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Research Paper

Comparative genomics and phylogenetic analysis of *Bacillus anthracis* strains isolated from domestic animals in Japan

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ABSTRACT

Anthrax, caused by *Bacillus anthracis*, is a severe zoonosis with a great impact on both human and animal health. In the present study, we identified the phylogenetic relationships among 16 Japanese strains of *B. anthracis,* including eight bovine strains, two equine strains, five swine strains, and one former vaccine strain, using in silico canonical single nucleotide polymorphism (canSNP) and core genome SNP analyses. The results of our in silico canSNP analysis suggest that these 16 Japanese strains could be divided into four lineages: i) one equine strain in A.Br.Ames, ii) one equine and six bovine strains in A.Br.001/002, iii) five swine and one bovine strain in A.Br.Aust94, and iv) one bovine and one vaccine strain in A.Br.008/011. A comparison with non-Japanese *B. anthracis* strains revealed a total of 3787 SNPs identified from the whole genome sequences of the Japanese strains; these SNP data were subjected to a phylogenetic analysis using the maximum parsimony (MP) method. Our core genome SNP analysis was also able to detect differences of a few chromosomal SNPs across clonal strains from the same cases that had different storage and passage histories.

Additionally, our whole genome SNP analysis clearly indicated that the Japanese swine anthrax cases of 1982 were caused by at least three independent strains; however, their phylogeny revealed no clear relationship with swine strains from other countries. The bovine strain belonging to the A.Br.008/011 lineage differed from a former Japanese vaccine strain by only 12 SNPs. Together with the phylogenic results and epidemiological circumstances, the diversity of strains reveals that the *B. anthracis* available in Japan probably resulted from multiple relatively recent import events, rather than reflecting the persistence of a more ancient ecologically established group.

1. Introduction

Bacillus anthracis, a Gram-positive spore-forming bacterial species, is the causative agent of the severe zoonosis anthrax. *B. anthracis* is a member of *Bacillus cereus* sensu lato, which comprises nine species of Gram-positive, endospore-forming bacteria including *B. cereus* and *B. thuringiensis* (Carroll et al., 2017). *B. cereus* is widely reported as a soil bacterium, and it also occurs in the rhizosphere of some plants (Vilain et al., 2006). Because of the wide presence of *B. cereus* in many food products, these bacteria are inevitably ingested in small numbers and thus contribute to the transitory intestinal flora. *B. thuringiensis* is generally regarded as an insect pathogen because of its ability to produce large crystal protein inclusions during sporulation; this is the only feature that can distinguish *B. thuringiensis* from *B. cereus* (Jensen et al., 2003). The frequent exchange of pathogenicity factors on plasmids via horizontal gene transfer in *B. cereus* and *B. thuringiensis* appears to be an important mechanism of their virulence evolution (Böem et al., 2015). In contrast, as a specialized animal and human pathogen, *B. anthracis* has most likely proceeded through a stepwise, reciprocal adaptation between its chromosomal and extrachromosomal genomes (Jensen et al., 2003). *B. anthracis* spores can survive for decades in the environment, such as in the soil, without proliferation (Dragon and Rennie, 1995; Hugh-Jones and Blackburn, 2009). Since *B. anthracis* is a relatively new species, having diverged only very recently from *B. cereus*, this pathogen has much less genetic variation than many other bacterial species with similar generation times (Keim et al., 2009; Van Ert et al., 2007a). For this reason, *B. anthracis* is genetically and phenotypically extremely homogeneous. Therefore, conventional methods such as serology, biochemical tests, and phage typing are not appropriate to differentiate *B. anthracis* strains (Keim et al., 2009; Pilo and

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Abbreviations: MLVA, multiple locus variable-number tandem repeats analysis; SNP, single nucleotide polymorphism.; canSNP, canonical single nucleotide polymorphism.; MP, maximum parsimony.; HRM, high-resolution melting.

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Frey, 2011; World Health Organization, 1998). Fortunately, whole genome sequencing (WGS) has provided unprecedented levels of resolution and phylogenetic accuracy using single nucleotide polymorphisms (SNPs) (Pearson et al., 2004, 2009).

Canonical single nucleotide polymorphisms (canSNPs) have since provided a shortcut to WGS because canSNP assays can be built to target the SNPs that define clades (Birdsell et al., 2012; Keim et al., 2004; Van Ert et al., 2007b). Presently, the in silico assessment of established canSNPs from WGS can offer a rapid method of assigning isolates to clades. Comparative genome analyses, using core genome SNPs, have revealed phylogenetic lineages from worldwide anthrax cases (Derzelle et al., 2015a, b, 2016a, b; Girault et al., 2014a; Sahl et al., 2016; Simonson et al., 2009; Vergnaud et al., 2016), as well as from injectional anthrax cases (Keim et al., 2015; Price et al., 2012).

In Japan, there were several anthrax outbreaks each year among bovine and equine animals before the 1980s. However, anthrax has become quite rare of late; the most recent incidents occurred in 1994 and in 2000 in humans and bovine livestock, respectively. We had previously analyzed 12 *B. anthracis* strains in Japan by performing a multiple locus variable-number tandem repeats analysis (MLVA 25) (Okutani et al., 2010) and 80-tag SNP typing (Kuroda et al., 2010), and we found that all the strains belonged to either the A3a or the A3b cluster. Thereafter, we reported the whole genome sequences of three other strains, which belonged to the A3b cluster (Okutani et al., 2015). The whole genome sequences of strains BA103 (bovine origin, A3b cluster by MLVA 25), Shikan-NIID (equine origin, A3b cluster by MLVA 25), and BA104 (swine origin, A3a cluster by MLVA 25) were used for a global comparative genetic analysis using whole genome SNPs (Derzelle et al., 2015a, b, 2016a, b). In the present study, we conducted WGS of additional *B. anthracis* strains that were isolated from animals between 1928 and 1991 as well as one vaccine strain. We also phylogenetically assessed these strains using canSNPs and core genome SNP analyses and compared their sequences with those of *B. anthracis* strains from other countries.

Some of the strains used in the genetic analyses from our previous work had obscure epidemiological information, e.g., the isolation year, isolation origin, or infection sources were unknown. This limitation prevented us from furthering our understanding of the relationship between epidemiological impact and genetic characteristics for those strains. To avoid similar limitations in the present study, we selected and focused on strains for which epidemiological information about the source and isolation location in Japan was available. Eight bovine strains had been obtained from independent anthrax cases between 1969 and 1991, and five swine strains had been isolated from the sporadic swine anthrax cases in 1982. Combining these with two equine strains and one vaccine strain provided a total of 16 strains to analyze in the present study. Because canSNP analysis had not yet been applied to these Japanese strains of *B. anthracis*, we first determined the canSNP lineages of these strains to reveal their relative phylogenetic positions. A core genome SNP analysis was then performed using the extracted SNPs from whole genome sequence data for comparison with those of *B. anthracis* strains from other countries.

2. Materials and methods

2.1. DNA extraction

The 16 strains of *B. anthracis* used in the present study are listed in Table 1. All these strains are part of a national collection. Strains BA102–BA111 were previously used for an MLVA analysis (Okutani et al., 2010); they were also analyzed in the present study, except for strains BA106 and BA110. Two equine strains (Shikan-NIID and Morioka), one bovine strain (Akita-80), and the former vaccine strain PAII were all stored in the National Institute of Animal Health (NIAH), Tsukuba, Ibaraki prefecture. Vaccine strain PAII was originally provided by the Pasteur Institute in 1897. A stock of this strain has been stored at the NIAH, Japan since 1950. Strains Tokushima1, Gunma1, 6166, and 6172 were stored in Kitasato University, Towada, Aomori prefecture. Strains 6166 and 6172 had been isolated from swine anthrax in Shizuoka prefecture. Strains 6166 and BA104 were isolated from the same anthrax case that occurred on July 8, 1982, and strains 6172 and BA105 were both isolated from a single case on July 17, 1982 (Amano et al., 1987). Live *B. anthracis* were cultured in biosafety level 3 (BSL-3) facilities according to the Act on the Prevention of Infectious Diseases and Medical Care for Patients with Infectious Diseases, as authorized by the biological risk management committee of the National Institute of Infectious Diseases.

The whole genomes of strains Shikan-NIID, BA103, and BA104 had previously been sequenced and reported as AP014833.1, DRR000183, and DRR000184, respectively (Okutani et al., 2010, 2015). The remaining strains, whose whole genomes were sequenced in the current study, were incubated overnight at 37 °C on sheep blood agar plates (Eiken Chemical, Tokyo, Japan) for DNA preparation. One loop of cells from each of the agar plates was inoculated into Luria-Bertani (LB) broth, and each overnight culture was lysed in lysis buffer (20 mmol/L Tris HCl, pH 8.0, 2 mmol/L EDTA [pH 8.0], 1.2% Triton X 100, and 0.2% SDS) and achromopeptidase (Wako Chemical, Tokyo, Japan) at a final concentration of 20 mg/mL After a 30-min incubation at 37 °C, proteinase K was added at a final concentration of 1.0 mg/mL, and the samples were then incubated overnight at 56 °C. An RNase A solution was added at a final concentration of 20 mg/mL, after which the samples were incubated at 25 °C for 2 min. DNA was extracted using phenol-chloroform, centrifuged for 20 min at 15,000 × *g*, and recovered in the aqueous phase (Sambrook and Russell, 2006). After the addition of two volumes of ice-cold 95% ethanol, the precipitated DNA was collected by centrifugation at 15,000 × *g* for 20 min. The DNA was then dissolved in MilliQ water or TE buffer (10 mmol/L Tris and 1.0 mmol/L EDTA [pH 8.0]). The quantity and quality of DNA were analyzed using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific), respectively, before the DNA was sequenced. The loci of the previous MLVA results were revised because tandem repeat numbers of the *vrrB2* gene were recounted in accordance with the proposed 2014 rule (Thierry et al., 2014).

2.2. Whole genome sequencing (WGS) analysis and phylogenetic analysis

DNA libraries were prepared using a NEBNext DNA library prep Master Mix kit for Illumina (New England Biolabs Japan, Tokyo, Japan) with index primers from NEBNext Multiplex Oligos for Illumina (New England Biolabs Japan) in accordance with the manufacturer's instructions. The libraries were applied to Illumina MiSeq (Illumina Inc., San Diego, CA, USA) by 300 paired-end sequencing with a MiSeq reagent kit v3 (600 cycles). *B. anthracis* Ames Ancestor (GenBank accession number NC_007530.2) was used as a reference strain for the chromosomal sequence in core genome SNP discovery, which was performed by applying the raw sequence data into the GcoGSA-BA pipeline (Yamashita et al., 2015). The concatenated SNP sequences were aligned by MUSCLE using MEGA7 (Kumar et al., 2016), and the phylogenetic analysis was conducted in MEGA 7 using the MP method. The MP tree was obtained using the Subtree-Pruning-Regrafting algorithm (Nei and Kumar, 2000), and the consistency index, the retention index, and the composite index for all sites were calculated by MEGA 7 (Archie, 1989; Farris, 1989; Nei and Kumar, 2000). Whole genome sequences of *B. anthracis* strains that had originated worldwide and were obtained from a public database to be used for comparison in the present study are listed in Table 2. Reliable SNP sites with at least five times coverage depth were selected from the mapped sites. The SNP positions are listed in the Supplementary Tables.

2.3. In silico canSNP analysis

The resulting reads, excluding the failed and duplicate ones, were

Table 1
Profiles of *B. anthracis* strains used in this study, along with their canSNPs, accession numbers, cluster and tandem repeats from MLVA analysis.

Strain name	Year/Month	Source	Isolated from	*In-silico* canSNP group	Accession Number	Cluster by MLVA25[d]	*vrrB2*	CG3	pXO2-at
Shikan-NIID	1928	equine	Tokyo	A.Br.Ames	AP014833.1	A3b	53	2	7
Morioka	before 1940	equine	Iwate	A.Br.001/002	DRR128181	A3b	53	2	–
Akita-80	1980	bovine	Akita	A.Br.001/002	DRR128182	A3b	53	2	9
BA102	1983	bovine	Miyagi	A.Br.001/002	DRR128183	A3b	53	2	9
BA103	1991	bovine	Miyagi	A.Br.001/002	DRR000183	A3b	53	2	9
BA108	1987	bovine	Shiga	A.Br.001/002	DRR128186	A3b	53	2	7
BA111	1985	bovine	Okayama	A.Br.001/002	DRR128188	A3b	53	2	8
Tokushima1	before 1984	bovine	Tokushima	A.Br.001/002	DRR128189	A3b	53	2	8
BA104	1982/July[a]	swine	Shizuoka	A.Br.Aust94	DRR000184	A3a	57	2	18
6166	1982/July[a]	swine	Shizuoka	A.Br.Aust94	DRR128192	A3a	57	2	16
BA105	1982/July[b]	swine	Shizuoka	A.Br.Aust94	DRR128184	A3a	57	2	9
6172	1982/July[b]	swine	Shizuoka	A.Br.Aust94	DRR128191	A3a	57	2	9
BA107	1982/Feb	swine	Okinawa	A.Br.Aust94	DRR128185	A3a	57	2	9
BA109	1969	bovine	Mie	A.Br.Aust94	DRR128187	A3a	57	2	9
Gunma1	before 1984	bovine	Gunma	A.Br.008/011	DRR128190		57	1	8
PAII[c]	1950	vaccine strain	PAII	A.Br.008/011	DRR152630		57	1	8

[a] These strains were isolated from the same incident in Shizuoka prefecture on July 8, 1982.

[b] These strains were isolated from the same incident in Shizuoka prefecture on July 17, 1982.

[c] Strain PAII was a former live vaccine strain in Japan. For further details, please refer to the Materials and Methods section.

[d] Referred by Okutani et al., 2010.

mapped to a *B. anthracis* Ames Ancestor chromosomal reference sequence using the CLC Genomics Workbench v9.5.3 (Qiagen, Hilden, Germany). Genotypes were determined based on the 13 published canonical SNPs (Van Ert et al., 2007b) along with some additional SNPs that were described later (Girault et al., 2014b).

2.4. SNP discrimination by high-resolution melting (HRM) assays

Japanese strain-specific diagnostic SNPs within the A.Br.001/002 lineage were identified from the whole genome SNP data (Supplemental Table for Fig. 2). We designed primers corresponding to these SNPs for HRM assays using Primer3Plus software [https://primer3plus.com/cgi-bin/dev/primer3plus.cgi]. The positions of these SNPs on the Ames Ancestor chromosome and the primer sequences used are listed in Table 3. Amplification was performed on a LightCycler® 480 (Roche Life Science, Penzberg, Germany) using the LightCycler® 480 High-Resolution Melting Master (Roche Life Science). Briefly, the reaction mixture consisted of 0.2 μM of each primer, 1 × LightCycler® 480 HRM master mix, and 2.5 mmol/L $MgCl_2$ in a 10-μl final volume. The run-parameters were: 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 58 °C, and 20 s at 72 °C. Samples were then heated from 65 °C to 88 °C, at a rate of 1.0 °C/s with 25 acquisitions/°C. The experiments were repeated four times, and the resulting HRM data were analyzed using the LightCycler® 480 Software (v1.5.1).

2.5. Availability of sequence data

Whole genome data of the strains sequenced in the present study were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers DRR128181 to DRR128192 and DRR152630.

3. Results

3.1. Anthrax in Japan and WGS of Japanese B. anthracis strains

We characterized 16 Japanese *B. anthracis* strains by paired-end WGS to infer their epidemiological and genetic relationships. Sequencing produced 0.19–7 million reads per strain that were applied to the GcoGSA-BA pipeline for acquiring core genome SNPs from chromosomes. The canSNP group of each of these strains was verified in silico and used to measure the genetic distances and variations of the strains. Chromosomal SNPs were identified from the sequence data of 90 *B. anthracis* strains, including Japanese strains and those originating worldwide, that were preset in the GcoGSA-BA pipeline against the Ames Ancestor reference genome. Fig. 1 shows the phylogenetic tree that was generated by the MP method based on these chromosomal SNPs using *B. cereus* AH820 as an outgroup to root the tree. The resulting MP tree indicated that the 16 Japanese strains from the present study could be divided into four major branches correlating with the canSNP groups A.Br.Ames, A.Br.001/002, A.Br.Aust94, and A.Br.008/011. All the strains previously reported as belonging to the A3a cluster based on an MLVA 25 analysis (Okutani et al., 2010) were classified into the A.Br.Aust94 lineage, and all the strains previously assigned to the A3b cluster by MLVA 25 were classified into the A.Br.001/002 and A.Br.Ames lineages (Table 1). The bovine strain from Gunma prefecture and the former Japanese vaccine strain PAII were classified into the A.Br.008/011 lineage. This is the first report of Japanese *B. anthracis* strains belonging to the A.Br.008/011 lineage.

3.2. Phylogenetic analysis of the A.Br.001/002 and A.Br.Ames lineages

Strain Shikan-NIID was isolated in 1928 (Table 1) from one of the war-horses bred at the Military Academy of the former Japanese army in Tokyo, and strain Morioka isolated in Iwate prefecture was also isolated from a war-horse of the army. Iwate prefecture was one of the major horse breeding areas at the time. The Shikan-NIID strain is the only strain from this study found to belong to the A.Br.Ames lineage (Table 1, Figs. 1 and 2A). We conducted a phylogenetic analysis with the MP method using 435 SNPs (Supplemental Table for Fig. 2A) from the chromosomes of strains belonging to the A.Br.Ames and A.Br.001/002 lineages (Fig. 2A). The Shikan-NIID strain was the most closely related to Danish strains K35/88 and K670/88 (Derzelle et al., 2015a), with 18 SNP differences. Within the A.Br.Ames canSNP lineage, the Shikan-NIID strain was observed to belong to its own branch, separate from the branches containing the Ames Ancestor or the related Danish strains (Fig. 2A). Strain Shikan-NIID possessed an A01/A-DK SNP-derived allele that was shared with the Danish isolates K670/88 and K35/88 (Derzelle et al., 2015a).

The six bovine strains and one equine strain assessed in the present study belonged to the A.Br.001/002 lineage and were divided into three phylogenetic groups by SNP515111, SNP240050, and SNP88730 based on the Ames Ancestor genome: A01 and A02, which were both previously described by Girault et al. (Girault et al., 2014b), and A03, which is based on SNP88730 (Fig. 2A). The A01 group included four Japanese strains, of which strains BA102, Morioka, BA103, and Akita-80 were isolated in the northern part of Japan (from the Miyagi, Iwate,

Table 2
Whole-genome-sequenced *B. anthracis* strains (from public databases) used for comparison in this study.

Strain	Country	canSNP-defined lineage	Source	Year	Accession number
A1055	United States of America	C.Br.A1055	soil	missing data	NZ_AAEO01000000
BF1	Germany	B.Br.004	cow	1997	AMDT01000000
CNEVA	missing data	B.Br.CNEVA	missing data	2009	NZ_AAEN01000000
KrugerB	South Africa	B.Br.KrugerB	missing data	missing data	NZ_AAEQ01000000
A0465	France	B.Br.004	cattle	1997	NZ_ABLH01000000
A0026	England	A.Br.005/006	equine	1992	SRR2968152, PRJNA302749, SAMN04283797
A0006	Australia	A.Br.005/006	missing data	missing data	SRR2968150, PRJNA302749, SAMN04283803
A16Rchina	China	A.Br.Ames	missing data	missing data	CP001974.1, CP001975.1
Han	China	A.Br.Ames	bovine	2012	CP008854.1
K3588	Denmark	A.Br.Ames	bovine	1988	ERR930298
K67088	Denmark	A.Br.Ames	bovine	1988	ERR930297
A0248	United States of America	A.Br.Ames	human	1968	CP001598.1,CP001599.1,CP001597.1
A0389	Indonesia	A.Br.001/002	goat	missing data	NZ_ABLB01000000
Sterne	SouthAfrica	A.Br.001/002	missing data	missing data	NC_005945
A	Denmark	A.Br.001/002	bovine	1935	ERR930302
B	Denmark	A.Br.001/002	bovine	1935	ERR930303
C	Denmark	A.Br.001/002	bovine	1960	ERR930304
K929	Denmark	A.Br.001/002	bovine	1966	ERR930299
ANSES08–20	France	A.Br.001/002	bovine	2008	NZ_JHCB00000000.2
ANSES_052	France	A.Br.001/002	bovine	1953	ERR1841046
ANSES_054	France	A.Br.001/002	bovine	1981	ERR1841047
ANSES_058	France	A.Br.001/002	bovine	1954	ERR1841049
A1102	Argentina	A.Br.003/004	pig	missing data	SRR2968196
K8215	Argentina	A.Br.003/004	bovine	1996	NZ_LGIG00000000.1
V770-NP-1R	United States of America	A.Br.003/004	cow	1951	CP009598.1, CP009597.1, AZQO01000000.1
A0001	Turkey	A.Br.Aust94	missing data	missing data	SRR2968189
A0103	Turkey	A.Br.Aust94	cow	missing data	SRR2968193
A0148	Turkey	A.Br.Aust94	human	1990	SRR2968194
A0002	Turkey	A.Br.Aust94	missing data	missing data	SRR2968144
A0083	Germany	A.Br.Aust94	soil	missing data	SRR2968155
A0404	India	A.Br.Aust94	human	missing data	SRR2968164
Aust94	Australia	A.Br.Aust94	cow	1994	NZ_AAES01000000
K2883	India	A.Br.Aust94	human	missing data	LFYH01000000.1
A0386	Scotland	A.Br.Aust94	human	missing data	SRR2968163
Ohio_ACB	United States of America	A.Br.Aust94	pig	missing data	CP009339.1, CP009340.1, CP009341.1
200031027	United States of America	A.Br.Aust94	cow	1957	JTAR01000000.1
A3716	Namibia	A.Br.Aust94	zebra	2006	SRR2968149
K1285	Namibia	A.Br.Aust94	zebra	missing data	LFYF01000000.1
9080G	Country of Georgia	A.Br.Aust94	cattle burial ground	1998	NZ_CM002399.1, NZ_CM002400.1, NZ_CM002398.1
52G	Country of Georgia	A.Br.Aust94	cow	2009	GCA_000559005.1
8903G	Country of Georgia	A.Br.Aust94	cattle burial ground	1997	NZ_CM002402.1, NZ_CM002403.1, NZ_CM002401.1
A0088	South Africa	A.Br.Aust94	missing data	missing data	SRR2968133
A0252	Zimbabwe	A.Br.Aust94	missing data	missing data	SRR2968160
A0455	Mozambique	A.Br.Aust94	cow	1965	SRR2968165
A0224	Australia	A.Br.Aust94	flies	missing data	SRR2968158
A0656	China	A.Br.Aust94	soil	1982	SRR2968145
A0659	China	A.Br.Aust94	soil	1982	SRR2968146
A1035	Albania	A.Br.008/011	sheep	missing data	SRR2968140
A1032	Albania	A.Br.008/011	goat	missing data	SRR2968139
A1033	Albania	A.Br.008/011	cow	missing data	SRR2968213
A11193	Bulgaria	A.Br.008/011	cattle	1960_80	SRR2968197
A11194	Bulgaria	A.Br.008/011	cattle	1960_80	SRR2968186
A0062	Poland	A.Br.008/011	missing data	missing data	SRR2968154
A0362	Norway	A.Br.008/011	cow	missing data	SRR2968203
A0628	China	A.Br.008/011	soil	1981	SRR2968206
A0324	Slovakia	A.Br.008/011	missing data	missing data	SRS966987
Tsiankovskii	Russia	A.Br.008/011	vaccine live stock	1960	NZ_ABDN02000000
Cvac02	China, but likely not original	A.Br.008/011	missing data	2012	CP008853.1
3154	Bulgaria	A.Br.008/011	soil	1960–80	ANFF00000000.1
3166	Bulgaria	A.Br.008/011	soil	1960–80	ANFG00000000.1
carbosap	Italy	A.Br.008/011	missing data	missing data	ANAO01000000
A0157	United States of America	A.Br.008/011	pig	1952	CP010342.1, CP010343.1
Ba4599	Scotland	A.Br.008/011	2009	1940_1945	AGQP01000000
UR-1	Germany	A.Br.008/011	human	heroin	ALNY00000000.1
A0174	Canada	A.Br.009	cow	2012	NZ_ABLT01000000
A0193	United States of America	A.Br.009	cow	1995	NZ_ABKF01000000
USA6153	United States of America	A.Br.009	missing data	missing data	NZ_AAER01000000
Gmb1	Gambia	A.Br.011/009	cow	missing data	CAVE010000000
Sen2Col3	Senegal	A.Br.011/009	ostrich	2010	CAVC010000000
Sen3	Senegal	A.Br.011/009	sheep	2010	CAVD010000000
H9401	South Korea	A.Br.005/008 & 005/007	human	2010	CP002091.1, CP002092.1, CP002093.1
Vollum	United States of America	A.Br.Vollum	human	1994	CP009326.1, CP009327.1, CP009328.1
A0488	UK	A.Br.007	cattle	1951	NZ_ABJC01000000
AH820	Norway		periodontal pocket	1956	NC_011773.1

Table 3
Japanese-specific SNPs and primer sequences used in our HRM analysis.

Position[a]	SNP	canSNP subgroup	Forward primer (5′- 3′)	Reverse primer (5′- 3′)	Mean Tm (°C)	PCR size (bp)
88,730	C to T	A.Br.001/002	GGATGGCCCTGATTCTGATATTG	TCTGTTTAGTTTCTTCTTCATTTTGCA	74.94 ± 0.11 (C) 74.38 ± 0.14 (T)	107
401,315	A to G	A.Br.001/002	CGAAGCAAGATAAAGACTTTGTGAAA	TCAGCAGCTTCTTTCGGCTCTT	77.99 ± 0.04 (A) 78.49 ± 0.02 (G)	91

[a] Position on the Ames Ancestor chromosome (NC_007530.2).

and Akita prefectures; Table 1 and Fig. 3D). The only strain belonging to the A02 group was strain BA108, which was isolated from a bovine source in the Shiga prefecture in 1987. The Tokushima1 and BA111 (both bovine) strains, divided by SNP88730, were isolated from the western part of Japan in the Tokushima and Okayama prefectures, respectively (Table 1 and Fig. 3D). It is possible that a certain number of strains belonging to the A.Br.001/002 lineage might have been prevalent nationwide at that time in Japan.

3.3. Phylogenetic analysis of the A.Br.Aust94 lineage

In the current study, *B. anthracis* strains from Okinawa (strain BA107) and Shizuoka prefectures (strains BA104, BA105, 6166, and 6172) were available for an analysis of their genetic relationships. All five of these strains were of swine origin and belonged to the A.Br.Aust94 lineage (Fig. 2B).

In our *B. anthracis* collection, there were two pairs of strains with the same origin but different storage and passage histories, namely strains BA104 and 6166 and strains BA105 and 6172 (Table 1). Strains BA104 and 6166 were isolated from a place 20 km away from the isolation location of strains BA105 and 6172, both in Shizuoka prefecture. The SNP profiles of strains BA104 and 6166 and of strains BA105 and 6172 revealed that strains BA104 and 6166 differed by only one SNP (SNP 1250307) whereas strains BA105 and 6172 differed by two SNPs (SNP 580754 and 1,952,624) within the 1060 chromosomal SNPs (Supplemental Table for Fig. 2B). An MLVA analysis of strains BA104 and 6166 revealed different numbers of tandem repeats (18 and 16 repeats, respectively) within pXO2 (Table 1). This discordance might be due to the strains having different numbers of passages during their storage. The tandem repeat number of *vrrB2*, one of the chromosomal gene markers of the MLVA 8 analysis, was 13 for both strains BA105 and 6172, whereas the other analyzed swine strains had 14 *vrrB2* tandem repeats (Table 1). The phylogeny in Fig. 2B suggests that the *B. anthracis* strains from the Japanese swine cases were genetically different; strains BA104 and 6166 shared the same branch with strain 200031027 from the USA (A.Br.099), strains BA105 and 6155 shared the same branch with Australia94 (A.Br.015), and strain BA107 shared the same branch with an Indian strain (A.Br.097). The swine *B. anthracis* strains Ohio_ACB (USA, A.Br.Aust94) and A1102 (Argentina, A.Br.003/004) seemed to have no clear genetic connection with the three Japanese *B. anthracis* swine strains (A.Br.Aust94), as shown in Figs. 1 and 2B. The BA109 strain, isolated from a bovine source in 1969, was found to belong to an independent genetic branch divided by A.Br.034 and was distant from all other Japanese swine strains (Fig. 2B); however, it was quite closely related to the Ohio_ACB strain.

3.4. Phylogenetic analysis of the A.Br.008/011 lineage

A phylogenic analysis conducted using the MP method and a canSNP analysis revealed that the Gunma1 strain, isolated from a bovine source in the Gunma prefecture, belonged to the A.Br.008/011 lineage and shared a branch with the PAII strain (Figs. 1 and 2C). We found that the Gunma1 strain differed from the PAII strain by 12 SNPs. Additionally, the repeat number of the CG3 marker in MLVA 8 for the Gunma1 strain was one (Table 1), whereas the CG3 marker repeat number for the other Japanese *B. anthracis* strains was two. These two Japanese strains were genetically close to strains Tsiankovskii and Cvac02, both of which were anthrax vaccine strains (Fig. 2C).

3.5. Diagnostic SNP genotyping assay

Girault et al. (2014b) reported that HRM assays allow the unambiguous discrimination of strains from diverse canSNP genotypes. We investigated two diagnostic SNPs that defined two A.Br.001/002 lineages and could be developed into HRM assays. The two expected alternate alleles exhibited distinct melting curves and melting temperatures (Tm). Differences in Tm ranged between 0.50 and 0.56 °C between both allelic states (Table 3). As introduced by Girault et al. (Girault et al., 2014b), the HRM assays in the present study could allow the unambiguous grouping of each allele and an accurate discrimination of the A.Br.001/002 strains from other globally diverse canSNP genotypes.

4. Discussion

In the present study, we analyzed the genetic background of past Japanese isolates of *B. anthracis* using whole genome sequences. These Japanese isolates were classified into four canSNP lineages: A.Br.Ames, A.Br.001/002, A.Br.Aust94, and A.Br.008/011 (Fig. 1). Eight strains of bovine or equine origin were found to belong to the A.Br.Ames and A.Br.001/002 lineages. All the strains from the swine anthrax outbreak in 1982 were found to belong to the A.Br.Aust94 lineage, and one bovine strain and one former vaccine strain were both classified into A.Br.008/011. Core genome SNP typing revealed the phylogenetic positions and genetic diversities of the Japanese *B. anthracis* isolates. Based on the phylogeny results, two bovine Japanese isolates of the A.Br.001/002 lineage (BA111 and Tokushima1) were grouped into the new A03 subgroup based on SNP88730 (Fig. 2A). Also phylogeny can discriminate differences between strains from the same A.Br.Aust94 lineage swine anthrax case (Fig. 2B). Former vaccine strain PAII was found to be closely related to the bovine strain Gunma1 as well as to vaccine strains (used in other countries) that belong to the A.Br.008/001 lineage (Fig. 2C).

Historically, anthrax was not a rare disease in Japan before the 1940s; during that period, hundreds of cases in bovine and equine species and more than 10 cases in swine were reported annually (Fig. 3A). From 1955 to 1965, bovine anthrax was considered to be caused by the dicalcium phosphate from imported bovine bone (contaminated with *B. anthracis* spores) that was used as supplemental feed for bovine animals (Imano et al., 1968); the number of anthrax cases declined after withdrawing the supplement.

Continuous anthrax vaccination also seems to have been effective in decreasing the number of bovine anthrax cases (Fig. 3A). In addition, there was a dramatic drop in the number of anthrax-infected horses after war-horse breeding ended (Fig. 3C), suggesting that this change was also a factor for the decline in the number of equine anthrax cases. Improvements in animal hygiene and a strict quarantine on the import of animal products might have additionally contributed to reducing the frequency of further contamination by *B. anthracis* spores from other countries. For example, the import of bone meal powder gradually

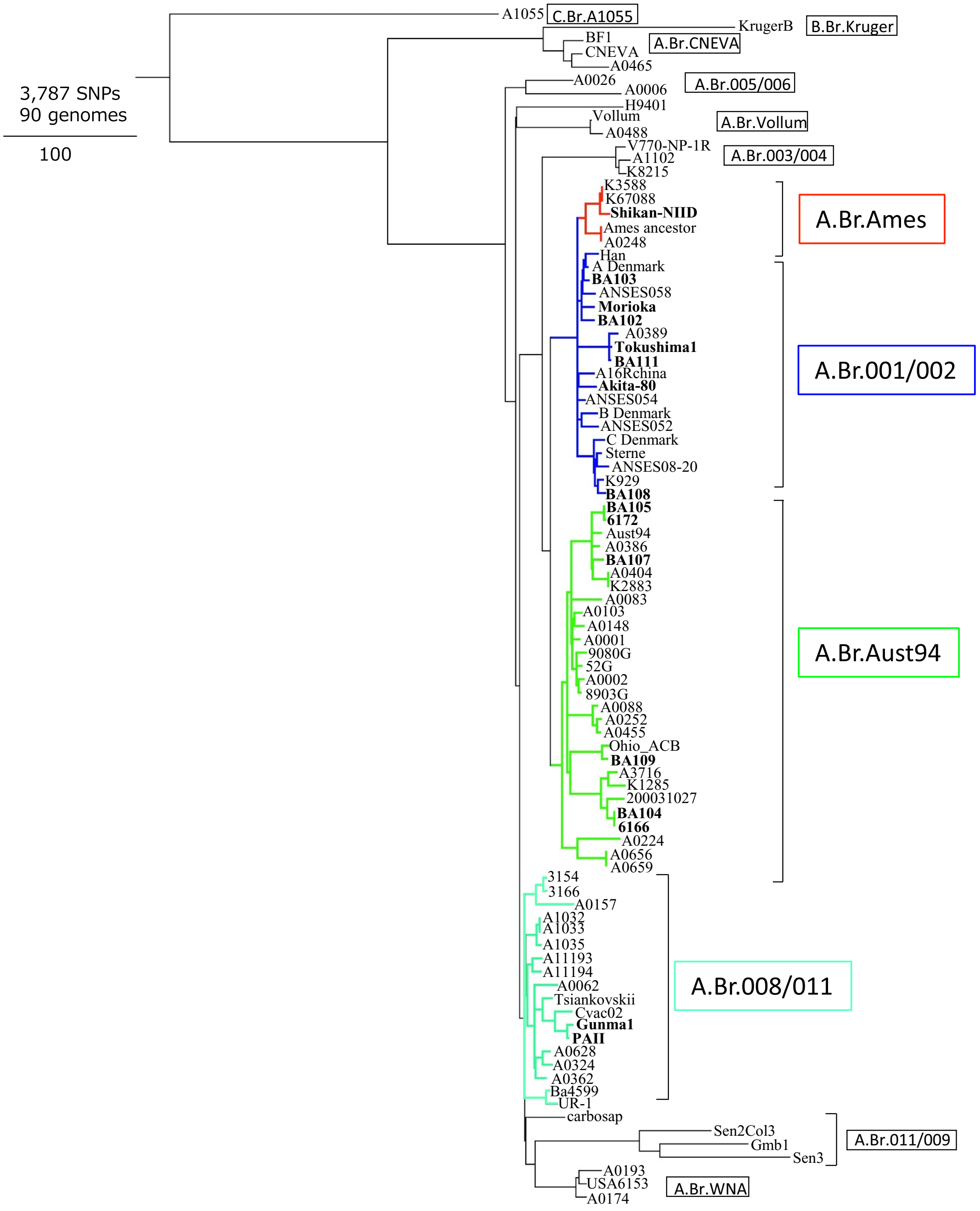
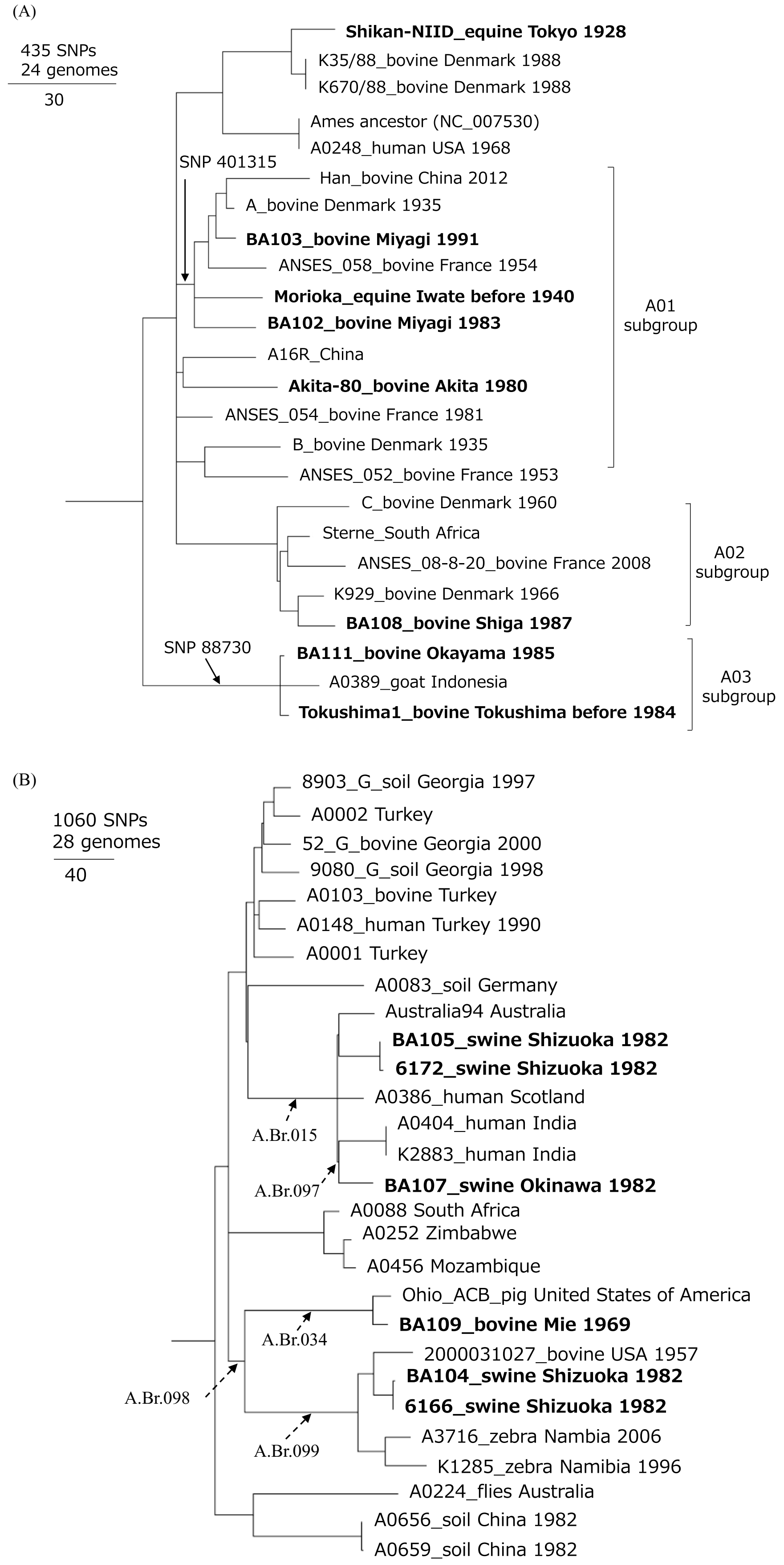


Fig. 1. Phylogenetic tree of 90 anthrax strains. The evolutionary history of anthrax strains was inferred using the maximum parsimony (MP) method. The consistency index is 0.964, retention index is 0.929, and composite index is 0.895, for all sites and parsimony-informative sites. In total, 3,787 SNPs were analyzed. The colors indicate the canSNP lineage: blue (A.Br.001/002), red (A.Br.Ames), green (A.Br.Aust94), and light blue (A.Br.008/011). The tree has been rooted using the AH820 *B. cereus* strain as an outgroup. Japanese strains are indicated by bold text. All the major or known lineages within *B. anthracis* are included in this figure and are listed in Table 2.

decreased beginning in the late 1980s, and it has been completely prohibited since 2001; this policy was implemented as a protective measure against bovine spongiform encephalopathy (BSE) and might help in preventing new spore infiltrations from abroad. Japan has had only one case of animal anthrax since 2001, which occurred in a cow in Miyazaki prefecture (Fig. 3A).

Most horses that were bred in Japan before WWII were war-horses, and the breeding of war-horses was an important activity of the Japanese army circa 1930. In 1928 when strain Shikan-NIID was isolated at Military Academy in Tokyo, there were 186 bovine, 49 equine, and 13 swine anthrax cases reported nationwide in Japan (Fig. 3A). Many French-origin Percherons or other European-origin broodmares and stallions were imported to Japan from various European countries. Although the percentage of pure European-blood horses among the total horses bred in Japan during 1906–1923 was under 30%, the percentage of these horses reached about 97% in 1935 (Okazaki, 2014). Therefore, war-horses might have been exposed to *B. anthracis* spores of European origin.

The vaccination of livestock animals appears to have been another important factor for reducing the number of anthrax-infected animals in Japan (Fig. 3A). Since 1976, the anthrax vaccine administered to animals in Japan has been produced using a spore solution of strain 34F2 (World Health Organization, 1998) (a pXO2-deficient strain), as shown in Fig. 3A. Before the introduction of the 34F2 strain, Japan used a live



(caption on next page)

Fig. 2. Phylogenetic tree of anthrax strains, separated by lineages. The evolutionary history of anthrax strains was inferred using the maximum parsimony (MP) method. The tree was rooted using the A1055 strain as an outgroup. (A) The A.Br.Ames and A.Br.001/002 lineages. These lineages currently contain 24 genomes and 435 SNPs. The consistency index is 0.973707, retention index is 0.928230, and composite index is 0.903824, as calculated by MEGA 7. The subgroups of A01 and A02 were classified by SNP515111 and SNP240050 (Girault et al., 2014b) and subgroup A03 was classified by SNP88730, all based on the Ames Ancestor genome. (B) The A.Br.Aust94 lineage. This lineage currently contains 28 genomes and 1,060 SNPs. The consistency index is 0.996088, retention index is 0.992987, and composite index is 0.989102. (C) The A.Br.008/011 lineage. This lineage currently contains 13 genomes and 350 SNPs. The consistency index is 0.947269, retention index is 0.777778, and composite index is 0.736765. Dotted arrows indicate the position and name of published diagnostic SNPs, whereas solid arrows indicate the position and name of new diagnostic SNPs within the A.Br.001/002 lineage that were identified in the present study. Japanese strains are indicated by bold text.

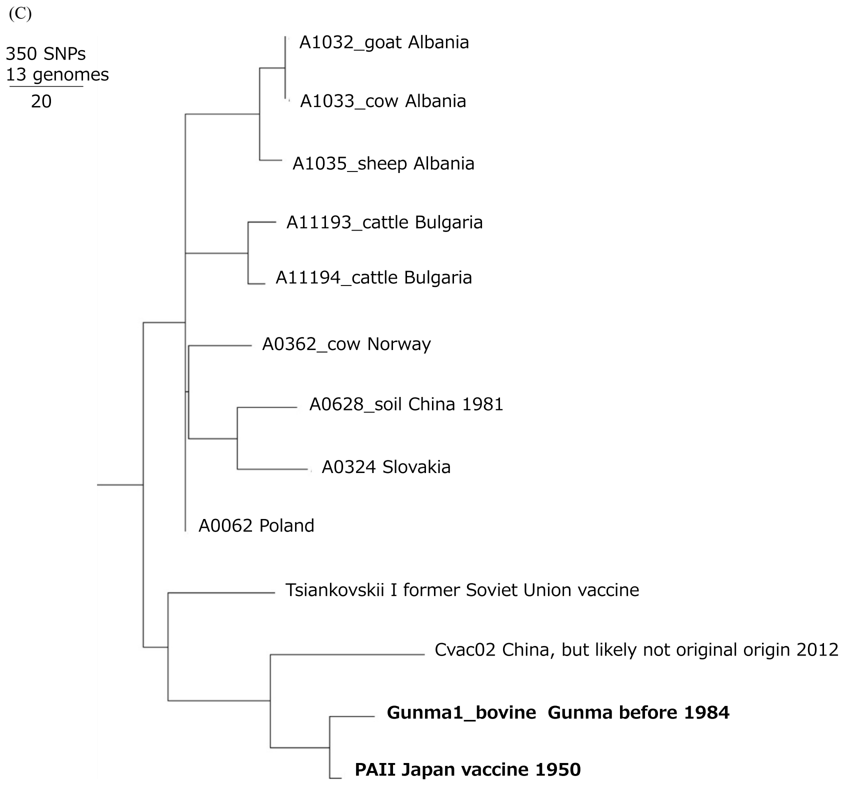


Fig. 2. (*continued*)

vaccine strain, PAII, which was originally provided to the NIAH in Japan in 1897 by the Pasteur Institute in Paris. In 1904, the NIAH began anthrax vaccination in cows and horses in response to an urgent need for controlling animal anthrax. A stock of the PAII strain used in the current study has been stored in the NIAH since 1950. This strain was attenuated by culturing at 42–43 °C, and the resulting spore solution, mixed with pathogenic plasmid-deficient strains, was used for vaccination. The PAII vaccine strain sometimes showed a reversal of pathogenicity in vaccinated animals. Thus, because vaccination with strain PAII was widely performed before 1976, it is possible that the bovine strain Gunma1 could be one of the revertant derivatives from the PAII vaccine strain (Figs. 2C and 3A).

Swine is known to be resistant to anthrax, and, if a pig does become infected with anthrax, the animal is less likely to show obvious symptoms and the resulting disease is often chronic (World Health Organization, 1998). In 1982, sporadic anthrax cases occurred in swine in several prefectures of Japan, including Okinawa, Shizuoka, Mie, and Aichi (Fig. 3B). Because swine are usually resistant to anthrax, the swine anthrax cases in 1982 were suspected to have some common infectious source (Amano et al., 1987). The strains BA104, 6166, BA105, 6172, and BA107 were isolated from female Landrace pigs, all of which displayed symptoms of the enteritis type of swine anthrax; neither the pharyngeal nor the septicemic type of swine anthrax were reported in the 1982 outbreak. These pigs, all healthy in appearance, were brought into a slaughterhouse, and their disease conditions were identified postmortem.

An epidemiological investigation of the two Shizuoka anthrax cases revealed that: 1) the two affected farms had both purchased feed from the same company, and 2) imported bone meal powder was an ingredient in each of these feeds (Amano et al., 1987). None of the farms or surrounding areas had any history of anthrax, and the pigs were all kept in concrete houses with very little chance of soil contact. These facts suggest that the compound feeds given to the pigs might have been the infectious source; however, no *B. anthracis* was isolated from the feeds during the investigation of the case. Previously, bone meal powder, imported to Japan from counties including Australia, the USA, and India, was industrially processed into animal products and their feeds without adequate sterilization; the contaminated bone meal powder might have been the cause of infection in the animals. These results suggest that anthrax in Japan might have been caused by *B. anthracis* spore contaminants from imported animal products.

5. Conclusions

The vaccination of livestock animals, prohibition of bone meal powder import, and quarantine of animal products all seem to have been effective factors that drastically reduced the number of anthrax cases in Japan. Since 2011, our group has been collecting soil from more than 20 places in Japan, and only *B. cereus* and *B. thuringiensis* have been detected to date (Okutani, Unpublished data). As an island

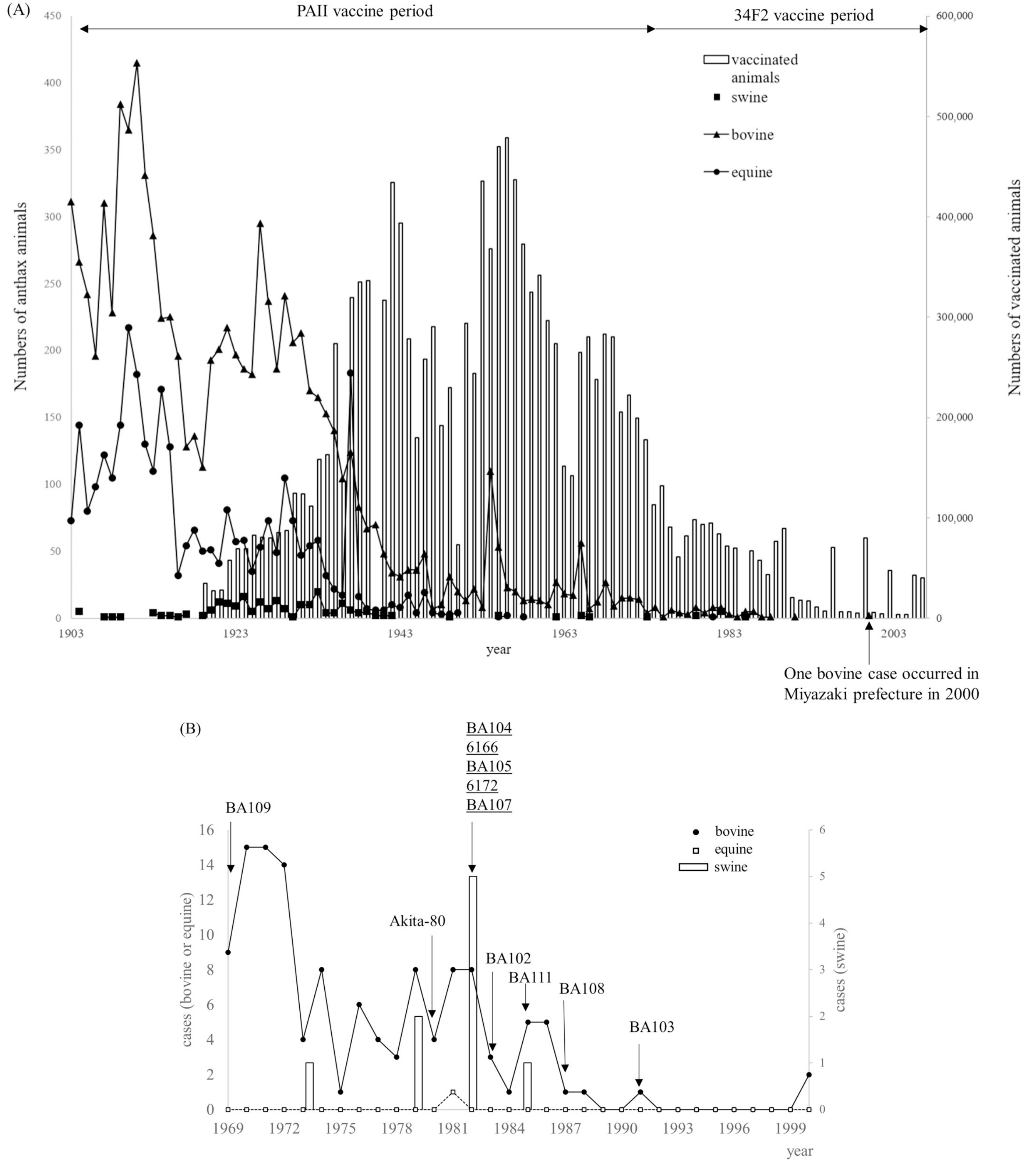


Fig. 3. Temporal and spatial analysis of animal anthrax incidence in Japan. (A) Variation in the number of animal anthrax cases (swine, bovine, and equine) and the number of vaccinated animals in Japan from 1903 to 2007. (B) Graph of the swine, bovine, and equine anthrax cases from 1969 to 2000, showing data from the Statistics of Animal Hygiene, Ministry of Agriculture, Forestry, and Fisheries, Japan. Arrows indicate the strains used in the present study in the year of isolation. Strains with obscure information concerning the isolated year are boxed, and strains of swine origin are underlined. (C) Variation in the number of livestock animals (bovine and equine) raised in Japan from 1903 to 1999. (D) Geographical distribution of the isolation prefectures for each of the 15 anthrax strains from animals in Japan that were assessed in the present study. The colors indicate the canSNP lineage: blue (A.Br.001/002), red (A.Br.Ames), green (A.Br.Aust94), and light blue (A.Br.008/011). The five strains of swine origin are underlined.

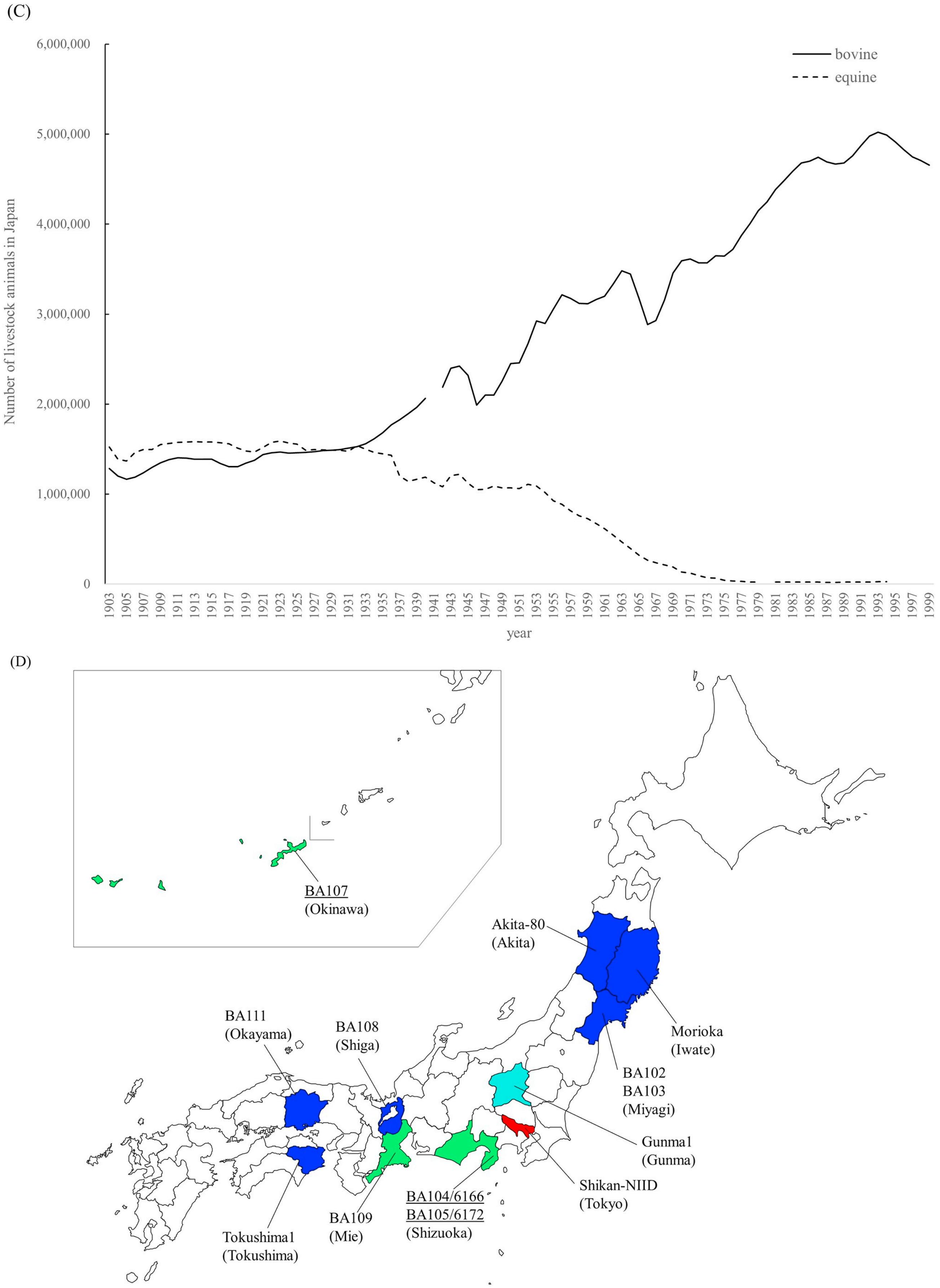


Fig. 3. (*continued*)

country, Japan does not allow new anthrax-infected animals to spontaneously migrate into the country; therefore, its geographic location further helps to prevent a re-emergence of anthrax in Japan.

Together with the phylogenic results and epidemiological circumstances, the observed low diversity of *B. anthracis* strains reveals that the *B. anthracis* strains in Japan probably resulted from multiple relatively recent import events, rather than reflecting the persistence of a more ancient ecologically established group. The strain-level classification, determined using core genome SNPs from whole genome sequences, provided by this study acts as a comparison point that may improve understanding of the genetic background of newly detected anthrax cases in the future.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.022.

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