

Water as Source of *Francisella tularensis* Infection in Humans, Turkey

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Francisella tularensis DNA extractions and isolates from the environment and humans were genetically characterized to elucidate environmental sources that cause human tularemia in Turkey. Extensive genetic diversity consistent with genotypes from human outbreaks was identified in environmental samples and confirmed water as a source of human tularemia in Turkey.

Tularemia is a disease caused primarily by 2 subspecies of *Francisella tularensis*: *F. tularensis* subsp. *tularensis*, which is restricted to North America; and *F. tularensis* subsp. *holarctica*, which is found widely throughout the northern hemisphere but is the only subspecies in most of Eurasia (1). Through whole-genome sequencing and canonical single-nucleotide polymorphism (canSNP) genotyping, *F. tularensis* subsp. *holarctica* has been divided into 4 major genetic groups (B.4, B.6, B.12, and B.16) consisting of multiple subgroups (Figure 1) (1–3). Geographic distribution of these subgroups in Europe, Japan, and the USA are well described (1–3).

The phylogeography of *F. tularensis* in Asia is poorly understood because of undersampling in many regions, but recent studies have revealed new insights. A report has described rich phylogenetic diversity of the bacterium in China (4), including the rare B.16 group (biovar *japonica*). Previously, B.16 was known only in Japan (1) and Turkey (6). Sweden reportedly has the highest overall phylogenetic diversity among regions worldwide (2).

In Turkey, tularemia cases in humans have increased since 2009 (7), but little is known about environmental sources. Tularemia was first reported in Turkey in 1936 and then was sporadically reported for several decades (7). After improved surveillance, the number of tularemia cases

increased in the 1980s and led to registration of tularemia as a reportable disease in 2004 (7,8). Incidence has continued to increase since then (7), and tularemia is now considered a reemerging zoonotic disease in Turkey.

Patients with oropharyngeal signs and symptoms account for ≈90% of tularemia cases in Turkey (8), and cases emerge seasonally from August–March (7). Seasonality of incidence of cases is presumably associated with consumption of contaminated water (9), but confirming sources is difficult. Reports of confirmation of *F. tularensis* from water samples by PCR (10) or culture (6) are rare, and definitive studies that link water to tularemia in humans are lacking. How water sources become seasonally contaminated is also unknown, but contamination could be caused by rodents. Recently, *F. tularensis* was confirmed by PCR from 2 mice captured in Thrace (11), but in Turkey, confirmation has not been obtained from ticks or mosquitoes, which are known vectors of *F. tularensis* (1,4).

Genetic characterization of clinical samples from tularemia outbreaks in Turkey in 2011 showed that multiple phylogenetic groups cause disease in multiple regions across Turkey (5); however, no environmental samples were assessed in that study. We report our findings from genetically characterized samples positive for *F. tularensis* from environmental and human sources located in multiple active tularemia areas in Turkey. Our results provide new insights into *F. tularensis* transmission from environmental sources to humans.

The Study

To examine environmental reservoirs that could be possible sources for human infections, during 2010–2013, we sampled water sources and rodent populations from suspected sites where transmission of *F. tularensis* infection could occur in Turkey. To survey and compare phylogenetic diversity of environmental samples and clinical samples, we examined 33 clinical samples of mostly oropharyngeal tularemia cases from approximately the same sites where environmental samples were collected. DNA was extracted (DNeasy Blood & Tissue Kit, QIAGEN GmbH, Hilden, Germany) from 6 water, 1 rodent spleen, and 33 human samples (online Technical Appendix Table 1, <http://wwwnc.cdc.gov/EID/article/21/12/15-0634-Techapp.pdf>).

The extractions were confirmed *F. tularensis*-positive by using PCR and targeting the *tu14* gene (12). Analysis

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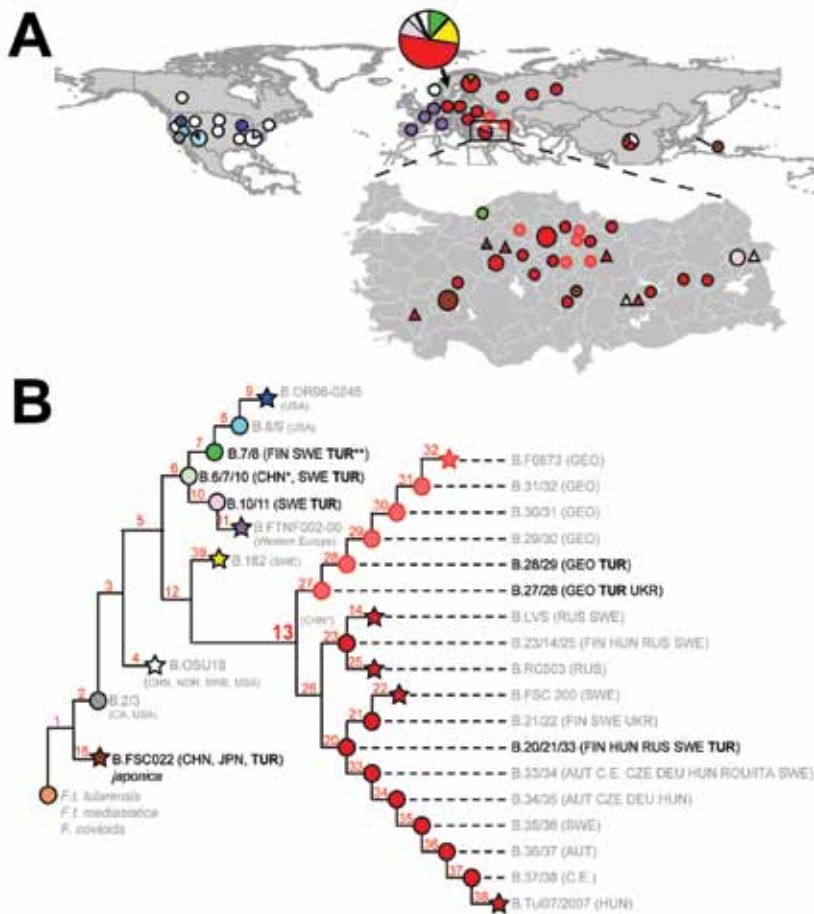


Figure 1. Phylogeography of *Francisella tularensis* subsp. *holarctica*. **A)** Global distribution of known phylogenetic groups determined on the basis of previous studies (2–4); enlarged map of Turkey shows locations of phylogenetic groups identified among the 40 samples positive for *F. tularensis* examined in this and previous studies (5). Circle size indicates number of samples (small circles, 1–3; medium circles, 4–6; large circles, 7–9). Colors of circles (human samples) and triangles (environmental samples) represent the phylogenetic subgroups to which these samples were assigned (see panel B). Subgroup B.16 (biovar *japonica*) is represented by the dot inside the brown circles and triangles. **B)** Phylogenetic tree for *F. tularensis* subsp. *holarctica* constructed on the basis of current canonical single-nucleotide polymorphism genotyping. Red numbers indicate nomenclature of canonical single-nucleotide polymorphism groups. Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. Countries of origin for samples assigned to relevant phylogenetic groups are as follows: AUT, Austria; CE, central Europe, unknown country; CHN, China; CZE, Czech Republic; DEU, Germany; FIN, Finland; GEO, Georgia; HUN, Hungary; ITA, Italy; NOR, Norway; ROU, Romania; RUS, Russia; SWE, Sweden; TUR, Turkey; UKR, Ukraine; USA, United States. CHN* indicates approximate phylogenetic placement because of a lack of resolved information on single-nucleotide polymorphisms (4). TUR** indicates identification from a previous study (5).

by using 21 published canSNP assays, as previously described (5), assigned these samples to 3 major phylogenetic groups and distinct subgroups: B.16 ($n = 11$); B.6 (2 subgroups: B.6/7/10, $n = 1$; and B.10/11, $n = 6$); and B.13 (2 subgroups: B.27, $n = 5$; and B.20/21/33, $n = 17$) (Figure 1; online Technical Appendix Table 1). Of the subgroups, 3 were previously unknown in Turkey: B.6/7/10, B.10/11, and B.16. The 7 environmental samples collected included most of the known phylogenetic diversity in Turkey and represented the 3 major groups: B.16, B.6 (B.6/7/10 and B.10/11), and B.13 (the group previously known to be in Turkey). Of the subgroups identified, all but B.6/7/10 were also found in the human samples.

To determine detailed associations between environmental and human clinical samples, we examined the genetic diversity among these samples by using multilocus variable number of tandem repeats analysis (MLVA) (13). All samples contained a single MLVA genotype (online Technical Appendix Figure, panels A–C); no mixed allele calls were observed at any of the examined variable number of

tandem-repeats loci. Three different environmental samples (F0922, F0910, and F0916) had canSNP and MLVA genotypes that were identical to those of clinical samples (online Technical Appendix Table 1). In 2 instances (F0910 and F0916), the environmental sample and its respective genetically identical clinical sample(s) were recovered from different geographic regions, resulting in identical genotypes being found in different localities and suggesting that close genotypes are dispersed widely in Turkey. One environmental sample (F0922) had genetic, geographic, and temporal data (online Technical Appendix Figure, panel A) concordant with data from human samples. This water sample shared identical canSNP and MLVA genotypes with 5 clinical samples recovered 2 weeks previously at the same locality, strongly suggesting that the human cases are linked with this infected water source.

The genetic characterization of *F. tularensis* from environmental sources provides insights into transmission of tularemia from the environment to humans, but little is known about how water is contaminated. The seasonal

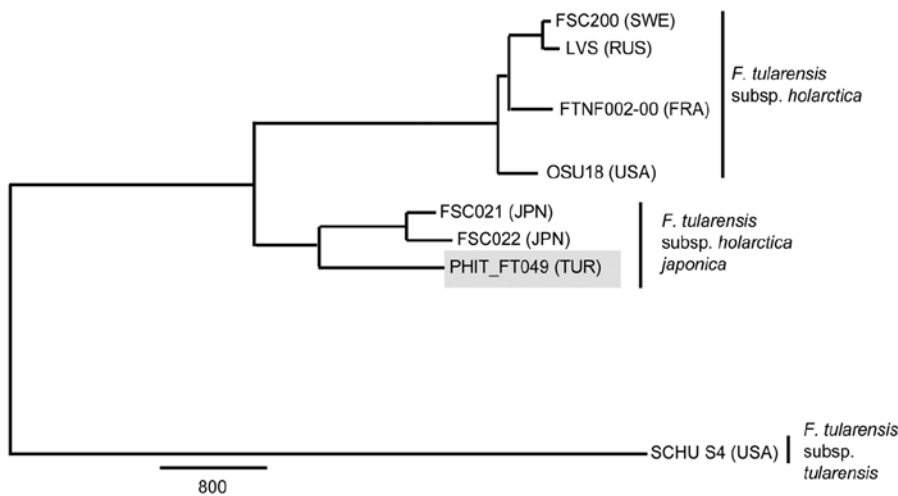


Figure 2. Maximum-parsimony phylogeny constructed by using 10,443 putative single-nucleotide polymorphisms discovered from whole-genome sequences of 8 *Francisella tularensis* strains. Gray shading indicates the B.16 (biovar *japonica*) strain from Turkey (PHIT_FT049). Detailed methods are described in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/articles/21/12/15-0634-Techapp.pdf>). Reference strains were retrieved from GenBank (online Technical Appendix Table 2). Countries of origin are indicated as follows: FRA, France; JPN, Japan; RUS, Russia; SWE, Sweden; TUR, Turkey; USA, United States. Scale bar indicates single-nucleotide polymorphisms.

nature of human outbreaks suggests that water sources are not constant reservoirs but rather are contaminated by another source. Rodents were identified as reservoirs (21% tularemia positive) in Bulgaria, where mainly oropharyngeal tularemia is endemic (14). We found a rodent sample (F0910) with canSNP and MLVA genotypes identical to an oropharyngeal clinical sample (F0898) (online Technical Appendix Table 1), a finding consistent with water contamination that originates from animal sources. However, the converse is also possible: animals could become infected by contaminated water.

Analysis of the 7 environmental *F. tularensis* subsp. *holarctica* samples from Turkey revealed extensive phylogenetic diversity that represents most known major groups in the world. Three of the 4 major *F. tularensis* subsp. *holarctica* phylogenetic groups (B.4, B.6, B.12, and B.16) are found in Turkey, including the highly basal B.16 group (biovar *japonica*) (Figure 1). This finding indicates that no single phylogenetic type is dominant in Turkey, unlike in Western Europe (3). Diversity was also represented in the clinical samples, suggesting that all major groups have similar capacities to cause disease, as other studies have suggested (15).

To gain insights into the evolutionary origin of the B.16 group, we examined the phylogenetic relationships among 3 published B.16 strains: 1 from Turkey (PHIT-FT049) (6) and 2 from Japan (FSC021 and FSC022) (GenBank accession nos. CP007148.1, SRX147922, and DS264138.1, respectively; Figure 2). We generated a global core-genome SNP phylogeny (online Technical Appendix) for these 3 B.16 strains and 5 strains from other groups (online Technical Appendix Table 2). As expected, PHIT-FT049 clusters with the Japanese B.16 strains from Japan and shares 448 putative SNPs; however, it is also distinct from the 2 strains from Japan, which together share 640 putative SNPs

(Figure 2). The distinctiveness of the B.16 strain from Turkey strongly suggests that it has an evolutionary history different from that of the Japanese strains. The MLVA phylogeny of B.16 strains (online Technical Appendix Table 1) reveals greater diversity among the 8 strains from Japan than among the 8 strains from Turkey. These data show that the B.16 strains from Turkey and Japan are highly distinct, and the greater diversity in strains from Japan supports the possibility that the place of ancestral origin of the B.16 group is Asia.

Conclusions

Phylogenetically diverse strains of *F. tularensis* subsp. *holarctica* are environmentally established in Turkey and cause human disease. The strains in Turkey now include many phylogenetic groups previously found only in Scandinavia or Asia.

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Dr. Kilic is a professor and a principal investigator of *F. tularensis* at the Public Health Institution of Turkey, National Tularemia Reference Laboratory, Ankara, Turkey. His research interests include the evolution, epidemiology, and control of bacterial zoonoses.

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Check out EID's 20-year-anniversary timeline and find an array of fascinating seminal moments in the journal's history.

History Was Made
The first issue of the *Emerging Infectious Diseases* journal launched in February of 1995 – 20 years ago next month. Click here to see the CDC/NCID Focus announcement of this rare historic event.



We've come a long way in 20 years.
Click here to check out the complicated "state of the art" instructions to access the online version of the EID journal. These instructions first appeared in the January of 1995 of CDC/NCID Focus.



The first issue of the *Emerging Infectious Diseases* journal was released in February 1995 – A quarterly that covered the period of January–March. The journal remained a quarterly until 1999, at which time it expanded to a bimonthly publication.



Emerging Infections: Microbial Threats to Health in the United States
In the early 1990s, Joshua Lederberg was a champion and advocate for emerging infectious diseases. He strongly believed that a need existed for a vigorous CDC response. With this goal in mind, he met with leadership at CDC and co-chaired one of the first meetings in the early 1990s to strategize on how to meet these threats.



EID: 20 Years of Publication
This February marks the 20th anniversary of the first issue of *Emerging Infectious Diseases* (EID). The mission of the monthly print and online, open access, peer-reviewed CDC journal has remained the same over the past two decades.



Dr. D. Peter Drotman, editor-in-chief of the *Emerging Infectious Diseases* journal is talking with Dr. James Hughes, professor of medicine and public health at Emory University. Dr. Hughes was, for many years, the director of the National Center for Infectious Diseases at CDC. They discuss the 20 year history of the EID journal in this podcast.



Color Has Arrived
Color and a graphic image were added to EID journal covers after two years of publication. Past cover colors were mostly grey and white with tables of contents. Founding EID managing editor, Polyzene Pitteris, said "I knew that we had to do better with the cover of the journal – make it more attractive and interesting so that readers would recognize it and pick it up!"



This October 1999 article is the most cited article in the history of EID.



Etymologia—a new EID section added in 2005
Etymologia is concerned with the origin of words, how they've evolved over time, and changed in form and meaning as they were translated from one language to another.



March, 1998, CDC partners with the Council of State and Territorial Epidemiologists, the American Society for Microbiology, and the National Foundation for CDC along with 62 other co-sponsors, to convene the inaugural International Conference on Emerging and Infectious Diseases.



<http://wwwnc.cdc.gov/eid/page/20-year-timeline>

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Water as Source of *Francisella tularensis* Infection in Humans, Turkey

Technical Appendix

Details of Samples and Reference Strains in This Study

Detailed Methods for Constructing the Phylogeny in Figure 2

Published reference genome assemblies (Technical Appendix Table 2) were downloaded from GenBank (1). Assemblies were aligned against the reference genome, *F. tularensis* subsp. *holarctica* OSU18, by using MUMer (2). The reference genome was also aligned against itself; regions that aligned >1 time represent duplication events and were filtered from downstream analyses. Single-nucleotide polymorphisms compared with the reference were concatenated, and a maximum-parsimony phylogeny (Figure 2) was inferred on a concatenation of ≈15,000 single-nucleotide polymorphisms by using Phangorn (3).

Technical Appendix Table 1. Details of samples from study of *Francisella tularensis* infection, Turkey

Original ID*	NAU ID†	County/Region	City	Source	Sample Type	Date	SNP subgroup‡
PHIT-FT049, F283§	F0915	Central Anatolia	Ankara	Water	DNA extract from isolate cultured from water	3/12/2012	B.16
F059	F0892	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/19/2010	B.16
F060	F0893	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/19/2010	B.16
F062	F0894	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/19/2010	B.16
F063	F0895	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/19/2010	B.16
F064	F0896	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/19/2010	B.16
F069	F0899	Central Anatolia	Kayseri	Human lymph node	DNA extract from clinical sample	2/24/2010	B.16
F071	F0900	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/26/2010	B.16
F072	F0901	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	2/26/2010	B.16
F085	F0902	Aegean	Afyonkarahisar	Human lymph node	DNA extract from clinical sample	3/19/2010	B.16
F272	F0912	Central Anatolia	Kayseri	Human lymph node	DNA extract from clinical sample	2/8/2012	B.16
F015	F0884	Central Anatolia	Çankırı	Human throat swab	DNA extract from isolate cultured from human	1/8/2010	B.28/29
F026	F0885	Black Sea	Amasya	Human throat swab	DNA extract from isolate cultured from human	1/18/2010	B.28/29
F217	F0907	Eastern Anatolia	Sivas	Human throat swab	DNA extract from isolate cultured from human	4/12/2011	B.28/29
F043	F0890	Central Anatolia	Yozgat	Human lymph node	DNA extract from isolate cultured	1/27/2010	B.28/29

Original ID*	NAU ID†	County/Region	City	Source	Sample Type	Date	SNP subgroup‡
F039	F0889	Black Sea	Tokat	Human lymph node	from human DNA extract from isolate cultured from human	1/22/2010	B.27/28
F303-s291, F291 F049	F0923	Aegean	Denizli	Water	DNA extract from isolate cultured from water	12/12/2013	B.20/21/33
F065	F0891	Central Anatolia	Kirsehir	Human throat swab	DNA extract from isolate cultured from human	2/8/2010	B.20/21/33
F067	F0897	Black Sea	Tokat	Human lymph node	DNA extract from isolate cultured from human	2/19/2010	B.20/21/33
F236	F0908	Black Sea	Ordu	Human throat swab	DNA extract from isolate cultured from human	4/12/2011	B.20/21/33
F282	F0914	Central Anatolia	Sivas	Water	DNA extract from isolate cultured from water	3/6/2012	B.20/21/33
F244	F0910	Central Anatolia	Ankara	Rodent/spleen	DNA extract from isolate cultured from rodent	11/14/2011	B.20/21/33
F037	F0888	Central Anatolia	Corum	Human lymph node	DNA extract from isolate cultured from human	1/20/2010	B.20/21/33
F027	F0886	Black Sea	Amasya	Human throat swab	DNA extract from isolate cultured from human	1/18/2010	B.20/21/33
F033	F0887	Black Sea	Amasya	Human throat swab	DNA extract from isolate cultured from human	1/18/2010	B.20/21/33
F237	F0909	Eastern Anatolia	Elazig	Human lymph node	DNA extract from isolate cultured from human	8/16/2011	B.20/21/33
F091	F0903	Central Anatolia	Yozgat	Human lymph node	DNA extract from isolate cultured from human	4/13/2010	B.20/21/33
F159	F0904	Central Anatolia	Kayseri	Human blood	DNA extract from isolate cultured from human	1/29/2011	B.20/21/33
F285	F0916	Eastern Anatolia	Malatya	Water	DNA extract from isolate cultured from water	4/5/2012	B.20/21/33
F163	F0905	Central Anatolia	Kayseri	Blood	DNA extract from isolate cultured from human	2/2/2011	B.20/21/33
F252	F0911	Eastern Anatolia	Mus	Human throat swab	DNA extract from isolate cultured from human	12/15/2011	B.20/21/33
F176	F0906	Eastern Anatolia	Bingöl	Human throat swab	DNA extract from isolate cultured from human	2/21/2011	B.20/21/33
F278	F0913	Eastern Anatolia	Malatya	Water	DNA extract from isolate cultured from water	2/21/2012	B.6/7/10
F293	F0917	Eastern Anatolia	Agri	Human throat swab	DNA extract from isolate cultured from human	2/13/2013	B.10/11
F294	F0918	Eastern Anatolia	Agri	Human throat swab	DNA extract from isolate cultured from human	2/14/2013	B.10/11
F295	F0919	Eastern Anatolia	Agri	Human throat swab	DNA extract from isolate cultured from human	2/14/2013	B.10/11
F297	F0920	Eastern Anatolia	Agri	Human throat swab	DNA extract from isolate cultured from human	2/14/2013	B.10/11
F292	F0921	Eastern Anatolia	Agri	Human throat swab	DNA extract from isolate cultured from human	2/14/2013	B.10/11

Original ID*	NAU ID†	County/Region	City	Source	Sample Type	Date	SNP subgroup‡
F296	F0922	Eastern Anatolia	Agri	Water	DNA extract from isolate cultured from water	2/28/2013	B.10/11

*Strain identification (ID) from Northern Arizona University, Flagstaff, AZ, USA.

†Strain ID from Public Health Institution of Turkey Microbiology Reference Laboratories.

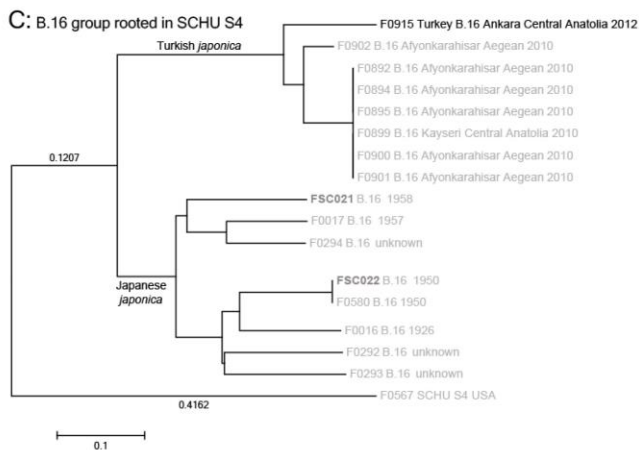
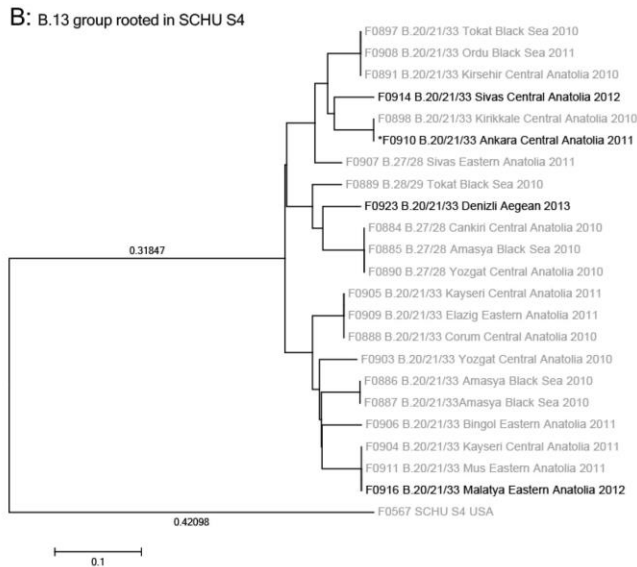
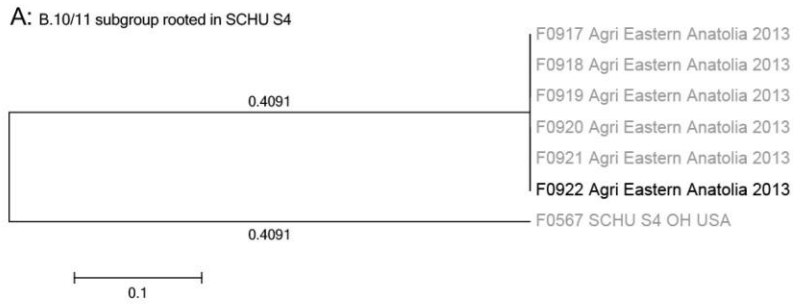
‡Subgroup (4).

§Published as GenBank accession no. CP007148.1 (National Center for Biotechnology Information, Bethesda, MD, USA).

Technical Appendix Table 2. Reference strains used in study of *Francisella tularensis* infection, Turkey

Reference strain	WGS accession no.
FSC022	AAYD00000000.1
FSC021	SRX147922
PHIT_FT049	CP007148.1
FSC200	NC_019551.1
LVS	NC_007880.1
FTNF002-00	NC_009749.1
OSU18	NC_008369.1
Schu S4	NC_006570.2

*WGS, whole genome shotgun sequencing data, National Center for Biotechnology Information, Bethesda, MD, USA.



Technical Appendix Figure. Multilocus variable number of tandem repeats analysis (MLVA) trees constructed on the basis of distance matrix. Environmental samples (water and * rodent source) are indicated with bolded font. Scale bar indicates genetic distance. A) MLVA phylogeny for the B.10/11 group, which is rooted by using the SCHU S4 strain published in GenBank. B) MLVA phylogeny for the B.13 group, which is rooted by using the SCHU S4 strain. C) MLVA phylogeny for the B.16 group, which is rooted by using the SCHU S4 strain.

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