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Veterinary Microbiology 196 (2016) 1–8

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Whole genome SNP analysis of bovine *B. anthracis* strains from Switzerland reflects strict regional separation of Simmental and Swiss Brown breeds in the past

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ARTICLE INFO

Article history:
Received 2 May 2016
Received in revised form 11 August 2016
Accepted 9 October 2016

Keywords:
Bacillus anthracis
Whole genome sequencing
Single nucleotide polymorphism
Molecular typing
Comparative genomics

ABSTRACT

Bacillus anthracis is an evolutionarily young species that presents an extremely low genetic diversity due to its slow mode of propagation, determined by short replication phases and long sporulation periods. In our ongoing efforts to elucidate phylogenetic relationships between European *B. anthracis* isolates, the genomes of five strains from Switzerland belonging to lineages B.Br.CNEVA and A.Br.Aust94 were sequenced. Comparative analysis with additional, available genomes from both lineages, were used to reconstruct the substructure of these populations. Genome-wide single-nucleotide polymorphism analysis revealed two phylogeographical different groups among the Swiss B.Br.CNEVA strains (central and eastern Switzerland), that define the oldest most recent common ancestor of the B.Br.CNEVA lineage currently known. Age-old practices of livestock selection, breeding and preservation of unique traits of local breeds in Alpine valleys have likely favored differentiation of regional *B. anthracis* populations over centuries and the emergence of genetically distinct strains in an otherwise similar environment.

1. Introduction

Anthrax has a long common history with animals and humans. It has been one of the infectious diseases with major mortality among livestock for several millenium (Turnbull, 2002). During the first part of the 20th century, active control measures and large animal vaccination programs succeeded in reducing the incidence of the disease in Europe. Many European countries are now probably free of anthrax, but the disease persists in regions around the Mediterranean Sea and in Eastern Europe where it almost exclusively affects animals at pasture (Schmid and Kaufmann, 2002; Derzelle and Thierry, 2013). In Switzerland, anthrax was eradicated in cattle in the 1960s by strictly prohibiting the burial of dead animals or slaughtered waste and requiring the burning of the carcasses of animals that died from diseases.

Bacillus anthracis, the etiological agent of anthrax, is a spore-forming bacterium belonging to the *Bacilus cereus* group. The pathogen spends the majority of its life cycle as a quiescent spore that can persist in soil for decades. All mammals are known to be susceptible to anthrax, but *B. anthracis* primarily affects herbivores, causing acute, often fatal disease in cattle. A tripartite toxin consisting of the edema factor, the lethal factor, and the protective antigen, which serves as adhesive unit for the latter two, is able to cause edema and cell death. Furthermore, the production of a polyglutamic acid capsule allows the organism to escape the immune system and spread in the host organism. Ruminants become infected by ingestion of soil-borne spores while browsing or grazing (Turnbull, 2002; Hugh-Jones and Blackburn, 2009).

As spore-forming bacteria, opportunity for accumulating DNA mutations is limited by the episodic short reproductive cycles interrupted by long dormant phases. *B. anthracis* constitutes therefore a highly monomorphic species with relatively little genetic variation, including single nucleotide polymorphisms (SNPs) (Keim et al., 2009; Pilo and Frey, 2011). With the completion of a growing number of genome sequences, genome-wide comparison of multiple strains has led to major progress in the understanding of the global population structure of the species (Pearson et al., 2004; Van Ert et al., 2007; Simonson et al., 2009). The *B. anthracis* population is composed of three main clades (A, B

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http://dx.doi.org/10.1016/j.vetmic.2016.10.014

and C), with further subdivisions into 13 lineages and genetic groups (*e.g.* C.Br.A1055, B.Br.001/002, B.Br.KrugerB, B.Br.CNEVA, A.Br.001/002, A.Br.Ames, A.Br.Australia94, A.Br.003/004, A.Br.Vollum, A.Br.005/006, A.Br.WNA, A.Br.011/009 and A.Br.008/011) (Van Ert et al., 2007). Clade A has known the most dramatic dispersal around the world, with A-lineages recovered from across the five continents. These strains account for the majority (85%) of anthrax cases reported around the world (Keim et al., 2000; Van Ert et al., 2007). Clade B is divided into two genetically distinct branches with geographically limited repartition. Branch B.Br.001/002 (including the derived B.Br.Kruger lineage) is ecologically established in Southern Africa, in particular in the Kruger National Park (South Africa), where it co-exists with strains from clade A, especially of lineage A.Br.Vollum (Smith et al., 2000). The B.Br.CNEVA branch is exclusively found in Europe. Its strains are generally isolated from livestock (Gierczynski et al., 2004; Pilo et al., 2008; Garofolo et al., 2011; Antwerpen et al., 2012; Steiner et al., 2013; Girault et al., 2014a). The C clade (C.Br.A1055) is an uncommon genetic group of unknown origin. Only four C.Br.A1055 strains are currently known in US collection (Van Ert et al., 2007).

In this study, genome-wide SNP analysis was used to subtype five bovine strains from Switzerland collected in the period 1952–1962. They represent the latest anthrax cases from cattle reported in Switzerland that were preserved. Comparative genomics were conducted using a total of 50 additional strains to get insight into the worldwide phylogenetic placement of the Swiss strains. The trees generated provide new insight on the evolutionary history, local settlement and differentiation of the European-specific B.Br.CNEVA branch and the worldwide A.Br.Aust94 group.

Table 1
Whole genome sequences of *B. anthracis* strains used in this study.

Strain	Country	canSNP	year	source	Accession number
Ames Ancestor	USA	A.Br.Ames			NC_007530.2
A0389	Indonesia	A.Br 001/002			NZ_ABLB00000000.1
BA103	Japan	A.Br 001/002	1991	Bovin	DRR000183
08_08_20	France	A.Br.001.002	2008	bovin	NZ_JHCB00000000.2
BA104	Japan	A.Br.Australia94	1982	swine	DRR000184
9080 G	Georgia	A.Br.Australia94	1998	soil	NZ_CM002398.1
Australia 94	Australia	A.Br.Australia94	1994	bovine	NZ_AAES00000000.1
JF3853	Switzerland	A.Br.Australia94	1952	Bovine	ERR899845
2000031027	USA	A.Br.Australia94	1957	Bos taurus	NZ_JTAR00000000.1
K1285	Namibia	A.Br.Australia94	1996	zebra	NZ_LFYF00000000.1
K1409	Denmark	A.Br.Australia94	1974	cattle	ERR930300
K2883	India	A.Br.Australia94	1997	human	NZ_LFYH00000000.1
K4834	Australia	A.Br.Australia94	1997	bovine	NZ_LFYJ00000000.1
A.Br.003	UK, scotland	A.Br.Australia94		human	NZ_JMPV00000000.1
52 G	Georgia	A.Br.Australia94	2009	bovin	NZ_CM002395.1
8903 G	Georgia	A.Br.Australia94	1997	soil	NZ_CM002401.1
A1039	Bolivia	A.Br 003/004	1999	Bos taurus	NZ_LAKZ00000000.1
K8215	Argentina	A.Br 003/004	1996	bovine	NZ_LGIG00000000.1
A1075	Chile	A.Br 003/004		Bos taurus	NZ_LBFE00000000.1
K3	South Africa	A.Br 005/006		human	NZ_CP009331.1
H29	Zambia	A.Br 005/006	2012	human	DRR014739
CZC5	Zambia	A.Br 005/006	2011	hippopotamus	NZ_BAVT00000000.1
PAK-1	Pakistan	A.Br 008/011	1978	sheep	NZ_CP009325.1
Turkey32	Turkey	A.Br 008/011	1991	human	NZ_CP009315.1
Ba4599	UK,Scotland	A.Br 008/011	2009	Human (heroin)	NZ_AGQP00000000.1
99–100	France	A.Br.011/009	1999	bovine	NZ_JHDR00000000.2
Scotland476	UK, Scotland	A.Br 011/009	2006	Animal skin (drum)	SRR2094254
Pollino	Italy	A.Br 011/009	2014	Bos taurus	NZ_CP010813.1
A0193	USA	A.Br WNA		bovine	NZ_ABKF00000000.1
Canadian_bison	Canada	A.Br WNA			NZ_CP010322.1
USA6153	USA	A.Br.WNA			NZ_AAER00000000.1
A0488	UK	A.Br.Vollum	1935	cattle	NZ_ABJC00000000.1
Vollum	UK, Scotland	A.Br.Vollum	1963	cow	NZ_CP007666.1
K1129	Pakistan	A.Br.Vollum	1995	Hair goat	NZ_LGIF00000000.1
JF3887	Switzerland	B.Br.CNEVA	1960	Bovine	ERR899847
JF3888	Switzerland	B.Br.CNEVA	1962	Bovine	ERR899848
JF3852	Switzerland	B.Br.CNEVA	1953	Bovine	ERR899844
JF3854	Switzerland	B.Br.CNEVA	1957	Bovine	ERR899846
00−82	France	B.Br.CNEVA	2000	Bovine	NZ_JHDS00000000.2
CNEVA-9066	France	B.Br.CNEVA	1992	Bovine	NZ_AAEN00000000.1
A0465	France	B.Br.CNEVA	1997	bovine	NZ_ABLH00000000.1
BF1	Germany	B.Br.CNEVA	2009	cow	NZ_AMDT00000000.1
RA3	France	B.Br.CNEVA	1998	bovine	NZ_CP009697.1
Kruger B	South Africa	B.Br.Kruger			NZ_AAEQ00000000.1
A0442	South Africa	B.Br.001/002			NZ_ABKG00000000.1
SVA 11	Sweden	B.Br.001/002	2011	Cow	NZ_CP006742.1
Zimbabwe89	Zimbabwe	B.Br.001/002			NZ_JMPU00000000.1
HYU01	Korea	B.Br.001/002	2009	soil	NZ_CP008846.1
BA1035	South Africa	B.Br.001/002		human	NZ_CP009700.1
A1055	USA	C.Br.A1055			NZ_AAEO00000000.1
2002013094	USA	C.Br.A1055	1956	soil	NZ_CP009902.1
2000031021	USA	C.Br.A1055		soil	NZ_CP007618.1
2000031052	USA	C.Br.A1055	1956	Bos Taurus	NZ_JSZS00000000.1
AH820	Norway	*B. cereus*			NC_011773.1

2. Materials and methods

2.1. Bacterial strains and biosafety procedures

Five *B. anthracis* strains isolated from bovine anthrax outbreaks occurring in Switzerland between 1952 and 1962 were used in this study (Table 1). Genomic DNAs were obtained from vegetative cells grown 16 h at 37 °C on 5% horse blood agar plates by scraping the agar surfaces to remove bacterial colonies. DNA was purified using the QIAGEN® Genomic-tip 100/G columns and QIAGEN® Genomic DNA Buffer Set. After isopropanol precipitation, genomic DNA was suspended in 400 µl of 10 mM Tris HCl (pH 8) for 2 h at 50 °C. Subsequently DNA preparations were filtered through a 0.2 µm Acrodisc® syringe filter (Pall Corporation, Ann Arbor, MI, USA) to

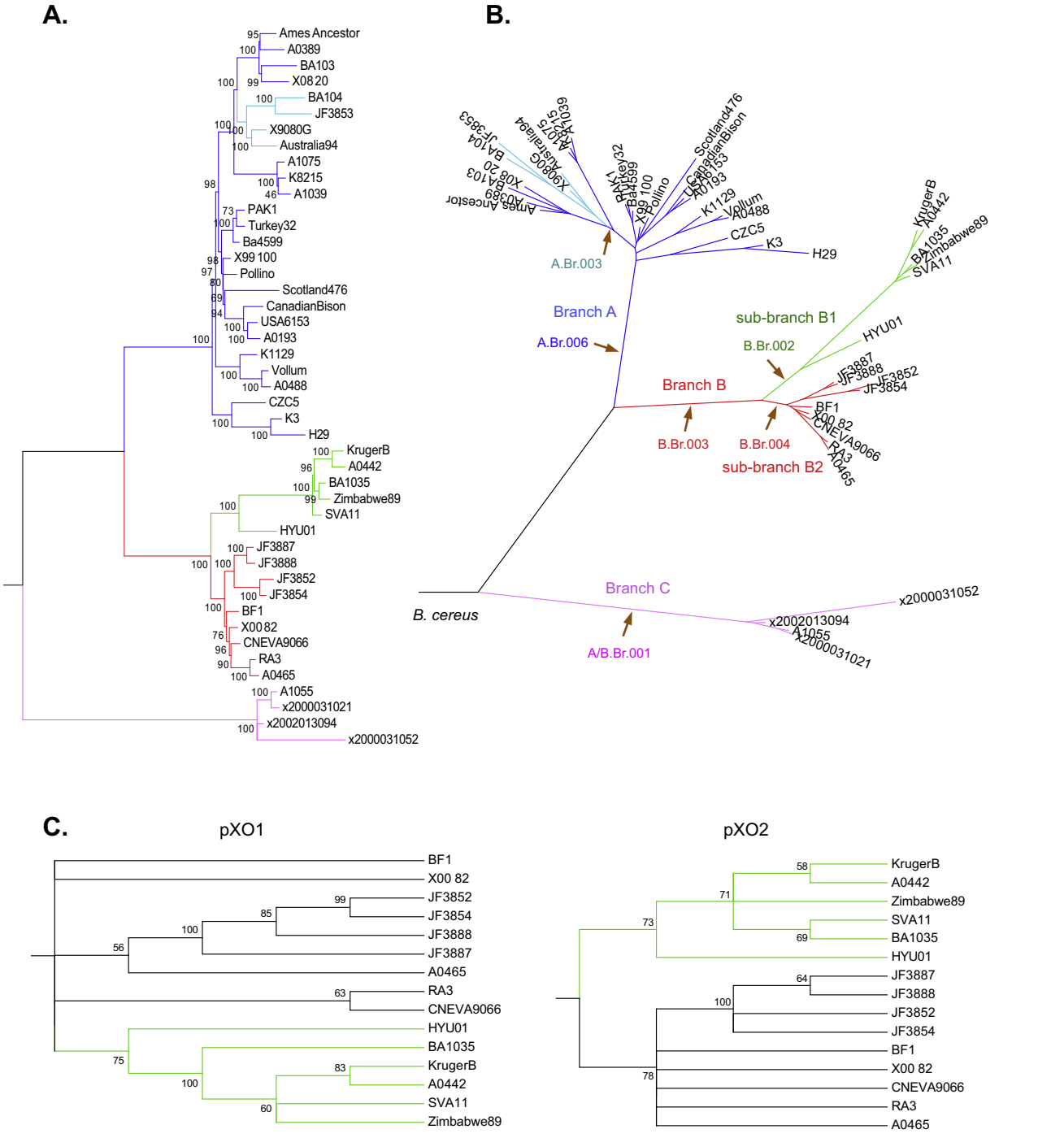

Fig. 1. Position of fifteen sequenced B-branch-affiliated strains on the *B. anthracis* phylogenetic tree based on whole-genome SNP analysis. Phylogenetic relationships between 45 *B. anthracis* strains were inferred by Maximum Likelihood. A-B. ML trees are drawn to scale, with branch lengths measuring the number of substitutions per site. Bootstrap scores (100 replicates) are shown next to the branches. The clustering into the 3 major phylogenetic branches is color-coded: branch C in pink, branch B in red (B.Br. CNEVA lineage) or green (B.Br.002/001 lineage), and branch A in blue. The A.Br.Aust94 lineage is shown in light blue. Brown arrows indicate the position and name of published canSNPs specific to these branches: A/B.Br.001 (C clade); B.Br.002, B.Br.003 and B.Br.004 (B clade); A.Br.006 and A.Br.003(A clade). The AH820 *B. cereus* strain was used as outgroup to root the phylogenetic trees. C. Bootstrap consensus dendrograms of strains belonging to the B branch based on plasmidic SNPs. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. There were a total of 7150 chromosomal SNPs, 385 pXO1 and 407 pXO2 SNPs in the final dataset. Evolutionary analyses were conducted in MEGA6.

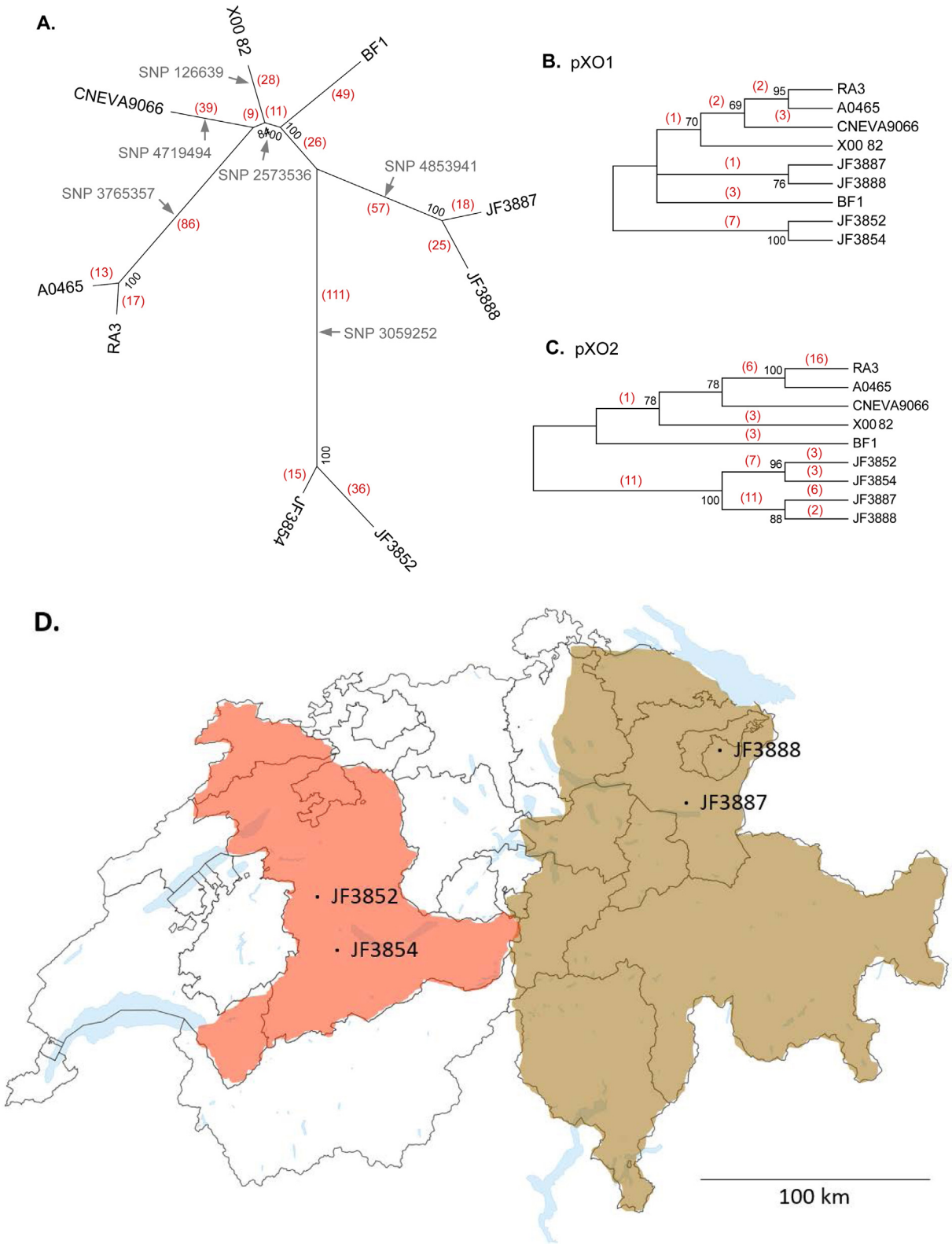

Fig. 2. Phylogenetic analyses of the B.Br.CNEVA lineage using the finished RA3 genome as reference. Phylogenetic relationships between nine B.Br.CNEVA strains were inferred by Maximum Likelihood. A. The ML tree is drawn to scale, with branch lengths measured in the number of substitutions per site (*i.e.* 550 chromosomal SNPs). The percentage of replicate trees in which the associated strains clustered together are showen (bootstrap values for 100 iterations). A black arrow indicates the branching position of the A.Br.Aust94 JF3853 strain (isolated in Switzerland in 1953) used as outgroup. Position and name of diagnostic SNPs within the B.Br.CNEVA lineage are shown in grey. The number of SNPs specific to each branch or node is reported in parenthesis and color-coded in red. B-C. Bootstrap consensus dendrograms based on 18 pXO1 SNPs (B) and 94 pXO2 SNPs (C). Branches corresponding to partitions reproduced in less than 68% bootstrap replicates were collapsed. Evolutionary analyses were conducted in MEGA6. D. Map of Switzerland showing the distribution of the two major cattle breeds in the 1960[ies]. Areas depicted in brown show the areas with Brown Swiss breed, in red with nearly exclusively Simmental breed. Note the areas follow mostly cantonal borders (depicted by thin black lines). Lakes are shown in pale blue. The origins of the different

warrant full removal of remaining *B. anthracis* cells or spores. Viability testing was systematically performed before DNA was taken out of the BSL-3 facility.

2.2. Whole genome sequencing and SNPs discovery

Total DNA were subjected to paired-end whole genome sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). The number of reads that passed Illumina quality filters varied from 0.9 to 1.4 million. Chromosomal, pXO1 and pXO2 sequences of the Ames Ancestor strain or the RA3 strain were used as references for genome and plasmids assembly [GenBank: AE017334.2, AE017336.2 and AE017335.3, CP009697.1, CP009696.1 and CP009695.1, respectively]. Paired-end reads were mapped to the Ames Ancestor genome using the Bowtie 2 tool and its default parameters (Langmead and Salzberg, 2012). Whole-genome SNPs discovery and variant calling were performed for each alignment using the SAMtools package (Li et al., 2009). Individual lists of SNPs were compiled and data filtered to remove SNP positions at ribosomal operons and 31 VNTR loci.

2.3. Comparative genomics *and* in silico *canSNP genotyping*

In order to identify additional strains affiliated to the A.Br. Aust94 lineage, all publicly available *B. anthracis* genomes were retrieved from database and *in silico* genotyped. Briefly, the complete or draft genome assemblies from a hundred of strains were used to generate artificial sets of overlapping, 200 bp-long reads (with a 4-fold genome coverage). Each set of reads were aligned to the Ames Ancestor sequences using the same pipeline as described for the paired-end reads of the Swiss isolates. Genotypes were next determined based on 12 published canonical SNPs (Van Ert et al., 2007) and a few additional SNPs (Birdsell et al., 2012; Girault et al., 2014b; Khmaladze et al., 2014). Sixty strains, representing the genetic diversity observed in *B. anthracis*, were further selected for phylogenetic analysis and comparison (Table 1).

2.4. Phylogenetic analysis

Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) using the *B. cereus* AH820 strain as outgroup and the whole genome SNP data set as input. Phylogenetic relationships were inferred using distinct methods, *i.e.* Maximum Likelihood, Maximum Parsimony and Neighbor-Joining. Bootstrap scores (100 replicates) were computed for each minimum spanning tree. The global evolutionary history of the species was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. ML trees with the highest log likelihood are shown in Figs. 1 and 2. Initial tree(s) for the heuristic search were obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimates using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value.

2.5. SNP discrimination assays by HRM

We designed High Resolution Melting (HRM) assays for three novel specific SNPs using Primer 3^{+} software [http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/]. The positions of these SNPs in the Ames Ancestor genome and the primers sequences used are listed in Table 2. Amplification was performed on the ViiA7™ Real-Time PCR System (Life Technologies) using the LightCycler® 480 High Resolution Melting Master Mix (Roche Diagnostics) as previously described (Derzelle, 2015).

2.6. Availability of supporting data

The sequence data of the whole genomes of strains JF3852, JF3853, JF3854, JF3888 and JF3887 have been deposited in the European Nucleotide Archive under project name PRJEB9467 and accession numbers ERR899844 to ERR899848.

3. Results-Discussion

3.1. Whole genome SNPs discovery and global phylogenomic analysis

Five Swiss strains, previously assigned by MLVA-8 and canSNP typing to the rare European B.Br.CNEVA sub-branch (n = 4) or the worldwide spread A.Br.Aust94 lineage (n = 1) (Pilo et al., 2008), were characterized by whole genome SNP analysis. Paired-end sequencing were performed using the Illumina technology, yielding 0.5–1.7 million reads per strain. Filter-passed reads were mapped to the Ames Ancestor reference genome for comparative analysis and SNP discovery. Data available for 40 additional genomes, from diverse geographic origins and lineages of *B. anthracis*, were also included (*e.g.*, 25 strains belonging to the nine A groups, eleven to the two branches of clade B, four to clade C). The genome sequence of *Bacillus cereus* strain AH820 was used as outgroup for phylogeny rooting (Table 1).

A total of 7150 chromosomal SNPs were identified among *B. anthracis* genomes and used to draw the phylogeny of the Swiss strains. Additional SNPs located on both virulence plasmids were also discovered (*i.e.* 385 and 407 SNPs for pXO1 and pXO2, respectively). Fig. 1 illustrates the maximum likelihood trees generated based on the identified SNPs. A and B clades showed a long and common history before their divergence into separated branches. The first radiation observed within each clade (separation between sub-branches B.Br.001/002 and B.Br.CNEVA, or emergence of the oldest African A.Br.005/006 group from the rest of the A group) has apparently occurred approximately at the same period of time in history, at a distance of 362 and 350 SNPs from the A-B most recent common ancestor (MRCA), respectively (Fig. 1B). Based on a molecular clock model, the divergence of the two B sub-branches has been proposed to have occurred about 4661–9322 years before present (YBP) (Van Ert et al., 2007). Dating divergence time in bacterial lineages is difficult, even more problematic for species such as *B. anthracis* that spends the majority of its life cycle as quiescent spore. Although roughly approximate, this age estimate coincides with the emergence of agriculture and farming in the Fertile Crescent region, some 11,000 years ago, and subsequent human migrations of early farmers into Europe over the next several thousand years (Zeder, 2008; Gangal et al., 2014). The Near East is a likely place for the initial introduction of the B clade into Eurasia from the African continent, which is the probable cradle of this bacterial species. The observation that Asian strains, represented by the Korean soil isolate HYU01 (Kim et al., 2014), are branching out from the B.Br.001/002 group leading to southern African strains early after the B sub-branches split (at a distance of 111 *versus* 424 SNPs) is consistent with this hypothesis (Fig. 1). However, it has to be taken into account that this branch could have been introduced to Asia more recently by contaminated animals or animal products.

African strains of the B.Br.001/002 lineage are characterized by relatively long branches (451–535 SNPs) as compared to the

B. anthracis strains are noted by the strain number. Data taken from: (Statistische Erhebungen und Schätzungen über Landwirtschaft und Ernährung. Bearbeitet vom Schweizerischen Bauernsekretariat. 43. Jahresheft, Ausgabe 1966).

Table 2
Specific canSNPs and primer sequences used for HRM analysis.

SNP Position[a]	SNP state	Target sub-group	Forward primer (5′-3′)	Reverse primer (5′-3′)
126639	A to G	B2/F-Alps	CCACAAGGTGGAATTATTACTAAAGA	GGTTCACCTGTTTTCGGATCT
3765357	A to G	B2/F-Pyr	GGTGGTTTCGGATATGCACT	AAAGGTGCTGGGGTAGTAAGG
4719494	G to A	B2/F-MC	TTAGATCTCGTTTTCGGTTCC	CAATGAGTGTACGGCTCCAA
2573536	A to G	B2/F	CATATATTTTCACCTCTTTTATGAACA	GATAAAAGGCTGTCGGATGG
3059252	G to A	B2/SW1	TGCGAAGTAAAATACCATCTGGA	AGCGTTATGAAGTAGGGGAGAGA
4853941	G to A	B2/SW2	TGCTTTAGAACGAGGAAATGATG	AATAGTTTGGCGTTGTCATGCT
394707	G to	A.Br.035	CGGGAACTGGTTGTTTACTAACTT	CGCTACTGCAGCCTTTACATAAT

[a] Localisation on the Ames Ancestor chromosome (GenBank accession no. AE017334.2).

Korean HYU01 strain (266 SNPs) or all European B.Br.CNEVA isolates (95–202 SNPs), suggesting an evolutionary rate at least twice faster in this part of the world. Anthrax transmission frequencies can greatly influences the substitution rates. This means that higher mutation accumulation may often be associated with a wider range of infections' opportunities. In Europe, but also Korea, Bhutan or the Caucasus region where B.Br.001/002 isolates have been reported (Eremenko et al., 2012; Jung et al., 2012; Thapa et al., 2014), anthrax mostly affected domesticated animals, especially cattle. Consequently, host availability and diversity substantially differ from the situation encountered in Southern Africa where large herds of susceptible ungulate and a larger number of modern bovids can be found.

3.2. B.Br.CNEVA phylogenetic analysis

Fifty-two point mutations were specific to the B.Br.CNEVA lineage (Fig. 1, Supplementary data). The nine available genomes were resolved into six phylogenetic groups showing patterns of differentiation at regional scale. The most basal node was defined by the split of the Swiss strains (JF3852, JF3854, JF3887, JF3888), shortly (6 SNPs) before the branching out of the German BF1 strain at a distance of 9 SNPs from the MRCA of the French isolates, including the terminal CNEVA9066 reference genome. Swiss strains further evolved into two clearly separated groups, sharing only 6 specific SNPs. Strains isolated in 1953 and 1957 in the canton of Berne, in the center of the country, clustered together (JF3852, JF3854). They appeared to have evolved faster (102 *versus* 51 SNPs) than the second group of strains (JF3887, JF3888) composed of isolates collected in an area separated by mountain ridges 150–200 km eastwards in 1960 and 1962 (Fig. 1, Supplementary data). As previously reported (Girault et al., 2014a), French strains were similarly resolved into three phylogeographic groups: the Alps (str. 00_82), the Massif Central (str. CNEVA9066) and the Pyrenees (str. RA3 and A0465), according to the regions of France where they are established. The Southern Bavarian strain BF1, collected near the Austrian border, formed its own genetic group. Evolutionary trees computed based on plasmidic SNPs identified on both pXO1 and pXO2 virulence plasmids did not confirm its relative placement closed to the French specimens, while corroborating the respective grouping of Swiss isolates (Fig. 1C).

To obtain a better resolution of the phylogenetic relationships observed between strains of the B.Br.CNEVA lineage, the nine whole-genome sequenced strains were further aligned to the only finished genome of the lineage, those of strain RA3, isolated in 1997 in the French Pyrenees. A total of 550 chromosomal, 18 pXO1 and 94 pXO2 SNPs were identified. Supplementary Table 1 lists the polymorphic genes that may contribute to the genetic specificities of the two Swiss groups. Positions of the 9 genomes relative to each others are illustrated on Fig. 2. The six regional clusters (central Switzerland, eastern Switzerland, Southern Bavaria, French Alps, Massif Central and French Pyrenees) previously highlighted were confirmed, as well as MRCA for the two Swiss groups or for the Bavarian and French clusters. Evolutionary distances between nodes (9–26 SNPs) were shorter compared to those to the tips towards each branch (extending for approximately 28–147 SNPs), suggesting long *in situ* differentiation time span. This phylogeographic pattern is consistent with our assumption of an initial introduction into Europe of an ancestral B-population from the Near East, with progressively derived local populations on an East-West trajectory. The earlier farmers who colonized Europe during the Neolithic transported all major livestock species (sheep, goat, cattle and pig). By around 5000 YBP almost all populations in mainland Europe practiced farming and cattle breeding (Zeder, 2008).

In contrast to the A-lineages that spread extensively around the world, presence of the B.Br.CNEVA branch is relatively localized in Europe. In addition to France, Southern Germany and Switzerland, B.Br.CNEVA strains have only been reported from Bosnia and Herzegovina, Croatia, Slovenia, Northern Italy, Slovakia and Poland. The trans-Alpine axis, made of pastoral valleys rich in grassland, might have represented a particularly favorable environment for spore survival and host-propagation for *B. anthracis*. The long-lasting implementation of livestock selection and breeding along the valleys and mountain slopes of the Alps could have facilitated the successful ecological establishment and persistence of the pathogen in farming areas. Geographic isolation might have contributed to the genetic diversity currently observed among B.Br.CNEVA isolates. Co-habitation of *B. anthracis* with its hosts, represented by regional breeds of cattle (as exemplified by Simmental and Swiss Brown in Switzerland), is a plausible assumption. The Simmental and the Brown Swiss are among the oldest of all breeds of cattle in the world. These two bovine races originate, respectively, from the canton of Berne and the eastern cantons of Schwyz, Zug, St. Gallen, Glarus, Lucerne, and Zurich. The canton of Schwyz was the scene of most of the early improvement of the Swiss Brown (often referred to as Brown Schwyzer in Switzerland). Unimproved cattle similar to the Brown Swiss have been in this territory for a considerable period of time (around 6000 YBP according to some historians) (http://www.ansi.okstate.edu/breeds/cattle/brownswiss/index.html). The Simmental breed is recorded since the Middle Ages. There is evidence of large, productive red and white cattle found in ecclesiastical and secular property records of western Switzerland. The Simmental name is derived from their original location, the Simme Valley in the canton of Berne. These red and white animals were highly sought because of their rapid growth development, outstanding production of milk, butter, and cheese`, and for their use as draft animals (http://www.ansi.okstate.edu/breeds/cattle/Simmental). Both bovine races were strictly kept locally for centuries.

3.3. A.Br.Aust94 phylogenetic analysis

The last Swiss genome (strain JF3853) belongs to the A.Br.Aust94 lineage. Members of this group have been identified throughout the five continents. The A.Br.Aust94 lineage is probably

not indigenous to Europe (Van Ert et al., 2007). Rather, the presence of these isolates in some European countries is likely to be due to the (more recent) importation of contaminated animal products from Asia or Africa. The JF3853 strain was isolated from cattle during an outbreak occuring in central Switzerland in december 1952. Anthrax outbreaks occurring in atypical seasons (winter) are often linked to the consumption by livestock of cut forage harvested from contaminated soils, or feed products derived from processing of infected carcasses (Fasanella et al., 2014). As ecological establishment of non-indigenous strains are rare, JF3853 presumably represents an imported anthrax case from Asia or Africa.

Genotyping the JF3853 genome using a set of recently published A.Br.Aust94-specific canSNPs (Birdsell et al., 2012; Khmaladze et al., 2014) resulted in its assignment to the most basal A.Br.014/013 node of the A.Br.Aust94 lineage (Fig. 3). Strain JF3853 shares the derived allele for canSNP A.Br.014 (SNP 1971191). It possesses ancestral alleles for SNPs A.Br.013 (SNP 3101332), A.Br.026 (SNP 3640599), A.Br.015a (SNP 182717) or A.Br.016 and A.Br.017, defining the two known branches leading to either the Turkish and Georgian A.Br.Aust94 strains or the terminal reference Australia94 genome, respectively (Khmaladze et al., 2014). The A.Br.014/013 node has been reported to contain many strains from Africa, especially Namibia or South of Africa, China and Australia (Khmaladze et al., 2014), but none of them were sequenced at that time. To obtain an accurate placement of the Swiss strain along the *B. anthracis* SNP tree, we further screened public databases for additional A.Br.Aust94 genomic sequences. The whole genome SNP phylogenetic tree drawn from 13 A.Br.Aust94 strains placed the JF3853 genome at the top of a new branch that branches off the basal node of the lineage, *i.e.* A.Br.014 (Fig. 3). Four novel nodes (A.Br.034–A.Br.037) along the branch terminating in the "exotic" Swiss strain (located at a distance of 221 SNPs) were identified. SNPs specific to these genetic groups are listed in Supplementary data. The A.Br.034 subgroup contains strains isolated from Japan (BA104) (Kuroda et al., 2010), USA (2000031027, OhioACB) and Namibia (K1285) in addition to the Swiss JF38533 isolate. Additional sequencing and phylogeography studies of the A.Br.Aust94 group are needed to better understand how all these strains fit into regional and global phylogeographic patterns.

3.4. Specific SNP discrimination genotyping assay

Three novel canSNPs that define the newly identified sub-clusters in lineage B.Br.CNEVA (SNP B2/SW1-Simmental and SNP B2/SW2-Brown Swiss) and A.Br.Aust94 (SNP A.Br.035) were developed into HRM assays (Table 2). Four other assays specific to the French subgroups of B.Br.CNEVA were previously designed (Girault et al., 2014a). The two expected alternate alleles for each canSNP exhibited distinct melting curves and melting temperatures, allowing unambiguous discrimination of each strains within their respective genetic sub-groups.

4. Conclusions

This work provides some new insights into the origin and evolution of the B.Br.CNEVA lineage of *B. anthracis*. An initial introduction of this lineage in Europe associated with early farmers' migrations during the Neolithic period is suggested, *via* a generally western trajectory across Europe. Co-habitation with local breeds of cattle such as Simmental and Swiss Brown in Switzerland; Tarentaise, Salers, Gascogne or Béarnaise in France (respectively in the Alps, Massif Central and Pyrenees); Fleckvieh or Pinzgauer in Austria and southern Bavaria; Piedmontese in Northwest Italy is proposed to have driven regional differentiation, yielding phylogeographical distinct sub-branches distributed along a corridor following the 700 miles of the Alps, stretching from the coastline of France to Bosnia and Herzegovina.

Conflicts of interest

None.

Acknowledgments

Whole genome sequencing was supported by the Next Generation Sequencing Platform of the University of Bern, and the Vital-IT high-performance computing center of the Swiss Institute of Bioinformatics. We are grateful to Gaudenz Dolf, Vetsuisse, University of Bern, for his valuable help with the distribution of Swiss cattle breeds during the last century.

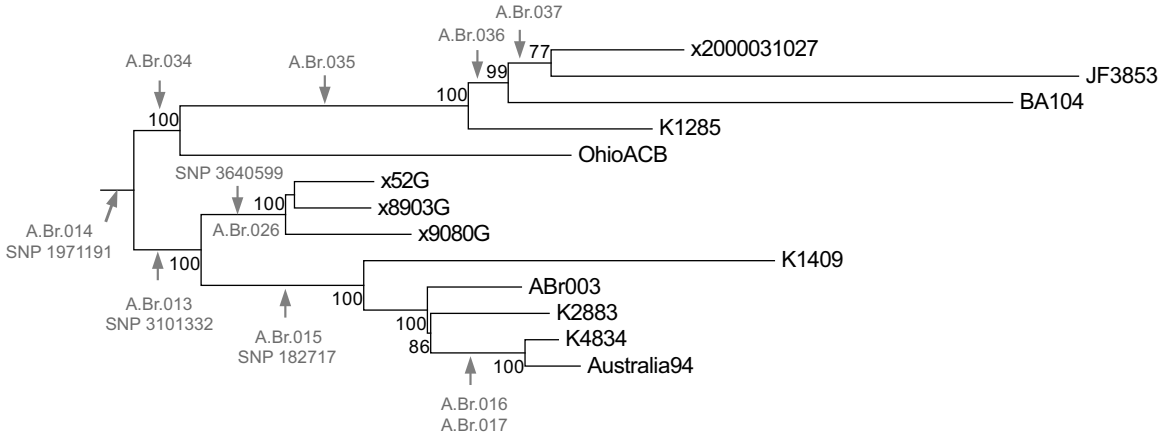

Fig. 3. Phylogenetic analyses of the A.Br.Aust94 lineage based on whole-genome SNP analysis. Phylogenetic relationships between thirteen A.Br.Aust94 strains were inferred by Maximum Likelihood. The ML dendrogram is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicates trees in which the associated strains clustered together are showed (bootstrap values for 100 iterations). Position and name of diagnostic SNPs and nodes within the A.Br.Aust94 lineage are shown in grey. Evolutionary analyses were conducted in MEGA6.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2016.10.014.

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