17), Portugal (5), and Serbia (GenBank accession numbers HM629448 and HM629449). The discrimination between FLEs and *F. tularensis* without gene sequencing is difficult, and the validation of new specific molecular markers is important (11).

Here we report on the detection and molecular characterization of two new, so far undescribed FLEs in three different tick species that seem to lack RD1, an important molecular marker for the discrimination of pathogenic *F. tularensis* subspecies.

A total of 472 ticks removed from human ($n = 32$) or animal ($n = 264$) hosts or collected from the environment ($n = 176$) during 2005 to 2008 were screened for the presence of *F. tularensis* and FLEs. The ticks originated from rural or urban areas of nine major districts in Bulgaria. Identification to species level was performed by using standard taxonomic keys and confirmed by mitochondrial 12S rDNA partial gene sequencing (2, 8). The ticks were either pooled according to region, source, species, developmental stage, and gender or analyzed individually. Each of the resulting 111 pools contained up to six imagoes (an average of two imagoes) or up to 10 nymphs (an average of six nymphs). The most prevalent tick species were *Rhipicephalus sanguineus* (31.4%; $n = 148$), *Dermacentor marginatus* (29.5%; $n = 139$), and *Ixodes ricinus* (25.4%; $n = 120$), followed by *Hyalomma marginatum marginatum* (5.9%; $n = 28$), *Dermacentor reticulatus* (3.6%; $n = 17$), *Rhipicephalus bursa* (3.4%; $n = 16$), *Rhipicephalus turanicus* (0.6%; $n = 3$), and *Hyalomma aegyptium* (0.2%; $n = 1$). Total nucleic acid extraction was done using the QIAamp viral RNA minikit (Qiagen, Germany), which was chosen on the basis of its good performance in a kit comparison (the RNA was used in another study).

Initially, all samples were screened with primers 153F/1281R (F stands for forward, and R stands for reverse) amplifying 1,151 bp of the *Francisella* 16S rRNA gene as previously described (1), which also amplifies the 16S rRNA gene of FLEs. The 16S rRNA gene-positive samples were further analyzed by PCR with tul4BF/tul4BR primers amplifying 838 bp of the *lpnA* (*tul4*) gene encoding the *Francisella* 17-kDa membrane lipoprotein as well as by RD1 assay capable of discriminating between different *F. tularensis* subspecies (3, 16). PCR extension temperatures were modified from the original reports (2, 3, 16) (65°C for the 12S rRNA gene assay, 68°C for the tul4B assay, and 65°C for the RD1 assay). DNA sequencing, multiple alignments, and generation of phylogenetic trees were conducted as previously described (12).

In total, 12 tick samples or pools, including *H. m. mar-*

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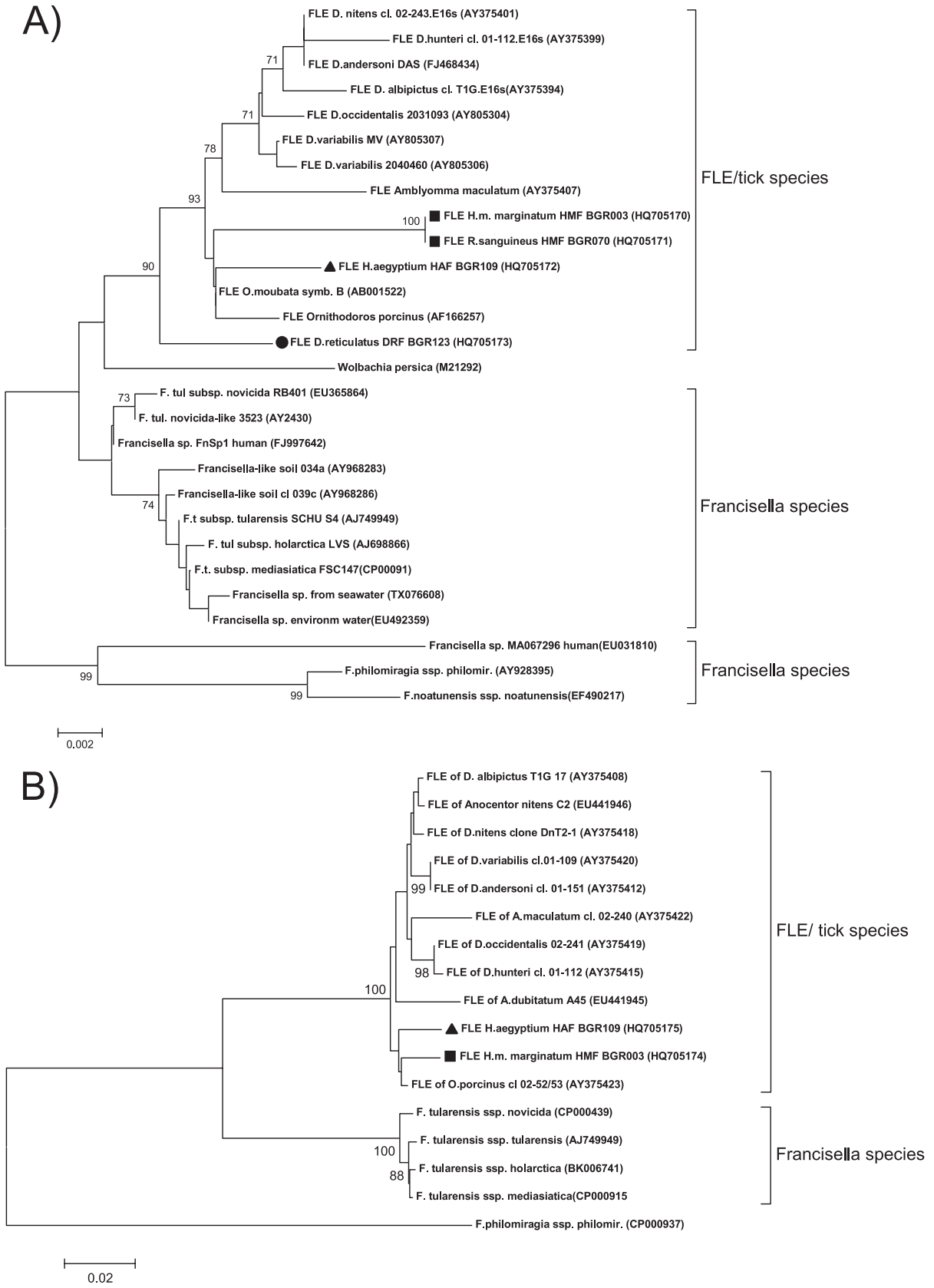

FIG. 1. Neighbor-joining phylogenetic trees of the 16S rRNA (A) and *tul4* (B) genes of various *Francisella* spp. Bootstrap values (1,000 replications) above 60 are shown. GenBank accession numbers are given in parentheses. The scale bar and number show the number of substitutions per nucleotide position. The sequences characterized in this study are designated with symbols as follows: squares, HMF; triangles, HAF; circles, DRF. Abbreviations: cl., clone; symb., symbiont; F. tul and F.t., *Francisella tularensis*; ssp., subspecies; environm, environmental.

ginatum (9 pools containing 16 ticks), *H. aegyptium* (1 tick), *R. sanguineus* (1 pool containing 2 ticks), and *D. reticulatus* (1 pool containing 3 ticks) were positive for *Francisella* 16S rRNA amplicons. All 16S rRNA sequences clustered within the monophyletic clade of previously described FLEs rather than with *F. tularensis* (Fig. 1A) (16).

Three distinct FLE genotypes were distinguished in total. Two of the three were found to be without a homologue in GenBank. The 16S rRNA gene FLE sequences from the *H. m. marginatum* and *R. sanguineus* ticks were identical and comprised a distinct genotype (subsequently referred to as HMF). HMF was detected in both *H. m. marginatum* males (6 samples) and females (3 samples) that were removed from various domestic animals or collected from the environment as well as in one pool of three *R. sanguineus* males. The closest related GenBank entries were FLEs of the soft ticks *Ornithodoros moubata* and *Ornithodoros porcinus* with 99% sequence identity, corresponding to 11 and 13 different nucleotides, respectively (Fig. 1A). As reported for other FLEs, this new FLE was also detected in two different tick species supporting the hypothesis of an independent evolution of FLEs and tick hosts (12, 16). The prevalence of HMF in *H. m. marginatum* ticks ranged from 32% (assuming 1 positive tick per pool) to 57% (assuming 16 positive ticks in the 9 pools). In *R. sanguineus*, the HMF prevalence ranged from 0.7% (1 positive tick) to 1.4% (2 positive ticks).

The second new FLE genotype was detected in a single female *H. aegyptium* tick (subsequently referred to as HAF) removed from a human. Phylogenetic analysis showed that HAF was more closely related to the FLE from *O. moubata* (99% and seven nucleotide differences) than HMF (Fig. 1A). As only one *H. aegyptium* tick was collected, the prevalence of HAF cannot be estimated with confidence.

The third FLE genotype (subsequently referred to as DRF) was found in a pool of three *D. reticulatus* males removed from an animal host. DRF differed in only two nucleotide positions from the previously reported *D. reticulatus* FLE from Hungary and Portugal (Fig. 1A) (5, 17). The prevalence of DRF in *D. reticulatus* ticks ranged from 5.8% (assuming 1 positive tick per pool) to 17.6% (assuming 3 positive ticks per pool).

The detected FLEs showed a specific geographic distribution. All HMF samples were from the same two neighboring regions in central and southern Bulgaria, whereas HAF and DRF originated from one eastern region and one northern region in Bulgaria, respectively. Interestingly, FLEs were detected only in a fraction of ticks collected in the same region, suggesting that FLEs are facultative and nonessential for the survival of the tick host and probably have diverged from a transmissible ancestor in the recent geologic past (16).

To further characterize the new FLEs, two additional molecular markers, RD1 and *tul4*, were analyzed (3, 16). *tul4* was successfully amplified from six of the 12 FLE-positive samples (Fig. 1B). The *tul4* sequences of HMF and HAF clustered separately from all known FLEs and further supported the 16S rRNA gene assay results (Fig. 1B). Despite the high sensitivity of the assay (10), no RD1 amplicons were obtained from any of the FLE-positive samples, suggesting that they lack this region or at least have a significantly different RD1 sequence. Our finding suggests that *F. tularensis* can be readily distinguished from the three FLEs described in this study by RD1. If RD1 is absent also in other FLEs, this marker could be of interest for a sequence-independent broad differentiation of *F. tularensis* from FLEs (11).

Further studies are needed to assess the pathogenic potential of FLEs, e.g., by comparing the genomes of nonpathogenic endosymbionts with their pathogenic relatives, as was recently done for *Rickettsia* species (6). Symbionts that were previously considered not pathogenic may thus turn out to be pathogenic, as was shown for *Rickettsia helvetica* and *Rickettsia slovaca* (15).

In conclusion, our findings add two new FLEs, found in three different ticks namely, in *Hyalomma marginatum marginatum*, *Hyalomma aegyptium*, and *Rhipicephalus sanguineus* to an increasing diversity of *Francisella* species. These two new taxa seem to be facultative secondary endosymbionts of ticks.

Nucleotide sequence accession numbers. The GenBank accession numbers for the new sequences obtained in this study are HQ705170 to HQ705175.

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