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Phylogeography and epidemiology of *Brucella suis* biovar 2 in wildlife and domestic swine

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ABSTRACT

Swine brucellosis due to *Brucella suis* biovar 2 (bv2) is enzootic in wild boar and hare in continental Europe and may cause major economic losses to the pig industry, mainly in free-ranged pig farms. The high nucleotide identity found among the *B. suis* biovar 2 isolates has long hindered the full understanding of the epidemiology and the phylogeography of the disease. Here, we used multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) and whole-genome analysis to identify single-nucleotide polymorphisms (SNPs) in order to gain insights from the largest *B. suis* bv2 dataset analyzed so far composed of domestic pigs and wildlife isolates collected throughout Europe since the 1970s. We found four major clades with a specific phylogeographic pattern. The Iberian clade contains isolates exclusively from the Iberian Peninsula. The Central European clade includes most isolates from France, Northern Italy, Switzerland and an important proportion of those of Northern Spain. The Eastern European clade clustered isolates from Croatia and Hungary mainly but also from areas of France, Germany, Italy and Poland. Finally, a separated Sardinian clade grouped three isolates from this island. At fine scale, MLVA demonstrated an endemic status of the infection in Europe and it allowed tracking a large outbreak formed by different farms from Spain linked to the same infection source. The whole genome SNP analysis showed that the strains form genetically distinct clades, shared between wild boar and pigs, in agreement with the MLVA clades. Interestingly, all hare isolates clustered together within two groups composed exclusively of wildlife isolates. Our results support the hypothesis that maintenance and spread of *B. suis* bv2 in Europe is a dynamic process linked to the natural expansion of wild boar as the main wild reservoir of the infection, while spread over long distances is found largely dependent on anthropogenic activities.

1. Introduction

Brucellosis is a major infectious disease of domestic pigs caused by three (biovars 1, 2 and 3) of the five *Brucella suis* biovars. The infection often leads to long-term reproductive failure, causing significant economic losses to the swine industry worldwide (EFSA, 2009; OIE, 2018; Olsen et al., 2012). Geographic distribution and host range differ among *B. suis* biovars. Biovars 1 and 3 affect mainly domestic and feral

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pigs (*Sus scrofa domesticus)* and wild boar (*Sus scrofa)* and cause zoonotic infections (Godfroid et al., 2005, 2013). Contrary to these biovars, biovar 2 is rarely pathogenic for humans and appears to be restricted to continental Europe, where the infection caused is indeed considered as an emerging problem (EFSA, 2009; Lagier et al., 2005; Mailles et al., 2017; Paton et al., 2001).

Wild boar and European brown hare (*Lepus europaeus*) are the only proven wildlife reservoirs of *B. suis* biovar 2 (herein bv 2) so far (Bergagna et al., 2009; Cvetnić et al., 2009; Godfroid, 2002; Grégoire et al., 2012; Muñoz et al., 2010). Although very unfrequently, *B. suis* bv *2* can also infect cattle but its epidemiological significance is unknown (Andersen and Petersen, 1995; Jaÿ et al., 2014; Szulowski et al., 2013).

Despite sporadic outbreaks, the European Union countries are considered officially free of porcine brucellosis. However, as *B. suis* bv 2 is widespread in wildlife, domestic pigs are at risk of acquiring brucellosis by contact with the wildlife reservoirs, that happens particularly in outdoor breeding systems or back yard herds (EFSA, 2009). Occasionally, the infection can be introduced also in intensive pig farms by entry of infected replacements and/or semen. Once introduced in a given herd, direct mucosal exposure to aborted tissues, genital secretions and contaminated food or water are the main routes of intra-herd transmission (Alton, 1990; Olsen et al., 2012).

Conventional microbiological and biochemical methods for biotyping of *Brucella* isolates are being progressively replaced by molecular techniques. Some of the latter offer high reproducibility and confidence of intra and inter-laboratory standardization and have been included in the OIE Terrestrial Manual (OIE, 2018). Namely, discrimination among main *Brucella* species (*B. suis* included) can be performed with a single step multiplex PCR named Bruce-ladder (López-Goñi et al., 2008). Moreover, the multiplex PCR known as Suis-ladder (López-Goñi et al., 2011) can be used for the differentiation of the five *B. suis* biovars. Alternative approaches based on single nucleotide polymorphisms (SNPs) have been proposed also for identifying the *B. suis* biovars (Whatmore and Gopaul, 2011). For epidemiological purposes, different molecular methods such as restriction fragment length polymorphism (RFLP) ((Cloeckaert et al., 1995; Vizcaíno et al., 1997)), applied to genes known to be polymorphic in *Brucella* (Cloeckaert et al., 1995; Vizcaíno et al., 1997) have been also developed. Nevertheless, due to the low genetic diversity of *Brucella* spp., all these approaches give not enough resolution for the sub-species differentiation. Multiple Locus Variable-number tandem-repeat Analysis (MLVA) (Le Flèche et al., 2006) or Multilocus Sequence Analysis (MLSA) (Whatmore et al., 2016) have been used to identify polymorphisms within *Brucella* species. Genotyping of *B. suis* bv 2 isolates from domestic pigs and wildlife indeed allowed the identification of wild boar as the main reservoir of infection for domestic pigs. However, the precise role of the European brown hare in the epidemiological cycle of *B. suis* bv 2 remains to be clarified (Duvnjak et al., 2015; Ferreira et al., 2017a; Garcia-Yoldi et al., 2007; Kreizinger et al., 2014; Vergnaud et al., 2018). Some studies reveal the existence in *B. suis* bv 2 of different genotypes and genetic clades with specific geographic location, especially the so called Iberian lineage, found to be exclusive of the Iberian Peninsula (Ferreira et al., 2017a; Garcia-Yoldi et al., 2007; Muñoz et al., 2010; Vergnaud et al., 2018). However, factors related to the specific geographic and host distribution of *B. suis* bv 2 remain to be identified.

The whole-genome SNP analysis, provides a high discriminatory power among *Brucella* genotypes and it is highly effective for establishing phylogenetic relationships between both closely and distantly related isolates (Garofolo et al., 2017; Georgi et al., 2017; Janowicz et al., 2018; Keim et al., 2004; Pearson et al., 2009; Tae et al., 2012; Tan et al., 2015). Therefore, the whole-genome SNP analysis provides complementary information to the MLVA typing and could be used to define stable molecular markers based on a proper selection of SNPs (*e.g.* conserved canonical SNPs).

Our study aims to expand knowledge on swine brucellosis epidemiology in Europe by characterizing a representative *B. suis* bv 2 strain collection from different European host populations using both MLVA and WGS genomic approaches. We describe a new MLVA panel specifically designed for *B. suis* bv 2 typing and use advanced phylogeographic analysis methods to clarify the epidemiological relationships and sources of infection in pigs.

2. Materials and methods

2.1. B. suis biovar 2 strains

A total of 519 *B. suis* bv 2 strains isolated from domestic pigs, wild boar, European hares, cattle and humans in France, Italy, Poland, Romania, and Spain between 1978 and 2015 were genetically characterized in this study. Supplementary material related to the strain collection analyzed is provided in Table S1. All isolates were confirmed as *B. suis* bv 2 using standard microbiological and biochemical procedures (OIE, 2018) and both Bruce-ladder (López-Goñi et al., 2008) and Suis-Ladder (López-Goñi et al., 2011) molecular methods.

The French strains (n = 211) comprised of isolates from pigs (n = 104), wild boar (n = 45), hares (n = 54), cattle (n = 3), humans (n = 2) and three strains from undetermined wildlife hosts (hare or wild boar) from 52 Departments. The Spanish panel (n = 146) included strains from domestic pigs (n = 96), wild boar (n = 48) and hares (n = 2) from 11 Provinces. The 96 isolates in the Spanish pigs' panel belonged to 38 farms most of them sharing the same semen or breeding stock provider. The Italian collection (n = 123) included isolates from wild boar (n = 114) and pigs (n = 9) collected from six Regions. Polish (n = 12) and Romanian (n = 27) isolates from wild boar, pigs and hares were also included in the study (see Table S1 for a more precise description).

For DNA extraction, all B. *suis* bv 2 original isolates were subcultured either in *Brucella* medium base (BAB; Oxoid, Hampshire, UK) or trypticase soy agar (TSA; Difco, NJ, USA) and incubated in a 5–10% CO_2 atmosphere at 37 °C during 48 h to assess the purity of cultures and the absence of dissociation. Bacterial DNA was extracted from single colonies using QIAamp® DNA minikit (QIAGEN, Hamburg, Germany), Maxwell® 16 Tissue DNA Purification Kit using Maxwell® 16 Instrument (Promega, Madison, WI, USA) or High Pure DNA Template Preparation kit (Roche Diagnostics, France) according to the manufactures' instructions.

2.2. Brucella suis MLVA analysis

Individual DNA samples from the 519 isolates were typed with the MLVA-16 panel using either the single-plex PCR and agarose gel electrophoresis method described elsewhere (Al Dahouk et al., 2007; Le Flèche et al., 2006) or the multiplex PCR and capillary electrophoresis on an ABI 3500 instrument with POP 7 polymer described by Garofolo et al. (Garofolo et al., 2013). Normalization of raw data generated by both methods was assessed using corrections derived from a comparative panel of 51 strains submitted to whole genome sequencing (see below). Moreover, our dataset was completed by including 332 previously published MLVA-16 profiles from *B. suis* bv 2 strains isolated from wild and domestic species in Germany (22 wildboar, 3 pigs and 1 hare) (Le Flèche et al., 2006), France (6 wildboar, 11 pigs, 11 hares and 1 cattle) (Le Flèche et al., 2006), Croatia (1 wildboar and 74 pigs) (Duvnjak et al., 2015), Portugal (56 wildboar, 32 pigs, 1 cattle and 2 sheep) (Ferreira et al., 2017a), Poland (2 hares) (Le Flèche et al., 2006), Hungary (55 wildboar, 8 pigs and 5 hares) (Kreizinger et al., 2014) and Switzerland (30 wildboar, 7 pigs and 4 hares) (Abril et al., 2011) (see Table S1 for details).Genetic diversity and partitions of each locus were determined using the Simpson diversity index (SDI) calculated on a dataset comprising of only one strain per MLVA-16 genotype using online calculation tool (http://www.comparingpartitions.info).

Clustering and congruence analyses were conducted with BioNumerics 7.6.3 (Applied Maths NV, Belgium) using data as

character dataset with categorical distance coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) or MST (Minimum Spanning Tree) methods. Clonal complexes were retrieved with the most stringent (conservative) definition (Spratt et al., 2004), where all members assigned to the same group differed only by one locus with at least one other member of the group. MLVA allelic profiles of Spanish isolates with known epidemiological links were analyzed using goeBURST algorithm in PHYLOViZ 2.0. MST was generated using default settings (Nascimento et al., 2017).

To determine relationships between genetic clades and countries, the Spearman coefficient was calculated using RELATE function in primer7 software with 10,000 random permutations. To determine the phylogeographic distribution of the clades, the analysis of similarity (ANOSIM) (Clarke, 1993) was carried out using primer7 software version 7.0.13. The whole dataset was analyzed first for distribution among different countries. The distribution of clades within each country (whenever feasible) was then tested by Pearson's Chi-squared test (χ^2) with Yates' continuity correction test using "Province" input as variable.

2.3. Whole genome sequencing (WGS) and SNP analysis

To strengthen MLVA results, SNPs were identified from WGS to determine the *B. suis* bv 2 population structure. The WGS was applied to 51 *B. suis* bv 2 isolates selected from our dataset to be representative of the MLVA phylogeny and geographic origin (Table S1). Additional 22 *B. suis* bv 2 genomes (strains isolated from wildboar, pigs, hares or unknown hosts in Belgium, Bulgaria, Czechoslovakia, Denmark, France, Hungary, Germany, Italy, Portugal and Spain between 1963 and 2009) available from the public database GenBank (accessed October 2017) were also included in the SNP analysis (Table S2).

Library preparation was performed using the Nextera XT Library Prep kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's manual. The libraries were sequenced using Illumina NextSeq 500 platform producing 150bp paired-end reads. After de-multiplexing and removal of the adapters, reads were trimmed to remove from 5′ end and 3′ end to discard nucleotides with quality score less than 20. Reads shorter than 70 bp and average Phred mean quality < 24 were automatically discarded. SNPs were discovered using In Silico Genotyper (ISG) version 0.16.10-3 (Sahl et al., 2015) using default filters to remove SNPs from duplicated regions, read coverage less than 10X and base call proportion less than 90%. ISG uses BWA (Li and Durbin, 2009) as aligner and GATK (McKenna et al., 2010) as SNP caller. SNPs were called based on alignment to the reference *B. suis* biovar *2* strain ATCC 23445 (GenBank Accession Numbers NC_010169.1; NC_010167.1).

Phylogenetic trees were constructed using maximum parsimony with *B. suis* biovar 1 reference strain 1330 (NC_004310.3, NC_004311.2) as an out-group to root the tree in PAUP* 4.0b (Wilgenbusch and Swofford, 2003). Branch support was assessed with 1000 bootstrap replicates and a consistency index was generated to assess the level of homoplasy, and Φ-test was conducted in SplitsTree to test recombination (Huson and Bryant, 2006). Finally the population structure was assessed using a Bayesian approach implemented in BAPS 6.0 software (the module hierarchical BAPS (hierBAPS) (Corander et al., 2003).

2.4. Accession numbers

All generated reads were submitted to National Center for Biotechnology Information (NCBI) under the bio-project accession number PRJNA497793.

3. Results

3.1. MLVA analyses

To establish a minimal MLVA informative panel for *B. suis* bv 2, we calculated the genetic diversity index and partitions for each of the 16 loci (Table S3). Bruce06, Bruce11, Bruce43, Bruce45, Bruce21 and Bruce16 showed very low (under 0.5) Simpson's Diversity Indexes (SDI). Bruce08, Bruce12, Bruce42, Bruce55, Bruce18 and Bruce19 loci showed 4–8 alleles and then average SDI values (from 0.508 to 0.770). However, the remaining loci (Bruce04, Bruce07, Bruce09 and Bruce30) showed a high number of alleles (12–27) with SDIs close to 1, confirming their variability. We defined a new MLVA panel for *B. suis* bv 2 selecting only the loci that showed 4 or more diverse alleles. Thus this new panel (that we named MLVA-11_{suis2}) blending stable and polymorphic markers, comprised the following 11 loci: Bruce08, Bruce11, Bruce12, Bruce42, Bruce55, Bruce18, Bruce19, Bruce04, Bruce07, Bruce09 and Bruce30. The MLVA-11_{suis2} and MLVA-16 identified for the 851 *B. suis* bv2 isolates examined the same number of partitions (579 genotypes) with a Pearson correlation equal to 99.5%.

The UPGMA dendrogram generated with the MLVA-11_{suis2} profiles identified four major clades with identity of at least 92.5% (Figure S1shows a detailed dendrogram with relevant information of each strain). Plotting these clades on the geographic maps (Fig. 1) suggested a link between phylogeny and geographic distribution of the isolates. Based on the location of the most prevalent clades they were named as: *i*) Iberian, containing strains isolated exclusively from the Iberian Peninsula; *ii*) Central European, comprising mostly isolates from France, Northern Italy, Northern Spain and Switzerland,; *iii*) Eastern European, featuring mostly isolates from Croatia, Germany, Hungary and Poland; and *iv*) Sardinian, containing three strains from Sardinia. Pig isolates from Poland and Romania were excluded from the phylogeographic assessment since they were proven to have an Iberian origin according to both PCR-RFLP analysis (Muñoz et al., 2010) and well-identified trade movements in the affected herds.

The correlation analysis resulted in a significant relationship between the genotype and the geography, supporting the above geographic distribution (Spearman correlation coefficient $\rho = 0.176$, $p = 0.0001$). The ANOSIM analysis revealed that the three main genetic clades were composed of distinct geographic groups with R-values ranging from 0.251 to 0.372 ($p = 0.001$). A clear phylogeographic separation was evidenced between Eastern European and Iberian clades, and between Iberian and Central European clades (R-values of 0.372 and 0.336, respectively). However, some geographic overlap was observed for Central European and Eastern European clades ($R = 0.251$).

The Central European clade was highly prevalent in Italy, France and Switzerland, while the Eastern European clade predominated in Croatia, Germany and Hungary. Finally, the Iberian genotypes clearly outnumbered other MLVA types in both Portugal and Spain (Fig. 1).

The analyses within countries showed that in Italy the Central European and Eastern European clades were clearly separated geographically ($\chi^2 = 50.297$; $p = 1.322e\text{-}12$), with the Central European clade found mostly in the North (Piemonte) and the Eastern European clade in the Apennine mountains (Emilia Romagna, Marche, Abruzzo, Lazio). In Spain the Iberian clade was exclusive and widespread all over the country ($\chi^2 = 35.409$; $p = 2.673e\text{-}09$) while in the Northern Provinces (above the Ebro's valley) overlapped with the central European clade. No significant geographical segregation was found within France, where the Central European genotype was prevalent.

The clonal complex (CC) analysis separated the isolates into 23 groups for the Iberian clade, 35 for the Central European and 33 for the Eastern European (Fig. 2A, Table S1). With the exception of nine cases (CCs 1, 3, 36, 38, 39, 40, 41, 46 and 50), the clonal complexes were exclusive from individual countries (Fig. 2C).

Two of the most prevalent CCs (CC1 and CC3), included the majority (58.5%; n = 134)) of isolates composing the Iberian clade

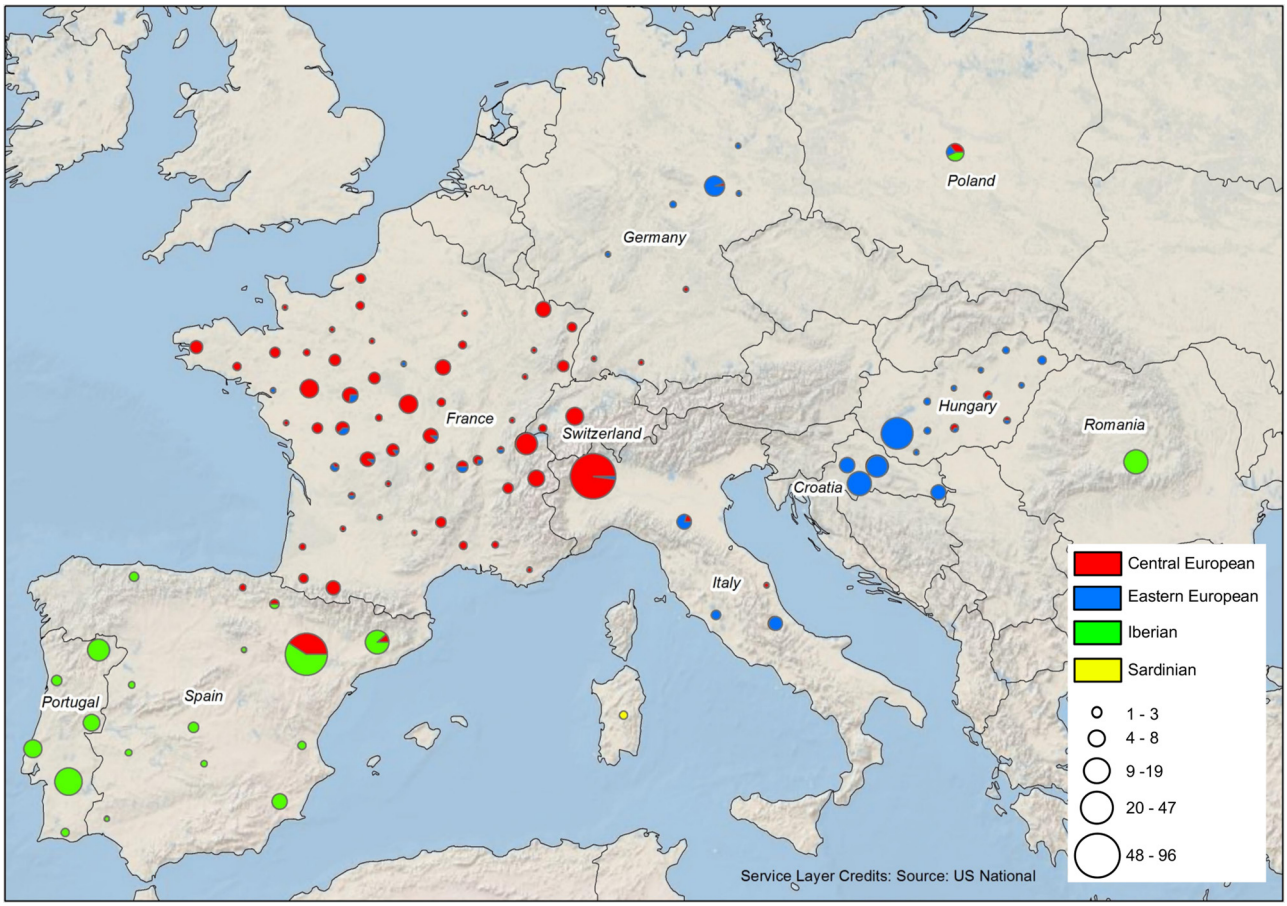

Fig. 1. Geographic distribution of MLVA clades in Europe. Geographical mapping of *B. suis* bv 2 according to the MLVA-11$_{suis2}$ was obtained by ArcGIS® software by Esri using the geographical coordinates found from "province" or "country" entries. Colors within the mapped circles correspond to the clade color designations in the MLVA phylogeny: Iberian clade in green, Central European clade in red, Eastern European clade in blue and Sardinian clade in yellow. Esri. " Natural Earth physical " [basemap]. Scale Range: 1:591,657,528 down to 1:2,311,162.″ Natural Earth physical Map". December 12, 2009. http://goto.arcgisonline.com/maps/World_Physical_Map. (February 13, 2019) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Fig. 2A). Most of the CC1 Spanish strains were isolated from domestic pigs in herds located in different regions (Table S1). Interestingly, all these CC1 affected herds were related epidemiologically to a single genetic holding (farm 15, Fig. 4) which provided semen and/or live replacements to all affected herds. Moreover, CC1 included several wild boar isolates (two from Spain and one from a bordering region of Portugal) as well as 27 strains from a single Romanian herd and 6 additional strains from a Polish herd (Fig. 2C), both linked epidemiologically (through trade movements) with the same Spanish genetic provider (farm 15). The CC3 was the second largest complex of the Iberian clade and grouped strains isolated mainly from pigs in Portugal. This CC3 was also found in wild boar from Portugal, and in Spanish outdoor pig herds in areas bordering Portugal (Fig. 2, panels B and C). Interestingly, CC3 included a single cattle isolate from Portugal (see Table S1 for details). Other minor Iberian CCs also revealed epidemiological links (Fig. 4 and Table S1). The Iberian CC4 grouped two strains isolated from pig farms in different regions. However, these farms were epidemiologically linked to the same genetic provider (farm 15), as confirmed in farm 18 by the presence of CC1. Finally, both CC8 and CC72 were found in epidemiologically related herds (one was the seed stock of the other).

Within the Central European clade, CC37 was the largest one (Fig. 2A), composed by 56.1% (n = 69) of the Italian strains (Fig. 2C), all isolated exclusively from wild boar (Fig. 2B). Most of the isolates (n = 37) were collected in Piemonte in the "*La mandria*" regional park (extending for 6,570 ha). Twenty-four Central European CCs were found in France (Fig. 2, panels A and C), six of which were also found in at least one bordering country (Italy, Switzerland or Spain). Three pig herds located in Northern areas of Spain were infected with Central European strains. Two of these herds formed the CC55 and CC93 (with two isolates each), that corresponded to outdoor and intensively reared pigs, respectively (Fig. 2 panels B and C and Fig. 4).

In the Eastern European clade, no shared CCs among countries were found. Five complexes (CC17, CC19, CC23, CC26, and CC21) grouped 41.4% of the strains isolated in Hungary, Croatia and Germany. France and Italy yielded three and six CCs, respectively.

Most of MLVA profiles from hare isolates could not be assigned to any CC, with the exception of CC29 (Eastern European) and CC57 (Central European), that grouped hare isolates from France and Hungary, respectively, and CC32 (Eastern European strains from France) and CC35 (Central European strains from Switzerland), composed of isolates from both hare and wild boar (Fig. 2, panels B and C).

Surprisingly, none of the strains isolated in atypical hosts in France (4 from cattle and 2 from human) clustered in any CCs with pig or wildlife isolates (Figure S1).

3.2. Whole genome sequencing

Fifty-one *B. suis* bv 2 isolates were selected to cover maximum genetic diversity as defined by MLVA-11$_{suis2,}$ and widest geographic spread (Figure S1). Additionally, 22 public available genomes were included to build up a SNP-based reference phylogeny (Fig. 3). The SNP analysis revealed 3,838 putative polymorphisms. The phylogeny constructed through parsimony, using *B. suis* biovar 1 strain 1330 as an out-

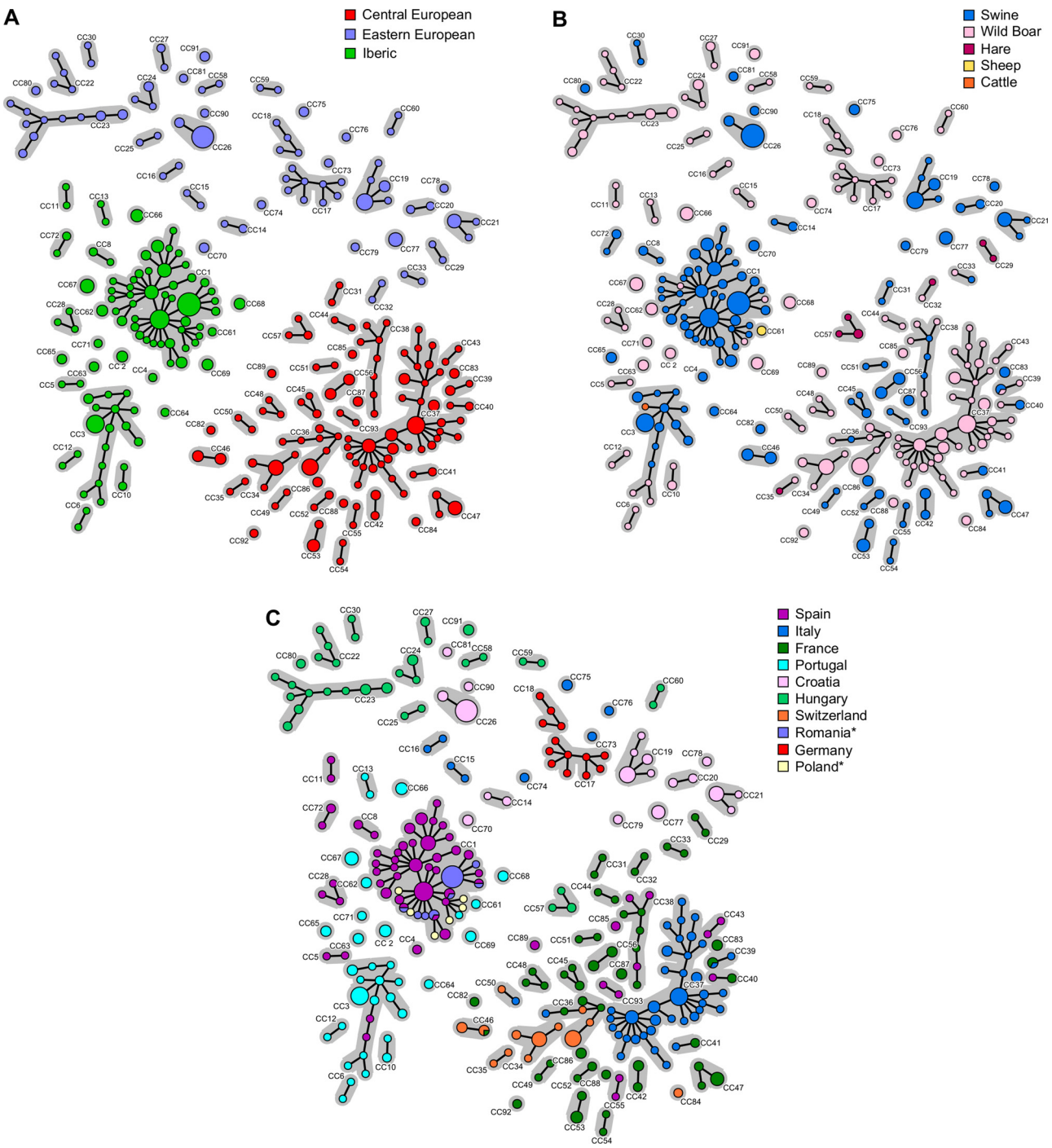

Fig. 2. Minimum spanning tree depicting the different Clonal Complexes found for each genetic clade, each circle corresponds to a particular genotype. (2 A): The circles are colored with respect to the MLVA-11_{suis2} clades; (2B): The circles are colored with respect to host-species; (2C): The circles are colored with respect to the country sources.

group control genome, revealed a very high (0.993) consistency index. The recombination was evaluated using the phi-test based on the SNP alignment and resulted not significant statistically ($p = 0.99$), demonstrating that recombination did not affect our interpretation. As shown in Fig. 3, the BAPS analysis split our dataset into six populations based on the secondary level of clustering. Subpopulations were arranged in monophyletic groups, confirming the robustness of the major clades of the SNP phylogeny, which were also supported by 100% bootstrap scores.

The SNP analysis demonstrated that the Sardinian, Eastern European and Iberian clades corresponded to BAPS1, 2 and 3 respectively, while the Central European clade was split, further into 3 groups (BAPS4-6). The BAPS1 group (Sardinian clade) comprised a basal clade, which contained only strains from Sardinia. BAPS2 group (Eastern European clade) was composed of strains from Italy, France, Germany, Denmark and Bulgaria, while the BAPS3 group (Iberian clade) grouped strains isolated in Spain and Portugal exclusively. Interestingly, isolates from hares were restricted mainly to BAPS4 and BAPS5, with the BAPS4 composed of two isolates only (one from France and one from Germany). BAPS5 and BAPS 6 contained strains from Italy and France together with other European strains.

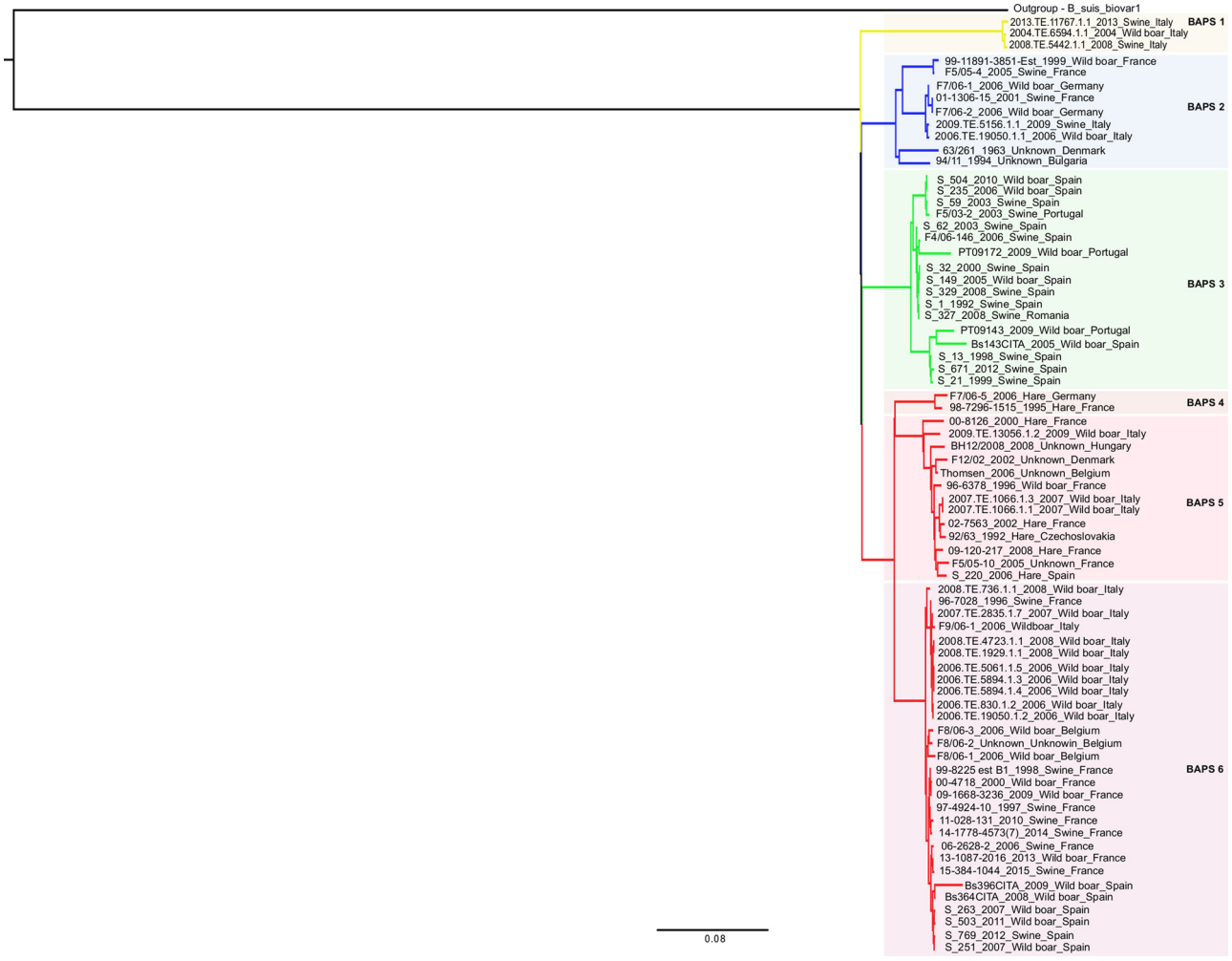

Fig. 3. Maximum parsimony tree for *B. suis* bv 2. BAPS group are identified with shaded areas. For each strain is reported the strain identification, the year of isolation, the affected host, and the country of the isolation.

4. Discussion

Swine brucellosis caused by *B. suis* bv 2 is considered an emerging sanitary problem in pigs in Europe (EFSA, 2009). Despite the existence of well-identified wildlife reservoirs, the epidemiological cycle of this infection remains to be fully clarified.

Scanty information is available on the efficacy of high-resolution molecular typing techniques to determine the phylogeography and the epidemiology of *B. suis* bv 2 infections (Di Sabatino et al., 2017; Duvnjak et al., 2015; Ferreira et al., 2017a, b; Garcia-Yoldi et al., 2007; Kreizinger et al., 2014; Vergnaud et al., 2018). These molecular investigations have been mainly focused on isolates from both wild boar and domestic pigs, with scarce inclusion of hares. Here MLVA and WGS-SNP analysis were used to analyze the largest *B. suis* bv 2 strain dataset so far, isolated across Europe between 1978 and 2015 from domestic pigs and wildlife reservoirs (including brown hares) as well as from some atypical hosts (sheep and cattle) and humans. The results obtained after characterizing 519 *B. suis* bv 2 strains (Table S1) were combined with a selection of those already available to draw a phylogeographic map of *B. suis* bv 2 infections in Europe (n = 332). Moreover, using a collection of Iberian strains from domestic herds, the MLVA-11_{suis2} panel proved to be useful for tracing back *B. suis* bv 2 outbreaks.

Previous genotyping studies based either on MLVA-16 (Panel 1, 2 A and 2B markers) or MLVA-11 (Panel 1 and 2 A markers) evidenced a lack of polymorphism of Bruce 06, 43, 45, 21 and 16 loci in *B. suis* bv 2 isolates (Duvnjak et al., 2015; Ferreira et al., 2017a; Garcia-Yoldi et al., 2007). Accordingly, these non-informative loci were excluded and the resulting MLVA-11_{suis2} was confirmed as adequate to detect genetic differences among closely related isolates, thus being a cost-effective alternative for *B. suis* bv 2 genotyping studies.

Overall, the main genetic clades defined by MLVA-11_{suis2} and particularly, the demonstration of a well differentiated Iberian clade, were in accordance with previous publications (Duvnjak et al., 2015; Ferreira et al., 2017a; Garcia-Yoldi et al., 2007; Kreizinger et al., 2014; Vergnaud et al., 2018). However, our study allowed the identification of four *B. suis* bv 2 clades showing specific geographic distribution (Iberian, Central European, Eastern European and Sardinian); whereas other studies (focused either on more restricted geographic areas or using different clustering algorithms) identified only two geographic clades (Ferreira et al., 2017a; Vergnaud et al., 2018) or did not find clear geographic clustering (Kreizinger et al., 2014).

WGS SNP analysis supported MLVA findings and allowed the identification of six subpopulations in our dataset, as inferred using Bayesian approach. Despite the low number of strains tested, the Sardinian cluster (BAPS1) branched out from the basal phylogenetic group suggesting its evolution in a confined geographic location, physically separated from the other *B. suis* bv 2 strains. On the contrary, the populations of the Eastern European (BAPS2), the Iberian (BAPS3), and the Central European (BAPS4, BAPS5 and BAPS6) clades formed well-structured populations. The geographic markers within *B. suis* bv 2 were backward supported by the SNP analysis which validated our

VNTR data.

The European wild boar is considered the most relevant wildlife reservoir of *B. suis* bv 2, playing a critical role for the transmission of infection to pigs. European wild boar are differentiated into four main lineages: *Sus scrofa scrofa* (distributed in Central Western Europe), *Sus scrofa attila* (Eastern Europe), *Sus scrofa meridionalis* (Sardinia and Corsica) and *Sus scrofa lybicus* (Southern Balkans) (Groves and Grubb, 1993). Despite the existence of some specific populations (see comments below), wild boar from the Iberian Peninsula share similar microsatellite markers with the European *Sus scrofa* lineages, but are different from both Central Western and Eastern European lineages (Alves et al., 2010). The phylogenetic diversity of *B. suis* bv 2 clearly parallels the distribution of wild boar lineages in Europe, where the Eastern European clade spread coincides with the distribution range of one of the largest wild boar population in the forested areas of Germany and Eastern neighboring countries (EFSA, 2010), while the Central European and the Iberian clades are dominant in France and Iberian Peninsula, respectively.

The presence of three separate B. *suis* bv 2 clades (Central European, Eastern European and Sardinian) in Italy could be related to the different origins of wild boar populations in this country. The native Italian wild boar (*S. scrofa meridionalis)* became extinct in the mainland Italy in the eighteen century due to excessive hunting while it can still be found in the main Sardinia island (Larson et al., 2005; Vernesi et al., 2003). The divergence of the basal B. *suis* bv 2 Sardinian clade suggests that it might have evolved separately within this local wild boar lineage. In the last century, feral pigs arriving from France repopulated the northwestern areas of the mainland Italy, being a likely source of introduction of the *B. suis* bv 2 Central European clade. Conversely, the presence of the Eastern European clade in the central Apennines could be related to the recent restocking campaigns with wild boar from Eastern Europe (Di Sabatino et al., 2017; Scandura et al., 2011).

Diverse wild boar lineages have been also identified in the Iberian Peninsula (Alves et al., 2010). Our data confirms the existence of two main *B. suis* bv 2 clades in this region (Central European and Iberian). In accordance with previous studies (Ferreira et al., 2017b; Muñoz et al., 2010), *B. suis* bv 2 Iberian clade is distributed all over the Iberian Peninsula while the Central European clade remains restricted to the northern regions above the Ebro's valley. Natural entry of wild boar from France through the Pyrenees or the common practice of restocking among hunting grounds (M.C. Arnal, unpublished findings) could account for the introduction of Central European *B. suis* bv 2 strains in Spain. This hypothesis is further supported by the identification of CC38 in wild boar from both France and Spain. Moreover, Ferreira and colleagues hypothesized allopatric speciation as a plausible origin of the diverse *B. suis* bv 2 clades in the Iberian Peninsula (Ferreira et al., 2017b).

Although the brown hare is considered also a *B. suis* bv 2 wild reservoir, its role in maintaining and spreading the infection to wild boar and pigs is not well understood. Because of the high density of this animal and the absence of wild boar in the affected area, hares were hypothesized as the most probable source of two *B. suis* bv 2 outbreaks in pigs in France (V. Mick, unpublished results) and Denmark (Andersen and Petersen, 1995). In fact, some authors found identical genotypes in both hare and swine populations (Duvnjak et al., 2015). However, other studies reported genetic divergence among hare and wild boar *B. suis* bv 2 isolates, with no shared genotypes between both wild species (Ferreira et al., 2016; Kreizinger et al., 2014). Our MLVA11$_{suis2}$ analysis confirmed also the absence of shared genotypes between hare and swine (Fig. 2B). Although geographical clustering was not clearly proven for hares in our study, hare isolates were often found close to both Central European and Eastern European wild boar isolates, and rarely found in proximity to pig isolates (Figures S1 and 2B). Bayesian model-based clustering strengthened these findings, since all hare isolates clustered together within two BAPS (4 and 5) grouping wildlife isolates with BAPS 4 composed of hare isolates exclusively (Fig. 3). In the Iberian Peninsula *L. europaeus* has been shown to carry *B. suis* bv 2 (Lavin et al., 2006). However, this hare species is not very abundant and its distribution range is restricted to the northern regions (usually above the Ebro's valley). In fact, most of the Iberian Peninsula is densely populated by the Iberian hare (*L. granatensis*) (Palacios and Mejide, 1979). Interestingly, a study conducted on 261 Iberian hares did not show any evidence of brucellosis (Martínez Durán, 2014). Altogether, the above findings confirm that European brown hare is a wild reservoir of *B. suis* bv 2 infections but its epidemiological role in the transmission to domestic pigs remains unclear. Further studies focusing on WGS of *B. suis* bv 2 isolated from larger hare populations across Europe are required to clarify the role of this animal in the epidemiology of *B. suis* bv 2.

Transmission to atypical species like domestic ruminants and humans seems to be accidental. Current MLVA-11$_{suis2}$ clustering analysis confirmed that Portuguese strain from cattle was close to swine and wild boar isolates included in the same CC3 (Fig. 2B), suggesting transmission through shared pastures from wild boar to livestock reared in traditional outdoor breeding systems. In contrast, our CC analysis did not link any of the French bovine strains (all them included in the Central European clade) to other animal species, but the UPGMA dendrogram (Figure S1) showed relatedness to one pig and one human isolate. Interestingly, the two patients included in this study were immunocompromised wild boar hunters exposed to wildlife, but not to domestic pigs (Mailles et al., 2017). One patient was infected by an Eastern European strain that appeared genetically close to wild boar and hare isolates (Figure S1), while the other one clustered with livestock isolates (swine and cattle). The CC analysis did not prompt any hypothesis about the human infection source.

In the fine scale epidemiological analysis conducted to define clonal complexes (Fig. 4), most Spanish isolates from pigs were grouped in CC1, despite being isolated in different farms and distant regions over a period of five years. Interestingly, all these farms were shown to be related epidemiologically to the same semen genetic provider (farm 15), which was found to be infected with a CC1 strain. This suggests that artificial insemination with infected semen from this provider was the cause of this important outbreak in Spain. Moreover, three wild

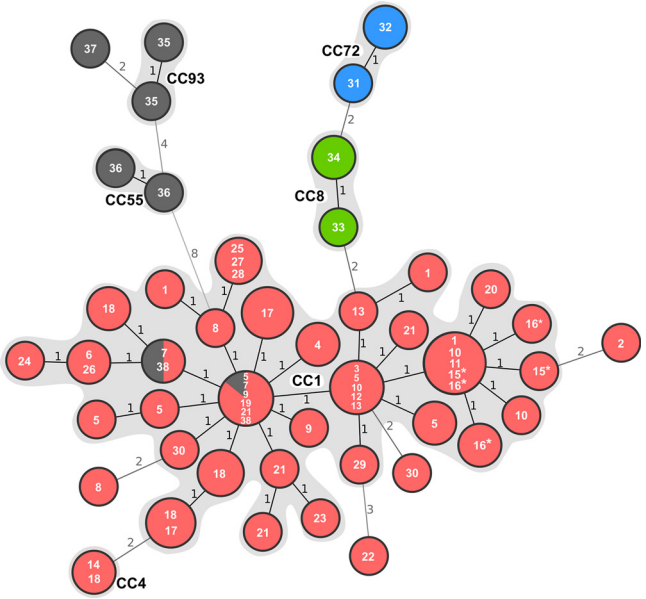

Fig. 4. Minimum Spanning Tree (MST) of some Iberian *B. suis* biovar 2 isolates from domestic pig herds based on MLVA-11$_{suis2}$ profiles. Farms with known epidemiological links are represented with the same node color and grey nodes corresponding to no known links. Farms known to provide genetic material are depicted with (*). Clonal complexes (CCs) are shaded in grey. The branch labels correspond to the number of discriminative alleles.

boar isolates were included in this CC1 (Fig. 2B) suggesting that this wild reservoir could be the primary source of infection for domestic pigs. In fact, in a similar study conducted in Portugal (Ferreira et al., 2017a) a total of 25 swine isolates were grouped in CC3, which included also three strains from wild boar.

Individual diagnosis of swine brucellosis based on serological tests (the most common routine diagnostic method) is extremely unspecific due to the widespread presence of false positive serological reactions caused by cross-reacting gram negative bacteria infecting swine (Dieste-Pérez et al., 2015; EFSA, 2009; Jungersen et al., 2006). Accordingly, a misinterpretation of the results of serological tests could explain the entry of the infected boars in that particular genetic holding. Once the infection is established, brucellosis can spread from a single insemination center or a seed stock holding to a large number of breeding farms in a very short time interval, affecting as many as 50–80% of sows in each holding (Olsen et al., 2012).

In Italy, CC37 is the most important complex, and has been prevalent for many years in wild boar in the small-extended natural park "*La mandria*" nearby Turin, demonstrating the endemicity of *B. suis* bv 2 in this area. As seen in Fig. 2C the CC39, and CC41, were composed of strains isolated in Italy and France while the CC36 included French, Italian, and Swiss isolates, suggesting a close genetic relatedness among isolates from these countries.

In France, swine brucellosis was considered virtually eradicated in the 1970s because of the disappearance of the traditional small back yard farms and the development of the intensive pig breeding system. However, *B. suis* bv 2 re-emerged in the 1990s after the important increase of outdoor pig farms (Garin-Bastuji and Hars, 1999) and since 1993, *B. suis* bv 2 outbreaks have been reported frequently in these extensively reared farms (Bronner and Garin-Bastuji, 2009). The CC analysis showed that only 6 out of 22 CCs identified in France were shared with other countries thus suggesting that a vast majority of French CCs was of autochthonous nature. The CC38 (composed by strains isolated in both pigs and wild boar) and CC40 (pig strains) were composed by strains isolated in the French Pyrenean departments as well as in wild boar in the Northern areas of Spain close to the French border. The close geographical proximity accounts for these shared CCs.

Our MLVA-11_{suis2} analysis grouped the isolates from epidemiologically related farms successfully in the same or very close CCs, thus supporting the value of this analysis for tracing back the epidemiology of infections induced by *B. suis* bv 2. In contrast to that reported in a *B. melitensis* bv 3 outbreak in which several strains coming from different hosts showed identical MLVA profiles (Holzapfel et al., 2018), our study evidenced that small MLVA variants can be identified within the same *B. suis* bv 2 outbreak. Indeed, isolates showing variations in one or two different loci were found within the same farm (Fig. 4). By contrast, we identified two very distant and epidemiologically unrelated pig outbreaks that were caused by *B. suis* bv 2 strains showing differences in 2 loci only. This was the case of isolates included in CC1 and CC8, in which the infections were due to different strains, as proven by both the epidemiological data and the different RFLP profiles identified (Table S1). Thus, before making conclusions on the apparent relatedness among isolates showing one or two MLVA differential loci, confirmation would require of further molecular tests (RFLP, SNPs or WGS) and a precise knowledge of the epidemiological history of the affected herds. Moreover, techniques such as MLVA and WGS require specific equipment and bioinformatics skills and are therefore recommended for fine scale epidemiological investigations. When routine identification at species and biovar level of *B. suis* isolates is the objective, this can be achieved effectively with simpler and cheaper PCR methods (López-Goñi et al., 2011, 2008) and furthermore, at least 5 different *B. suis* bv 2 genotypes (see Table S1) can be identified by PCR-RFLP as described elsewhere (Garcia-Yoldi et al., 2007; Muñoz et al., 2010).

5. Conclusions

The high-resolution typing techniques used allow to identify phylogeographic structure, as well as local transmission events in a fine scale epidemiological analysis. Our results demonstrate that, regardless of the effect of genetic drift on *B. suis* VNTR loci, MLVA-11_{suis2} is an effective tool to identify swine outbreaks of common origin. SNP analysis shows higher resolution and increases the confidence when resolving space-temporal relationship between related isolates. With the definition of stable analytical protocols, WGS is a good candidate to replace other high-resolution techniques in modern epidemiology.

It can be concluded that maintenance and spread of *B. suis* bv 2 in Europe is a dynamic process, which depends on the natural expansion of the wild boar as the principal wild reservoir of infection. *B. suis* bv 2 is maintained in multiple, genetically distinct, and geographically defined populations, likely via the wild boar meta-populations. The exact geographic landscape of these subpopulations is likely to be relaxed with new subclades emerging and becoming established, and others being transferred to new locations like the Central European clade in Spain and the Eastern European clade in central Italy, where they may be established temporarily or over the long-term. Long-distance spread of *B. suis* is largely dependent on anthropogenic activities, such as the international trade and translocation or restocking of wild animals for hunting purposes, that may lead to the sudden emergence of an exotic clade in a previously unaffected territory.

Author Contributions Statement

PM, VM, JB and GG conceived and coordinated the study and wrote the paper. BGB, MT, EDG, FDM conceived the study. PMM, VM, LS, AJ, MM, MC, CR, SA, MA, LS, MJ, KZ, MA, AD, SZ, MCZ, participated on field samples collection, bacteriological isolation and/or molecular characterization of *B. suis* bv2 strains. All the authors reviewed the draft and contributed to the final version.

Conflict of interest statement

The authors declare not to have conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.025.

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