1 **Origins and Global Context of *Brucella abortus* in Italy**

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Abstract

Background

Brucellosis is a common and chronic disease of cattle and other bovids that often causes reproductive disorders. Natural infection in cattle is caused by *Brucella abortus* and transmission typically occurs during abortions, calving, or nursing. Brucellosis is also a major zoonotic disease due to contamination of dairy products or contact with the tissues of infected animals. Brucellosis has been eradicated from most of the developed world in the last forty years but persists in many regions; *B. abortus* remains prevalent in portions of Africa, the Middle East, Asia, and Central and South America, as well as in the Mediterranean basin. Specifically, *B. abortus* has persisted in southern Italy in both cattle and water buffalo. Previous attempts at analyzing the genetic structure of *B. abortus* in Italy have been challenging due to the limited genetic variability and unresolved global population structure of this pathogen.

Results

We conducted genome-wide phylogenetic analysis on 11 representative strains of *B. abortus* from Italy, and compared these sequences to a worldwide collection of publically available genomes. Italian isolates belong to three clades basal to the global *B. abortus* lineage. Using six assays designed to identify substructure within the Italian clades in a collection of 261 isolates, one clade predominates throughout endemic districts in the country, while the other two clades are more geographically restricted to southern Italy.

Conclusions

Although related strains exist worldwide, *B. abortus* isolates from Italy are substantially different than those found in much of the rest of Europe and North America, and are more closely related to strains from the Middle East and Asia. Our assays targeting genetic substructure within Italy

50   allowed us to identify the major lineages quickly and inexpensively, without having to generate

51   whole genome sequences for a large isolate collection. These findings highlight the importance

52   of genetic studies to assess the status and the history of pathogens.

53

Introduction

Although eradicated throughout much of the developed world, bovine brucellosis continues to be common in Southern Italy [1]. *Brucella abortus*, the etiological agent, is a Gram-negative non-motile and non-sporulating bacterium that forms coccobacilli with oxidative metabolism [2]. At the beginning of the 20th century, *B. abortus* was recognized by Benhard Bang as the causative agent of epizootic abortion in cattle, but only later was this disease associated with Malta fever of goats caused by *B. melitensis* [3] and classified by Meyer in the genus *Brucella* [4]. The disease presents primarily as reproductive disorders in cattle, resulting in substantial economic losses to agriculture [5]. For example, the economic burden of bovine brucellosis in Latin America has been estimated at $600 million annually [6]. *Brucella abortus* is a significant zoonotic agent, and humans are typically infected by consumption of raw dairy products, at slaughter, or during veterinary care and animal husbandry [7].

The Italian government has conducted a country-wide eradication program for cattle brucellosis since 1994 [8]. Animal cases are currently limited to seven regions of southern Italy, with the highest prevalence of infection in areas of Sicily, Calabria, and Apulia regions [9]. This eradication program, along with strict regulations on cattle movements, has reduced the prevalence and geographical distribution of brucellosis in Italy [10]. In order to achieve the eradication of brucellosis, the identification of the sources of infection is essential, as is determining whether the same strains are circulating within a region or continuously reintroduced from other areas or countries. Additionally, the host species and source region are important for the epidemiology of human zoonoses.

Molecular epidemiology has been challenging in *Brucella* due to relatively high levels of nucleotide similarity throughout the genus [11]. Traditional molecular approaches involving

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77   biochemical testing are only able to categorize samples into broad phenotypic groups of biovars,

78   and for *B. abortus* eight different biovars have been described (biovars 1–7 and 9) [12]. More

79   recent genetic analyses using variable number tandem repeat (VNTR) markers have given much

80   higher resolution and can be highly informative in some cases [13-16], but homoplasy in VNTR

81   markers and a lack of sampling for some regions make it challenging to place *B. abortus* into a

82   true global phylogeny [17].

83   We used whole genome sequencing (WGS) to place 11 Italian *B. abortus* isolates onto a

84   global context. We also identified specific single nucleotide polymorphisms (SNPs) that define

85   the three major Italian clades in our study, and then developed clade-specific assays to genotype

86   our Italian collection of 261 isolates. This genotyping allowed us to rapidly and inexpensively

87   identify the lineages of *B. abortus* circulating in southern Italy and place Italian strains in a

88   global phylogeny. In Italy, where brucellosis is a major public health concern, the information

89   retrieved by this genotyping will also support the epidemiological investigations in case of

90   human infections.

91

92   **Results**

93   Whole genome comparisons generated approximately 9,000 putative SNPs that were used to

94   construct a maximum parsimony phylogenetic tree. Italian isolates of *B. abortus* were part of

95   three clades that are basal to the most common worldwide lineage, which we refer to as the

96   biovar 1, 2, 4 lineage (hereafter biovar 1/2/4), that is ubiquitous in North America and parts of

97   Europe (Fig. 1). Isolates from western Italy and a closely related sample from France form a

98   "West Italia" subclade defined by 83 SNPs. A broader clade that also includes isolates from

99   Poland, Spain, and China is defined by 91 SNPs. Members of the West Italia subclade and

100 broader clade are closely related to the biovar 1/2/4 lineage, which is consistent with the

101 biochemical designation of most of these isolates as biovar 1.

102 The other Italian isolates are part of an even more basal and considerably more diverse group

103 of *B. abortus* genomes. Unlike the biovar 1/2/4 lineage, this basal group is comprised largely of

104 isolates from biovars 3, 5, 6, 7, 9 that are broadly distributed across Europe, Asia, and Africa.

105 Substantial diversity among African isolates suggests this continent may be the region of origin

106 of *B. abortus* as a species (JTF, unpubl. data). Inconsistencies in biovar assignment compared to

107 their placement in the phylogenetic tree suggest that biovar classification in *B. abortus* does not

108 consistently reflect genetic relationships or that biochemical determination of biovars is

109 unreliable.

110 Six of our isolates from across Italy form a "Trans Italia" subclade along with isolates from

111 Mongolia, southern Europe, and Egypt, which are defined by 44 SNPs. The broader clade also

112 includes an isolate from Turkey. The 169 SNPs exclusive to this clade suggests a fairly long time

113 period separates these isolates from the common ancestor they share with genomes from Uganda

114 and Iraq. Isolates from eastern Italy form an 'East Italia" subclade, with 157 SNPs exclusive to

115 the branch. The broader clade contains an isolate from Greece, which shares 145 exclusive SNPs

116 with the East Italia cluster. Members of the East Italia subclade are closely related, although

117 these three isolates came from three different regions.

118 We developed 13 Melt-MAMA assays to genotype a larger collection of 261 samples and to

119 better define the population substructure of *B. abortus* in Southern Italy. Six of the Melt-MAMA

120 assays produced consistent results and corresponded to two assays each for the West Italia, Trans

121 Italia, and East Italia subclades and the broader clades to which they belong (Table 1). Our

122 genotyping indicated that 13 Italian isolates came from the West Italia subclade, 214 isolates

123 were part of the Trans Italia subclade, and 34 isolates were part of the East Italia subclade

124 (Supplementary material). We then mapped the geographic distributions of the clades to

125 determine their phylogeographic patterns. The West Italia genotypes consisted entirely of

126 isolates from biovar 1 detected in the three western Italian districts of Campania, Basilicata, and

127 Calabria (Fig. 2). The East Italia group was found mostly in the eastern regions of southern Italy:

128 Apulia, Molise and Abruzzo (Fig. 2). Finally, the Trans Italia group was distributed throughout

129 endemic regions of Southern Italy, suggesting its dominance in the region (Fig. 2).

130

131 **Conclusion**

132 Whole genome sequencing and comparison to a global collection of genomes allowed us to

133 determine the evolutionary history of *B. abortus* in Italy. Despite the strains being isolated

134 exclusively in 2011, the use of multi-locus variable number tandem repeat (VNTR) analysis

135 (MLVA) in a previous study allowed us to select and then sequence a diverse collection of

136 isolates from throughout endemic districts in Southern Italy [17]. Challenges with VNTRs

137 limited our phylogenetic inference for *B. abortus* for the country. However, genome-wide

138 phylogenetics using SNPs identified that Italian strains come from three major clades, all of

139 which are substantially different than the common biovar 1/2/4 lineage found in much of Europe

140 and North America. Genotyping assays supported these findings in a larger collection of 200

141 isolates from Italy.

142 We believe that the relatedness of Italian strains to lineages from other parts of Europe, Asia,

143 and Africa suggests that *B. abortus* in Italy has a very different evolutionary history than the

144 dominant lineage of biovar 1/2/4 that was brought to the New World by various Europeans in

145 infected cattle. Two or three different domestication events of wild cattle in Eurasia have

7

146 occurred [18], and then cattle, with their associated diseases, were introduced by humans

147 throughout the world [19]. Italian farms contain several European cattle breeds, but also contain

148 several primitive cattle breeds such as Podolica, Marchigiana, Chianina, and Romagnola, which

149 are all directly linked to the ancient cattle *Bos primigenius* from Eurasia [20]. Current brucellosis

150 programs strictly control cattle movements, but these programs have been only recently

151 established, and *B. abortus* has likely been influenced by several centuries of introductions and

152 disseminations of infected cattle throughout Italy.

153 The newly developed Melt-MAMA assays specific to the three Italian clades and subclades

154 allowed us to screen a large collection of isolates and quickly and inexpensively assign them to

155 one of three genetic groups. Future testing of additional isolates using these six SNP assays

156 during eradication programs will allow them to be typed more rapidly and confidently than

157 biotyping, and could serve Italian diagnostic laboratories as a first line assay. Genotyping

158 coupled with knowledge of herd movement through use of risk maps, spatial modeling, and

159 Social Network Analysis (SNA) techniques should enhance the reliability of the Italian

160 brucellosis eradication plan. Although a multiplex PCR approach has been developed recently

161 [14], and the MLVA-16 has been shown to be robust and informative [21], these techniques

162 remain cumbersome for data production and standardization. Perhaps a more reasonable

163 approach would be to pair the SNP assays with a limited number of VNTR loci to achieve a

164 higher resolution for molecular epidemiology purposes. We recognize the capabilities of whole

165 genome sequencing, but we are also aware that this technology is not yet accessible to all labs

166 and requires skills that are not present in many diagnostic laboratories. Nonetheless, for some

167 outbreak investigations, MLVA-16 or whole genome sequencing may be essential when

168 complete epidemiological traceback is required. Ultimately, our study demonstrates the utility of

169 WGS SNPs paired with extensive epidemiologic data for analyzing the distribution of *B. abortus*

170 isolates throughout endemic regions.

171

172 **Methods**

173 **Aims of the study**

174 The study was conducted to genetically characterize Italian *B. abortus* isolates from 261 animal

175 cases in southern Italy from 2011 to 2014. The overall goal was to trace the origin and the global

176 connections of bovine brucellosis in Italy and to develop a rapid genotyping method for novel

177 isolates.

178

179 *B. abortus* **isolates**

180 Large ruminants (cattle and water buffalo) and small ruminants (sheep and goat) testing positive

181 for brucellosis in the serological tests carried out in the context of the national brucellosis

182 eradication campaign are slaughtered, and the epidemiological investigation is supported through

183 the isolation and characterization of *Brucella* spp. from selected tissues or body fluids. The

184 preferred tissues for culture are lymphatic glands (i.e. mammary and genital lymph nodes, and

185 spleen), uterus, and udder. Direct and enrichment *Brucella* cultures were carried out with these

186 tissue samples following the OIE procedures. The suspected colonies were assigned to the

187 species *B. abortus* and the relative biovar (1-7, 9) using PCR and traditional serotyping [22]. All

188 cultured isolates were stored in the Italian collection with associated epidemiological data. To

189 achieve maximum diversity necessary for these types of projects, we followed the guidelines

190 Pearson et al. [23] to select 11 phylogenetically and geographically diverse *B. abortus* strains

191 (Table 2) for whole genome sequencing using results from our VNTR study [17].

192

Whole genome sequencing and SNP detection

194 We sequenced the isolates with an Illumina MiSeq to produce paired 300 bp reads with a 400 bp

195 insert size (GenBank PRJNA284953) [24]. SNPs were determined with NASP version 1.0.0

196 [25], using default filters to remove SNPs from duplicated regions, read coverage less than 10X,

197 base call proportion less than 90%, and loci not orthologous in all samples. NASP uses BWA

198 [26] as the aligner and GATK [27] as the SNP caller. This analysis allowed us to compare these

199 11 genomes to 179 genomes of *B. abortus* from GenBank, accessed via PATRIC [28], that have

200 been examined as part of a global analysis of *B. abortus* (JTF, unpubl. data). Phylogenetic trees

201 were constructed using maximum parsimony in PAUP* 4.0b [29]. Branch support was assessed

202 with 1000 bootstrap replicates and a consistency index was generated to assess the level of

203 homoplasy.

204

Melt-MAMA PCR assays

206 We designed SNP-specific assays that targeted six branches as shown in table 1 leading to

207 specific Italian clades according to a maximum parsimony tree (Fig. 1). Downstream real time

208 PCR analyses using Melt-MAMA were performed on 261 *B. abortus* isolates isolated in Italy

209 from the endemic territories during the period 2011–2014 (Supplementary material). Briefly,

210 Melt-MAMA allows sensitive genotyping assays to be developed that distinguish samples

211 containing a single nucleotide difference, which can be robust determinants of specific lineages

212 in clonal bacteria [30]. The assays satisfied the following five conditions as previously described

213 [31], including: (i) Two 3′ forward primers containing sequences complementary to the allelic

214 sequence for each SNP; (ii) a common reverse primer; (iii) an additional mismatch in the

10

215   forward primer in anti-penultimate and penultimate position alternately; (iv) a GC-clamp in

216   position 5´ for one of the forward primer; (v) amplicon of reduced size (60–120 bp). Relative

217   primer concentrations were tested in ratios of 1:1, 4:1, and 1:4 during optimization of the PCR

218   reaction conditions. Assays were considered valid when they gave consistently different melt

219   profiles for samples containing SNPs for ancestral versus derived alleles.

220

221   **Declarations**

222   **List of abbreviations**

223   PCR: Polymerase chain reaction; VNTR: Variable number tandem repeat; MLVA: Multi Locus VNTRs

224   Analysis; BSL: biosafety level 3 laboratory; SNPs: single nucleotide polymorphisms. Melt-MAMA: Melt

225   analysis of mismatch amplification mutation assays.

226   **Ethics approval and consent to participate**

227   This study is a retrospective molecular investigation of the *Brucella* collection from the National and OIE

228   Reference Laboratory Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise. No human or

229   animal data were used therefore informed consent was not required.

230   **Consent for publication**

231   Not applicable.

232   **Availability of data and materials**

233   The genomes are available at GenBank under the project PRJNA284953

234   **Competing interests**

235   The authors declare that they have no competing interests.

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Authors' contributions

241 GG and JTF have designed the study and drafted the final manuscript, EDG, FDM, CC and PC

242 contributed in planning the project and critically reviewed the manuscript, IP, AA, LS, KZ,GDD and MA

243 performed the WGS, Melt-MAMA and conventional tests for typing the *Brucella* isolates, GG, JTF and

244 KPD conducted the bioinformatics analyses. All authors read and approved the final manuscript.

Acknowledgments

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356 **Fig. 1.** Fig. 1. Maximum parsimony tree for *B. abortus*, with genomes from Italian isolates
357 highlighted in red. Bootstrap support values were 100 for all major branches, with only the
358 shallowest clades having less support (data not shown). Numbered branches indicate the number
359 of SNPs identified for the clade (left most) and subclade (right most) level groupings for the
360 West Italia (top), Trans Italia (middle) and East Italia (bottom) subclades. Samples are identified
361 by the strain name and where known, the biovar (bv) number, animal source, country of origin (3
362 letter ISO codes), and year of isolation.

363
364
365 **Fig. 2.** Geographical mapping of the Italian isolates according to Melt-MAMA genotyping. GIS
366 coordinates separated by groups are shown as follow: green for the West Italia group (A), yellow
367 for the East Italia (B) and red for the Trans Italia (C).
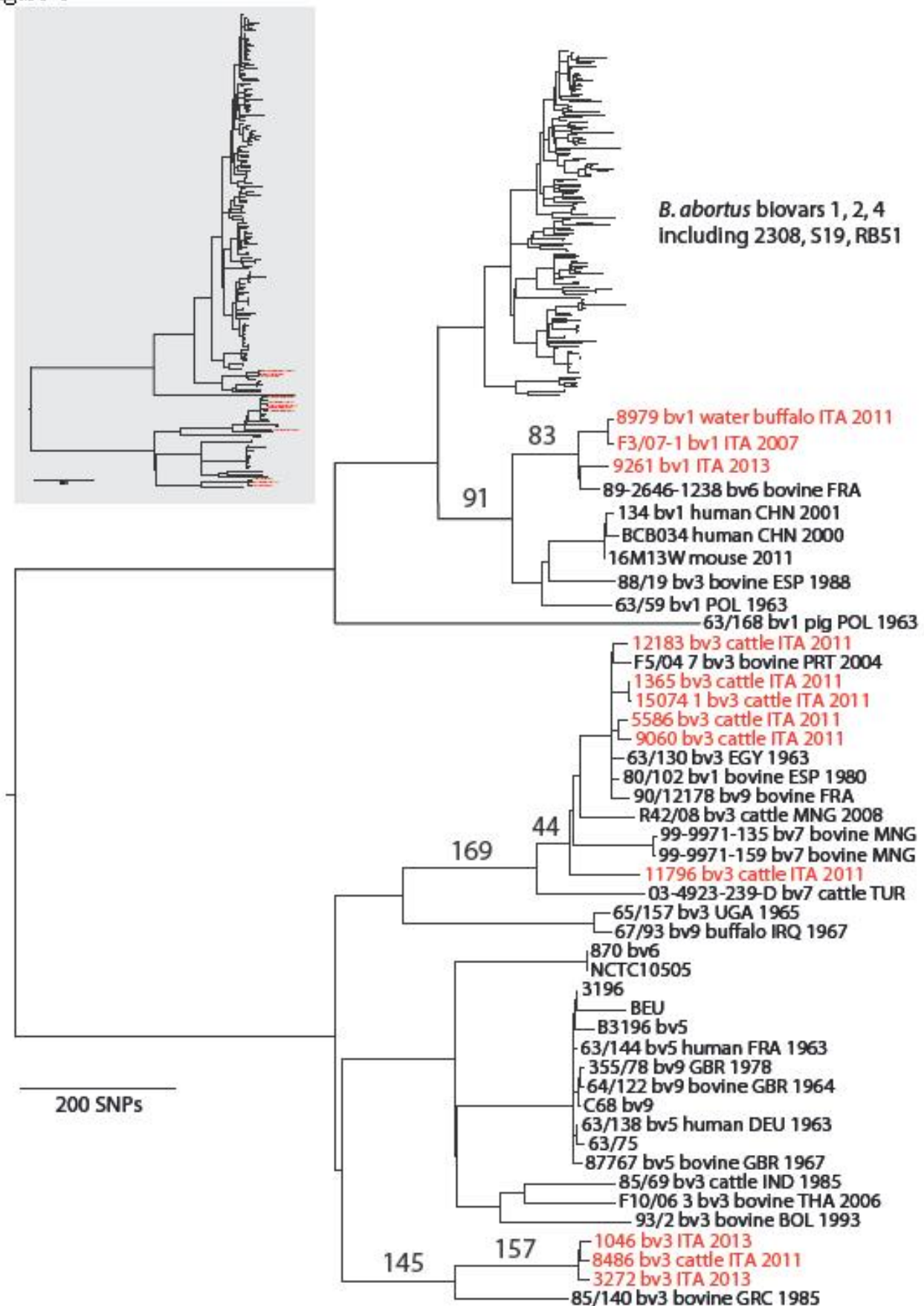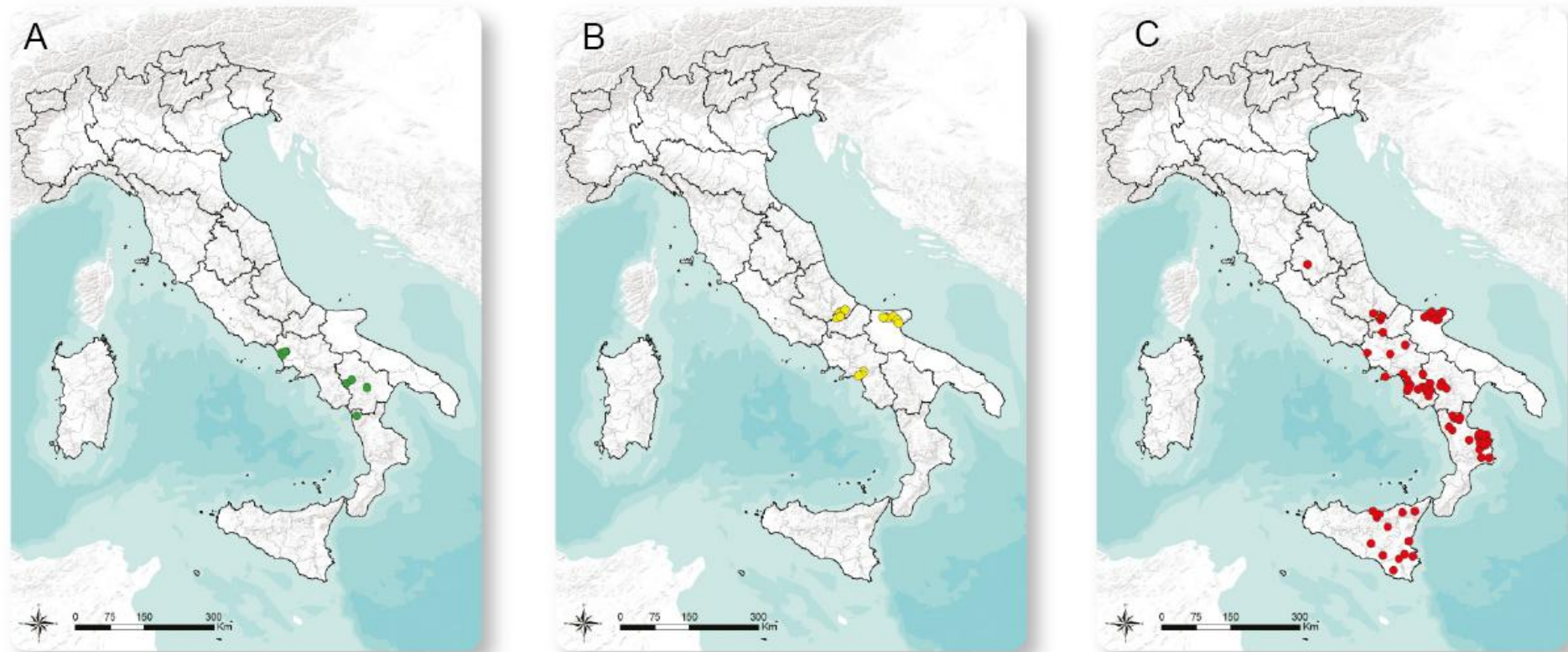
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Figure 1

Figure 2

Table 1. Characteristics of Melt-MAMA primers designed for six major branches containing Italian *Brucella abortus* isolates. Genome position is in the reference strain 2308 with chromosome number indicated in parentheses.

Branch	Location in *B. abortus* 2308	SNP allele	Melt-MAMA primer	Melt-MAMA Primer sequences (5′ to 3′)	PCR mix Concentration	Annealing Temp.	Melting Temp.
Branch 1 WestItalia	951620 NC_007618.1	C/T	A	GCGACCGCATCCTCTAaTC	0.4 µM		80.8º C
			D	cggggcggggcggggGCGACCGCATCCTCTAcTT	0.1 µM	56º C	83.4º C
			C	CATGGTAGGCAGGCTGGTT	0.5 µM		
Branch 2 WestItalia	1336380 NC_007618.1	A/G	A	CGAAGTTTCCCGACACCcA	0.4 µM		81.2º C
			D	cggggcggggcggggCGAAGTTTCCCGACACCtG	0.1 µM	56º C	83.9º C
			C	GATGAAGAAATCGATGAGCG	0.5 µM		
Branch 1 EastItalia	104052 NC_007618.1	G/T	A	TGGAGGACGAAGTCTTCGaTG	0.25 µM		78º C
			D	cggggcggggcggggGGAGGACGAAGTCTTCGcTT	0.25 µM	56º C	82.9º C
			C	ATCCTCGACAGTTGCGGC	0.5 µM		
Branch 2 EastItalia	360724 NC_007618.1	C/A	A	TACCGCGGCCCGTCTAtC	0.25 µM		81.6º C
			D	cggggcggggcggggTACCGCGGCCCGTCTAaA	0.25 µM	56º C	83.7 º C
			C	AATCAAAGCGGCTTGCCTC	0.5 µM		
Branch 1 TransItalia	740090 NC_007618.1	T/C	A	CAAAAGCGGACGGTCAgAT	0.4 µM		78.9º C
			D	cggggcggggcggggTCAAAAGCGGACGGTCAtAC	0.1 µM	56º C	80.9º C
			C	CCCTCAACCCAGACGTTCG	0.5 µM		
Branch 2 TransItalia	864308 NC_007618.1	C/T	A	AGGCATTACCGTTTCAGATTtC	0.4 µM		78.5 º C
			D	cggggcggggcggggAGGCATTACCGTTTCAGATTaT	0.1 µM	56º C	81.9º C
			C	TCTTTCCCGGCTCCGAAT	0.5 µM		

Abbreviations: A: ancestral R: derived C: common

Details of primers concentration used in each reaction with proper annealing temperature is given. Melting temperatures detected for both the amplicons derived by the specific primer binding is given according to the experiments run on Applied Biosystems® 7500 Real-Time PCR System.

Table 2. Epidemiological data for 11 *Brucella abortus* isolates that were whole genome sequenced. All samples were collected in 2011 and were molecularly identified as *B. abortus*.

id	Region	Province	Town	Host	Biovar
1046	Abruzzo	CH	Roccaspinalveti	cattle	3
5586	Calabria	KR	Pallagorio	cattle	3
8486	Campania	SA	Giffoni Valle Piana	cattle	3
9060	Calabria	CS	Scala Coeli	cattle	3
8979-3	Campania	CE	Cancello ed Arnone	water buffalo	1
11796	Sicilia	ME	Tusa	cattle	3
12183	Campania	SA	Corleto Monforte	cattle	3
15074/1	Puglia	FG	San Marco in Lamis	cattle	3
1365/1	Molise	IS	Carovilli	cattle	3
3272	Puglia	FG	Apricena	cattle	3
9261	Calabria	CS	Papasidero	cattle	1