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Research paper

Comparative genomics of *Bacillus anthracis* from the wool industry highlights polymorphisms of lineage A.Br.Vollum

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ABSTRACT

Background: With the advent of affordable next-generation sequencing (NGS) technologies, major progress has been made in the understanding of the population structure and evolution of the *B. anthracis* species. Here we report the use of whole genome sequencing and computer-based comparative analyses to characterize six strains belonging to the A.Br.Vollum lineage. These strains were isolated in Switzerland, in 1981, during iterative cases of anthrax involving workers in a textile plant processing cashmere wool from the Indian subcontinent.
Results: We took advantage of the hundreds of currently available *B. anthracis* genomes in public databases, to investigate the genetic diversity existing within the A.Br.Vollum lineage and to position the six Swiss isolates into the worldwide *B. anthracis* phylogeny. Thirty additional genomes related to the A.Br.Vollum group were identified by whole-genome single nucleotide polymorphism (SNP) analysis, including two strains forming a new evolutionary branch at the basis of the A.Br.Vollum lineage. This new phylogenetic lineage (termed A.Br.H9401) splits off the branch leading to the A.Br.Vollum group soon after its divergence to the other lineages of the major A clade (i.e. 6 SNPs). The available dataset of A.Br.Vollum genomes were resolved into 2 distinct groups. Isolates from the Swiss wool processing facility clustered together with two strains from Pakistan and one strain of unknown origin isolated from yarn. They were clearly differentiated (69 SNPs) from the twenty-five other A.Br.Vollum strains located on the branch leading to the terminal reference strain A0488 of the lineage. Novel analytic assays specific to these new subgroups were developed for the purpose of rapid molecular epidemiology.
Conclusions: Whole genome SNP surveys greatly expand upon our knowledge on the sub-structure of the A.Br.Vollum lineage. Possible origin and route of spread of this lineage worldwide are discussed.

1. Introduction

Bacillus anthracis, the causative agent of anthrax, forms endospores that can remain dormant for years in many types of soil all over the world. *B. anthracis* commonly infects wild and domesticated herbivorous mammals that ingest or inhale spores while grazing (Hugh-Jones and Blackburn, 2009). Once the endospores enter the host, they germinate and then spread by the circulation to the lymphatics. The poly-γ-D-glutamic acid capsule produced by the pathogen disguises *B. anthracis* from immune surveillance and allows its unimpeded growth in the host. When the bacteria gain access to the circulating blood, they multiply and typically kill the animal within a few days or weeks. Production of the powerful tripartite protein toxins by *B. anthracis*, lethal toxin and edema toxin in cooperation with the shared protective antigen that serves as the receptor binding subunit, causes cell death and tissue swelling. Anthrax is often fatal in cattle, sheep and goats. West Africa is the most affected area of the world, followed by the traditional anthrax belt that stretches from the Middle East into Central Asia (Hugh-Jones and Blackburn, 2009).

Occupational exposure to infected animals or their contaminated products, such as hides, wool, hair, or bones, is the usual pathway of exposure for humans, besides consumption of meat from infected animals. In the nineteenth and early twentieth centuries, many workers who dealt with wool and animal hides were routinely exposed to anthrax spores. Spores were brought into factories with organic matter (blood clots and skin fragments) that was contaminating the animal fibers. This industrial anthrax, including a dangerous form of inhalational anthrax, became known as “woolsorter's” or “ragpicker's” disease. It was

Abbreviations: DNA, deoxyribonucleic acid; SNP, single nucleotide polymorphism; canSNP, canonical single nucleotide polymorphism; VNTR, variable-number tandem repeat; MLVA, multiple-locus variable-number tandem repeat analysis; WGS, whole-genome sequencing; NGS, next generation sequencing; PCR, polymerase chain reaction; HRM, high resolution melting; Tm, melting temperature; NCBI, National Center for Biotechnology Information; SRA, Sequence Read Archive; SPR, Subtree-Pruning-Regrafting; ML, Maximum Likelihood; NL, Neighbor-Joining; MP, Maximum Parsimony.

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a serious threat after the 1830s, when various Eastern wools such as mohair or Cashmere goat hair were imported for use as textile fibers (Brachman et al., 1966; Carter, 2004).

Today, reports of anthrax cases related to handling of animal products in industrial settings are extremely rare in Europe, largely due to widespread animal vaccination during the 20th century, improved decontamination procedures and the fact that raw wool and goat hair processing have almost disappeared from Western industrialized countries. In Europe, only a very few factories still exist in regions with historical textile industries, where sheep wool and goat hair originating from areas where anthrax is endemic remain at high biological risk. In Switzerland, between 1978 and 1981, small outbreaks of human anthrax occurred in a plant that processed synthetic fibers and cashmere wool from Pakistan. Within 3 years, 25 workers had contracted anthrax. Twenty-four cases had the cutaneous form and one had inhalation anthrax from which all the patients recovered (Pfisterer, 1991; Winter and Pfisterer, 1991). We have previously reported the MLVA-8 and canSNPs typing of some of the strains isolated from clinical cases, goat hairs, and air filters from this factory (Pilo et al., 2008). All isolates were found to cluster with the A4 genotype and to belong to the sublineage A.Br.Vollum. The minor variations found between some of these strains (strains could be differentiated by two VNTR allelic difference on the pXO1 and pXO2 markers) raised questions about the epidemiological significance of the differentiation (Pilo et al., 2008).

Classically, the lifecycle of *B. anthracis* is described by short vegetative bursts in infected hosts, which is estimated to last 20–40 bacterial generations (Keim et al., 2004), alternating with long periods of dormancy as an environmental spore until disease is re-established. The capacity of *B. anthracis* spores to germinate and establish populations of vegetative cells in the environment is a matter of discussion in the scientific community. Recent laboratory findings tend to indicate that *B. anthracis* may have some ability to interact with members of the grassland-soil community, including plants, invertebrates and protists (Dey et al., 2012; Saile and Koehler, 2006; Schuch and Fischetti, 2009). In any case, *B. anthracis* evolves very slowly and represents a genetically extremely homogenous, monomorphic species, which makes it an ideal organism to study vertical evolution over extended periods of time. The global structure of the *B. anthracis* population is divided into three major genetic clades (A, B and C), with further subdivisions into 12 main lineages (Pilo and Frey, 2011; Van Ert et al., 2007). The type strain of the species, strain Vollum (NCTC10340^T; ATCC14578^T), is affiliated to the A.Br.Vollum lineage (corresponding to MLVA cluster A4). The geographically broad distribution of this lineage throughout the world is thought to be a consequence of human activities in commerce and industrialization. Indeed, the occurrence of A.Br.Vollum strains in Western industrialized regions has often been tied to the international trade of spore-infected items, in particular wool or goat hair from the Middle-East and central Asian area (Pilo et al., 2008; Van Ert et al., 2007; Wattiau et al., 2008).

To infer the epidemiological relationships existing between strains and gain further resolution into the population sub-structure of the A.Br.Vollum lineage, six strains associated with the wool-processing factory outbreaks were sequenced. Genetic characterization was performed by whole-genome SNP analysis and canonical SNP genotyping (Keim et al., 2004; Van Ert et al., 2007), which best roots the phylogenetic relationship of *B. anthracis* strains. Comparative genomics were conducted using twenty-eight additional strains belonging to lineage A.Br.Vollum and 47 diverse strains identified among the hundreds of *B. anthracis* genome sequences available in public databases.

2. Methods

2.1. Bacterial strains, DNA extraction and biosafety procedures

Six *B. anthracis* strains belonging to A.Br.Vollum were sequenced in this study (Table 1). They were collected during anthrax outbreaks that have occurred in northern Switzerland over three years (1978–1981) in a textile factory (Pfisterer, 1991; Pilo et al., 2008; Winter and Pfisterer, 1991). All *B. anthracis* manipulations were performed in a biosafety level 3 laboratory using class II type A2 biosafety cabinet.

DNA was 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 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

DNA was 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 0.5 to 1.7 million. Chromosomal, pXO1 and pXO2 sequences of the Ames Ancestor strain were used as reference for genome and plasmids assembly [RefSeq: NC_007530.2, 129 NC_007322.2 and NC_007323.3, 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. Contiguous SNPs were also excluded from the analysis.

2.3. Comparative genomics and in silico canSNP genotyping

In order to identify additional strains affiliated to the A.Br.Vollum lineages, all publicly available *B. anthracis* genomes were retrieved from database and in silico genotyped. Briefly, the complete or draft genome assemblies from a hundred strains were used to generate sets of artificial, overlapping, 200 bp-long reads (with a 4-fold genome coverage). Each set of reads was aligned to the Ames Ancestor sequences using the same pipeline as described above for the six Swiss isolates. Genotypes were next determined based on 13 published canonical SNPs (Van Ert et al., 2007) and a few additional SNPs. The locations of these SNPs along the Ames Ancestor genome are listed in Table 3. Seventy-five strains, representing the genetic and geographic diversity observed in *B. anthracis*, were further selected for phylogenetic analysis and comparison (Tables 1 and 2). Besides the 28 A.Br.Vollum isolates we identified, the sample includes four genomes belonging to each of the eleven other canSNP lineages or groups described so far. Strains harbouring both virulence plasmids (pXO1 and pXO2) were preferred.

2.4. Phylogenetic analysis

Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016) using the *B. cereus* AH820 strain as outgroup and the whole genome SNP data set as input. Phylogenetic relationships were inferred using three distinct methods, i.e. Maximum Likelihood, Maximum Parsimony and Neighbor-Joining. The Tamura 3-parameter model was statistically selected as the best-fit nucleotide substitution model using MEGA7 tools, and applied for modelling how DNA sequences change over evolutionary time. The Subtree-Pruning-Regrafting (SPR) algorithm was used as heuristic model for tree inference. Bootstrap scores (250 replicates) were computed for each phylogenetic reconstruction.

The global evolutionary history of the species was inferred by using the Maximum Likelihood (ML) method. Initial ML trees were obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimates using the Maximum Composite Likelihood

Table 1
A.Br.Vollum whole-genome sequences genotyped in this study.

Strain	Country	canSNP	Year	Source	Accession number
JF3783	Switzerland	A.Br.Vollum	1981	Human infected	ERR899824
JF3784	Switzerland	A.Br.Vollum	1981	Air filter	ERR899825
JF3785	Switzerland	A.Br.Vollum	1981	Raw goat hair	ERR899826
JF3786	Switzerland	A.Br.Vollum	1981	Raw goat hair	ERR899827
JF3787	Switzerland	A.Br.Vollum	1981	Cashmere goat hair	ERR899828
JF3788	Switzerland	A.Br.Vollum	1981	Human infected	ERR899829
CDC2000031008	USA	A.Br.Vollum		Air filter	NZ_JTAP00000000.1
CDC 2000032892		A.Br.Vollum			NZ_JSZZ00000000.1
Vollum1B	USA	A.Br.Vollum	1951	Human	NZ_CP009328.1
CDC684	USA	A.Br.Vollum			NC_012581.1
A0488	UK	A.Br.Vollum	1935	Cattle	NZ_ABJC00000000.1
Vollum	UK, Scotland	A.Br.Vollum	1963	Cow	NZ_CP007666.1
SK102	USA	A.Br.Vollum	1976	Wool (Pakistan)	NZ_CP009464.1
K1129	Pakistan	A.Br.Vollum	1995	Hair goat	NZ_LGIF00000000.1
CDC 2000031709	USA	A.Br.Vollum	1956	Hair goat	NZ_JSZU00000000.1
CDC 2000032967		A.Br.Vollum			NZ_JTAB00000000.1
CDC 2000032968		A.Br.Vollum			NZ_JTAC00000000.1
CDC 2000032951		A.Br.Vollum	1958	Human	NZ_JTAA00000000.1
CDC 2000031031	USA	A.Br.Vollum	1957	Human	NZ_JSZP00000000.1
CDC 2002734238	USA, North Carolina	A.Br.Vollum	1978	Environment	SRR2340986
CDC 2002721563	USA, North Carolina	A.Br.Vollum	1988		SRR2339961
CDC 2002734099	USA, Carolina	A.Br.Vollum	1976	Human	SRR2340349
CDC 2002721537	USA, Rhode Island	A.Br.Vollum	1974	Cashmere goat hair	SRR2339757
CDC 2002013077	USA, South Carolina	A.Br.Vollum	1960		SRR2339626
CDC 2002734233	USA, Massachusetts	A.Br.Vollum	1976	Yarn, environment	SRR2340910
CDC 2002734264	USA, Massachusetts	A.Br.Vollum	1976	Yarn, environment	SRR2339406
CDC 2002734177	USA, New Hampshire	A.Br.Vollum	1978	cattle hair	SRR2340486
CDC 2002734058	USA, New Hampshire	A.Br.Vollum	1978	wool	SRR2340350
CDC 2002734097	USA, New Jersey	A.Br.Vollum	1976	yarn	SRR2340464
CDC 2002721538	USA, Pennsylvania	A.Br.Vollum	1974	Saddle blanket	SRR2339728
CDC 2002013191	USA, Washington	A.Br.Vollum	1974	Blanket	SRR2339728
CDC 2002013132	Pakistan	A.Br.Vollum		Soil	SRR2339634
CDC 2002013124	USA, Texas	A.Br.Vollum	1988		SRR2339631
CDC 2002013096		A.Br.Vollum	1957		SRR2339629
H9401	Korea	A.Br.Vollum	1994	Human	NC_017729.1
CDC 2000031038		A.Br.Vollum	1957	Environment	NZ_JSZQ00000000.1

approach, and then selecting the topology with superior log likelihood value (default parameters). Neighbor-Joining trees were computed using the Maximum Composite Likelihood method based on the Tamura 3-parameter model and a dataset from which the *B. cereus* strain was deleted. Maximum Parsimony trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

2.5. SNP discrimination assays by HRM

Diagnostic SNPs within the A.Br.Vollum lineage were identified from the whole genome SNP discovery data (see Supplementary data - Lists of SNPs specific to the A.Br.Vollum and A.Br.H9401 lineages) by searching for SNPs with allelic states shared only by the different A.Br.Vollum subgroups.

High Resolution Melting (HRM) assays for some specific SNPs were designed using Primer 3^+ software (Untergasser et al., 2007). Their positions along the Ames Ancestor genome and the primers sequences used are listed in Table 3. Amplification was performed on the ViiA7™ Real-Time PCR System (Life Technologies) using the LightCycler® 480 High Resolution Melting Master Mix (Roche Diagnostics). The reaction mixture consisted of 0.2 μM of each primer, 1 × LightCycler® 480 HRM master mix and 2.5 mM $MgCl_2$ in a 10-μl final volume. The following parameters were used: 10 min at 95 °C were followed by 40 cycles consisting of 10 s at 95 °C, 10 s at 58 °C and 20 s at 72 °C. Samples were next heated to 95 °C for 30 s, cooled down to 65 °C for 1 min and heated from 65 °C to 88 °C at a rate of 1 °C/s with 25 acquisitions/°C. HRM data were analyzed by the ViiA7™ Software (version 1.2.1).

2.6. Availability of supporting data

The sequencing data of strains JF3783, JF3784, JF3785, JF3786, JF3787 and JF3788 have been deposited in the European Nucleotide Archive under project name PRJEB9466 and accession numbers ERR899824 to ERR899829.

3. Results

3.1. Whole genome sequencing and in silico genotyping

In 1981, six strains were collected during an outbreak that affected several workers of a wool-processing factory located in northern Switzerland (Pfisterer, 1991; Pilo et al., 2008). These isolates were sampled either directly at the processing plant or from patients that got infected (Table 1). We characterized in detail these strains by paired-end whole genome sequencing, using the Illumina technology, to understand how the different cases were epidemiologically related. Sequencing produced 0.5 to 1.7 million reads per strain, after applying the quality filter of the base-calling pipeline. Filter-passed reads were aligned to the Ames Ancestor reference genome for whole genome SNP discovery. Their placement within the A.Br.Vollum canSNP lineage (characterized by a derived allele for the A.Br.007 SNP position (Keim et al., 2004)) was confirmed in silico.

These strains were imported to Europe from Pakistan through the trade of cashmere goat hair bales. In order to provide context to the outbreak's strains, all publicly available sequences of *B. anthracis* (complete or draft genomes, as well as raw reads from Short Read Archive (SRA)) were screened against a set of previously established canonical SNP markers (Derzelle, 2015; Van Ert et al., 2007) and in silico

Table 2
Whole-genome sequences used in this study for comparison.

Strain	Country	canSNP	year	source	Accession number
Ames Ancestor	USA, Texas	A.Br.Ames	1981	Bovin	NC_007530.2
K670/88	Denmark	A.Br.Ames	1988	Bovin	ERR930297
Shikan NIID	Japan	A.Br.Ames	1928	Horse	**AP014833.1**
A0248	USA	A.Br.Ames			NC_012659.1
A0389	Indonesia	A.Br 001/002			NZ_ABLB00000000.1
BA103	Japan	A.Br 001/002	1991	Bovin	DRR000183
A16	China	A.Br 001/0022			NZ_CO001970.1
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	Cattle	NZ_AAES00000000.1
K1409	Denmark	A.Br.Australia94	1974	Cattle	ERR930300
CDC2000031023		A.Br 003/004	1957	Bos taurus	NZ_JTAQ00000000.1
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
K836	Denmark	A.Br 005/006	1968	Cattle	ERR930301
E	Denmark	A.Br 005/006	1937	Cattle	ERR930306
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
Tsiankovskii-1	Soviet Union	A.Br 008/011	1960	Vaccine	NZ_ABDN00000000.2
99–100	France	A.Br.011/009	1999	Bovine	NZ_JHDR00000000.2
Carbosap	Italy	A.Br.011/009		Vaccine	NZ_JPIG00000000.1
Scotland476	UK, Scotland	A.Br.011/009	2006	Animal skin (drum)	SRR2094254
Pollino	Italy	A.Br.011/009	2014	Bos taurus	NZ_CP010813.1
CDC 2000032832		A.Br WNA			NZ_JSZX00000000.1
A0193	USA	A.Br WNA		Bovine	NZ_ABKF00000000.1
Canadian_bison	Canada	A.Br WNA		Bison	NZ_CP010322.1
USA6153	USA	A.Br WNA			NZ_AAER00000000.1
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.BrCNEVA	1997	Bovine	NZ_ABLH00000000.1
BF1	Germany	B.Br.CNEVA	2009	Cow	NZ_AMDT00000000.1
Kruger B	South Africa	B.Br.Kruger			NZ_AAEQ00000000.1
A0442	South Africa	B.Br.001/002		Kudu (antelope)	NZ_ABKG00000000.1
SVA 11	Sweden	B.Br.001/002	2011	Cow	NZ_CP006742.1
Zimbabwe89	Zimbabwe	B.Br.001/002			NZ_JMPU00000000.1
A1055	USA, Louisiana	C.Br.A1055		Soil	NZ_AAEO00000000.1
CDC 2002013094	USA	C.Br.A1055	1956	Soil	NZ_CP009902.1
CDC 2000031021	USA	C.Br.A1055		Soil	NZ_CP007618.1
CDC 2000031052	USA	C.Br.A1055	1956	Bos Taurus	NZ_JSZS00000000.1
AH820	Norway	*B. cereus*	1995	Human	NC_011773.1

genotyped (Table 3). This approach enabled us to identify 28 additional strains from diverse origins affiliated to the A.Br.Vollum lineage, including three strains from Pakistan, i.e. strains K1129 (Chun et al., 2012), SK-102 (Johnson et al., 2015) and CDC2002013132 (Table 1). Fourty-seven other strains (35 belonging to various lineages within clade A, eight of clade B and four of clade C) were also selected for further analysis (Table 2). Noteworthy, it was observed that, if marker A05 (Girault et al., 2014b) is not assayed, as is the case in most canSNPs investigations, strains such as Korean H9401 or the unspecified CDC2000031038 might be wrongly assigned to the African A.Br.005/006 lineage (characterized by a derived allele for the A05 SNP position, but only by default in the initial set of 13 canSNPs described by Van Ert et al. (2007)).

3.2. Phylogenetic clustering based on whole genome SNP analysis

The *B. cereus* AH820 genome, a strain closely linked to the *B. anthracis* species (Helgason et al., 2000), was used as outgroup for further evolutionary analyses. Comparative analysis of these genomic sequences was carried out. A total of 39,894 chromosomal SNPs were identified among this total dataset and used to draw the phylogeny of these 82 strains. Figs. 1 and 2 illustrate the minimum spanning trees generated by using Maximum Likelihood method based on chromosomal (Fig. 1 and Supplementary Fig. S1) or pXO1 and pXO2 (Fig. 2) whole genome SNP discovery. As expected, the thirty-four A.Br.Vollum-affiliated strains clustered together. Interestingly, the analysis revealed that both the Korean H9401 and unspecified CDC2000031038 strains, featuring an ancestral allele for the A.Br.007 canSNP position (Table 3), are clustering in close proximity with the A.Br.Vollum lineage (Fig. 1). They emerged from the branch leading to the A.Br.Vollum lineage at a short distance (6 SNPs) from the internal node corresponding to their most recent common ancestor. The distance to the tip of this new lineage (tentatively termed A.Br.H9401) is approximately 300 SNPs. The branch leading to both A.Br.H9401 and A.Br.Vollum lineages appeared soon after the divergence of the most basal A lineage, the African A.Br.005/006 group. Similar topology was confirmed by Maximum Parsimony and Neighbor-Joining methods (Supplementary Fig. S2). Phylogenetic trees based on pXO1 sequences (Fig. 2A) also reflected quite similar relationships, although more polytomies were noted in the A clade. In contrast, the pXO2 data poorly reconstructed the *B. anthracis* population structure of this major clade (Fig. 2B).

A total of 2546 chromosomal SNPs differentiate all A.Br.Vollum and A.Br.H9401-affiliated strains within the *B. anthracis* species and resolved the A.Br.Vollum lineage into two genetic sub-groups (Fig. 3, Supplementary Fig. S3). The six strains associated with the wool-processing plant outbreak causing human infections clustered together with strains

Table 3
canSNP signatures defining *B. anthracis* sub-lineages and clusters.

canSNP position[a]	canSNP name	C.Br.A1055	B.Br.KrugerB	B.Br.001/002	B.Br.CNEVA	A.Br.Ames	A.Br.001/002	A.Br.Aust94	A.Br.003/004	A.Br.Vollum	A.Br.H9401	A.Br.005/006	A.Br.008/009	A.Br.008/011	A.Br.011/009	A.Br.WNA
182106	A.Br.001	T	T	T	T	**C**	T	T	T	T	T	T	T	T	T	T
947760	A.Br.002	G	G	G	G	**A**	**A**	G	G	G	G	G	G	G	G	G
1493280	A.Br.003	A	A	A	A	**G**	**G**	**G**	A	A	A	A	A	A	A	A
3600786	A.Br.004	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T
162509	A.Br.006	C	C	C	C	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
266439	A.Br.007	T	T	T	T	T	T	T	T	**C**	T	T	T	T	T	T
3947375	A.Br.008	T	T	T	T	T	T	T	T	T	T	T	**G**	**G**	**G**	**G**
2589947	A.Br.009	A	A	A	A	A	A	A	A	A	A	A	A	A	**G**	**G**
1455402	B.Br.001	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T
1056740	B.Br.002	G	**T**	**T**	G	G	G	G	G	G	G	G	G	G	G	G
1494392	B.Br.003	G	**A**	**A**	G	G	G	G	G	G	G	G	G	G	G	G
69952	B.Br.004	T	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T
3698013	A/B·Br.001	**G**	A	A	A	A	A	A	A	A	A	A	A	A	A	A
2552486	A.Br.011	G	G	G	G	G	G	G	G	G	G	G	G	G	**A**	**A**
405303	A05	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
4752196	AVH	C	C	C	C	C	C	C	C	**T**	**T**	C	C	C	C	C

canSNPs that define a particular sub-lineage or sub-group are underlined. The corresponding allele is in bold.
[a] Localisation on the Ames Ancestor chromosome.

K1119 (Chun et al., 2012), CDC2002734233 and CDC2002013132, three strains that could be traced back to Pakistan or to trade of cashmere goat hair bales. All six strains are individually characterized and differ from each other by several SNPs, especially the JF3784 isolate (with 46 unique SNPs) (Fig. 3). All other A.Br.Vollum strains were located in nodes along the terminal branch leading to strains A0488 and Vollum, as previously described for strain CDC684, Vollum (Okinaka et al., 2011) and CVI-23932 (not included in this study) (Derzelle et al., 2015). The geographic origin of most of these isolates, that represents imported cases of anthrax, are unfortunately unknown.

3.3. Specific SNP discrimination genotyping assays

Diagnostic SNPs that define these two A.Br.Vollum sub-groups were selected and developed into real-time PCR assays (Table 4, Fig. 3). Novel SNP discrimination assays based on High-Resolution Melting (HRM) technology were designed. The two expected alternate alleles exhibited distinct melting curves and melting temperatures (Tms). Differences in Tm ranged from 0.7 to 1 °C between both allelic states (Table 4). The assays allow unambiguous grouping of each allele and accurate discrimination of the A.Br.Vollum strains from other globally diverse canSNP genotypes.

4. Discussion

In 2007, seven whole genomes of *B. anthracis* were initially used to define 13 canonical SNPs representing key phylogenetic positions along the *B. anthracis* phylogenetic SNP tree. This set of canSNP markers divides the worldwide *B. anthracis* population into three major clades (A, B and C) and 12 clonal groups or lineages (Van Ert et al., 2007). Today, clustering of strains is refined and improved by additional sequencing projects as the cost and time required for sequencing is constantly declining and the number of published genomes rapidly increasing. However, although certain geographical regions are extensively sampled (Girault et al., 2014a; Kenefic et al., 2009; Khmaladze et al., 2014; Simonson et al., 2009), others are still poorly studied, such as the Indian subcontinent.

In contrast to other lineages of clade A (e.g. A.Br.Ames, A.Br.WNA, A.Br.008/009 or A.Br.Aust94) (Girault et al., 2014a; Kenefic et al., 2009; Khmaladze et al., 2014; Price et al., 2012; Simonson et al., 2009), there is currently no relevant publication about the A.Br.Vollum lineage. In this study, we provide some new insights on the genetic diversity and sub-population structure of this broadly spread lineage. We demonstrate that strains associated with contaminated goat hairs from Pakistan, belong to a newly described sub-branch within the A.Br.Vollum lineage, distinct to the one leading to the reference A0488 strain (Van Ert et al., 2007). We provide evidence that the genetic diversity existing between strains isolated in 1981 in Switzerland argues against the persistence of a single, unique continuing contamination source inside the factory. It rather suggests iterative infections caused by separate contamination events upon exposure to various strains originating from the same geographic area.

Molecular epidemiologic studies of *B. anthracis* have often demonstrated that particular genotypes usually match particular geographic regions (Derzelle et al., 2015; Girault et al., 2014a; Kenefic et al., 2009; Khmaladze et al., 2014; Simonson et al., 2009). The diversity unrevealed in this study within the A.Br.Vollum lineage likely reflects common regional strain patterns. Today, lineage A.Br.Vollum appears to be dominant in Pakistan and Afghanistan and may also be ecologically established in Iran (Keim et al., 2004; Price et al., 2012; Van Ert et al., 2007). In the central Asian republic of Kazakhstan, most isolates belong to the A.Br.008/009 group and only a few specimens belonged to the A.Br.Vollum cluster (Aikembayev et al., 2010). In Georgia and the South Caucasian-Turkish region, a majority of strains are affiliated to the A.Br.Australia 94 cluster (Khmaladze et al., 2014). Collections from

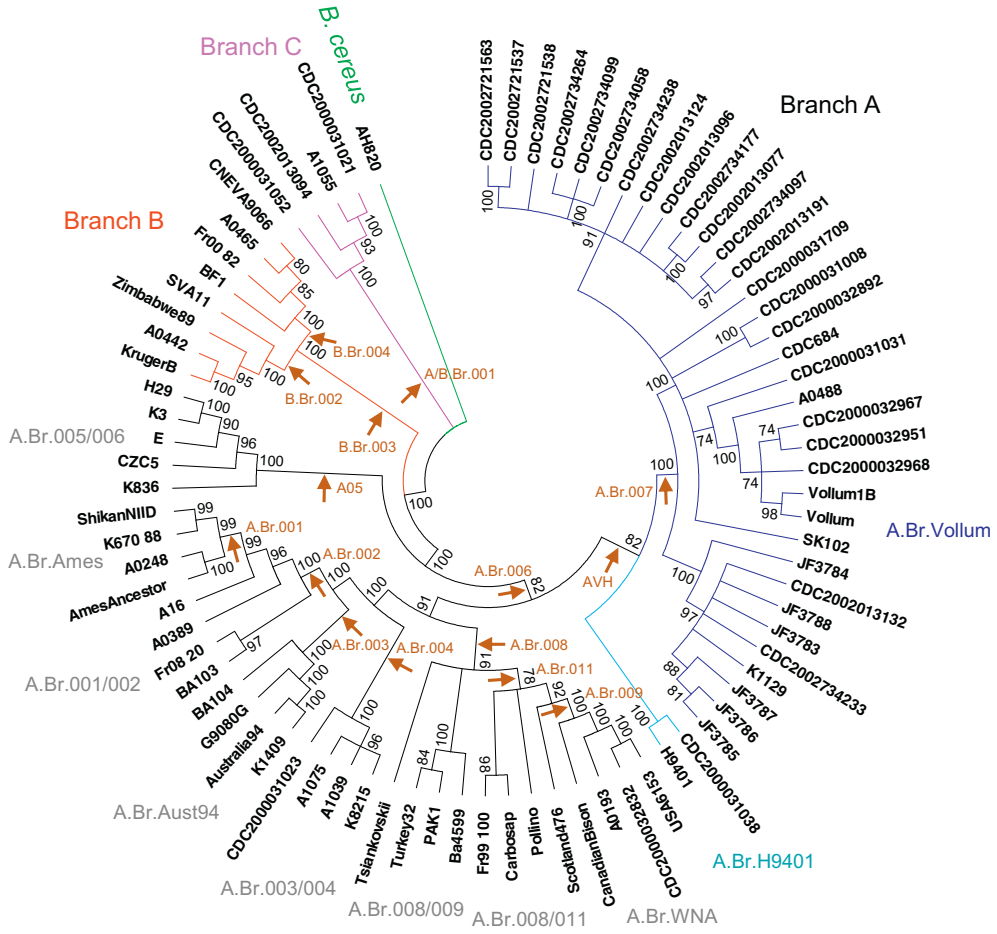

Fig. 1. Position of sequenced A.Br.Vollum-affiliated strains on the B. anthracis phylogenetic tree based on whole-genome SNP analysis. Phylogenetic relationships between 81 *B. anthracis* strains were inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed. Bootstrap scores from 250 replicates are shown next to the branches. The clustering of strains into the 3 major phylogenetic branches is color-coded: branch C in pink, branch B in red and branch A in black. The tree has been rooted using the AH820 *B. cereus* strain as outgroup (in green). The A.Br.Vollum lineage is indicated in blue, while the new proposed A.Br.H9401 lineage is in light blue. Brown arrows indicate the position and name of some published SNPs specific to various canSNP groups: A/B.Br.001 (C clade); B.Br.002, B.Br.003 and B.Br.004 (B clade); A.Br.006 (A clade), A05 (A.Br.005/006 group), A.Br.007 (A.Br.Vollum), A.Br.008 and A.Br.011 (A.Br.008/011 and A.Br.011/009 groups), A.Br.009 (A.Br.WNA), A.Br.004 (A.Br.003/004, A.Br.Australia94, A.Br.001/002 and A.Br.Ames), A.Br.003 (A.Br.Aust94, A.Br.001/002 and A.Br.Ames), A.Br.002 and A.Br.001 (A.Br.001/002 and A.Br.Ames), AVH (A.Br.H9401). There were a total of 39,894 SNPs in the final dataset. Evolutionary analyses were conducted in MEGA7.

India and western China are also dominated by genotypes belonging to the A.Br.Aust94 sub-lineage (Simonson et al., 2009).

The occurrence of lineage A.Br.Vollum throughout the world is thought to be due to the extensive trade of unprocessed animal fibers from Pakistan/Afghanistan, such as mohair and cashmere (Keim et al., 2000; Pilo et al., 2008; Van Ert et al., 2007; Wattiau et al., 2008). Cashmere goat hair was processed in many wool factories during the last century. Trading of commercial quantities of goat down between Asia (Nepal and Kashmir) and continental Europe began in the 19th century (McGregor, 2002; Ripley and Dana, 1861). Today, China and Mongolia have become the largest producers of raw cashmere, while Afghanistan, Iran, Turkey and other Central Asian Republics produce significant but lesser amounts. The Turks were the sole producers of Angora goats (thought to originate from the mountains of Tibet) until the middle of the 19th century. Raw mohair was first exported to the European countries, especially to UK, around 1820. England then became the leading manufacturer of mohair products. In order to face the growing demand for raw mohair, Angora stocks were introduced into South Africa in 1838 (currently the largest mohair producer in the world), the United States in 1849, and Australia from 1856 to 1875 (Chisholm, 1911). The presence of bovine strains in historical collection of the United Kingdom (i.e. A0488 and Vollum) likely indicates past spill over from wool contaminants into the endemic bovine population in this country.

Taking into account (i) the role played by "wool trade" in the proliferation and dispersal of this lineage, (ii) the early divergence of the A.Br.Vollum off the A branch - the A.Br.Vollum lineage appeared soon after the divergence of the African, most basal lineage A.Br.005/006 – and (iii) the importance of the development of human civilization and animal domestication in the natural history of anthrax (Simonson et al., 2009; Van Ert et al., 2007), it is tempting to speculate that the A.Br.Vollum lineage might have originated from the introduction into Southwest Asia of an ancestral African strain and its ecological adaptation to new hosts, i.e. local, ancient breeds of goats and sheep. Goats appear to have already been herded and domesticated animals in Baluchistan at earliest known Neolithic period, back to ca. 10,000 years before present (Fuller, 2006). Wild Markor goats can still be found in the dry mountains of Afghanistan, Iran and Pakistan (Hays, 2009). Sheep were first domesticated in the mountains of Kazakhstan, Turkmenistan, Afghanistan and Iran, from Asiatic moufflon, at least 9000 ago (Fuller, 2006; Hays, 2009). Ancient sheep roamed pastures and grassland with people for at least 11,000 years in Iran and Pakistan (Fuller, 2006). Noteworthy, the closest relatives of those prehistoric sheep are, today, sheep kept off the Shetland Islands in Scotland.

The last interesting finding of this study was the placement of two strains, including H9401, an isolate obtained from a Korean patient

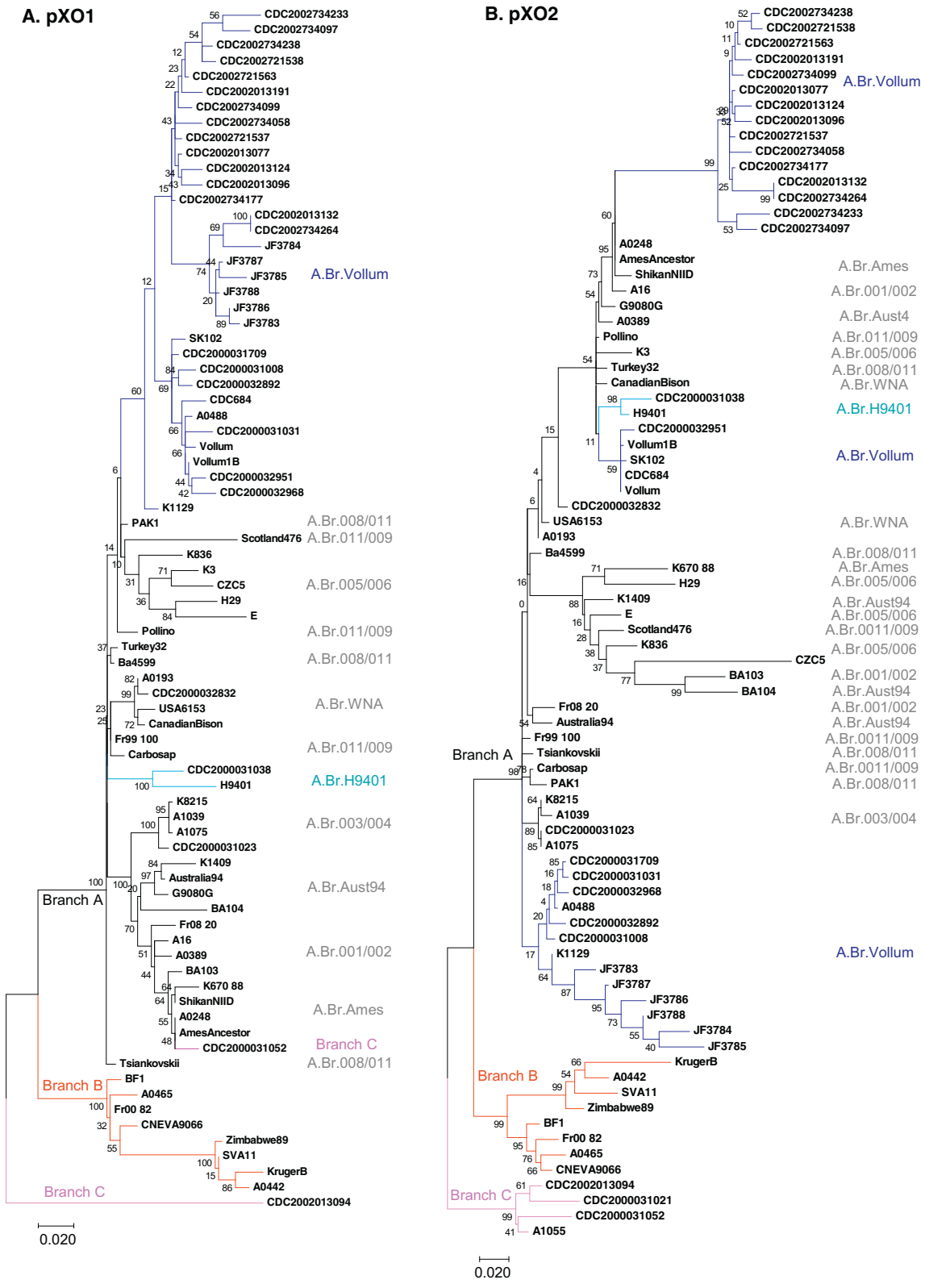

Fig. 2. Molecular phylogenetic analysis of pXO1 and pXO2 SNPs of A.Br.Vollum-affiliated strains by Maximum Likelihood method. Phylogenetic relationships inferred from SNP analysis of the pXO1 (n = 78, A) and pXO2 (n = 80, B) nucleotidic sequences based on the Tamura 3-parameter model. The ML tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Bootstrap scores from 250 replicates). The clustering of strains into the 3 major phylogenetic branches is color-coded: branch C in pink, branch B in red and branch A in black. The A.Br.Vollum lineage is indicated in blue, while the new proposed A.Br.H9401 lineage is in light blue. The three has been rooted using the C clade as outgroup. Clusters with values lower than 50% are not supported. There were a total of 529 (pXO1) and 546 (pXO2) SNPs in the final dataset. Evolutionary analyses were conducted in MEGA7.

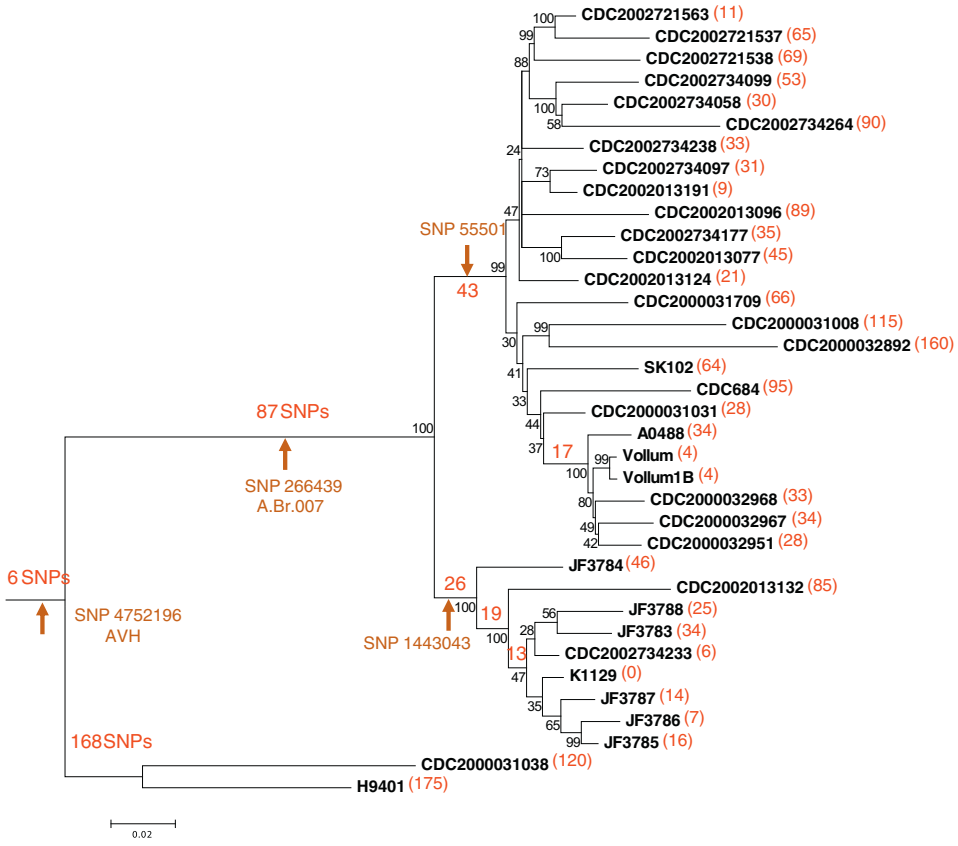

Fig. 3. Evolutionary analyses of the thirty-six B. anthracis strains belonging to the A.Br.Vollum and A.Br.H9401 lineages based on whole-genome SNP analysis. Phylogenetic relationships were inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (A). The ML tree with the highest log likelihood (−18,621.4449) is shown. The tree is drawn to scale, with branch lengths in the number of substitutions per site. Bootstrap values (250 iterations) are shown next to each branch. There were a total of 2546 SNPs positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Indicated in red are the numbers of common SNPs within a cluster. The numbers of unique SNPs is shown in parenthesis for each strain. Brown arrows indicate the position and name of diagnostic SNPs within the A.Br.Vollum and A.Br.H9401 lineages. Evolutionary analyses were conducted in MEGA7.

with gastrointestinal anthrax in 1994, within a new lineage phylogenetically related to A.Br.Vollum. Both strains neither share the derived genotype for the A.Br.007 canSNP, that defines the A.Br.Vollum lineage, nor the derived genotype for the A05 SNP position (Girault et al., 2014b) that characterizes the African A.Br.005/006 group. In silico analysis of ten recently published SNPs that reside along the Vollum branch (Okinaka et al., 2011) confirmed their phylogenetic placement near the most basal node defining Vollum-like strains, sharing 6 unique SNPs, including a derived allele at the 4,752,196 position (SNP AVH, Table 3), exclusively. Separation of the A.Br.H9401 lineage from the Vollum branch appears to have occurred very early in the past, shortly after the most basal node within the A clade (Fig. 1), suggesting that the A.Br.H9401 lineage might have been introduced into Korea from an ancestor that originated from the Fertile Crescent of Southwest Asia, millennia ago. The distance to the tip of the new A.Br.H9401 lineage is approximately 300 SNPs, consistent with a long and independent evolution of this lineage outside Afghanistan/Pakistan. Introduction into Korea of a *B. anthracis* A.Br.Vollum-like ancestor might date back to the eastward diffusion of crops and livestock species from Southwest Asia during the third millennium BC (Nomura et al., 2013; Saisho and Purugganan, 2007). However, more genomes of strains from the Korean peninsula need to be investigated to substantiate this notion. It is worth mentioning that the earliest recorded evidence of the Neolithic transition in East Asia, i.e. the eastward spread of barley in East Asia, is in a Korean archaeological site 2200 years BP, raising the possibility of an earlier diffusion of crops and pastoral animals to Korea by a northern Steppe route. The conventional diffusion of wheat and barley in China (where the *B. anthracis* A.Br.001.002 group dominates) was probably latter, via the central steppe and the "Silk Road" (Fuller et al., 2007; Saisho and Purugganan, 2007).

Table 4
Specific A.Br.Vollum SNPs and primer sequences used for HRM analysis.

Position*	SNP	Forward primer (5′-3′)	Reverse primer (5′-3′)	Mean Tm (°C)	PCR size (bp)
266439 (A.Br.007)	A to G	CCACAAGGTGGAATTATTACTAAAGA	GGTTCACCTGTTTTCGGATCT	73.9 (A) 74.9 (G)	80
55501	G to A	ACAGCATCTCATCTTGAATCAGAA	TTGAATAATTGTTCCCTCACCAA	76.7 (G) 76.0 (A)	70
1443043	C to T	GTCAAGCGACAACTGTTACGTCT	TGTACTTCCTCAGCCGCTTC	80.5 (T) 81.2 (C)	58

* Localisation on the Ames Ancestor chromosome (GenBank accession no. AE017334.2).

5. Conclusion

Comparative whole-genome sequencing offers a powerful way for in-depth characterization of any bacterial pathogen. It also provides an unbiased approach for informative SNPs discovery. In this study, a new picture of the genetic diversity within the population of *B. anthracis* strains of the A.Br.Vollum lineage has been established. The knowledge derived from this work adds unprecedented resolving power to current molecular typing tools and allows tracing of the origin and spread of anthrax during the 19th century.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.10.019.

Competing interests

The authors declare that they have no competing interests.

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