Type of the Paper (Article, Review, Communication, etc.)

Molecular epidemiology characteristics of *Brucella abortus* strains in China and their relationships with world lineages

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Abstract: *Brucella abortus* is a facultative intracellular Gram-negative bacterium that causes chronic persistent infections in humans and livestock. In this study, conventional bio-typing, multiple-locus variable-number tandem repeat analysis (MLVA), and whole-genome sequencing-single-nucleotide polymorphism (WGS-SNP) were used to investigate the molecular epidemiology characteristics of *Brucella abortus* strains in China and their relationships to world lineages. A total of 100 strains were collected from 1953 to 2013, suggesting that *B. abortus* circulated in China in the past five decades. Moreover, most strains were mainly distributed in the northwest area. During this period, seven biovars were found, indicating that *B. abortus* displayed a high diversity of biovars and that the main epidemic areas are in the northern provinces. Strains have high genetic diversity, and bruce07 is the most helpful locus for genotyping of this population. Moreover, 17 MLVA-11 genotypes were found; 13 of them are of known genotypes and four are unassigned genotypes, indicating that *B. abortus* in this study had several geographic origins. Still, strains from unassigned genotypes may originate from China. Many shared MLVA-16 genotypes were observed in strains from the same provinces in Northern China, which confirmed a brucellosis outbreak within northern regions. WGS-SNP analysis showed that eight Chinese strains formed a ladder-like phylogram (C. Ⅶ) with strains from nine countries, including Uganda, Iraq, Russia, Georgia, Spain, Italy, Egypt, Mongolia, and China; strains were introduced to these countries from a single source. Notably, Chinese strains were closely related to strains from Russia and Mongolia; frequent animal (cattle) trade and exchanges may promote this process. We will provide new and insightful information for strength surveillance and control of *B. abortus* brucellosis in China.

Keywords: *B. abortus*; molecular epidemiology; MLVA; WGS-SNP; China

1. Introduction

Brucellosis, one of the world's most common and significant zoonoses, affects various domestic and wildlife animals, and also humans [1]. Abortion and infertility in the infected animals cause hung economic loss, whereas prolonged low fever, fatigue, and sweat in humans resulted in significant public health concerns worldwide [2]. Bovines and small ruminants, which excrete bacteria in the milk and reproductive discharges, are major sources of infection for humans and other animals [3]. Contact with infected domestic and consumption of raw dairy products are the main routes for human infection [4]. Brucellosis is caused by species of the genus *Brucella*, which includes *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* [5]. Of these, *B. abortus*, *B. melitensis*, and *B. suis*

are the most vital agents for human brucellosis in China [6]. Our previous study showed that B. melitensis is a latent "travel bacterium" that spread and expanded from North China to South China [7]. However, the impact on the public health and development of *B. abortus*' animal husbandry should never be overlooked [8]. In Northern China, where the traditional agropastoral areas with more developed animal breeding industries are located, the brucellosis seroprevalence was greater than 10%：, the seroprevalence of brucellosis in Southern China reached only 5.5% [9]. Despite the relatively low seroprevalence (2.8%) in a serological survey of yaks, the estimated economic losses were substantial in Tibet. With no control program for brucellosis in yaks, the total annual economic loss in the study area was estimated at US$521, 043, mainly resulting from abortions and reduction of the milk and meat yield [10]. However, there is limited available information on the molecular epidemiology of brucellosis caused by *B. abortus*.

A bio-typing assay is time consuming and difficult to interpret due to limited standardization of the typing reagents, but it was useful for *Brucella* strain identification and species differentiation [11]. Moreover, multi-locus variable-number tandem repeat assays (MLVA) are a genetic approach with high discriminatory power in the *Brucella* genus, providing a fine-scale resolution among isolates and allowing the determination of the source of infection, geographical origin, and spread pattern of disease [12,13]. The whole-genome sequencing-single-nucleotide polymorphism (WGS-SNP) approach is used to establish phylogenetic relationships of *Brucella* isolates [14,15]. In this study, 100 strains were isolated from samples collected between 1953 and 2013 from various host animals throughout the countrywide in China. This study characterized these *Brucella* strains using bio-typing, MLVA, and WGS-SNP, to determine the distribution regions, genotyping characteristics, geographic origins, and phylogenetic relationships of *B. abortus* strains at the global level. The findings could support a nation-wide control and prevention program against brucellosis.

2. Materials and Methods

2.1. The source of Bacterial strains

The MLVA-16 characteristics, panel1, MLVA-11 genotypes, species/biovars, hosts, location, and year of *B. abortus* strains were collected, and Microsoft Excel (Microsoft, Redmond, CA, U.S.) was used for data cleaning. A total of 100 strains were obtained in this study; 65 were found in cattle, 20 in humans, 7 in sheep, 4 in yak, 3 in goat, and for 1 strain the host is unknown. Moreover, 36 strains of previously published from Inner Mongolia [16] were involved in this study. The isolates and biovar of the strains were identified using standard procedures [17]. All isolates were identified as *Brucella* species based on morphology and conventional identification methods according to standard bio-typing procedures, including the need for CO_2 for growth, H_2S production, sensitivity to thionin (10 and 20 µg/ml), basic fuchsin (20 µg/ml), and agglutination with mono-specific antiserum for and M antigens and phage lysis test (Tbilisi, Tb; Berkeley, Bk2; Weybridge; Wb) [18,19]. AMOS-PCR (*B. abortus* bv. 1, 2, and 4, B. melitensis, B. ovis, and B. suis bv. 1) [20] was used to verify further *B. abortus* bv. 1, 2, and 4.

2.2. *Brucella* MLVA-16 genotyping scheme

MLVA-16 in all strains was performed as previously described [21,22]. Briefly, the 16 primer pairs were divided into three panels: panel 1 (MLVA-8: eight loci including bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55), often used for species identification, panel 2A (three loci including bruce18, bruce19, and bruce21), and panel 2B (five loci including bruce04, bruce07, bruce09, bruce16, and bruce30), used for genotype comparison; MLVA-11 (panels 1 and 2A) (trace-back geographic origin of strains), and MLVA-16 (panels 1, 2A, and 2B). Fragments were sized after comparison with a ROX (carboxy-X-rhodamine)-labeled molecular ladder (MapMaker 1000; Bioventures Inc., Murfreesboro, TN, USA) and Gene Mapper software version 4.0 (Applied Biosystems). The fragment sizes were subsequently converted to repeat unit numbers using a published allele-numbering system [23].

2.3. Analysis of the MLVA data

The genetic diversity of *B. abortus* strains in this study was calculated based on the Hunter-Gaston diversity index (HGDI) according to a previously published method [24]. BioNumerics version 5.1

software (Applied math, Belgium) was used to analyze the MLVA-16 assay data (Table S1). Both the categorical coefficient and unweighted pair group methods were applied to the clustering analysis. MLVA-11 was used to investigate the geographical origins between our isolates [25,26] and 1,482 isolates from the MLVA bank (http://microbesgenotyping.i2bc.paris-saclay.fr/databases) (Table S2), and minimum-spanning trees were constructed using the goeBURST algorithm with PHYOVIZ 2.0 [27]. The MLVA-16 approach was applied to the genetically related investigation between strains from those reported in China (n = 100) (Table S1) and at the global level (n = 1525) (Table S3). Moreover, 62 *B. abortus* (Table S4) genomes were retrieved from GenBank and used for genomic comparison and phylogenetic analyses. The phylogenetic tree was constructed using the TreeBeST [28] based on maximum-likelihood phylogenies (PHYML) with 1,000 bootstrap replicates.

3. Results

3.1. The species/biovars, region distribution, and host profile of Chinese *B. abortus*

A total of 100 *B. abortus* samples were collected from 1953 to 2013 (11 in the 1950s, 9 in the 1960s, 5 in the 1970s, 38 in the 1980s, 1 in 1990, 4 in 2000s, and 32 in 2010s). A total of seven biovars were observed in this population, including *B. abortus* bv. 1 (n = 24), *B. abortus* bv. 2 (n = 2), *B. abortus* bv. 3 (n = 60), *B. abortus* bv. 5 (n = 1), *B. abortus* bv. 6 (n = 2), *B. abortus* bv. 7 (n = 1), and *B. abortus* bv. 9 (n = 10). *B. abortus* bv. 3 was the dominant species in China (Table 1). These strains were collected from 14 provinces (cities and autonomous regions) from 1953 to 2013, including 36 strains in Inner Mongolia, 14 in Xinjiang, 11 in Hebei, 10 in Heilongjiang, 6 in Gansu, and 5 in Zhejiang; the remaining regions contained 1 to 4 strains, 85% strains was distributed in northern, China. Moreover, 100 strains were obtained from five hosts, including 65 in cattle, 65%, followed by 20 in humans, 7 in sheep, 4 in yak, 3 in goat, and 1 in an unknown host (Table 1).

3.2. MLVA-16 genotyping results

Based on HGDI, three loci (bruce06, 08, and 12) showed no diversity (HGDI=0.0000) and bruce55 showed the highest diversity in all loci of panel 1, with a value of HGDI of 0.6515. The lowest value of HGDI in panel 2B was bruce16 (HGDI=0.2576). The HGDI value in other four loci were > 0.5705, and the HGDI value of panel1, MLVA-11, and MLVA-16 was 0.7820, 0.8196, and 0.9853, respectively (Table 2). In panel 1, 10 genotypes were observed, of which 7 are known, including 116 (n = 1), 30 (n = 4), 37 (n = 5), 112 (n = 13), 117 (n = 15), 28 (n = 17), and 36 (n = 39). The remaining three unassigned genotypes were Ba8-1 (n = 2) (4-5-3-12-2-1-3-2), Ba8-2 (n = 2) (4-5-3-1-2-3-1-3-2), and Ba8-3 (n = 2) (4-5-3-1-2-3-1-5-2) (Fig. 1).

Bases on MLVA-11, 17 genotypes were found, 13 of which were of known genotypes, namely, 71 (n = 1), 83 (n = 1), 181 (n = 1), 210 (n = 9), 331(n = 1), 327 (n = 3), 75 (n = 4), 78 (n = 4), 326 (n = 4), 77 (n = 5), 82 (n = 11), 328 (n = 12), 72 (n = 38), and 4 novel genotypes: Ba11-1 (n = 1), Ba11-2 (n = 1), Ba11-3 (n = 2), and Ba11-4 (n = 2) (Fig. 1). Genotype 72 was distributed in seven regions, the higher diversity of MLVA-11 genotype was observed in strain from Inner Mongolia, Xinjiang Province, Hebei Province, and Sichuan Province (Table 1). However, the novel MLVA-11 genotype was found in Ningxia, Heilongjiang Province, and Hebei Province (Table 1). Based on the complete MLVA-16 loci, 100 strains were divided into four groups (I–IV) and 65 MLVA-16 genotypes (G.T.) had a 50% similarity coefficient. Both biovar 1 and 2 fell into group I; the other five biovars 3, 5, 6, 7, and 9 were clustered in other three groups (I, III, and IV), of which group I contained six subgroups (a–f) (Fig. 1). The 54 strains consisted of 19 shared genotypes that contained 2–8 strains, and 46 strains of an independent genotype, each present a single strain. Eighteen out of 19 strain-shared genotypes comprised strains from the same province; only one shared genotype (GT48) contained five strains obtained from four provinces (Fig. 1).

3.3. Geographic origin profile of Chinese *B. abortus* strains on a global scale

Among 17 MLVA-11 genotypes, seven were shared genotypes (72, 75, 82, 83, 181, 210, and 328) comprising strains from 2 to 10 countries, and the remaining 10 genotypes were single genotypes that were exclusively found in Chinese strains. The genotype 72 (n = 38) was the predominant population and accounted for 38% (38/100), followed by genotype 328 (n = 12), 82 (n = 11), and 210 (n = 9), accounting for 12% (12/100) 11% (11/100), and 9% (9/100), respectively. MLVA-11 genotype 72 was

shared by strains from six countries, including France, Germany, Italy, Kazakhstan, Portugal, and China. Genotype 82 comprised strains from 10 countries, including Brazil, Costa Rica, France, Germany, Italy, Kazakhstan, Portugal, South Korea, the United States, and China. Genotype 210 consisted of strains from four countries, Costa Rica, Italy, United States, and China. Genotype 78 was shared by strains from England, the United States, and China. Genotype 83 was shared by strains from Brazil, Costa Rica, England, and China. Genotype 75 included strains from Brazil and China. Genotype 181 consisted of strains from Portugal and China. However, another 10 single genotypes were exclusive of strains from China (Fig. 2).

3.4. Worldwide phylogenetic analysis based on both MLVA-16 and WGS-SNP

The MLVA-16 comparison analysis showed that 1,625 strains were sorted into three groups (A–C), and Chinese strains (group A) were clustered together with strains from Italy, Kazakhstan, and Brazil, but had no sharing of the MLVA-16 genotype was observed (Fig. 3). The whole-genome SNP analysis showed that 65 worldwide *B. abortus* strains were grouped into seven clades (I –Ⅶ) (Fig. 4); 16 Chinese strains were divided into five clades (Clade I , III, Ⅳ, Ⅵ, and Ⅶ). Clade I was composed of two Chinese strains and one strain from Kenya; clade III included strains from China, Poland, and Spain; clade Ⅳ included strains from China, South Korea, and USA; clade VI included strains from China, Italy, and Greece. Clade Ⅶ was composed of 19 strains from nine countries, including Uganda, Iraq, Russia, Georgia, Spain, Italy, Egypt, Mongolia, and China. These strains formed a ladder-like phylogram. However, most strains (n = 8) from China were found in clade Ⅶ and were the most similar to strains from Russia and Mongolia (Fig. 4).

3.5. Figures, Tables and Schemes

Categorical
MLVA-*B. abortus*

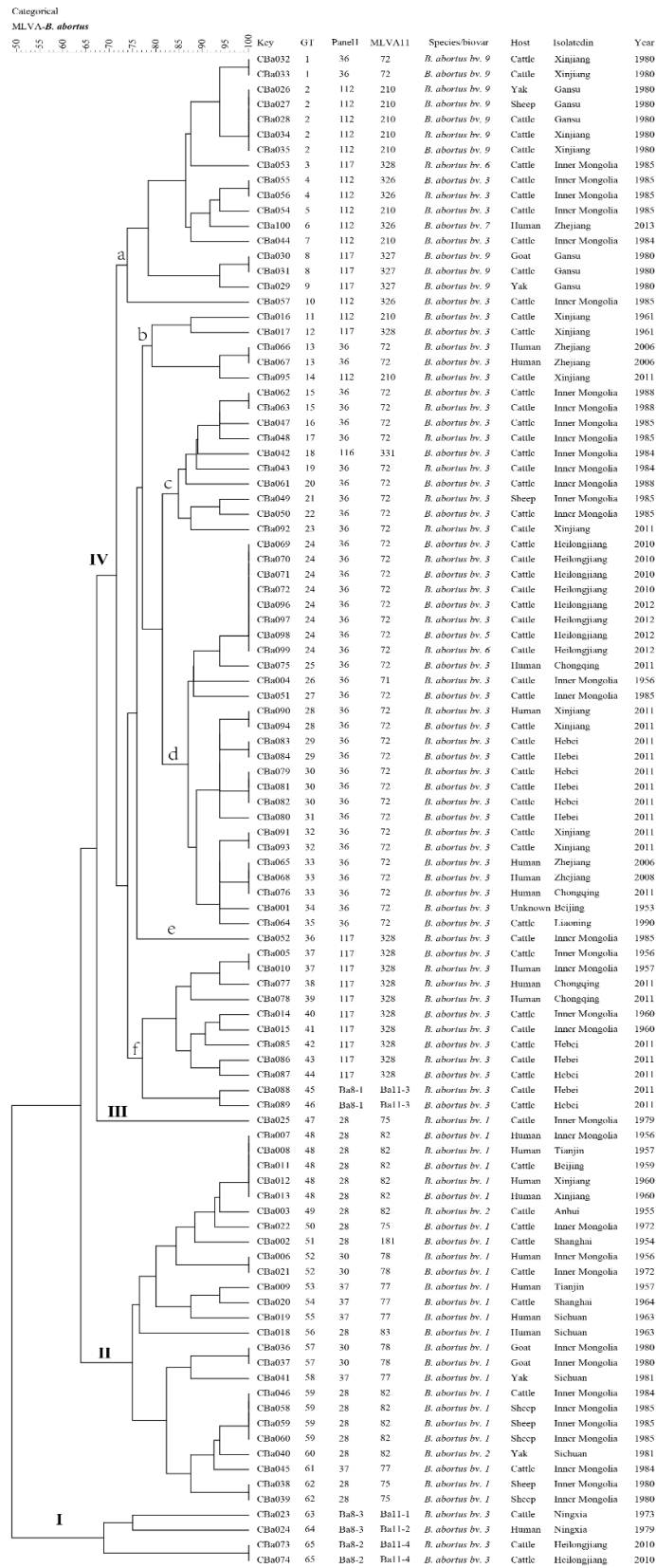| Key	GT	Panel1	MLVA11	Species/biovar	Host	Isolatedin	Year
CBa032	1	36	72	*B. abortus bv. 9*	Cattle	Xinjiang	1980
CBa033	1	36	72	*B. abortus bv. 9*	Cattle	Xinjiang	1980
CBa026	2	112	210	*B. abortus bv. 9*	Yak	Gansu	1980
CBa027	2	112	210	*B. abortus bv. 9*	Sheep	Gansu	1980
CBa028	2	112	210	*B. abortus bv. 9*	Cattle	Gansu	1980
CBa034	2	112	210	*B. abortus bv. 9*	Cattle	Xinjiang	1980
CBa035	2	112	210	*B. abortus bv. 9*	Cattle	Xinjiang	1980
CBa053	3	117	328	*B. abortus bv. 6*	Cattle	Inner Mongolia	1985
CBa055	4	112	326	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa056	4	112	326	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa054	5	112	210	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa100	6	112	326	*B. abortus bv. 7*	Human	Zhejiang	2013
CBa044	7	112	210	*B. abortus bv. 3*	Cattle	Inner Mongolia	1984
CBa030	8	117	327	*B. abortus bv. 9*	Goat	Gansu	1980
CBa031	8	117	327	*B. abortus bv. 9*	Cattle	Gansu	1980
CBa029	9	117	327	*B. abortus bv. 9*	Yak	Gansu	1980
CBa057	10	112	326	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa016	11	112	210	*B. abortus bv. 3*	Cattle	Xinjiang	1961
CBa017	12	117	328	*B. abortus bv. 3*	Cattle	Xinjiang	1961
CBa066	13	36	72	*B. abortus bv. 3*	Human	Zhejiang	2006
CBa067	13	36	72	*B. abortus bv. 3*	Human	Zhejiang	2006
CBa095	14	112	210	*B. abortus bv. 3*	Cattle	Xinjiang	2011
CBa062	15	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1988
CBa063	15	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1988
CBa047	16	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa048	17	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa042	18	116	331	*B. abortus bv. 3*	Cattle	Inner Mongolia	1984
CBa043	19	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1984
CBa061	20	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1988
CBa049	21	36	72	*B. abortus bv. 3*	Sheep	Inner Mongolia	1985
CBa050	22	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa092	23	36	72	*B. abortus bv. 3*	Cattle	Xinjiang	2011
CBa069	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2010
CBa070	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2010
CBa071	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2010
CBa072	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2010
CBa096	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2012
CBa097	24	36	72	*B. abortus bv. 3*	Cattle	Heilongjiang	2012
CBa098	24	36	72	*B. abortus bv. 5*	Cattle	Heilongjiang	2012
CBa099	24	36	72	*B. abortus bv. 6*	Cattle	Heilongjiang	2012
CBa075	25	36	72	*B. abortus bv. 3*	Human	Chongqing	2011
CBa004	26	36	71	*B. abortus bv. 3*	Cattle	Inner Mongolia	1956
CBa051	27	36	72	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa090	28	36	72	*B. abortus bv. 3*	Human	Xinjiang	2011
CBa094	28	36	72	*B. abortus bv. 3*	Cattle	Xinjiang	2011
CBa083	29	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa084	29	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa079	30	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa081	30	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa082	30	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa080	31	36	72	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa091	32	36	72	*B. abortus bv. 3*	Cattle	Xinjiang	2011
CBa093	32	36	72	*B. abortus bv. 3*	Cattle	Xinjiang	2011
CBa065	33	36	72	*B. abortus bv. 3*	Human	Zhejiang	2006
CBa068	33	36	72	*B. abortus bv. 3*	Human	Zhejiang	2008
CBa076	33	36	72	*B. abortus bv. 3*	Human	Chongqing	2011
CBa001	34	36	72	*B. abortus bv. 3*	Unknown	Beijing	1953
CBa064	35	36	72	*B. abortus bv. 3*	Cattle	Liaoning	1990
CBa052	36	117	328	*B. abortus bv. 3*	Cattle	Inner Mongolia	1985
CBa005	37	117	328	*B. abortus bv. 3*	Cattle	Inner Mongolia	1956
CBa010	37	117	328	*B. abortus bv. 3*	Human	Inner Mongolia	1957
CBa077	38	117	328	*B. abortus bv. 3*	Human	Chongqing	2011
CBa078	39	117	328	*B. abortus bv. 3*	Human	Chongqing	2011
CBa014	40	117	328	*B. abortus bv. 3*	Cattle	Inner Mongolia	1960
CBa015	41	117	328	*B. abortus bv. 3*	Cattle	Inner Mongolia	1960
CBa085	42	117	328	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa086	43	117	328	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa087	44	117	328	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa088	45	Ba8-1	Ba11-3	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa089	46	Ba8-1	Ba11-3	*B. abortus bv. 3*	Cattle	Hebei	2011
CBa025	47	28	75	*B. abortus bv. 1*	Cattle	Inner Mongolia	1979
CBa007	48	28	82	*B. abortus bv. 1*	Human	Inner Mongolia	1956
CBa008	48	28	82	*B. abortus bv. 1*	Human	Tianjin	1957
CBa011	48	28	82	*B. abortus bv. 1*	Cattle	Beijing	1959
CBa012	48	28	82	*B. abortus bv. 1*	Human	Xinjiang	1960
CBa013	48	28	82	*B. abortus bv. 1*	Human	Xinjiang	1960
CBa003	49	28	82	*B. abortus bv. 2*	Cattle	Anhui	1955
CBa022	50	28	75	*B. abortus bv. 1*	Cattle	Inner Mongolia	1972
CBa002	51	28	181	*B. abortus bv. 1*	Cattle	Shanghai	1954
CBa006	52	30	78	*B. abortus bv. 1*	Human	Inner Mongolia	1956
CBa021	52	30	78	*B. abortus bv. 1*	Cattle	Inner Mongolia	1972
CBa009	53	37	77	*B. abortus bv. 1*	Human	Tianjin	1957
CBa020	54	37	77	*B. abortus bv. 1*	Cattle	Shanghai	1964
CBa019	55	37	77	*B. abortus bv. 1*	Human	Sichuan	1963
CBa018	56	28	83	*B. abortus bv. 1*	Human	Sichuan	1963
CBa036	57	30	78	*B. abortus bv. 1*	Goat	Inner Mongolia	1980
CBa037	57	30	78	*B. abortus bv. 1*	Goat	Inner Mongolia	1980
CBa041	58	37	77	*B. abortus bv. 1*	Yak	Sichuan	1981
CBa046	59	28	82	*B. abortus bv. 1*	Cattle	Inner Mongolia	1984
CBa058	59	28	82	*B. abortus bv. 1*	Sheep	Inner Mongolia	1985
CBa059	59	28	82	*B. abortus bv. 1*	Sheep	Inner Mongolia	1985
CBa060	59	28	82	*B. abortus bv. 1*	Sheep	Inner Mongolia	1985
CBa040	60	28	82	*B. abortus bv. 2*	Yak	Sichuan	1981
CBa045	61	37	77	*B. abortus bv. 1*	Cattle	Inner Mongolia	1984
CBa038	62	28	75	*B. abortus bv. 1*	Sheep	Inner Mongolia	1980
CBa039	62	28	75	*B. abortus bv. 1*	Sheep	Inner Mongolia	1980
CBa023	63	Ba8-3	Ba11-1	*B. abortus bv. 3*	Cattle	Ningxia	1973
CBa024	64	Ba8-3	Ba11-2	*B. abortus bv. 3*	Human	Ningxia	1979
CBa073	65	Ba8-2	Ba11-4	*B. abortus bv. 3*	Cattle	Heilongjiang	2010
CBa074	65	Ba8-2	Ba11-4	*B. abortus bv. 3*	Cattle	Heilongjiang	2010

Figure 1. Dendrogram based on the MLVA-16 genotyping assay (UPGMA method) showing the relationships among the 100 *B. abortus* isolates. The columns show the identification numbers (Key), MLVA-16 genotypes (GT), MLVA-11 (panels 1 and 2A) genotypes, species biovars, hosts, geographical locations, and the year each strain was isolated.
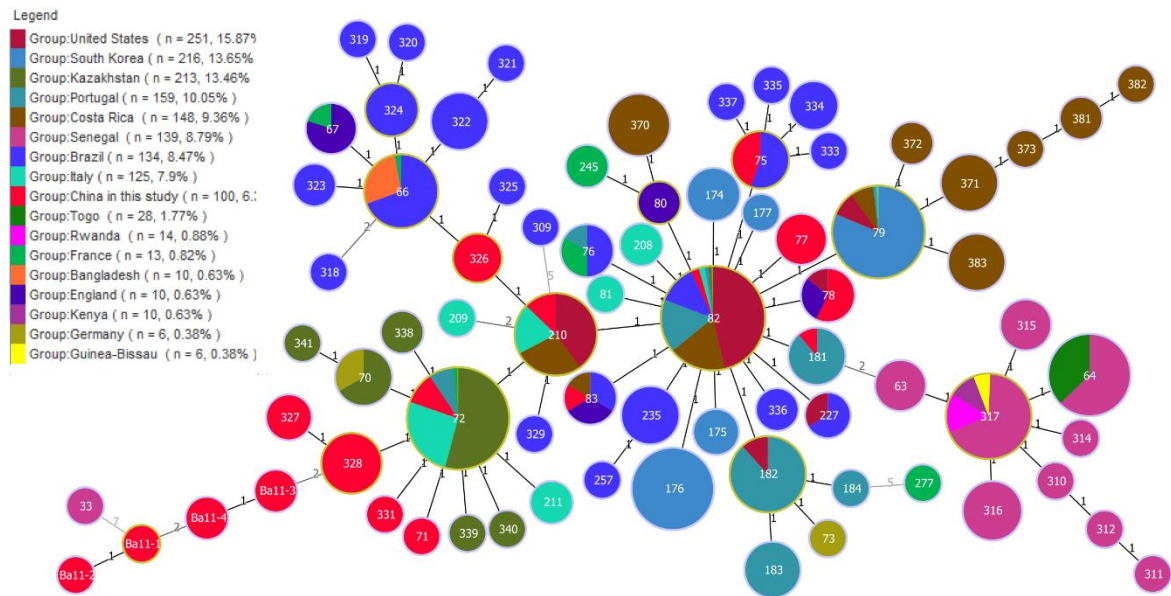
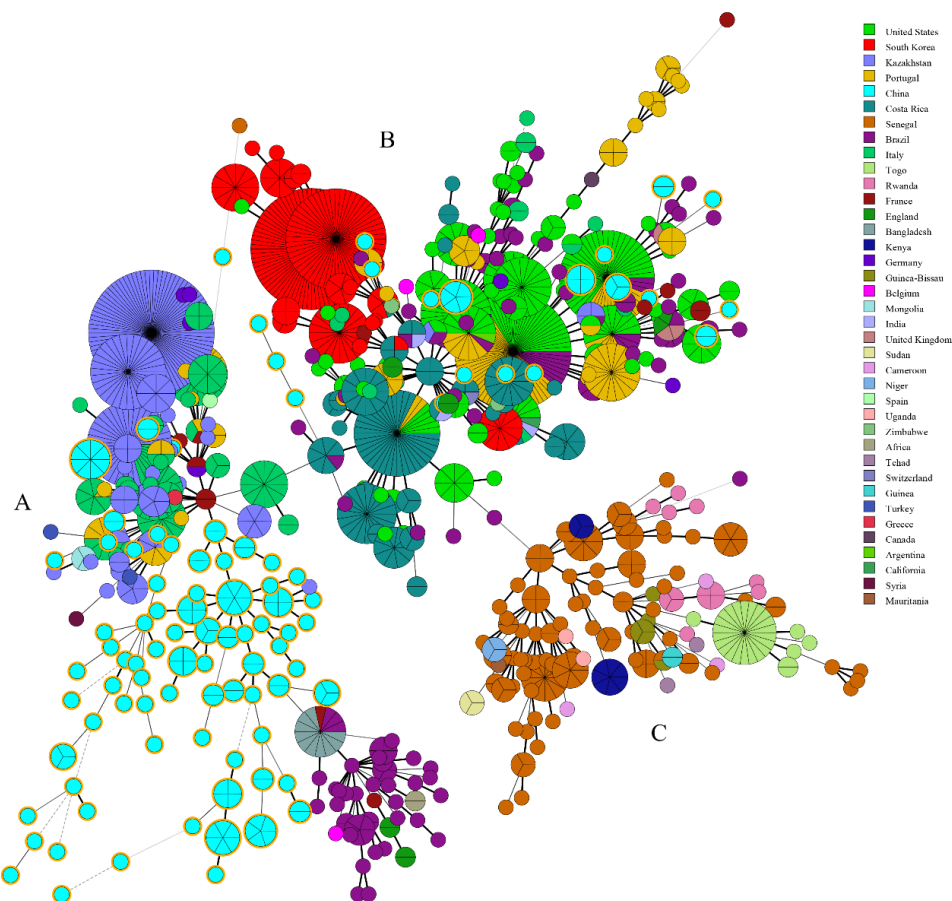Figure 2. Geographic origins of Chinese *B. abortus* strains based on MLVA-11.

Figure 3. Minimum spanning tree for *B. abortus* using MLVA-16 data with Chinese isolates (blue).
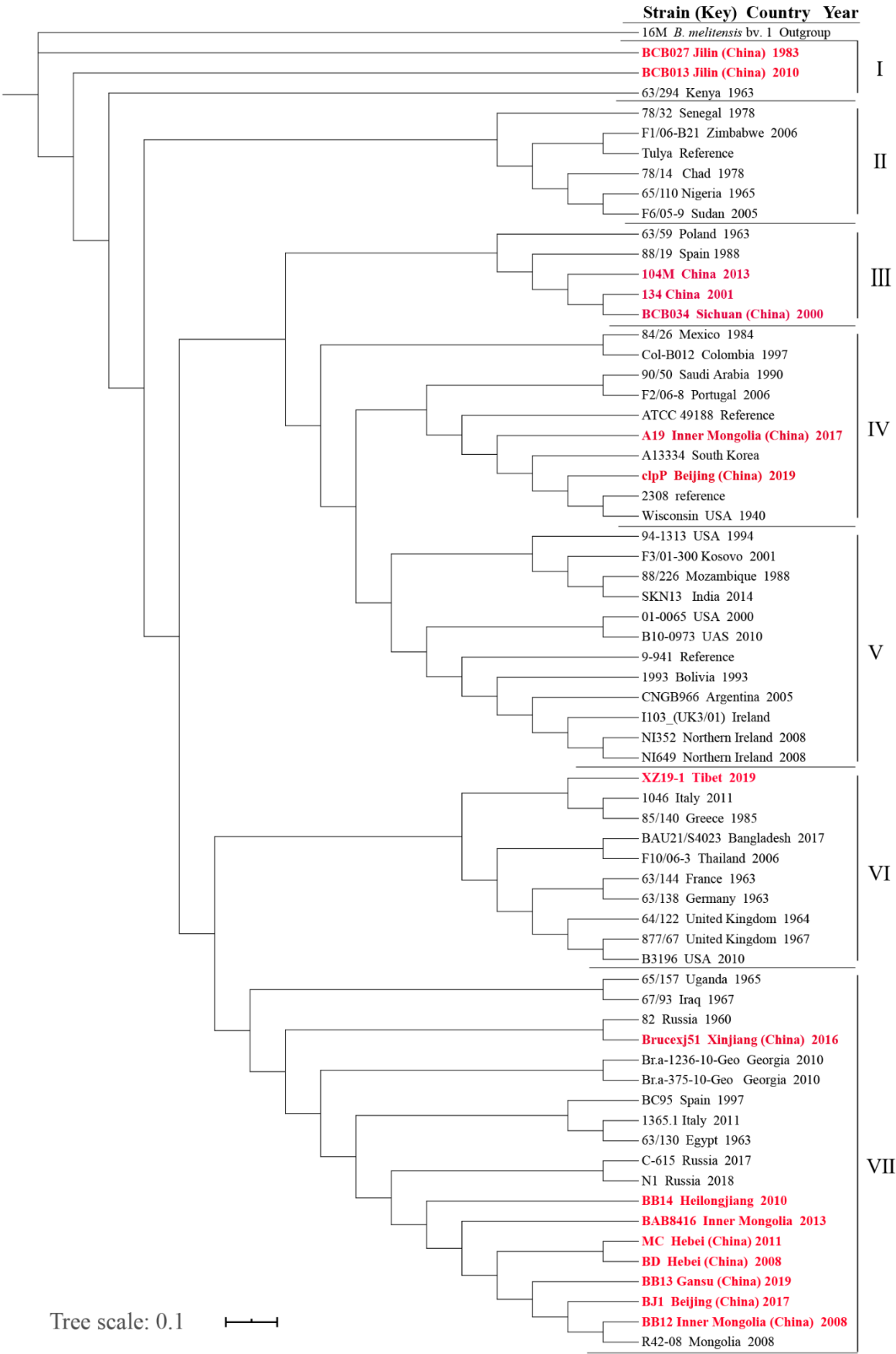
Figure 4. WGS-SNP phylogenetic analysis of *B. abortus* at a global level.

Table 1. Location, numbers, percentages (%), species, panel1, MLVA-11, hosts, and year of *Brucella abortus* in this study.

Provinces	No.	Species-biovar	Panel1	MLVA-11	Host	Year
Anhui	1	*B. abortus* bv. 2	28	82	Cattle	1955
Liaoning	1	*B. abortus* bv. 3	36	72	Cattle	1990
Beijing	2	*B. abortus* bv. 1, 3	28, 36	82,72	Cattle	1953, 1959
Ningxia	2	*B. abortus* bv. 3	Ba8-3	Ba11-1, 2	Human, cattle	1973, 1979
Shanghai	2	*B. abortus* bv. 1	28, 37	181, 77	Cattle	1954, 1964
Tianjin	2	*B. abortus* bv. 1	28, 37	82, 77	Human	1957
Chongqing	4	*B. abortus* bv. 3	36, 117	72, 328	Human	2011
Sichuan	4	*B. abortus* bv. 1, 2	28, 37	83, 77, 82	Yak, Human	1963, 1981
Zhejiang	5	*B. abortus* bv. 3, 7	36, 112	72, 326	Human	2006, 2008, 2013
Gansu	6	*B. abortus* bv. 9	112, 117	210, 327	Cattle, sheep, Yaks, Goat	1980
Heilongjiang	10	*B. abortus* bv. 3, 5, 6	36, Ba8-2	72, Ba11-4	Cattle	2010, 2012
Hebei	11	*B. abortus* bv. 3	36, 117, Ba8-1	72, 328, Ba11-3	Cattle	2011
Xinjiang	14	*B. abortus* bv. 1, 3, 9	28, 36, 112, 117	72, 82, 210, 328	Cattle, Human	1960, 1961, 1980, 2011
Inner Mongolia	36	*B. abortus* bv. 1, 3, 6	28, 30, 36, 37 112, 116, 117	71, 72, 75, 78, 82 210, 326, 328, 331	Cattle, sheep, Human, Goat	1956–1988

Table 2. Allelic types and HGDI of *B. abortus* for 16 loci in this study.

Panel and Locus	HGDI values
Bruce06	0.0000
Bruce08	0.0000
Bruce11	0.4034
Bruce12	0.0000
Bruce42	0.0776
Bruce43	0.1139
Bruce45	0.0396
Bruce55	0.6515
Bruce18	0.1701
Bruce19	0.2204
Bruce21	0.0396
Bruce04	0.6392
Bruce07	0.7905
Bruce09	0.7719
Bruce16	0.2576
Bruce30	0.5705
Panel 1	0.7820
MLVA-11	0.8196
MLVA-16	0.9835

4. Discussion

B. abortus infection was highly endemic in dairy herds in China [9]. The comprehensive molecular epidemiological and phylogenetic analysis of the *B. abortus* strains nationwide are useful for improving the surveillance and control program to determine the risk factors associated with brucellosis in humans. In this study, 100 *B. abortus* were collected from 1953 to 2013, indicating that *B. abortus* strains have circulated in China for the past several decades, but the strain numbers were lower than those of B. melitensis [29]. This observation is in agreement with a report that *B. abortus* was a secondary pathogen agent for brucellosis in China [6]. The seven biovars were observed in this study, and *B. abortus* bv. 3 was dominant species in China, suggesting that *B. abortus* in China has had a high diversity of species/biovars, which poses a potential challenge for animal vaccination. The 85% strains were distributed in traditional and domestic animals breeding developed provinces, providing helpful information for disease control and prevention. However, the distribution range of this species is different from that of the B. melitensis strains, which cover all mainland regions in China [30]. Similarly, the most frequently recorded *B. abortus* biovar is biovar 3 in the Republic of Kazakhstan. More than 90% of the overall *B. abortus* samples were isolated from the northern regions of East and West Kazakhstan [31]. Five kinds of hosts were observed in this population, and 65% of strains were from cattle. This indicated a narrower host spectrum than that of B. melitensis [32], which is a significant impact factor for strains with similar geographic distribution and is closely related to the low virulence of *B. abortus* strains. In Italy, the strains identified from cattle showed a high prevalence of *B. abortus* bv. 3 isolates (84.5%), followed by B. melitensis bv. 3 (9.9%) and *B. abortus* biovars 1 and 6 (5.5% and 0.1%, respectively) [33]. In Africa, *B. abortus* bv. 3 is the most commonly isolated strains in cattle [[34]. Moreover, there were no strains from swine and wild animals; further pathogen surveillance in other hosts is a priority.

The complete MLVA-16 approach exhibited excellent discriminatory power to *B. abortus* with a high HGDI value (0.953). Notably, five panel 2B markers had a high Hunter-Gaston index, and bruce07 is the most helpful locus for genotyping *B. abortus* in China. This result was consistent with B. canis data in China; there were at least two alleles for each of the 16 loci investigated by the MIVA approach [12]. However, this feature is different from the results of studies of B. melitensis diversity in China. The majority of loci from panel 1 and panel 2A showed low diversity, and the bruce04 locus displayed the highest diversity (HGDIs = 0.841) [35]. MLVA-16 panel has a high discriminatory power to *B. abortus* from Italy, with polymorphism levels of 0.939 based on the HGDI, compared to 0.380 and 0.380 for the MLVA-8 and MLVA-11 panels, respectively; and the highest variability was detected in the 2B panel for the bruce09 locus [36]. HGDI analysis of *B. abortus* bv. 3 from West Africa showed that the highest diversity indices were observed with markers composing panel 2 with three to nine alleles, especially at bruce16, which is known as one of the most variable loci [37]. Betsy J et al. reported that, historically, approximately 85% of *B. abortus* infections in the U.S. were caused by biovar 1, which would result in an HGDI≈0.2 [38]. The variability of HGDI features among strains from different regions may reveal different microevolution patterns of the *Brucella* population [39], suggesting that the strains described in this study were introduced from multiple geographic regions.

A total of 17 MLVA-11 genotypes were observed; the higher diversity of MLVA-11 genotype was observed in strains from Inner Mongolia, Xinjiang Province, Hebei Province, and Sichuan Province, suggesting that animal trade often occurred among these regions in the past. The seven shared MLVA-11 genotypes (72, 75, 82, 83, 181, 210, and 328) comprising strains from 2 to 10 countries suggest that the strains from each shared genotype had a common source, indicating that the *B. abortus* from this study had multiple geographic, cross-border transmission, probably due to exchange and trade of infected animal. Trade ties and livestock exchange between countries have a long history; archeological findings unraveled trade relations between the nomadic people who inhabited Central Asia and China long before the Common Era (C.E.) [40]. MLVA-11 genotype 72 was shared by strains from six countries, including France, Germany, Italy, Kazakhstan, Portugal, and China. MLVA-16 comparison analysis showed that Chinese strains were clustered together with Italy, Kazakhstan, and Brazil. A previously reported that, 4,500 years ago, seasonal nomadic pastoralist routes were formed from modern Southern Kazakhstan to Xinjiang Uygur Autonomous

Region, covering more than 70% of the high-mountain route of the silk road [41]. However, there is no trade-in live cattle between Kazakhstan and China in modern history, but actively developing livestock products [42]. Furthermore, four strains from Sichuan Province had three MLVA-11 genotypes (83, 77, and 82), two of which were shared (82 and 83) with strains from other regions, and one (77) is exclusive of strains from Sichuan Province. Although it was a historical area of *B. abortus* brucellosis, it had no shared genotype 72 [6]. The investigation of serology and bacteriology of *B. abortus* infection in this region is a priority. However, 10 single genotypes were exclusively found in Chinese strains, indicating that these strains originated from China lineages, but further investigation on the origin of the strains is recommended.

In this study, the 19 shared MLVA-16 genotypes included 54 strains, with each genotype representing 2–8 strains, and 18 out of 19 shared genotypes consisted of strains of the same province. Only one shared genotype (GT48) was present in five strains, and these were obtained from four different provinces, suggesting that the majority of brucellosis cases were outbreak epidemic within respective regions. This conclusion is in agreement with the geographic distribution of the strains and outbreak cases mainly focus on Northern China. Moreover, the remaining 46 independent genotypes of each single strain indicated that 46% (46/100) cases had epidemiology unrelated or sporadic characteristics [43]. Strengthening the surveillance of *B. abortus* animal infection applied serology, bacteriology, molecular assays in northern regions, China helps better understand the epidemiology of brucellosis.

The sixteen Chinese strains were divided into five clades by whole-genome SNP analysis, confirming that strains from this study had multiple ancestor strains. Remarkably, clade Ⅶ formed a ladder-like phylogram and consisted of strains from nine countries, including Uganda, Iraq, Russia, Georgia, Spain, Italy, Egypt, Mongolia, and China from a single ancestor. The *B. abortus* isolates from Italy are substantially different from those found in Europe and North America and are more closely related to strains from the Middle East and Asia [44]. Furthermore, most Chinese strains (n = 8) were the most similar to Russia and Mongolia; they were geographically close. They revealed that strains from a common ancestor were continuously circulating among these countries. Human brucellosis seroprevalence among rural people in Mongolia is high [45], and Mongolian *B. melitensis* isolates had high genetic similarity with Chinese strains, likely due to geographical proximity [46]. Moreover, some strains from Mongolia had closely related MLVA genotypes to strains from Russia [47]. In the North Caucasian Federal District, the largest number of new human brucellosis cases was notified in the Republic of Dagestan (59.3%) and the Stavropol Territory (27.4%), and the true prevalence of brucellosis greatly exceeds the official statistical data [48]. The frequent spread of these lineages from one country to another due to long-term trading partnerships between the three countries is a likely explanation for the data. Otherwise, due to the uncontrolled introduction of the agents via humans, infected animals, semen, and vectors have a high risk of *B. abortus* infection [49]. In endemic countries, combining the serodiagnosis and bacteriology, and molecular diagnosis for surveillance of brucellosis is essential. Molecular genotyping should be systematically applied to support control plans for control of brucellosis in China.

Moreover, our study has some limitations. First, the strains were collected from previous studies that might have been influenced by many aspects, such as the local economic situation and the diagnosis and surveillance status of brucellosis. Second, due to variability in the number of strains collected among different provinces and for different years in this study, a genome analysis of strains from more regions and hosts is recommended. Third, no related epidemiology data were collected, and analysis of animals moves, and exchange is lacking. Therefore, a whole-province survey on human and animal infections with *B. abortus* should be initiated.

5. Conclusions

In this study, a nationwide molecular analysis of *B. abortus* strains were performed. Our research showed that there was a high diversity of biovar and MLVA-11 genotypes among this population. *B. abortus* strains exhibited multiple geographic origin characteristics and the predominant genotype was shared with strains from many countries, indicating that the strains have multiple geographic origins. Chinese strains were similar to strains from Russia and Mongolia. These have been traditionally countries of cattle and sheep breeding industries. Our work contributes to a better understanding of the epidemiology of *B. abortus* brucellosis in China and also provides invaluable information that could be helpful to devise control strategies for the disease.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: LZG and ZZZ performed strains collected and analysis, WXM and LZG performed genotyping and cluster analysis and drafted the manuscript; WM conducted epidemiological investigations and data analysis; CBY and LZG participated in the design of the study and critically reviewed the manuscript; WXM and LZJ participated in the design of the study and managed the project. All authors read and approved the final version of the manuscript.

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Appendix A

Abbreviations
PCR: Polymerase chain reaction
MLVA: Multiple-locus variable-number tandem repeat analysis
VNTR: Variable-number tandem repeat analysis
HGDI: Hunter-Gaston discrimination index
SNPs: Single-nucleotide polymorphisms
WGS: Whole-genome Sequencing
MST: Minimum-spanning tree

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