

# VNTR-based epidemiological study of *Francisella tularensis* ssp. *holarctica* biovar II strains isolated from humans, European brown hares and red foxes in Austria from 1995 to 2010

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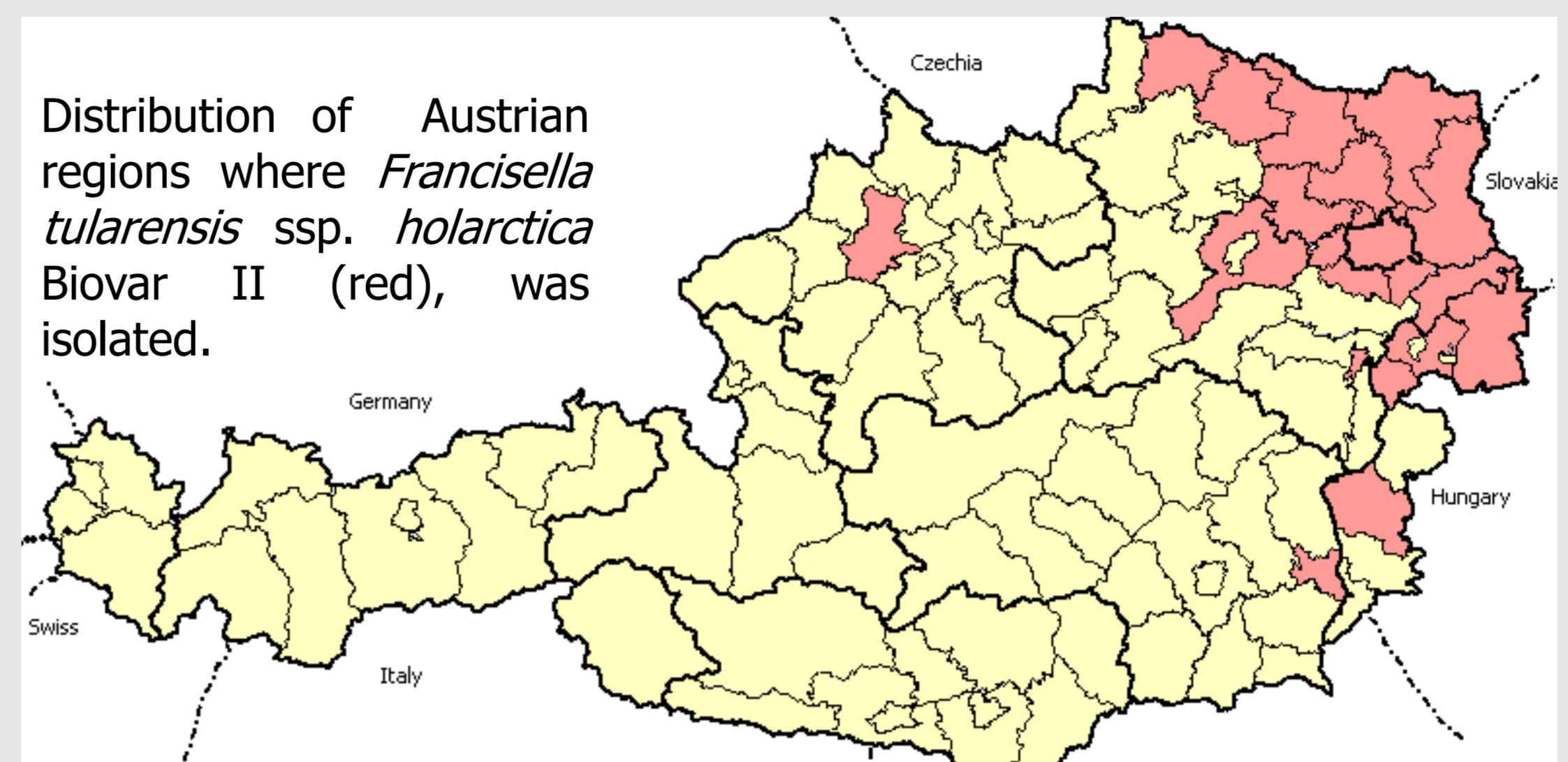
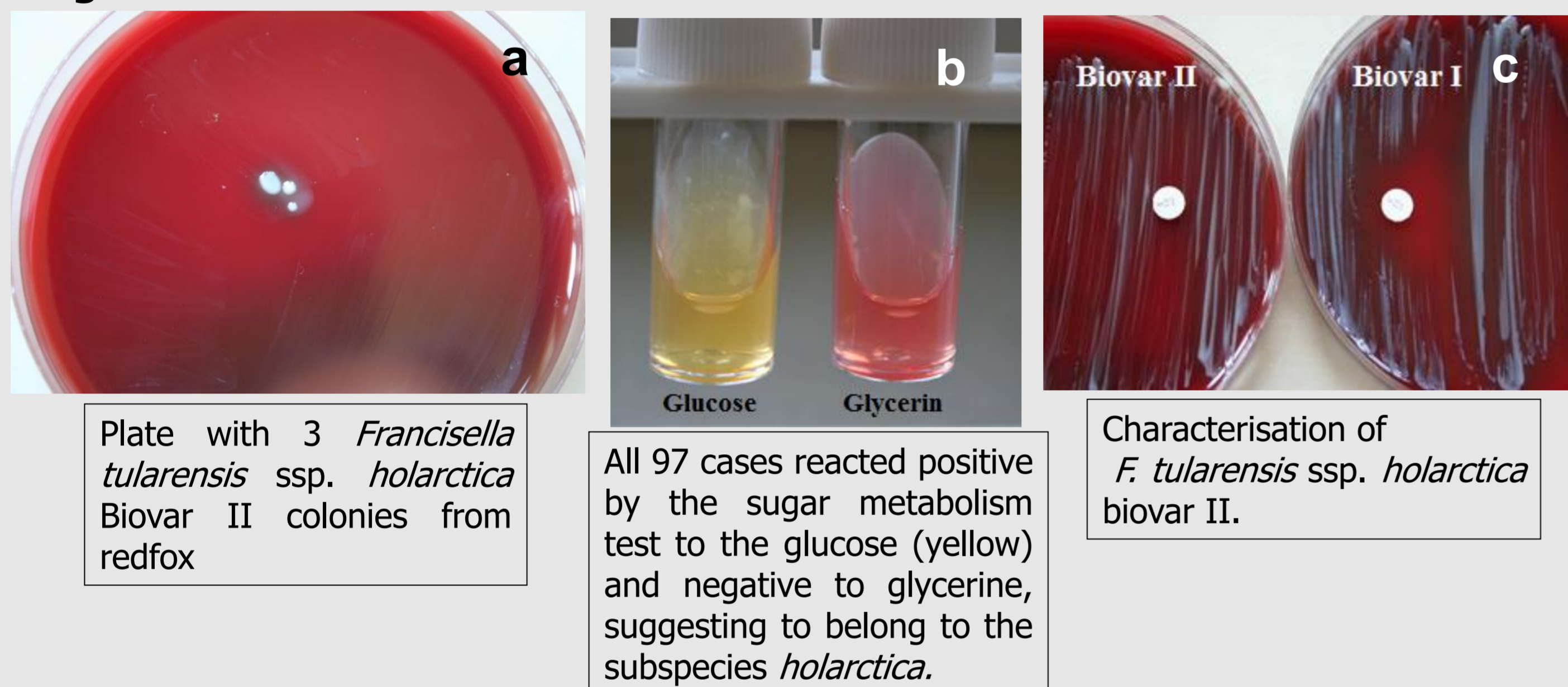
## Background

*Francisella tularensis* (*Ft*) is one of the most highly infectious bacteria known, affecting more animal species than other known zoonotic pathogens. The geographical distribution of tularemia in Austria is well known (1,2), however, the diversity and genetic relationships between strains isolated from humans and wild animals remained unknown. Therefore, VNTR analysis was used to study the genetic relationships within a collection of *Francisella tularensis* isolates, collected from 1995 to 2010, including representatives from different regions in Austria.

## Bacterial isolation and identification

Bacterial isolation from native samples was done in cystine-heart-agar plates (Fig. 1a). In 97 positive cases, strain identification and differentiation between subspecies (ssp.) *tularensis* and *holarctica* were further performed with the agglutination test and the sugar metabolism test respectively (1b). The agar diffusion test with erythromycin plates was used finally for determination of the Biovars (1c) (3). All isolates were identified as *F. tularensis* ssp. *holarctica* biovar II.

Fig.1



VNTR-loci displayed between 1 and 6 alleles (2 to 12 repeats) (Table 1) with SDI between 0 and 0.62 for M24 and M6 markers, respectively.

Phylogenetically, 9 big clusters were distinguished. The NJ cluster analysis revealed 17 genotypes, in 9 cases even to individual strain discriminations level (Table 2).

Eleven out of the 17 genotypes were already present until 1997, so distribution seems to be in many little natural foci, also called "hotspots". Some genotypes from 1995 could be found in 2009-2010. Six new genotypes were detected after 2005. The 4 most affected regional districts had 15/17 of the genotypes.

Isolate Nr.	Genotype	Region	M3	M6	M20	M21	M22	M24
63, 93	1	MB, OW	6	4	2	2	4	2
31	2	HL	10	4	2	2	4	2
61, 64, 87, 88, 98	3	MB (2), ND (2), HB	9	4	2	2	4	2
2, 3, 4, 19, 20, 24, 26, 35, 49, 51, 52, 62, 67	4	WT (3), GF (4), HL, KO (3), MB (2)	8	4	2	2	4	2
85	5	ND	8	2	2	2	4	2
7	6	GF	8	3	2	2	4	2
8, 15, 25, 43, 44, 69, 75, 76	7	GF (3), HL (2), MB, MD (2)	8	5	2	2	4	2
39	8	HL	6	5	2	2	4	2
77	9	MD	9	5	2	2	4	2
5, 12, 13, 14, 18, 22, 23, 32, 36, 42, 46, 47, 48, 50, 53, 56, 57, 59, 66, 71, 72, 73, 82, 86, 94	10	WT, GF (6), HL (3), KO (6), MB (3), PL (3), BL, ND, OW	7	5	2	2	4	2
41, 81, 84	11	HL, BL, W	7	3	2	2	4	2
11, 16, 17, 45	12	GF (3), HL	7	6	2	2	4	2
40	13	HL	7	4	2	3	4	2
27	14	GF	9	6	2	3	4	2
54	15	KO	6	4	2	3	4	2
1, 6, 10, 21, 29, 30, 33, 34, 37, 38, 55, 58, 60, 65, 68, 70, 74, 78, 79, 80, 83, 89, 90, 91, 92, 95, 96, 97	16	H, WT, GF (3), HL (5), KO, MB (5), WN, MD, WU (2), W, EU (3), OW (2), FF, GK	7	4	2	2	4	2
9	17	GF	12	3	2	2	3	2
<i>Francisella tularensis</i> ssp. <i>holarctica</i> Biovar I		reference strain	3	4	2	2	4	1
<i>Francisella tularensis</i> ssp. <i>tularensis</i>		reference strain	21	3	2	3	2	1
<i>Francisella tularensis</i> ssp. <i>mediasiatica</i>		reference strain	6	2	4	1	12	1
<i>Francisella tularensis</i> ssp. <i>tularensis</i>		reference strain	5	3	3	2	3	1

Table 2

VNTR-classification of *Ft* including the 17 genotypes found in Austria (randomly named from 1 to 17) and their regional distribution

## VNTR-based epidemiological analysis

Six most discriminative VNTR markers (Fig. 2) within the *Ft* genome (M3, M6, M20, M21, M22 and M24) were amplified by specific SYBR-Green Real-time-PCR and directly sequenced. Then, the Simpson's Diversity-Index (SDI) was calculated for each individual marker. Sequences were analyzed with reference sequences of *Francisella* spp. with the BioNumerics software (Table 2).

Finally, to give a better overview about the relationships cluster analysis of discrete character sets was generated, including a Categorical Similarity Coefficient combined with a Neighbor-Joining (NJ) Method and a Multidimensional Scaling.

Fig.2

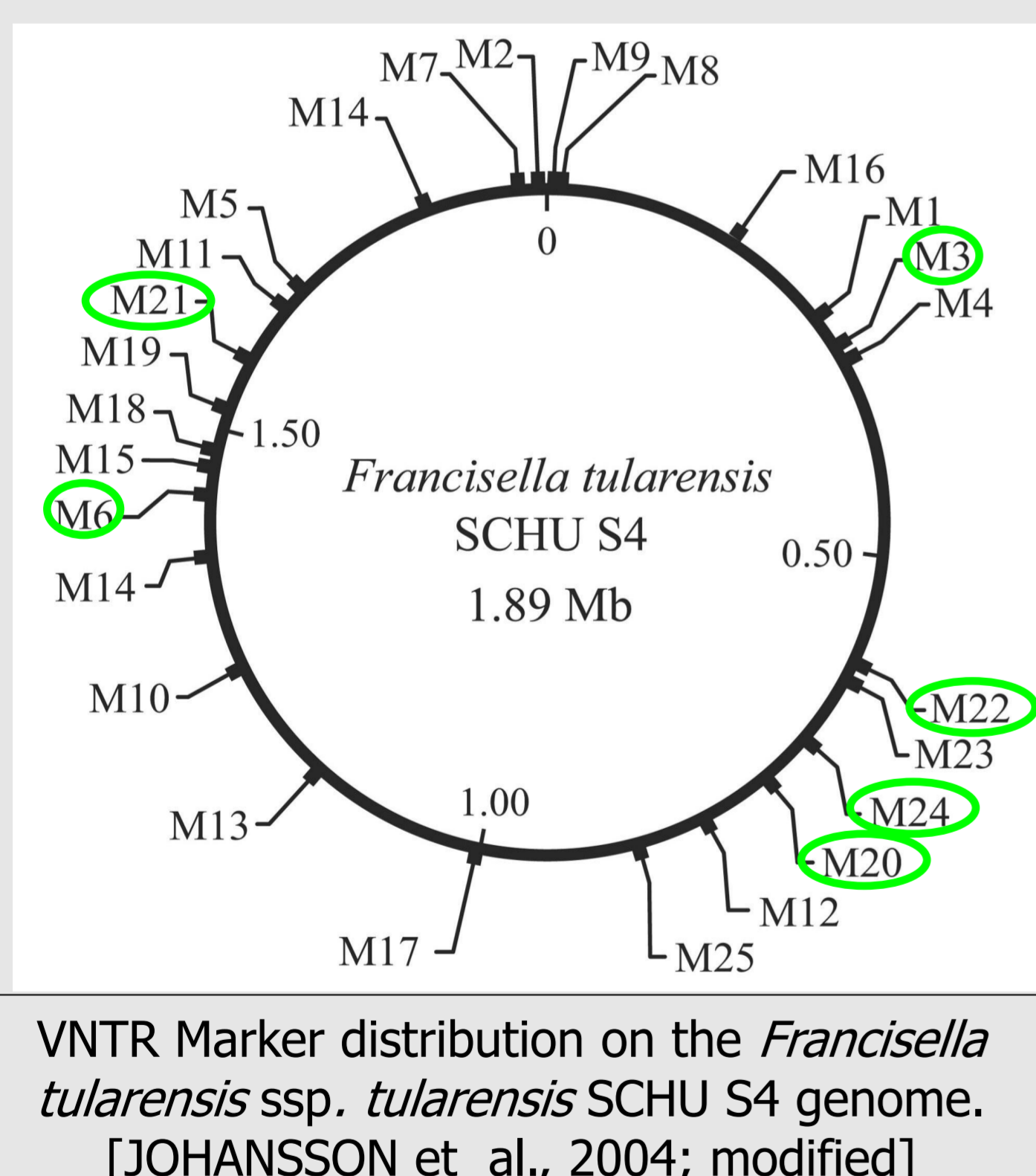
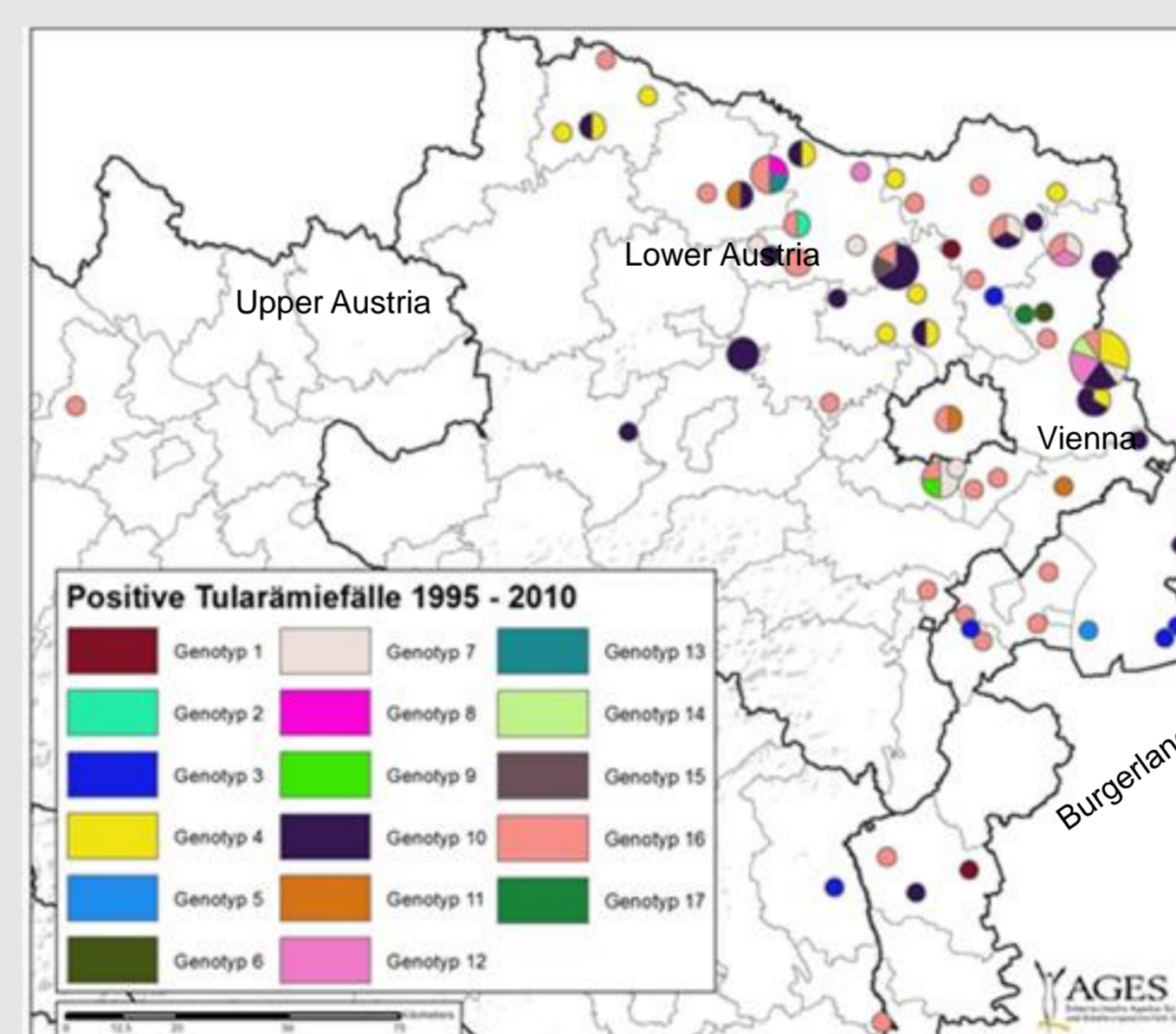


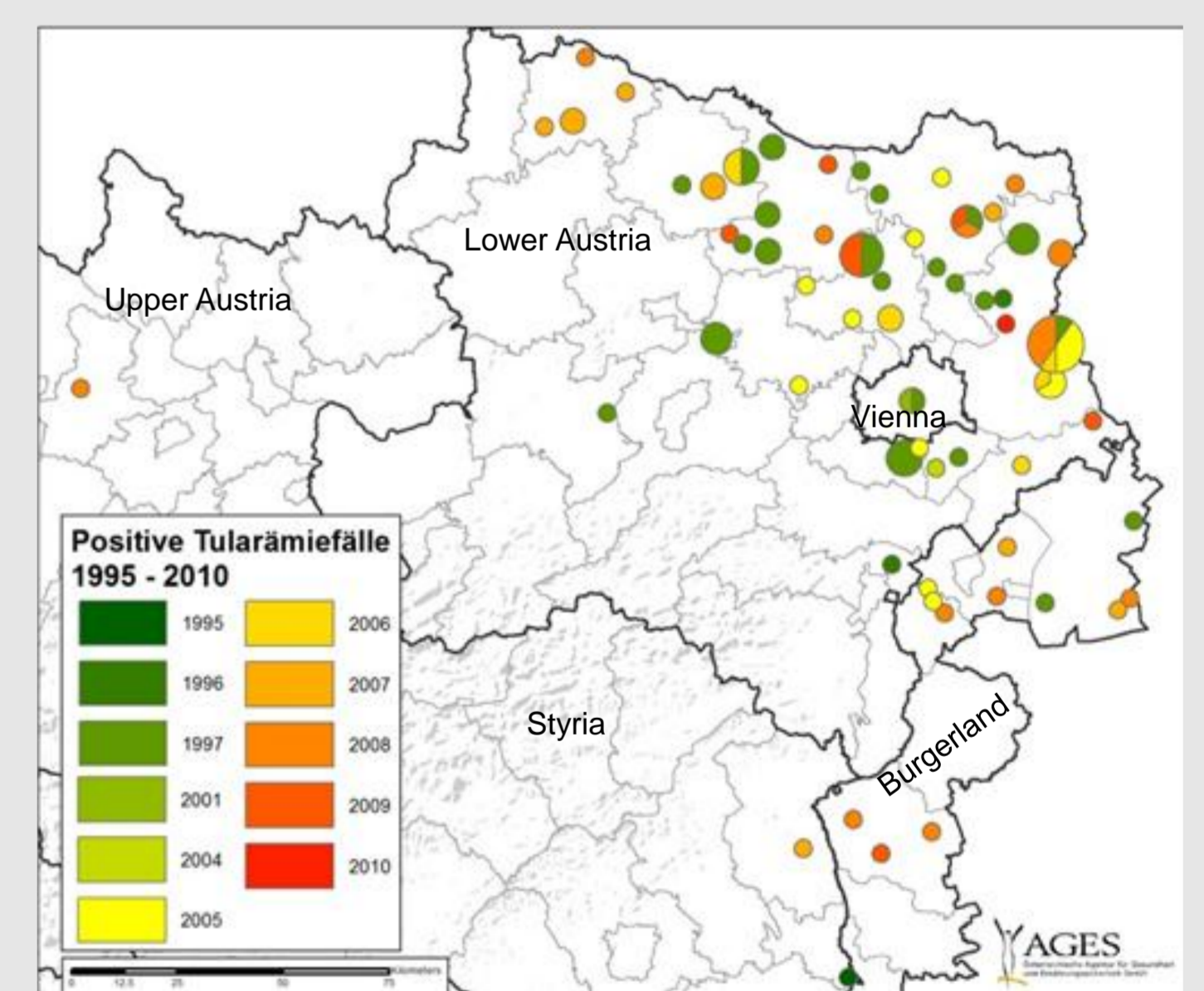
Table 1

VNTRs	Motiv	Repeat-Size
Ft-M3	AATAAGGAT	9
Ft-M6	TTGGTGAACCTTCTGCTCT	21
Ft-M20	ATTATTTGATC	12
Ft-M21	TCAATTA	7
Ft-M22	AAAAAT	6
Ft-M24	ATAAATTAATTTTGGATTA	21

VNTR repeat sequences [JOHANSSON et al., 2004]



Genotype distribution of *Ft* ssp. *holarctica* Biovar II



Distribution of *Ft* ssp. *holarctica* Biovar II positive cases between 1995 and 2010.

## Conclusion

In Austria, *Francisella tularensis* ssp. *holarctica* biovar II strains showed higher genetic diversity as reviewed in previous studies (4), possibly because they had been never included in any comparative study, and because of the numerous strains collected in a relative small geographical region. Red fox and human isolates were all clustered to previous hare isolates. Since the natural reservoir remains still unclear, future investigation about the role of the brown hare in Austria would be necessary.

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