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Molecular diversity of *Bacillus anthracis* in the Netherlands: Investigating the relationship to the worldwide population using whole-genome SNP discovery

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ABSTRACT

Bacillus anthracis, the causative agent of anthrax, has been widely described as a clonal species. Here we report the use of both canonical SNP analysis and whole-genome sequencing to characterize the phylogenetic lineages of *B. anthracis* from the Netherlands. Eleven strains isolated over a 25-years period (1968–1993) were paired-end sequenced using parallel sequencing technology. Five canSNP groups or lineages, i.e. A.Br.001/002 ($n = 6$), A.Br.Aust94 ($n = 2$), A.Br.008/011 ($n = 1$), A.Br.011/009 ($n = 1$) and A.Br.Vollum ($n = 1$) were identified. Comparative analyses, with a focus on SNPs discovery, were carried out using a total of 52 *B. anthracis* genomes. A phylogeographic "Dutch" cluster within the dominant A.Br.001/002 group was discovered, involving isolates from a single outbreak. Diagnostic SNPs specific to the newly identified sub-groups were developed into high-resolution melting SNP discriminative assays for the purpose of rapid molecular epidemiology. Phylogenetic relationships with strains from other parts of the world are discussed.

1. Introduction

Anthrax is an infectious disease caused by the Gram positive bacteria *Bacillus anthracis*. Animals, especially wild and domesticated ruminants are most susceptible, but humans can also be affected, by accident most of the time (WHO, 2008). *B. anthracis* is a pathogen that has an endospore as a part of its life cycle. This spore form is extremely resilient to heat, drought and chemicals. Spores can remain dormant in soils or in the environment for decades before given the chance to infect another host (Dragon and Rennie, 1995). Herbivores ingest or inhale spores while grazing (Hugh-Jones and Blackburn, 2009). Spores then outgrow into a vegetative state and develop a capsule that provides protection from the host's immune system. The bacteria also produce extremely potent toxins which are responsible for the disease symptoms. Rapid multiplication results in toxic shock and death of the host in a few days. Two plasmids, pXO1 and pXO2, carry these virulence factors (Uchida et al., 1985; Koehler, 2002; WHO, 2008).

B. anthracis has the particularity to solely replicate during short periods of time while infecting the host. Hence *B. anthracis* evolves very slowly and represents a genetically extremely homogenous, monomorphic species (Keim et al., 2009). The global population structure is composed of three major genetic clades A, B and C. These groups are presently subdivided into 13 distinct lineages robustly defined by key point mutations (canonical single nucleotide polymorphisms, canSNPs) (Marston et al., 2011; Van Ert et al., 2007).

Anthrax has been a well-known disease for centuries in Europe. Active control measures and large animal vaccination programs during the 20th century have been successful and have reduced the incidence of the disease drastically. Soil contamination in Western Europe was sharply reduced. Although the incidence of the disease may be undervalued (Fasanella et al., 2010), only a few sporadic cases in livestock are still reported in countries adjoining the Mediterranean Sea and in Eastern Europe. In the rest of the continent, new cases are very rare and generally linked to human activities that interfere with soil surface (e.g., works of drainage or excavation, digging, or cleaning out of dams and ditches) in areas with anthrax history (Derzelle and Thierry, 2013). Areas where anthrax has previously stroke are at risk for having recurring outbreaks, as recently observed in Sweden (Ågren et al., 2014).

In the Netherlands, no anthrax in animals has been reported for the last two decades. The last outbreaks in cattle occurred in 1993 in Velden (Limburg, South-East of the Netherlands) and 1991 in Winsum (Groningen, North-East of the Netherlands). However,

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recent finding of bones with viable *B. anthracis* spores at a construction site near Lent (Gelderland, central-East of the Netherlands) indicates that clinical anthrax in Dutch livestock might still be an actual risk (Koene et al., 2015). The Lent village where the bones were found in white pits has a long history of anthrax in cattle. The most recent outbreak in this area occurred in November 1987; an animal was found dead at one of the banks of the River Waal opposite Nijmegen. Laboratory confirmed cases of anthrax from the same meadows were also reported in 1973, 1976, and 1982. Archive information from the local livestock fund and local newspaper mentions anthrax casualties among cattle on a regular base as far back as 1886 (Koene et al., 2015). Human outbreaks in the Netherlands are rare. Since 1976 (when the disease has been made notifiable for humans), seven cases of human anthrax have been reported in the Netherlands. The most recent human infection in 1993 was associated with the post mortem examination of an anthrax infected cow in Velden.

The population structure of *B. anthracis* is known to be largely diversified in Europe, reflecting both ancient and recent introduction of the species in this region of the world (Derzelle and Thierry, 2013). In this report, canSNP typing and whole-genome sequencing were used to characterize eleven isolates from the Netherlands, dating from 1967 to 1993. Comparative genomics were conducted using 41 additional strains belonging to various canSNP lineages to get insight into the worldwide phylogenetic placement of Dutch strains. Identification of hundreds of SNPs retrieved from compiled NGS sequences provided the highest possible resolution for molecular epidemiology and phylogeny. Novel SNPs that specifically discriminate the different sub-groups of Dutch strains were identified and developed into PCR-based genotyping assays.

2. Materials and methods

2.1. DNA extraction

Genomic DNAs were obtained from vegetative cells grown at 37 °C on 5% horse blood agar plates. DNA was purified using the QIAGEN® Genomic-tip 100/G columns and QIAGEN® Genomic DNA Buffer Set as previously described (Girault et al., 2014a). Viability testing was systematically performed before DNA was taken out of the BSL-3 facility. An aliquot of each DNA preparation (a quarter) was spread on Petri dishes and grown at 37 °C for 24 h.

All *B. anthracis* manipulations were performed in a biosafety level 3 laboratory using class II type A2 biosafety cabinet.

2.2. CanSNP typing by high resolution melting (HRM)

CanSNP analysis was performed as previously described (Derzelle et al., 2011; Derzelle, 2015). This technique categorizes isolates into one of 12 sub-lineages (C.Br.A1055, B.Br.CNEVA, B.Br.KrugerB, A.Br.Vollum, A.Br.Aust94, A.Br.Ames, A.Br.WNA, B.Br.001/002, A.Br.005/006, A.Br.003/004, A.Br.001/002, A.Br.008/009). Additional canSNP that provides resolution within the A.Br.008/009, A.Br.001/002, A.Br.Vollum and A.Br.Aust94 groups were also used (Marston et al., 2011; Birdsell et al., 2012; Girault et al., 2014b; Khmaladze et al., 2014; Okinaka et al., 2011).

We designed HRM primers at four canSNPs specific to the Dutch phylogenetic sub-group of *B. anthracis* using Primer 3$^+$ software. The positions of this SNP in the Ames Ancestor genome [GenBank:AE017334.2] and the primers sequences used are listed in Table 1.

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).

2.3. Draft whole genome sequencing (WGS) and data analysis

Strains were sequenced at the IMAGIF sequencing platform (Imagif, Gif sur Yvette, France). Isolates were subjected to paired-end whole genome sequencing on the Illumina HiSeq2000 platform (paired-end data of 2 × 100 bp) (Illumina Inc., San Diego, CA, USA).

Ames Ancestor [GenBank:AE017334.2] was used as the reference genome for assembly. Ames Ancestor plasmid pXO1 [GenBank:AE017336.2] and pXO2 [GenBank:AE017335.3] were used as references for plasmids assembly. Short reads data sets were exported on the FastQ format and mapped to the Ames Ancestor genome and both pXO1 and pXO2 plasmidic sequences using BioNumerics version 6.6 (Applied Maths, Belgium) and Power assembler module asking for a similarity of at least 90%. A set of SNPs was deduced for each genome sequence data using BioNumerics Chromosome Comparisons module. Individual lists were compiled and data filtered to remove SNP positions at which one or more isolates displayed an ambiguous residue call or

Table 1
Specific canSNPs and primer sequences used for HRM analysis.

canSNP	Position[a]	SNP	Target lineage	Forward primer (5′-3′)	Reverse primer (5′-3′)
AVO/A2a′	41872	A to G	A.Br.Vollum	AAAGAACTTTGGGAAAAGATGGA	GACGATCTTCCGTTGTACGAATA
A02/A	4961938	G to A	A.Br.001/002	TTGTTAACGCAACATCTCTACGC	CATTTAATACGCCACCAATTACG
A02/B1-NL	4248098	G to A	A.Br.001/002	CGTAAACGATTCAACGATCATCT	GCGGAAATACATTACATGACTCC
A08/D	108749	C to A	A.Br.008/011	CAAGATGTGTTCATGGGAGATTTC	CTGCACCGTTAATTACGAATGTT
CVI-23932	3330126	G to A	A.Br.Vollum	AGTTCTTCCAAATGATTCGTGTAACT	GGCGGATTAACAGAAGGGG
CVI-56430	1920311	A to G	A.Br.Aust94	CGGGTTTAACGTATTCTAAGGATGT	AATTGTTGGAAGCGCTCTATATCA
CVI-un2	4720332	G to A	A.Br.008/011	GAGCCATTAACCGTGAGAAAGTT	GTTTGTAACGTCAGGAAGTTTGACT
A.Br.13	3101332	A to G	A.Br.Aust94	Reference: Birdsell et al., 2012	
A.Br.14	2824144	T to C	A.Br.Aust94	Reference: Birdsell et al., 2012	
A.Br.15	317219	A to G	A.Br.Aust94	Reference: Birdsell et al., 2012	
A.Br.26	3640599	C to T	A.Br.Aust94	Reference: Khmaladze et al., 2014	
A.Br.27	4355524	C to T	A.Br.Aust94	Reference: Khmaladze et al., 2014	
Unnamed	111199	A to G	A.Br.Vollum	Reference: Okinaka et al., 2011	
A11/3	1975689	G to A	A.Br.011/009 branch 3	Reference: Girault et al., 2014b	

[a] Localisation on the Ames Ancestor chromosome (GenBank Accession No. AE017334.2).

missing data. Ribosomal operons and VNTR loci were also excluded from the analysis, as well as contiguous SNPs (using a window-frame of 10 bp).

2.4. Whole genome phylogenetic analysis

The genomic sequences for 40 available *B. anthracis* strains used for comparison can be found in the NCBI microbial genome website at http://www.ncbi.nom.nih.gov. The whole genome-sequencing of strain IEMVT89 has been previously reported (Girault et al., 2014a).

A minimum spanning tree was drawn in BioNumerics by using the filtered whole genome sequencing SNP data as input. Nodes were numbered by BioNumerics. CanSNPs along the five branches revealed within the A.Br.Vollum lineage were identified from whole genome sequencing data by searching for SNPs with allelic states shared only by these different subgroups.

3. Results and discussion

3.1. canSNP typing and whole genome sequencing

Eleven *B. anthracis* strains from the CVI collection that were isolated in the Netherlands from 1967 to 1993 were selected for whole-genome sequencing (Table 2). All strains were first subjected to canonical single nucleotide polymorphism (canSNPs) analysis using high resolution melting to determine their placement within the larger *B. anthracis* phylogeny (Derzelle et al., 2011; Derzelle, 2015; Marston et al., 2011; Van Ert et al., 2007). Six of the eleven *B. anthracis* isolates from the Netherlands clustered within the A.Br.001/002 canSNP group. Two isolates showed the same canSNP genotype as the A.Br.Aust94 lineage. The three remaining isolates were affiliated to additional groups: the TransEurasian sub-groups A.Br.008/011 and A.Br.011/009, and the A.Br.Vollum lineage.

Dutch isolates were then characterized by paired-end whole genome sequencing. The Illumina sequencers produced millions of reads per strain after applying the quality filter of the Illumina base-calling pipeline. The filter-passed reads were aligned to the Ames Ancestor reference genome, resulting in more than a 50-fold sequencing depth on average and genome coverage higher than 99.7% (Table 2).

3.2. Extraction of whole strain-specific SNPs among B. anthracis strains

Comparative analysis of the genomic sequences of these isolates was carried out using genomic data of 41 additional *B. anthracis* strains of worldwide origin, 33 belonging to various lineages of clade A, seven of clade B and one of clade C (Table 3). A total of 6316 non-homoplasic chromosomal SNPs were identified among this total dataset and used to draw the phylogeny of the Dutch isolates in comparison with the major known clades and lineages. Fig. 1 illustrates the minimum spanning tree (MST) generated by the chromosomal whole-genome SNPs analysis. Additional SNPs located on both pXO1 and pXO2 plasmids were also identified (i.e. 207 and 172 SNPs for pXO1 and pXO2, respectively) (Fig. 2).

Table 3
Whole-genome sequences from public database used in this study.

Strain	Country	canSNP	Accession Number
Ames Ancestor	USA	A.Br.Ames	NC_007530.2
A2012	USA	A.Br.Ames	AAAC00000000.1
Ames	USA	A.Br Ames	NC_003997.3
A0248	USA	A.Br Ames	NC_012659.1
Sterne	South Africa	A.Br 001/002	AE017225.1
A0389	Indonesia	A.Br 001/002	ABLB00000000.1
BA103	Japan	A.Br 001/002	DRR000183 (SRA)
A16	China	A.Br 001/002	CP001970.1
ATCC14185	Israel	A.Br 003/004	AZQO00000000.1
CZC5	Zambia	A.Br 005/006	BAVT00000000.1
Tsiankovskii-1	Soviet Union	A.Br 008/011	ABDN00000000.2
Ba3154	Bulgaria	A.Br 008/011	ANFF00000000.1
Ba3166	Bulgaria	A.Br 008/011	ANFG00000000.1
Ba4599	Scotland	A.Br 008/011	AGQP00000000.1
UR-1	Germany	A.Br 008/011	ALNY00000000.1
Carbosap	Italy	A.Br 011/009	ANAO00000000.1
Sen2Col2	Africa	A.Br 011/009	CAVC000000000.1
Sen3	Africa	A.Br 011/009	CAVD000000000.1
Gmb1	Africa	A.Br 011/009	CAVE000000000.1
Australia 94	Australia	A.Br.Australia94	AAES00000000.1
9080 G	Georgia	A.Br.Australia94	AZUE00000000.1
52 G	Georgia	A.Br.Australia94	AZUF00000000.1
8903 G	Georgia	A.Br.Australia94	AZUD00000000.1
CDC684	USA	A.Br.Vollum	NC_012581.1
H9401	Korea	A.Br.H9401	NC_017729.1
A0488	UK	A.Br.Vollum	ABJC00000000.1
Vollum	UK	A.Br.Vollum	AAEP00000000.1
USA6153	USA	A.Br.WNA	AAER00000000.1
A0174	Canada	A.Br WNA	ABLT00000000.1
A0193	USA	A.Br WNA	ABKF00000000.1
99-100	France	A.Br.011/009	JHDR00000000
08-8/20	France	A.Br.001/002	JHCB00000000
00-82	France	B.Br.CNEVA	JHDS00000000
CNEVA-9066	France	B.Br.CNEVA	NZ_AAEN00000000.1
A0465	France	B.Br.CNEVA	NZ_ABLH00000000.1
BF1	Germany	B.Br.CNEVA	AMDT00000000.1
Kruger B	South Africa	B.Br.Kruger	AAEQ00000000.1
A0442	South Africa	B.Br.001/002	ABKG00000000.1
SVA 11	Sweden	B.Br.001/002	CP006742.1
A1055	USA	C.Br.A1055	AAEO00000000.1

3.3. A.Br.001/002 phylogenetic analysis

As previously described (Girault et al., 2014b), the whole-genome SNP analysis further resolved the A.Br.001/002 subpopulation (in this study represented by15 genomes) into two distinct

Table 2
Whole-sequenced strains, canSNP typing and NGS data.

Strain	Genome coverage[a]	Sequencing depth	Source	Years	canSNP group	Sub-group
CVI-un-1	99.99	151	Unknown	1968	A.Br.001/002	A02/A
CVI-un-2	99.98	132	Unknown	1976	A.B.008/011	Middle East
CVI-260187	99.42	143	Unknown	1987	A.Br.011/009	Branch 3
CVI-56430	99.57	137	Lent, dairy cattle strain	3-November-1987	A.Br.Aust94	A.Br.13/15/26
CVI- 23932	99.98	148	Dog	1989	A.Br.Vollum	AVO/A2
CVI-127491 V08551	99.90	140	Winsum, bull (case 1)	July 1991	A.Br.001/002	A02/B
CVI-128268	99.99	132	Winsum, bull (case 2)	14-August-1991	A.Br.001/002	A02/B
CVI-13185	99.99	163	Winsum, beef cattle (case 3)	19-October-1991	A.Br.001/002	A02/B
CVI-131959-5	99.99	154	Winsum, dairy cow (case 5)	28-October-1991	A.Br.001/002	A02/B
CVI-132064-1	99.99	140	Winsum, cattle (case 7)	30-October-1991	A.Br.001/002	A02/B
CVI-188678-1	99.98	146	Velden, outbreak dairy cattle	31-August-1993	A.Br.Aust94	A.Br.26/27

[a] Genome coverage are based on the Ames Ancestor genome size (5227419 pb).

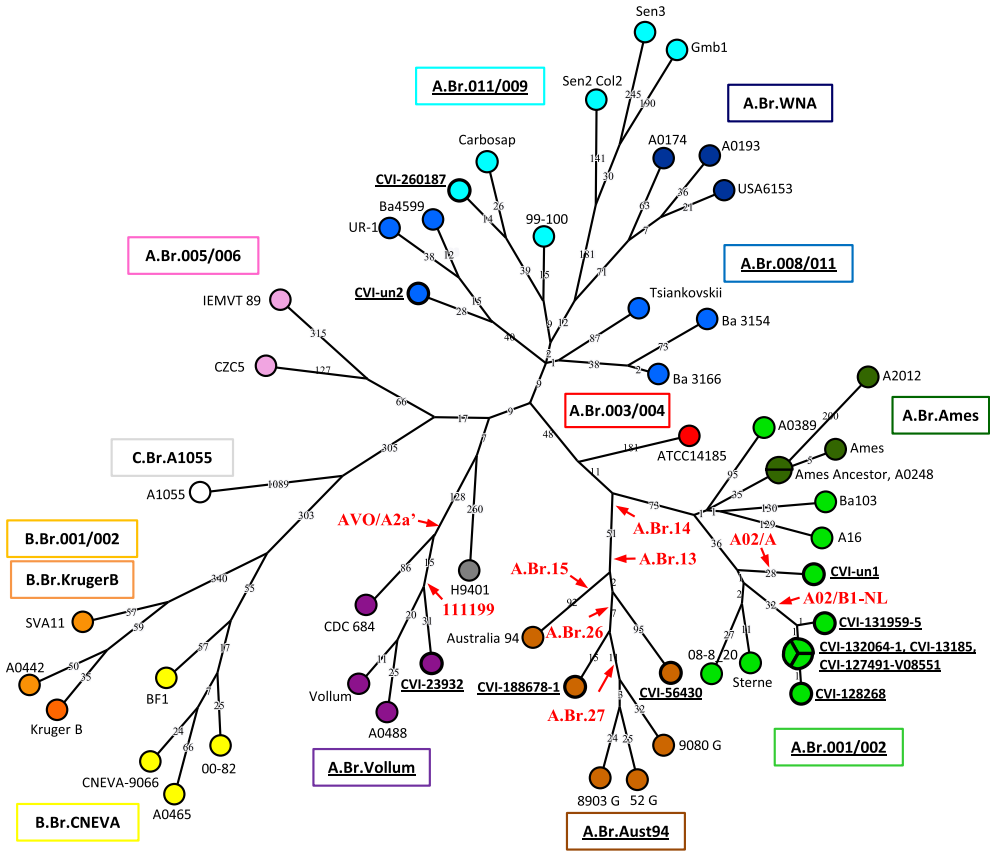

Fig. 1. Position of the eleven Dutch strains on the *B. anthracis* phylogenetic tree based on whole-genome SNP analysis. Minimum spanning tree based on 6316 chromosomal SNPs. The 13 different canSNP groups are color-coded: C.Br.A1055 in white, B.Br.CNEVA in yellow, B.Br.001/002 and B.Br.Kruger in orange, A.Br.011/009 in light blue, A.Br.008/011 in blue, A.Br.WNA in dark blue, A.Br.005/006 in pink, A.Br003/004 in red, A.Br001/002 in green, A.Br.WNA in dark green, A.Br.Aust94 in brown and A.Br.Vollum in purple. The position of the 11 newly sequenced isolates from the Netherlands (bold and underlined), the African IEMVT89 strain and 40 available whole genome-sequenced strains is marked. The length of each branch is proportional (logarithmic scale) to the number of SNPs identified between strains. Indicated in red is the position of some new or published SNPs specific to various canSNP sub-groups: A.Br.13, A.Br.14, A.Br.15, A.Br.26 and A.Br.27 (A.Br.Aust94); SNP 111199 and AVO/A2a′ (A.Br.Vollum); A02/A and A02/B1-NL (A.Br.001/002 subgroup A02); A08/D (A.Br.008/011) and A11/3 (A.Br.011/009 Branch 3). Total tree size is 6457, i.e. it contains approximately 2.2% of homoplasia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phylogenetic sub-groups, A01 and A02. The first one contains the A.Br.Ames lineage (strains Ames Ancestor, Ames, A2012 and A0248) and three strains isolated in China (A16), Japan (Ba103) (Kuroda et al., 2010) or Indonesia (A0389). The A01 sub-group (also termed "Ames sub-group") radiates very shortly after the A01-A02 divergence (1 SNP) into at least three sub-branches (Fig. 1).

The A02 sub-group (also termed "Sterne sub-group") includes the terminal reference Sterne vaccine strain. The six Dutch A.Br.001/002 isolates cluster within the A02 sub-group that also contains French A.Br.001/002 strains (08-8_20) isolated from the Doubs *department* in the North East of France (Girault et al., 2014a). Five of the Dutch A.Br.001/002 isolates are closely related to each other, differing by a maximum of three chromosomal SNPs. Similar SNP patterns were found using both virulence plasmids data (Fig. 2).These isolates were collected during a series of anthrax outbreaks on a livestock farm in Winsum (Groningen). The first case in July 1991 (CVI127491-V08551) involved the death of a bull. Apparently the possibility of anthrax was not taken into account and the carcass was opened on the farm, leading to contamination of the premises. Until November 1991 at least four additional outbreaks occurred among dairy and beef cattle on the farm: on August 14th (CVI128268), October 19th (CVI131185), October 28th (CVI131959) and October 30th (CVI-128268) (Table 2). These ultimately resulted in nine animal casualties. During this period the herd was vaccinated and animals with elevated body temperature were treated with antiserum produced by CVI. We identified 63 SNPs that differentiated these isolates from the sixth Dutch A.Br.001/002 isolate, of which 32 were unique to the above Dutch sub-cluster and 28 unique to the CVI-un1 strain. No information is available on the origin of this isolate from 1968.

The A.Br.001/002 group may have originated in China (Simonson et al., 2009) but is also distributed at higher latitudes in Western Europe. It accounts for a significant part of the European *B. anthracis* distribution in Denmark, Belgium, the United Kingdom, the Netherlands and was also found in northeastern France (Derzelle and Thierry, 2013; Girault et al., 2014a).

3.4. A.Br.008/009 phylogenetic analysis

The two TransEurasian (TEA) Dutch A.Br.008/009 isolates CVI-260187 and CVI-un2 were recovered in 1986 (A.Br.011/009) and 1976 (A.B.008/011) respectively. Unfortunately documentation on their origin is lacking. These isolates were found to be phylogenetically related to (i) the Carbosap vaccine and all French strains (including 99-100) belonging to the third branch of the A.Br.011/009 sub-groups previously described (Girault et al., 2014a); or to (ii) heroin-associated strains of the A.Br.008/011 sub-group isolated during the heroin outbreaks occurring among drug users in Europe from 2000 to 2013 (Price et al., 2012; Hanczaruk et al., 2014). The TEA group has been found in most European countries. It is well established in Southern and Eastern Europe and represents the dominant subgroup in Italy, Bulgaria, Hungary and

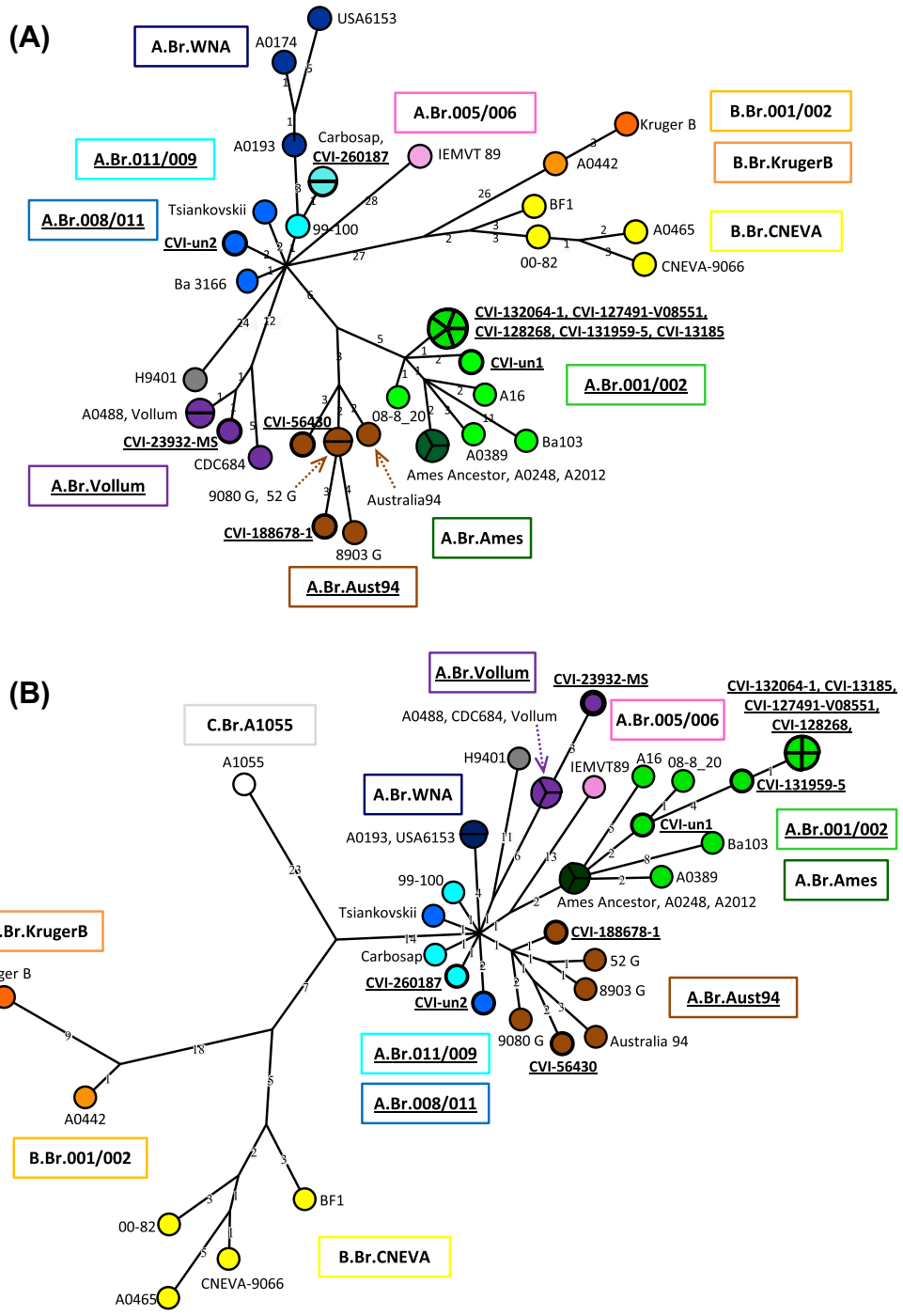

Fig. 2. Position of the Dutch strains on the *B. anthracis* phylogenetic tree based on plasmidic SNP analysis. Minimum spanning tree based on 207 pXO1 SNPs (A) and 172 pXO2 SNPs (B). The 13 different canSNP groups are color-coded: C.Br.A1055 in white, B.Br.CNEVA in yellow, B.Br.001/002 and B.Br.Kruger in orange, A.Br.011/009 in light blue, A.Br.008/011 in blue, A.Br.WNA in dark blue, A.Br.005/006 in pink, A.Br.003/004 in red, A.Br.001/002 in green, A.Br.Ames in dark green, A.Br.Aust94 in brown and A.Br.Vollum in purple. The position of the 11 newly sequenced isolates from the Netherlands (bold and underlined), the African IEMVT89 strain and 40 available whole genome-sequenced strains is marked. The length of each branch is proportional (logarithmic scale) to the number of SNPs identified between strains. Total tree size is respectively 210 and 175 SNPs for pXO1 and pXO2 trees, i.e. it contains approximately 1.5% of homoplasia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Albania (Derzelle and Thierry, 2013; Van Ert et al., 2007; Peculi et al., 2015).

3.5. A.Br.Aust94 phylogenetic analysis

To obtain an accurate placement of the two A.Br.Aust94 Dutch isolates from the Lent (CVI-56430) and Velden (CVI-188678-1) outbreaks, we further screened both genomes using a set of recently published A.Br.Aust94-specific SNP assays (Birdsell et al., 2012; Khmaladze et al., 2014). CVI-188678-1 was isolated from cattle during the last anthrax outbreak occurring in the Netherlands in 1993 on a dairy farm in Velden (Limburg) along the banks of the river Meuse. Construction activities at the site involved digging which supposedly brought spores to the surface. This isolate shares the derived allele for canSNP A.Br.026, at the most basal node of the branch leading to the recently published

52-G Georgian strain. Most Turkish A.Br.Aust94 isolates ($n = 32$) were assigned to this basal node (Khmaladze et al., 2014).

CVI-56430, originating from Lent with a long history of anthrax, fits in the A.Br.013/015 node of the A.Br.Aust94 lineage that also contains two isolates from Europe (i.e. Germany and the United Kingdom) and eight from the USA (Khmaladze et al., 2014). This genetic group is not ecologically established in these parts of the world. Rather, the presence of these isolates in Europe and the USA is likely to be due to the importation of contaminated animal products from Asia. The A.Br.013/015 sub-group gives rise to the lineages that contain all Georgian isolates, as well as the lineage leading to the terminal reference Australia 94 genome (Khmaladze et al., 2014).

3.6. A.Br.Vollum

The last Dutch isolate, CVI-23932, from 1989 was isolated from a dog that was submitted for post mortem examination on the suspicion of rabies. However bacteriological examination rapidly revealed anthrax. Unfortunately, no further information on the origin or source of infection is available. The isolate clusters in lineage A.Br.Vollum together with three other sequenced strains: Vollum, A0488 and CDC684 (Fig. 1). The Dutch specimen is somehow related to the A.Br.Vollum terminal reference genome A0488 and the Vollum strain, both isolated in the UK in the 1930s and 1960s. The A.Br.Vollum lineage reveals to contain a particularly large number of strains, mostly isolated from humans from various, most distantly related countries world-wide. But this lineage was traced back to strains originating from the Middle-East (Van Ert et al., 2007).

A.Br.Aust94 and A.Br.Vollum are among the minor lineages introduced in Europe that are believed to originate from Southeast Asia or the Middle East, as a consequence of human activities and international trade exchanges of animal products contaminated with *B. anthracis* spores. The link between the Asian origin of these lineages and their presence in the Netherlands is not surprising as the Dutch East India Company (*Vereenigde Oostindische Compagnie, VOC*) is often considered to have been the first multinational corporation in the world and the first company to issue stock. Between 1602 and 1796 the VOC sent almost a million Europeans to work in the Asia trade on 4785 ships, and netted for their efforts more than 2.5 million tons of Asian trade goods (Chambers, 2006; Gaastra, 2009).

3.7. Specific SNP discrimination genotyping assay

Two specific canSNPs (A02/B1-NL and A02/A) that define the Dutch A.Br.001/002 clusters were developed into HRM assays. Two other assays, specific to the A.Br.008/011 cluster including the heroin-associated isolates and CVI-un2 (A08/D) or to the A.Br.Vollum cluster including CVI-23932 and CDC 684 (19) (AVO/A2a′), were designed (Table 1, Fig. 1). Three SNP specific, respectively, to strains CVI-23932, CVI-56430 or CVI-un2 were also developed into assays. These novel diagnostic assays were validated against 250 *B. anthracis* DNAs in collection. The two expected alternate alleles exhibited distinct melting curves and melting temperatures, allowing unambiguous discrimination of the Dutch isolates within their respective genetic sub-groups (data not shown).

4. Conclusion

These data show that different lineages of *B. anthracis* have been involved in various outbreaks in the Netherlands in the period between 1967 and 1993. This is consistent with findings in other European countries (Derzelle and Thierry, 2013).

As previously reported, three sublineages appear to be more largely distributed in Europe and may be ecologically established on a sub-continental scale: the A.Br.008/009 (TransEurasian group), B.Br.CNEVA and A.Br.001/002 canSNP groups (Derzelle and Thierry, 2013; Girault et al., 2014b). Although B. Br.CNEVA was not represented among the Dutch isolates, two of the eleven isolates were appointed to the TransEurasian group and six belonged to the A.Br.001/002 group. Of the latter, genotyping neatly confirmed the close relationship of five isolates from a series of outbreaks on a single farm.

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