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Highlighting priority areas for bovine viral diarrhoea control in Italy: a phylogeographic approach

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Abstract

The prevalence and genetic diversity of bovine viral diarrhoea virus (BVDV) in a geographic area are largely influenced by live animal trade and management practices. Despite control and eradication programs currently underway in several European countries, the risk of BVDV spread within and among countries is still present. BVDV-1 is the predominant type circulating in European cattle population. In this study, a phylogeographic analysis was applied to the BVDV-1 highest prevalent subtypes in Italy to reconstruct the origin and spatial-temporal distribution and to trace main viral flows between different locations to highlight priority areas for BVDV control. A comprehensive dataset of BVDV-1b (n=173) and 1e (n=172) 5' UTR sequences was analyzed, including both novel and published sequences from Italy and from European countries bordering and/or with commercial cattle flows with Italy. A common phylogeographic pattern was observed for BVDV-1b and 1e subtypes: interspersed from multiple Italian areas and European countries was widespread until the end of the last century, whereas significant local clusters were observed starting from 2000. These findings support a continuous viral flow among different areas over long time scales with no evidence of significant geographical structure, while local transmission networks are limited to more recent years. Northern Italy has been confirmed as the area of origin of the main clades of both BVDV subtypes at national level, acting both as a crucial area for introduction and a maintenance source for other areas. Piedmont, Central and Southern Italian regions contributed to limited geographical distribution and local BVDV-1b and 1e persistence. On the whole, priority control measures for BVDV-1b and 1e in Italy should be focused on: i) implementation of BVDV systematic control in all Northern Italian regions to break the viral flow from larger to smaller animal populations; and ii) breaking the dynamics of infections in regions with self-maintenance of BVDV by voluntary control programs.

Key words: Pestivirus; Bovine viral diarrhoea virus; cattle; Phylogeography; Epidemiology; Italy

1. Introduction

Bovine viral diarrhoea virus (BVDV) is a widespread pathogen of cattle. Occurrence of disease, economic impact, its wide distribution and inclusion of BVDV in the OIE list increased the awareness towards this disease (Gunn et al., 2005). Control and eradication programs are underway in several European countries and Scandinavian countries are currently either free, or almost free from BVDV (Ståhl & Alenius, 2012).

Nowadays, most of the countries from the Mediterranean basin show an endemic diffusion of BVDV infection (Hurtado et al., 2003; Billinis et al., 2005; Luzzago et al., 2014; Aslan et al., 2015) and are not applying any systematic control at national level. In Italy, regional mandatory eradication programs are restricted to Trentino Alto-Adige, bordering Austria, whereas voluntary control programs are applied in few other northern regions (Piedmont, Veneto, Friuli-Venezia Giulia). Italy showed a high BVDV genetic diversity compared to other countries and the most likely explanation is livestock trade from European countries and among Italian regions (Luzzago et al., 2012; Giammarioli et al., 2015).

BVDV belongs to the *Pestivirus* genus of the *Flaviviridae* family. Genetic typing of BVDV isolates distinguishes two recognized species (BVDV-1 and BVDV-2) and a putative third bovine species, referred to as HoBi-like pestivirus or BVDV-3 (Liu et al., 2009). To date, 17 BVDV-1 subtypes have been identified and four additional novel subtypes have been proposed and revised (BVDV-1a to BVDV-1u). Such diversity within this species raised concern related to the emergence and spread of new BVDV variants, with possible implications for animal health and disease control (Giammarioli et al., 2015; Yeşilbağ et al., 2017).

The BVDV type and subtype prevalence in a given geographic area has been largely influenced by live animal trade and management practices. Long distance transmission between regions and

countries is mainly related to livestock trade of BVDV persistently infected (PI) animals or dams carrying a PI foetus (Lindberg and Alenius, 1999). The most recent evidences of BVDV spread was documented in England and Wales, which occurred due to restocking with cattle from continental Europe (Strong et al., 2013), and in Germany and Netherlands, following animal trade during the BVDV-2 outbreaks (Gethmann et al., 2015). Therefore, the risk of BVDV spread within and among countries is still high and present.

Genetic characterization of isolates and phylogeographical analysis gives a unique opportunity to trace routes of infection. In fact, phylogeography combines spatial and temporal analyses of isolates sampled at known times in different areas to reveal location and time of origin of infections and flow of geographic spread (Pybus & Rambaut, 2009, Zehender et al., 2013). The methods currently used of phylogeographical reconstruction are inherently limited by the availability of sequences that respect the minimum criteria of inclusion, namely time of collection and location of origin of the strains.

The BVDV 5' UTR region is a non-coding region of the genome, which has been used extensively for diagnostic purpose and virus typing (Vilcek et al., 2004). Therefore, a large number of 5' UTR sequences are available and publicly accessible from databases, with additional information on the collection date and geographical origin of the isolates necessary for advanced phylogenies.

Phylogeographic analysis built on the 5' UTR sequence are informative (Luzzago et al., 2012) especially over long time scales, such as decades, while regions with higher variability (e.g., Npro and E2 genes) allow resolution of phylogeny over shorter time scales (Chernick et al., 2014).

The aim of this study was to reconstruct the origin and spatio-temporal distribution of the highest prevalent subtypes in Italy (Luzzago et al., 2014) and in several European countries (Tajima et al., 2001; Jackova et al., 2008; Hornberg et al., 2009; Kuta et al., 2013) to highlight priority actions to be introduced to control BVDV. A phylogeographic analysis of a comprehensive dataset of BVDV-1b and 1e 5' UTR sequences was performed, including both novel and published sequences from Italy and other European countries bordering and/or with commercial cattle flows with our country.

2. Materials and Methods

2.1 *Samples and subtype assignment*

Samples sent to laboratories for routine testing because of suspected BVDV infection were used, therefore no animals were specifically sampled to perform this study. Twenty-six positive samples were obtained from 26 Italian cattle herds and were characterized by RT-PCR and sequencing as previously described (Luzzago et al., 2014). The sequences were classified by alignment with BVDV-1 reference strains retrieved from GenBank by using Clustal X with BioEdit software version 7.0 (freely available at <http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Phylogeny was estimated by the neighbor-joining algorithm (NJ) and the maximum likelihood (ML) method with 1,000 bootstrap replicates. The sequences of subtype -1b and 1e were selected and included in the following comprehensive datasets.

2.2 *BVDV-1b and 1e datasets*

Two datasets of sequences for each BVDV subtype were generated.

The first included Italian sequences only and was obtained aligning the novel sequences from this work with other Italian sequences retrieved from published peer-reviewed journals and characterized by locality and year of sampling collection. Inclusion criteria allowed to retrieve BVDV-1b and BVDV-1e sequences from Northern Italian areas: Piedmont (n=102), Lombardy (n=80), Emilia-Romagna (n=26), Veneto (n=13) (Falcone et al., 2003; Luzzago et al., 2001, 2012, 2014); from Central areas (n=12) (Giammarioli et al., 2008; Luzzago et al., 2014) and Southern Italy (n= 19) (Canella et al., 2012; Luzzago et al., 2012, 2014). The sampling dates ranged from 1995 to 2013 for BVDV-1b and from 1996 to 2013 for BVDV-1e (Table S1).

A second dataset (Italian plus European) was generated for each subtype by implementing Italian sequence' datasets with BVDV-1b and 1e sequences from European cattle, retrieved from public

database, restricting the geographic localization to countries bordering Italy and/or linked by commercial flows with Italy (<https://www.vetinfo.sanita.it>). Inclusion criteria for European sequences was availability of sample collection year. Moreover, in a group of sequences from the same Polish herd a single sequence was selected in case of 100% genetic identity. A total of 34 BVDV-1b and 38 BVDV-1e sequences were obtained from the following countries: Austria (n=8) (Hornberg et al., 2009; La Rocca & Sandvik, 2009), France (n=43) (Jackova et al., 2008; La Rocca & Sandvik, 2009), Germany (n=4) (Tajima et al., 2001), Poland (n=13) (Kuta et al., 2013), Slovenia (n=2) (Toplak et al., 2004), Switzerland (n=2) (Bachofen et al., 2008). The sampling dates of European sequences ranged from 1960 to 2011 for BVDV-1b and from 1994 to 2006 for BVDV-1e (Table S1).

2.3 Phylogeographic and phylodynamic analysis

2.3.1 Likelihood mapping analysis

The phylogenetic signal of each dataset was investigated by means of the likelihood-mapping analysis of 10,000 random quartets generated using TreePuzzle (Schmidt et al., 2002). All of the three possible unrooted trees for a set of four sequences (quartets) randomly selected from the dataset were reconstructed using the maximum likelihood (ML) approach and the selected substitution model. The posterior probabilities of each tree were then plotted on a triangular surface so that the dots representing the fully resolved trees fell at the corners and those representing the unresolved quartets in the centre of the triangle (star-tree) (Schmidt et al., 2002). Using this strategy, described in detail elsewhere (Strimmer & von Haeseler, 1997), the data were considered unreliable for phylogenetic inference when more than 30% of the dots were in the centre of the triangle.

2.3.2 Phylogenetic reconstruction

The analysis was performed for both BVDV-1 subtypes. The best-fitting nucleotide substitution

model was estimated by means of JModelTest (Posada, 2008), and selected a HKY model (Hasegawa et al., 1985) with gamma-distributed rates among sites. The phylogenetic tree, model parameters, evolutionary rates and population growth were co-estimated using a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST v.1.8.0 package (Drummond et al., 2012).

Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade. As coalescent priors, four simple parametric demographic models (constant population size, and exponential, expansion and logistic population growth) and a piecewise-constant model, the Bayesian skyline plot (BSP) under both a strict and a relaxed (uncorrelated log-normal) clock were compared (Drummond et al., 2012).

Two independent MCMC chains were run for 150 million generations with sampling every 15,000th generation for BVDV-1b dataset and for 300 million generations with sampling every 30,000th generation using BDVD-1e sequences, and were combined with the LogCombiner 1.80 included in the BEAST package. Convergence was assessed on the basis of the effective sampling size (ESS) after a 10% burn-in using Tracer software version 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). Only ESS's of ≥ 200 were accepted.

Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals, and the best-fitting models were selected using a Bayes factor (BF, using marginal likelihoods) implemented in BEAST (Suchard et al., 2001).

In accordance with Kass and Raftery (1995), only values of $2\ln\text{BF} \geq 6$ were considered significant. The trees were summarised in a target tree using the Tree Annotator program included in the BEAST package, choosing the tree with the maximum product of posterior probabilities (maximum clade credibility) after a 10% burn-in.

The time of the most recent common ancestor (tMRCA) estimates were expressed as mean and 95% HPD years before the most recent sampling dates, corresponding to 2013 in this study.

2.3.3 Bayesian phylogeographic analyses

The continuous-time Markov Chain (MCC) process over discrete sampling locations implemented in BEAST (Lemey et al., 2009) was used for the geographical analysis, implementing the Bayesian Stochastic Search Variable Selection (BSSVS) model which allows the diffusion rates to be zero with a positive prior probability. Comparison of the posterior and prior probabilities that the individual rates would be zero provided a formal BF for testing the significance of the linkages between locations: rates with a BF of >3 were considered well supported and formed the migration pathway. The final trees were visualised using FigTree version 1.4 (available at <http://tree.bio.ed.ac.uk/software>). The significant migration rates were analysed and visualised using SPREAD, which is available at <http://www.kuleuven.be/aidslab/phylogeography/SPREAD.html>. For phylogeographic analysis, the BVDV-1 Italian isolates were assigned to distinct regions from Northern Italy (Piedmont; Lombardy; Veneto; Emilia Romagna) and a macroarea consisting of Center and Southern Italy. The BVDV-1 European isolates were assigned to country of origin: BVDV-1b from Austria, Switzerland, Germany, France, Slovenia, Poland; BVDV-1e from Austria, Switzerland and France.

3. Results

3.1 BVDV subtype assignment and sequence datasets

The novel sequences were classified as BVDV-1a (n=1), 1b (n=13); 1d (n=2), 1e (n=8), and 1h (n=2) (Table S2).

On the whole, the BVDV-1b Italian and Italian plus European datasets were made of 139 and 173 sequences, respectively. One-hundred thirty-four and 172 BVDV-1e sequences constituted the Italian and Italian plus European datasets, respectively

3.2 Phylogeographic inference

3.2.1 Likelihood mapping

For BVDV-1b and 1e Italian datasets, the likelihood mapping of 10,000 random quartets showed that more than 79% and 86% of the dots were distributed at the corners of the likelihood map, while 15% and 10% in the central area, respectively, thus indicating that the datasets contained sufficient phylogenetic information (Figures S1, S2).

For BVDV-1b and 1e Italian and European datasets, 79% and 85% of the dots were distributed at the corners of the likelihood map, while 15% and 11% in the central area, respectively (Figures S3, S4).

3.2.2 Evolutionary rates estimation

The BF analysis showed that the uncorrelated lognormal relaxed clock fitted the data significantly better than the strict clock for all datasets (comparison between strict and relaxed clocks showed $2\ln\text{BF}$ 50.4 and 6.2 for Italian and Italian plus European BVDV 1b respectively; 93.8 and 59.7 for Italian and Italian plus European BVDV-1e, respectively). Under the relaxed clock the BF analysis showed that the constant model was better than the other models for BVDV-1b ($2\ln\text{BF}>7$ and 53.6, Italian and Italian plus European dataset, respectively) while the Bayesian skyline model was better than the other models for BVDV-1e ($2\ln\text{BF}>15.4$ and 16.5, Italian and Italian plus European dataset, respectively). The estimated mean evolutionary rates of the 5' UTR Italian sequences analysed were 1.003×10^{-3} subs/site/month (95% HPD: 6.09×10^{-4} ; 1.44×10^{-3}) for BVDV-1b and 7.73×10^{-3} subs/site/month (95% HPD: 3.45×10^{-3} - 1.35×10^{-2}) for the BVDV-1e subtype.

3.2.3 BVDV-1b phylogeographic analyses of Italian sequence dataset

The phylogeographic maximum clade credibility tree, summarizing all of the trees obtained during the MCMC search on BVDV-1b sequences from cattle in Italy, is shown in figure 1. A high level of spatial interspersed among regions was observed, with no evidence of significant geographical structure. The most probable location of the root of the tree was Lombardy, supported by a posterior probability (pp) significantly higher than that of the other locations (pp=0.44 vs pp=0.21 for

Piedmont, the second most probable location of the root). The tMRCA, corresponding to Lombardy root, was estimated 85 years ago (95% HPD 48-130 years ago) corresponding to 1928.

The phylogeographical tree showed two large significant clades ($pp=0.99$) including 17 (13%) and 24 (19%) Italian isolates, respectively. Lombardy represented the most probable origin of both clades and tMRCA estimations indicated a mean origin of these clades in 1975 and 1980. All the other significant clades ($n=38$) were smaller and encompassed between 2 and 9 isolates (table 1). Of these clades, 27 were homogeneous for the sampling location and the median year of origin was 2002 (1993-2010) while 11 included different geographic areas and the median year of origin was 1996 (1984-2006).

Bayesian phylogeography showed statistically supported links at the Bayes factor test ($BF > 3$) between Lombardy and Central-Southern Italy ($BF=59$), Lombardy and Emilia-Romagna ($BF=7$), Piedmont and Central-Southern Italy ($BF=4$).

The Bayesian Skyline plot showed that the effective number (N_e) of BVDV-1b infections at national level remained constant until the 1970s and then started to increase gradually, the curve reached a plateau between 1995 and 2005 and it sharply decreased until 2010 at a level similar to the beginning (figure 2).

3.2.4 BVDV-1b phylogeographic analyses of Italian plus European sequences dataset

Only one large significant main clade ($pp=1$), corresponding to the previously described Italian largest significant clade ($n=24$), was identified and included also three sequences from Poland, and one from Austria. All the other clades ($pp>0.8$) were smaller (between 3 and 9 isolates) and included mixed Italian and European (Austria, Poland and France) sequences (figure 5).

Using the evolutionary rates estimates reported above, we estimated a mean tMRCA for the tree root of 87.1 years ago (95% HPD 63-124 years ago), corresponding to 1926.

Germany represents the most probable origin of Italian BVDV-1b ($pp=0.53$ vs $pp=0.36$ for Italy, the second most probable location of the root).

Bayesian phylogeography showed statistically supported links at the Bayes factor test ($BF > 3$) between Italy and European countries. In particular significant links were observed between Italy and Austria ($BF = 3724$), Poland ($BF = 1092$), France ($BF = 95$), Slovenia ($BF = 16$), and Germany ($BF = 6$).

3.2.5 *BVDV-1e phylogeographic analyses of Italian sequence dataset*

The tree represented in Figure 3 showed a significant geographical structure over the last decade, with sequences segregating mainly on the basis of their geographical origin. After its origin, estimated on the basis of the tree-root tMRCA being in 1992 (1989-1995), BVDV-1e branched into three main significant clades ($pp > 0.7$): two including most of sequences from all the Italian areas and one smaller clade that included only isolates from Northern areas. The most probable location of the root of the tree was Lombardy, supported by a significantly higher posterior probability than other locations ($pp = 0.84$ vs $pp = 0.15$ for Emilia-Romagna, the second most probable location of the root). Lombardy also represents the most probable location of the three main clades (table 2). The majority of isolates from Piedmont clustered into two pure subclades connected to the largest clades by long branches typical of bottleneck phenomenon.

Bayesian phylogeography of BVDV-1e showed statistically well supported links at the Bayes factor test ($BF > 3$) between Emilia-Romagna and Central-Southern Italy ($BF = 10176$) and Piedmont and Veneto ($BF = 80$).

The Bayesian Skyline plot showed that the effective number (N_e) of BVDV-1e infections at national level increased from the beginning of 1990 until 1998. After this period the curve reached a plateau between 2000 and 2003 and then it decreased until 2008 and remained relatively constant at a level similar to the beginning (figure 4).

3.2.6 *BVDV-1e phylogeographic analyses of Italian plus European sequences dataset*

The Italian-European (Austria, France, Switzerland) tree resembled what was observed in the Italian BVDV-1e tree, with most of the sequences clustering into three significant clades ($pp > 0.7$) (figure 6). French sequences were widely interspersed. The mean tMRCA for the root was 38.4 years ago (95% HPD 25-56 years ago), corresponding to 1975.

The most probable origin of BVDV-1e was Italy ($pp = 0.72$ vs $pp = 0.15$ for France, the second most probable location of the root). Italy represented also the most probable origin of significant clades that included European sequences.

Bayesian phylogeography of BVDV-1e showed statistically supported links at the Bayes factor test ($BF > 3$) between Italy and France ($BF = 53$), Italy and Austria ($BF = 4$).

4. Discussion

BVDV-1b and 1e are the most prevalent and wide distributed subtypes in Italy and in bordering countries with established commercial cattle flows. Therefore, the phylogeographic analysis was focused on these BVDV-1 subtypes. The reconstruction of the most relevant spatial-temporal distribution patterns of BVDV-1b and 1e infections could highlight priority areas to address BVDV control in Italy.

The ancient origin of BVDV-1b was established by the results of tree root tMRCA estimation, suggesting an origin of this subtype dating back to a time span between 1889 and 1950, with a mean estimation falling in the end of the 20s of the last century. Moreover, our tMRCAs are included within the HDP 95% range of BVDV-1b recently estimated across all genes by other Authors (Chernick & van der Meer, 2017).

Phylogeographic analysis of the European dataset suggests that BVDV-1b originated outside of Italy, most probably in Germany. This result is in agreement with previous reports indicating Germany as the first European Country in which this subtype was described (Tajima et al., 2001).

Lombardy was the region of introduction of BVDV-1b subtype in Italy. From this region, BVDV-

1b subtype was distributed on a national level, as demonstrated by the significant links at the Bayes factor test between this region and other Italian regions, confirming previous findings based on a lower number of sequences (Luzzago et al., 2012). The observation of different highly significant clades in Italy suggests that the currently circulating strains originated in a period between 1975-1980. This result is supported by the population dynamics analysis with Skyline plot showing an increase in the effective number of infections from the 1970s until the end of the last century. In agreement with the population dynamics analysis, BVDV serological surveys in Italian herds demonstrated a gradual increase of BVDV prevalence among dairy herds in the same years (Cavirani et al., 1992).

The Italian strains showed a high level of geographical interspersion in the most deep nodes of the local tree. In addition, the observation of several significant clades encompassing Italy, Poland, France and/or Austria and the statistical supported links between Italy and European countries suggest that multiple exchanges of BVDV-1b between European countries, linked by animal trade, occurred until the end of the last century. Several significant clades were present only in the terminal nodes of the tree, probably representing recent networks in the local enzootic circulation of the virus. This observation, together with a decrease in the number of infections, observed by the Bayesian Skyline plot, suggests a recent change in the distribution pattern of BVDV-1b in Italy. This modified pattern of BVDV-1b infection is likely linked to changes in herd management, and activation or further strengthening of BVDV control strategies starting from the end of the last century. Indeed, in Italy BVDV was recognized as a relevant disease in cattle herds from the beginning of 1990, as reported by regional studies on the disease and planning of few local voluntary control programs (Lindberg et al., 2006). At the same time, several BVDV vaccines were available with an increase of commercialized vaccines both for beef and dairy cattle (Valla G., personal communication). We hypothesize that awareness in BVDV infection with control at local level and availability of vaccines, containing BVDV-1b, concurred to the decrease of this subtype. Subsequently, regional control strategies introduced in some Italian regions and in several European

countries from 2000 likely contributed to a further reduction of BVDV infections and to a prevalent BVDV-1b distribution within the same region, explaining the low interspersions among areas in recent years.

Concerning BVDV-1e, phylogeographic analysis based on Italian and European sequences suggests a more recent origin of this subtype compared to BVDV-1b (year 1975, with a confidential interval between 1957 and 1988), in agreement with the first reports of BVDV-1e infections in Europe in the 1990s (Vilcek et al., 2001). In our analysis and on the basis of the available sequences with known sampling location, Italy represents the first most probable European country of BVDV-1e origin, followed by France, consistent with the contemporary descriptions of this subtype in the two countries (Vilcek et al., 2001).

Lombardy region was confirmed as the region of diffusion of BVDV-1e subtype in Italy.

Phylogenetic and phylogeographic analyses showed an increase in the number of BVDV-1e infections together with an interspersions among different Italian regions and European countries in the past century. The observed interspersions were characterized by several clades encompassing sequences obtained from distant areas around 1990-2000 and were supported by the significant links between different Italian regions and between Italy and European countries. Among the significant clades observed between Italy and Austria, France and Switzerland, the most frequent interspersions were between French and Italian sequences, indicative of viral flow between these two countries.

The direction of these viral flows needs to be confirmed by the analysis of a more comprehensive genetic data set, but epidemiological data show a commercial link of cattle mainly from France to Italy (<https://www.vetinfo.sanita.it/>).

As already observed for BVDV-1b, Italian regional clusters of BVDV-1e were mainly observed in recent years together with a decrease in the number of infections. In Piedmont, Central and Southern Italy a strong spatial structure was observed, with the strains obtained in a single area that segregated together in significant regional subclades.

On the whole, a common phylogeographic pattern was observed for both BVDV-1b and 1e

subtypes in Italy: interspersions from multiple areas was widespread until the end of the last century, whereas significant regional clusters were mainly observed in recent years. These findings support a continuous BVDV-1b and 1e flow before 2000 among different areas over long time scales, with no evidence of significant geographical structure, whilst local circulation was prevalent in recent years. We hypothesize that this pattern of spatial-temporal distribution reflected the progressive introduction of BVDV control measures. The BVDV-1b and 1e interspersions until the 1990s were observed in a period of time characterized by the absence of any control measures both in Italy and in most European countries. In this period BVDV-1b and 1e were highly prevalent in cattle population according to the first identifications of the virus and to our phylogenetic inference. Therefore the risk of transmission events related to livestock trade between regions and countries was high.

In more recent years, the total cattle population declined with the biggest drop recorded from 1999 to 2000 associated to milk quotas (Eurostat, 2017). In Italy the decline of cattle heads is still ongoing with a relevant reduction (32.2%) of farms from 2000 to 2010 (Istat, 2017).

At the national level, we confirm our previous findings (Luzzago et al., 2012; Cerutti et al., 2016) that highlighted Northern regions as the area of origin of the main clades of both BVDV subtypes and we show that this pattern was mainly revealed before 2000. In particular, Lombardy, the Northern region with the highest national cattle population, acted as a crucial area for introduction of BVDV-1b and 1e to other regions. Epidemiological data confirmed that Lombardy was a region with a high prevalence of different BVDV subtypes (Giammarioli et al., 2015) and also showed a high frequency of livestock trade from and to other Italian regions (<https://www.vetinfo.sanita.it/>), in the absence of any regional BVDV control program. Other Northern regions, thanks to the implementation of control programs, likely reduced the contribution of BVDV-1b and -1e recent diffusion to other regions. In recent years, Piedmont and Central and Southern areas concurred mainly to a restricted geographical circulation of BVDV-1b and 1e, as demonstrated by significant local transmission networks, suggesting a local maintenance of BVDV infections. This hypothesis

was also supported by previous identification of several BVDV types and subtypes restricted to North-western, Central and Southern Italy (Luzzago et al., 2014; Giammarioli et al., 2015; Decaro et al., 2016).

5. Conclusions

The current situation in Europe concerning eradication and control programs for BVDV is rapidly evolving, with an increased number of countries applying systematic control measures at national level (Ståhl & Alenius, 2012; Moennig & Becher, 2015). In this respect, the introduction of a national BVDV program is advisable also in Italy in the near future.

In the meanwhile, considering the phylogeographic pattern of BVDV-1b and 1e as indicator of most important viral flows in Italy, the priority control measures should be focused on: i) implementation and harmonization of BVDV systematic control in all Northern Italian regions to break the viral flow from larger to smaller animal populations; and ii) breaking the dynamics of infections in regions with self-maintenance of BVDV by voluntary control programs.

The Italian pattern of BVDV-1b and 1e spread could also be useful for planning BVDV control and eradication programs in countries with a high risk of spread and maintenance of infections and that do not apply BVDV systematic measures, such as the Mediterranean basin areas.

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Conflict of interest statement

The authors declare no conflict of interest.

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Table 1 Characteristic of BVDV-1b Italian significant subclades stratified according to geographic area[†]

SUBCLADE (n=38)	Number of isolates	Median tMRCA[‡]	Locality	pp[§] range
LO (n=11)	2-4	13.25 (5.37-19.69)	Lombardy	0.92-1
PI (n=9)	2-7	7.44 (3.39-16.99)	Piedmont	0.77- 0.99
ER (n=1)	2	16.57	Emilia-Romagna	0.83
CS (n=6)	2	10.80 (4.33-12.68)	Center and Southern Italy	0.66-0.97
LO, PI, VE (n=1)	9	21.24	Lombardy	0.85
LO, CS (n=2)	2	18,95 (11.26-26.65)	Lombardy	0.61-0.68
LO, ER (n=1)	2	17.43	Lombardy	0.85
VE,CS (n=2)	3-4	20.98 (13.03-28.94)	Veneto, Center and Southern Italy	0.35-0.82
PI, CS (n=2)	2-3	8.35 (7.07-9.64)	Piedmont	0.41-0.44
PI, ER (n=1)	3	12.45	Piedmont	0.57
PI, LO (n=1)	7	16.04	Lombardy	0.95
PI, ER, CS (n=1)	3	18.40	Emilia-Romagna	0.40

[†] Italian isolates were classified by sampling region of Northern Italy: Lombardy (LO); Piedmont (PI); Veneto (VE); Emilia-Romagna (ER) and macroarea consisting of Center and Southern Italy (CS).

[‡]tMRCA: time of the most common ancestor

[§] pp: posterior probability

Table 2 Characteristic of BVDV-1e Italian significant subclades stratified according to geographic area[†]

SUBCLADE (n=15)	Number of isolates	Median tMRCA[‡]	Locality	pp[§] range
PI (n=3)	3-38	6.99 (4.85-8.44)	Piedmont	0.89-0.99
LO (n=3)	2	10.94 (6.46-13.26)	Lombardy	0.99-1
ER (n=2)	2	13.68 (12.79-14.57)	Emilia-Romagna	0.81-0.98
CS (n=2)	3-5	10.48 (10.33-10.63)	Center and Southern Italy	0.71-1
VE (n=1)	2	8.36	Veneto	0.96
VE, PI (n=2)	29-45	11.66 (11.28-12.05)	Veneto	0.84-0.91
ER , LO (n=1)	6	16.54	Emilia-Romagna	0.76
LO, ER, CS (n=1)	4	17.01	Lombardy	0.59
LO, ER, PI, VE (n=1)	7	18.58	Lombardy	0.93

[†] Italian isolates were classified by sampling region of Northern Italy: Lombardy (LO); Piedmont (PI); Veneto (VE); Emilia-Romagna (ER) and macroarea consisting of Center and Southern Italy (CS).

[‡]tMRCA: time of the most common ancestor

[§] pp: posterior probability

Figures

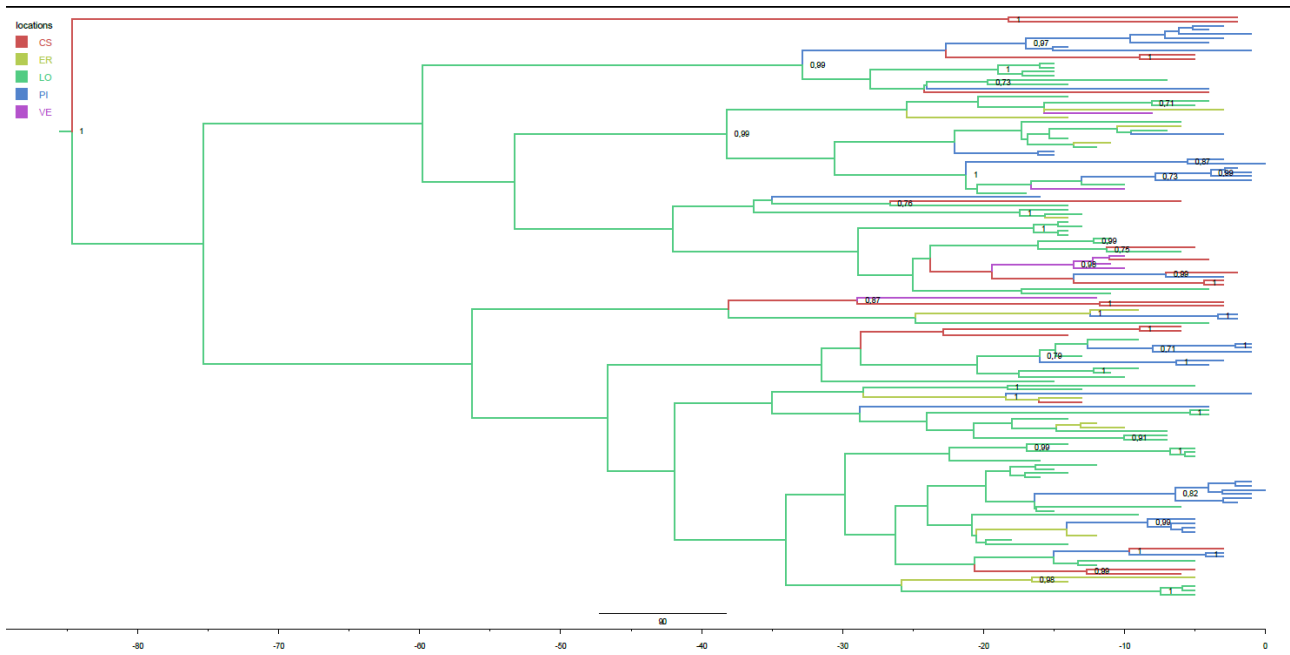


Figure 1. Time-scaled Bayesian maximum clade credibility phylogeographic tree of 139 Italian 5' UTR sequences of BVDV-1b from 1995 to 2013. Branches are colored according to the most probable region of origin, isolates were assigned to distinct regions from Northern Italy (PI=Piedmont; LO=Lombardy; VE=Veneto; ER=Emilia Romagna) and a macroarea consisting of Center and Southern Italy (CS), as summarized by the legend. Posterior probabilities of the clades (values ≥ 0.7) were indicated. The scale at the bottom of the tree represents time (years before present).

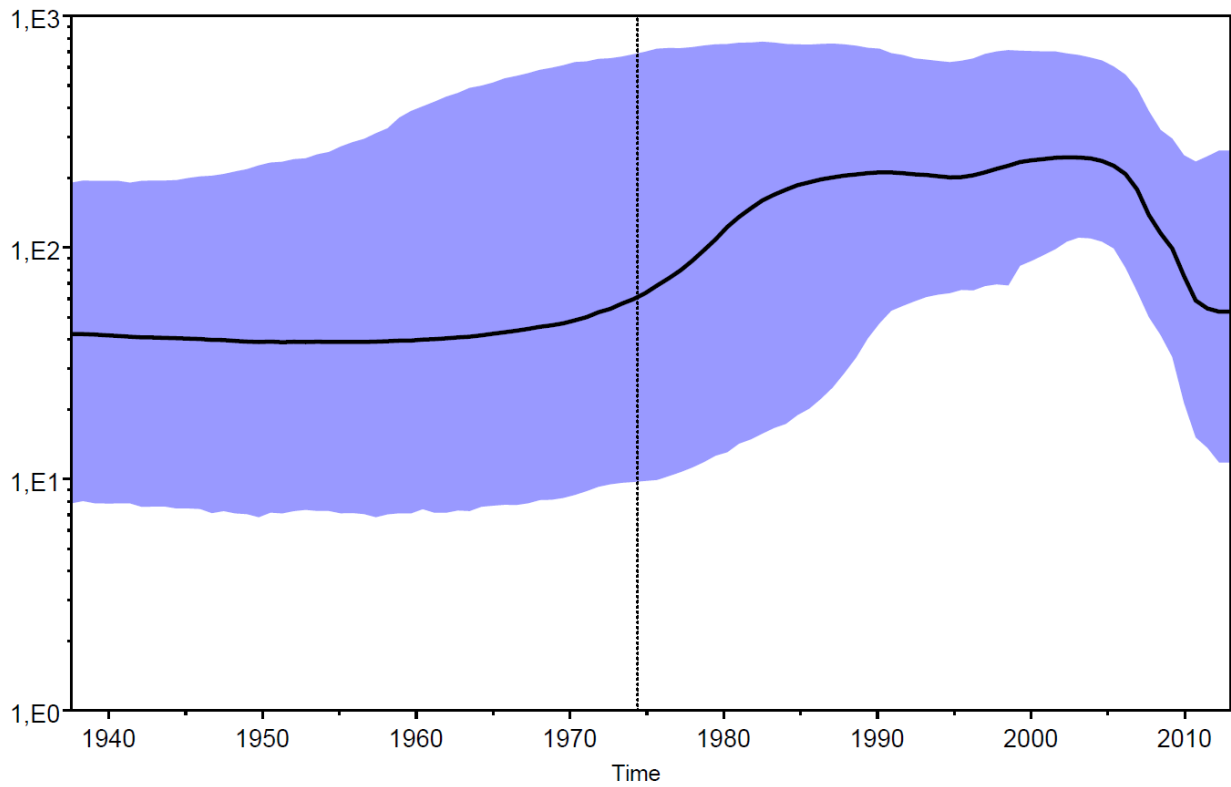


Figure 2. The Bayesian skyline plot obtained from the BEAST analysis on the alignment of Italian 5' UTR sequences of BVDV-1 b. The x axis is in units of years, and the y axis is equal to N_{es} (the product of the effective population size and the generation length in years). The thick solid line is the median estimate, and the colored area is the 95% HPD.

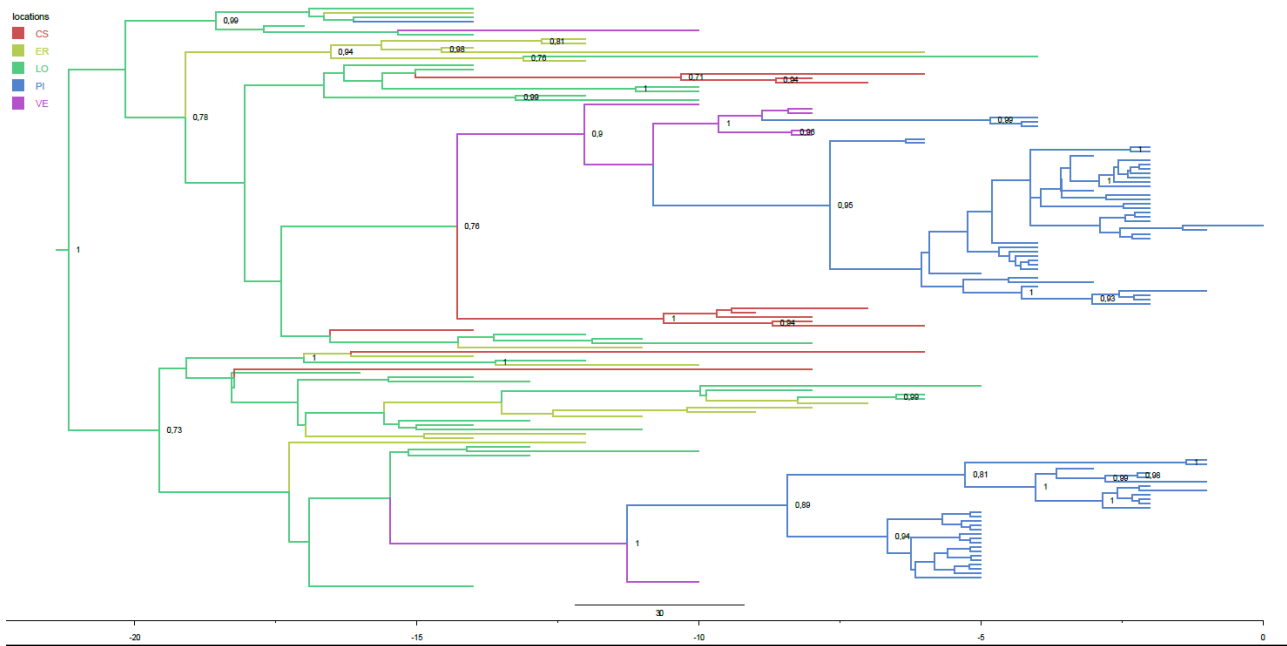


Figure 3. Time-scaled Bayesian maximum clade credibility phylogeographic tree of 139 Italian 5' UTR sequences of BVDV-1e from 1996 to 2013. Branches are colored according to the most probable region of origin, isolates were assigned to distinct regions from Northern Italy (PI=Piedmont; LO=Lombardy; VE=Veneto; ER=Emilia Romagna) and a macroarea consisting of Center and Southern Italy (CS), as summarized by the legend. Posterior probabilities of the clades (values ≥ 0.7) were indicated. The scale at the bottom of the tree represents time (years before present).

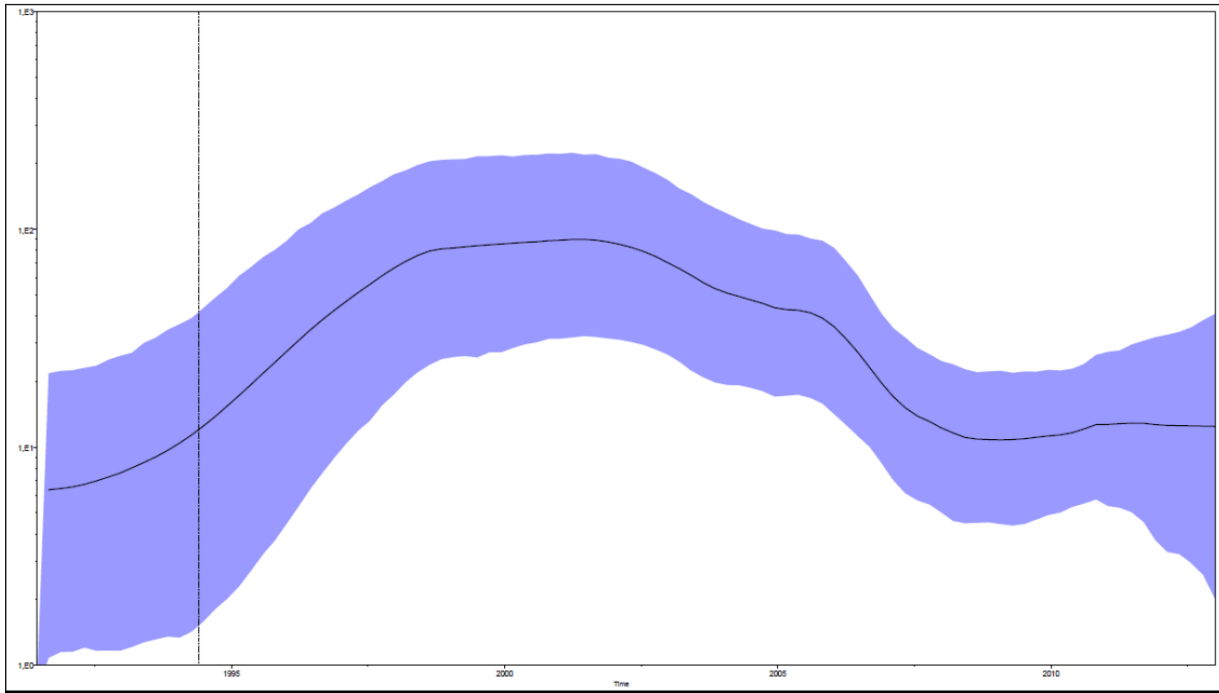


Figure 4 The Bayesian skyline plot obtained from the BEAST analysis on the alignment of Italian 5' UTR sequences of BVDV-1e. The x axis is in units of years, and the y axis is equal to N_{es} (the product of the effective population size and the generation length in years). The thick solid line is the median estimate, and the colored area is the 95% HPD.

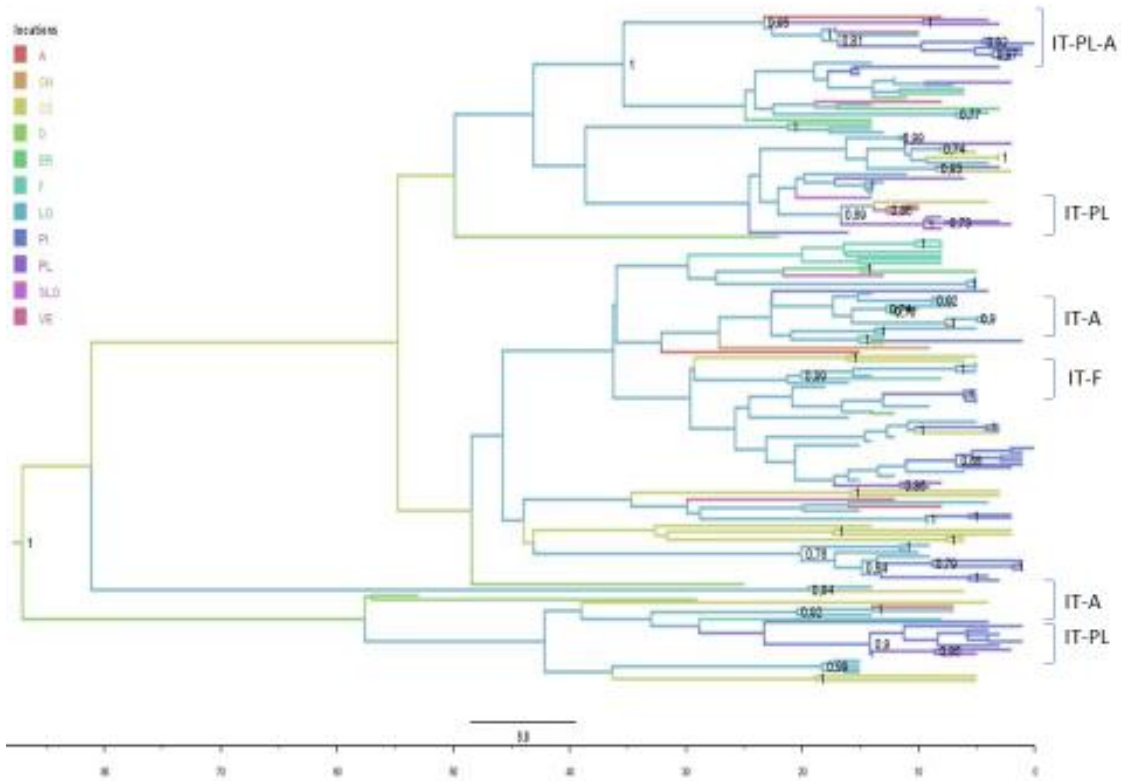


Figure 5 Time-scaled Bayesian maximum clade credibility phylogeographic tree of 173 Italian plus European 5' UTR BVDV-1b sequences. Branches are colored according to the most probable country of origin. European isolates were assigned to country of origin (A=Austria, CH=Switzerland, D=Germany, F=France, SLO=Slovenia, PL=Poland); Italian isolates were assigned according to figure 1. Posterior probabilities of the clades (values ≥ 0.7) were indicated. The scale at the bottom of the tree represents time (years before present).

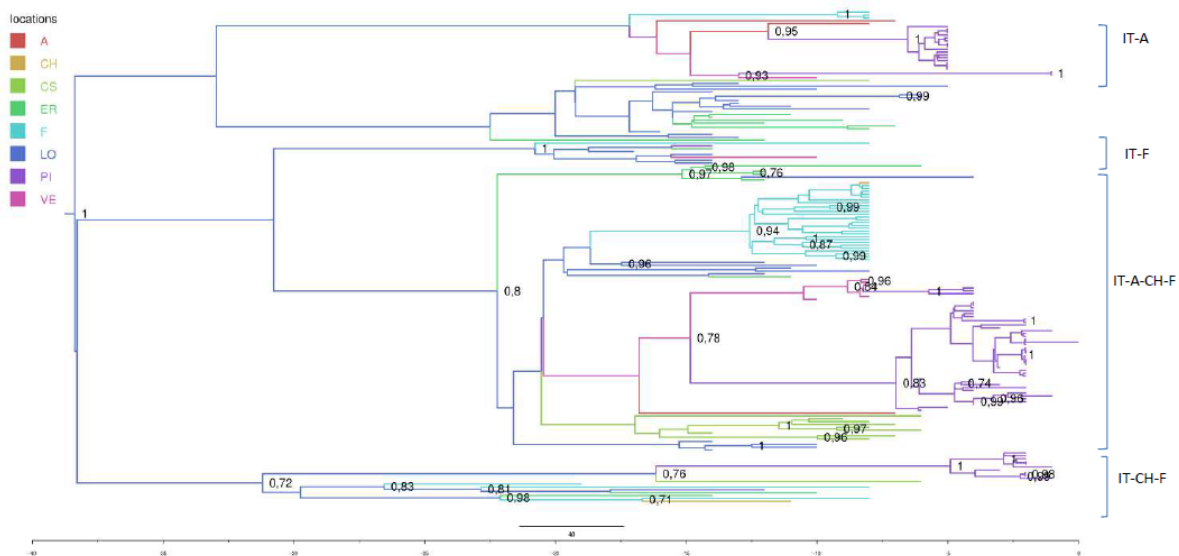


Figure 6 Time-scaled Bayesian maximum clade credibility phylogeographic tree of 172 Italian plus European 5' UTR BVDV-1e sequences. Branches are colored according to the most probable country of origin. European isolates were assigned to country of origin (A=Austria, CH=Switzerland, F=France); Italian isolates were assigned according to legend figure 3. Posterior probabilities of the clades (values ≥ 0.7) were indicated. The scale at the bottom of the tree represents time (years before present).

Supporting information

Table S1 BVDV subtype, Country and Accession number/Reference of BVDV-1b and BVDV-1e sequences retrieved from published peer-reviewed journals.

Table S2 BVDV subtype, Region and Accession number of BVDV novel sequences from this study.

Figure S1 Likelihood map of the BVDV-1b Italian dataset 5'UTR sequences. Each dot represents

the likelihoods of the three possible unrooted trees per quartet randomly selected from the data set. The numbers indicate the percentage of dots in the centre of the triangle. Fully resolved trees fell at the corners and the unresolved fell at the centre area.

Figure S2 Likelihood map of the BVDV-1e Italian dataset 5'UTR sequences. Each dot represents the likelihoods of the three possible unrooted trees per quartet randomly selected from the data set. The numbers indicate the percentage of dots in the centre of the triangle. Fully resolved trees fell at the corners and the unresolved fell at the centre area.

Figure S3 Likelihood map of the BVDV-1b Italian and European dataset 5'UTR sequences. Each dot represents the likelihoods of the three possible unrooted trees per quartet randomly selected from the data set. The numbers indicate the percentage of dots in the centre of the triangle. Fully resolved trees fell at the corners and the unresolved fell at the centre area.

Figure S4 Likelihood map of the BVDV-1e Italian and European dataset 5'UTR sequences. Each dot represents the likelihoods of the three possible unrooted trees per quartet randomly selected from the data set. The numbers indicate the percentage of dots in the centre of the triangle. Fully resolved trees fell at the corners and the unresolved fell at the centre area.