# Genome sequence analysis of a distinctive Italian infectious bursal disease virus

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**ABSTRACT** In a recent study, an emerging infectious bursal disease virus (IBDV) genotype (ITA) was detected in IBDV-live vaccinated broilers without clinical signs of infectious bursal disease (IBD). VP2 sequence analysis showed that strains of the ITA genotype clustered separately from vaccine strains and from other IBDV reference strains, either classic or very virulent. In order to obtain a more exhaustive molecular characterization of the IBDV ITA genotype and speculate on its origin, genome sequencing of the field isolate IBDV/Italy/1829/2011, previously assigned to the ITA genotype, was performed, and the sequences obtained were compared to the currently available corresponding sequences. In addition, phylogenetic and recombination analyses were performed. Interestingly, multiple amino acid (AA) sequence alignments revealed that the IBDV/Italy/1829/2011 strain shared several AA residues with very virulent IBDV strains as well as some virulence markers, especially in the VP1 protein. Nevertheless, sequence analysis demonstrated the presence of several residues typical of IBDV strains at a low degree of virulence in the IBDV/Italy/1829/2011 strain. Although homologous recombination and reassortant phenomena may occur naturally among different IBDV strains, no evidence of those events was found in the genome of the IBDV/Italy/1829/2011 strain, which was confirmed to be a genetically distinctive IBDV genotype.

**Key words:** infectious bursal disease virus, genotype ITA, genome characterization, phylogenetic analysis, recombination

2017 Poultry Science 0:1–8
http://dx.doi.org/10.3382/ps/pex278

## INTRODUCTION

Infectious bursal disease (**IBD**), also known as "Gumboro disease," is a worldwide, highly contagious poultry disease caused by the IBD virus (**IBDV**), which belongs to the *Avibirnavirus* genus within the *Birnaviridae* family (Van den Berg, 2000). The IBDV destroys the developing B-lymphocytes in the bursa of Fabricius, the major organ for the development of humoral immunity in birds. The destruction of the chicken bursa results in immunosuppression and has a notable economic impact on production due to increased susceptibility to secondary infections and a suboptimal response to vaccinations (Balamurugan and Kataria, 2006).

The IBDV is a non-enveloped, bisegmented, double-stranded RNA virus. The viral genome consists of two segments, called A (3.4 kb) and B (2.8 kb). Segment A contains 2 partially overlapping open reading frames (**ORF**), ORF1 and ORF2. The smaller ORF1 encodes the non-structural viral protein VP5 (17 kDa), dispensable for viral replication in vitro, but also involved in the pathogenicity of the virus (Lombardo et al., 2000). The larger ORF2 encodes the precursor polyprotein $NH_2$-VP2-VP4-COOH (108 kDa), which is autocatalitically cleaved by the protease VP4 into the viral capsid protein VP2 (40 to 45 kDa), the ribonucleoprotein VP3 (30 to 34 kDa), and the viral protease VP4 (28 to 30 kDa) (Ture and Saif, 1992; Mundt et al., 1995; Van den Berg, 2000). Several studies have been focused on the VP2 protein, as it is the major structural protein, induces neutralizing antibodies, and is responsible for the antigenic variation due to its hypervariable region, between amino acid (**AA**) residues 206 and 350. The VP3 protein does not contain virus-neutralizing epitopes, but is involved in virus replication and RNA packaging. The VP4 protein processes the precursor polyprotein (Nagarajan and Kibenge, 1997). Segment B contains one ORF, encoding the RNA-dependent RNA polymerase VP1 (90 kDa) and is responsible for viral replication and mRNA synthesis (Ture and Saif, 1992;


Received May 22, 2017.
Accepted August 31, 2017.
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The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database, and the accession numbers KY930929 and KX520665 have been assigned.

**Table 1.** Primers used to amplify and sequence IBDV/Italy/1829/2011 segments A and B.

| Primer (5′-3′) reverse transcriptase | Location of 5′ nucleotide | Primer (5′-3′) PCR | Location of 5′ nucleotide | Amplicon size (bp) |
|---|---|---|---|---|
| *Segment A* | | | | |
| Gum-5R CCCGGATTATGTCTTTGA[1] | | GA1F CTCAGGTCAGAGACCTCGAC[4] | 223 | 491 |
| | 1459 | GA1R GCCTGTCACTGCTGTCACAT[4] | 714 | |
| | | Gum-2F GCCCAGAGTCTACACCAT[1] | 717 | 742 |
| | | Gum-5R CCCGGATTATGTCTTTGA[1] | 1459 | |
| Gum-8R AGGGGACCCGCGAACGGATCC[1] | | GA4nF GAACCTGGTCACAGAATACG[4] | 1254 | 532 |
| | | GA4nR ATAGCGTGGCACCCTCTCT[4] | 1786 | |
| | | GA4Fmod CCGTAGTCGACGGGATTCT[4] | 1700 | 497 |
| | 3261 | GA4Rmod CAGTGGCGAGCTTGGTGC[4] | 2197 | |
| | | GA5F CYCAAYGCYTRTGGCGAGATT[4] | 2140 | 481 |
| | | GA5R GCTGTCCCGTACTTGGCTCTT[4] | 2621 | |
| | | GA6FmodCTCGCAAACGCACCACAAGC[4] | 2564 | 586 |
| | | GA6R CAGGAGCCTCACTCAAGGTC[4] | 3150 | |
| *Segment B* | | | | |
| 2 sense GAGAGCCGCCAATAGCCATG[d] | | G772+ CACCCGGTGAGGATGACAAGC[2] | 751 | 1109 |
| | | GumF2RmodGATCCCGAGATCTTTGCTGTA[2] | 1860 | |
| | 2755 | B1744forGTCCCCCTTGCACAACCAGGGTAC[4] | 1743 | 1080 |
| | | 2 sense GAGAGCCGCCAATAGCCATG[4] | 2823 | |
| | | T7BC1 GGATACGATGGGTCTGACCCT[3] | 1 | 885 |
| | | B898rev CATAGGTAGTCCACTTGATGAC[4] | 886 | |

[1]Jackwood et al., 2008.
[2]Kong et al., 2004.
[3]Boot et al., 2000.
[4]Primers manually designed.

Von Einem et al., 2004). Two distinct serotypes (1 and 2) of IBDV have been differentiated. Serotype 1 strains vary in virulence and are classified as very virulent (**vv**), classic, attenuated, or antigenic variant (Van den Berg, 2000). Serotype 2 strains are avirulent and do not induce protection against serotype 1 strains (Eterradossi and Saif, 2013).

The IBDV affects young chickens and is widespread in nearly all commercial poultry-producing countries around the world. In Italy, the first report of IBD dates back to 1965 (Rinaldi et al., 1965); since then, the disease has become endemic in Italy, and live vaccination of young birds was introduced (Coletti et al., 1983; Asdrubali and Franciosini, 1993). More recently, the genotyping of the Italian IBDV isolates allowed a clearer epidemiological picture. Predominant circulation of the vv strains was confirmed, and the presence of atypical classic strains was reported (Moreno et al., 2007; Moreno et al., 2010), despite the routine use of intermediate and intermediate plus live vaccines and inactivated booster in breeders.

In a recent study, an emerging IBDV genotype (ITA) was detected in IBD-live vaccinated broilers without clinical signs of IBD and with a history of poor growth performance. VP2 sequence analysis showed that strains of the ITA genotype clustered separately from vaccine strains and from other IBDV reference strains, either classic or vv, retrieved from the GenBank or previously reported in Italy (Lupini et al., 2016).

In order to obtain a more exhaustive molecular characterization of the IBDV ITA genotype and speculate on its origin, genome sequencing of the IBDV/Italy/1829/2011 field isolate, belonging to the ITA genotype on the basis of the VP2 sequence analysis (Lupini et al., 2016), was performed, and the sequences obtained were compared to the currently available homologous sequences.

## MATERIALS AND METHODS

### Virus

The IBDV/Italy/1829/2011 field strain had been isolated from the bursa of Fabricius of 3-week-old IBD-live vaccinated broilers, having poor growth performance but without overt clinical signs of IBDV. In the present study, the isolate was propagated once in 12 day-old specific pathogen-free (**SPF**) eggs via the chorioallantoic membrane route (Senne, 2008) in order to perform the molecular analysis. Virus titer was $10^{2.3}$ mean embryo infective dose/mL.

### Genome Sequencing

The total viral RNA was extracted using the QIAamp® Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Two reverse transcriptions for segment A and one for segment B were carried out, using ImProm-IITM Reverse Transcriptase (Promega, Milan, Italy) in a 10 $\mu$l final volume containing 2 $\mu$l of 5X reaction buffer, 1.2 $\mu$l of MgCl2 (3 mM), 0.5 $\mu$l of dNTP (0.5 mM), 0.5 $\mu$l of reverse primers (Table 1), 3.3 $\mu$l of nuclease-free water, and 2 $\mu$l of RNA. The RT mixtures were

incubated at 42°C for 60 min and were then maintained at 70°C for 15 minutes.

Segment A and segment B were amplified in 6 and 3 overlapping fragments, respectively. PCRs were carried out using Go Taq® DNA Polymerase (Promega, Milan, Italy) in a final volume of 25 $\mu$l containing 5 $\mu$l of 5X Go Taq® Flexi Buffer (Promega, Milan, Italy), 1.75 $\mu$l of MgCl2, 0.5 $\mu$l of dNTP (0.2 mM), 0.5 $\mu$l of each primer (Table 1), 12.5 $\mu$l of nuclease-free water, and 4 $\mu$l of cDNA. The PCR cycling parameters consisted of a precycle step at 95°C for 2 min followed by 35 cycles at 95°C for 1 min, primer-specific parameters, and finally at 72°C for one minute. The last cycle was followed by a final extension step of 72°C for 5 minutes.

The Wizard® SV Gel and PCR Clean-Up System (Promega, Milan, Italy) was used to purify the reverse transcriptase-polymerase chain reaction products, according to the manufacturer's instructions. Sanger sequencing was performed in both directions using the PCR primers (Bio-Fab Research, Rome, Italy).

### Sequence Analysis

Nucleotide and predicted AA sequences of the A and the B segments of the IBDV/Italy/1829/2011 strain were edited and assembled using Bioedit software. They were aligned and were then compared with 30 published representatives of all the molecular types of the IBDV strains retrieved from the GenBank database (Table 2), using Mega 6 (Tamura et al., 2013).

### Recombination and Phylogentic Analysis

A collection of 46 complete IBDV genome sequences was downloaded from GenBank. Alignments were carried out both for each segment separately and for a concatenation of the 2 using multiple alignment using fast Fourier transform (Katoh et al., 2013). A NeighborNet network based on the full genome was reconstructed using Splits Tree4v4.12.3 (Huson and Bryant, 2010), and the presence of recombination within the dataset also was tested using the Phi test implemented in the same software (Huson and Bryant, 2010). Recombination analysis was carried out on all datasets using Recombination Detection Program 4 (**RDP**) (Martin et al., 2010). Settings were adjusted according to the RDP manual. Recombination breakpoints were also estimated using Genetic Algorithm Recombination Detection (Kosakovsky et al., 2006). Phylogenetic trees were reconstructed using PhyML, a combination of nearest-neighbor interchange, and subtree pruning and regrafting was selected after removal of the recombinant region/sequences. Branch support was estimated with the fast non-parametric version of the approximate Likelihood-Ratio Test (Shimodaira–Hasegawa [SH]-aLRT) (Anisimova et al., 2011). Ancestral state reconstruction of AA was performed on VP1, VP2–3-4 polyprotein and VP5 using the analysis of phylogenetics and evolution package (Paradis et al., 2004) implemented in R, based on trees reconstructed on an extended collection of sequences.

Finally, a phylogenetic tree was reconstructed using a full collection of 1149 VP2 partial sequences. For computational reasons, the database was reduced by collapsing identical sequences, and the phylogenetic tree was reconstructed using Fasttree (Price et al., 2010).

**Table 2.** IBDV reference strains used in the sequence analysis.

| IBDV strains | Accession number | |
|---|---|---|
| | **Segment A** | **Segment B** |
| **Attenuated** | | |
| D78 | AF499929.1 | EU162090.1 |
| ViBursaCE | EU162089.1 | EU162092.1 |
| ViBursaG | EU162088.1 | EU162091.1 |
| Cu1 | D00867.1 | AF362772.1 |
| JD1 | AF321055.1 | AY103464.1 |
| 903 78 | JQ411012.1 | JQ411013.1 |
| CEF94 | AF194428.1 | AF194429.1 |
| Gt | DQ403248.1 | DQ403249.1 |
| CT | AJ310185.1 | AJ310186.1 |
| J1C7 | EF646853.1 | EF646854.1 |
| W2512 | / | AF083092.1 |
| IC-IBDV-Br | KC603936.1 | KC603936.1 |
| **Classic** | | |
| F52 70 | HG974565.1 | HG974566.1 |
| STC | D00499.1 | / |
| CS-2–35 | EF418033.1 | EU162093.1 |
| cro-pa-98 | / | EU184690.1 |
| Cu-1/ | / | AF362775.1 |
| GA-1 | EF418034.1 | EF162094.1 |
| **Very virulent** | | |
| Gx | AY444873.3 | AY705393.1 |
| SH99 | LM651365.1 | LM651366.1 |
| OKYM | D49706.1 | D49707.1 |
| GZ 96 | AY598356.1 | AY598355.1 |
| UPM94 273 | AF527039.1 | AF527038.1 |
| UK661 | X92760.1 | NC_0,04179.1 |
| Ks | DQ927042.1 | DQ927043.1 |
| PO7 | AY665672.1 | / |
| CAHFS_K669 | JN585293.1 | / |
| Hub-1 | AIG93145.1 | GQ449693.1 |
| **Variant** | | |
| Variant E | AF133904.1 | AF133905.1 |
| GLS | AY368653.1 | AY368654.1 |

## RESULTS AND DISCUSSION

Segments A and B of the IBDV/Italy/1829/2011 strain were successfully amplified, obtaining 2,937 nucleotides for segment A and 2,746 for segment B. The nucleotide sequences were entered into the GenBank database under the accession numbers KY930929 (segment A) and KX520665 (segment B). Segment A showed 96% nucleotide similarity with the representative classic strains and 95 to 94% with the attenuated and vvIBDV strains considered in the present study, respectively. Segment B showed 96% nucleotide similarity with the representative classic and attenuated strains, and 90% with vvIBDV strains.

All the nucleotide sequences were fully translatable into AA sequences. The comparison between the AA sequences of IBDV/Italy/1829/2011 and the corresponding sequences of the representative reference IBDV strains is reported (Figures S1-S12) and discussed.

## Segment A

The ORF1 of the IBDV/Italy/1829/2011 encoded the VP5, which has an important role in the release of viral progeny from infected cells and in the induction of apoptosis in vitro (Lombardo et al., 2000). AA sequence analysis of VP5 showed that IBDV/Italy/1829/2011 shared Ile 78 with the non-vvIBDV strains and Trp 137 with the vvIBDV strains (Supplementary Figures 1 to 4). These residues seem to be involved in virus virulence, as demonstrated in a previous report showing that AA Phe 78 and Trp 137 of the vvIBDV strains changed into Ile 78 and Arg 137 after attenuation by passages in the chicken embryo fibroblasts (Wang et al, 2007). In addition, Hernández et al. (2010) reported that Trp 137 is a conserved residue of 6 vvIBDV strains considering it a typical AA of vvIBDV.

The ORF2-encoded polyprotein consists, as previously mentioned, of VP2, VP4, and VP3. Overall, the predicted AA sequence of the polyprotein indicated that the IBDV/Italy/1829/2011 strain shared 7 AA residues with the non-vvIBDV strains (Leu 294, Ile 451, Lys 685, Pro 715, His 751, Ala 990, and Thr 1005) and one with the vvIBDV strains (Ser 299). Interestingly, 12 peculiar AA residues were found (Cys 45, Glu 53, His 220, Gln 222, Glu 253, Lys 256, Ser 260, Val 321, Thr 708, Phe 750, Ala 778, and Ile 875) (Supplementary Figures 5 to 8).

In particular, Nagarajan and Kibenge (1997) reported that VP2 showed a serine-rich heptapeptide region in position 326 to 332 (Ser-Trp-Ser-Ala-Ser-Gly-Ser), observed in virulent strains (Supplement Figure 8). The VP3 AA sequence showed Ala 990 as non-vvIBDV (Supplementary Figures 5 to 7). Wang et al. (2010) demonstrated that mutations at position 990 would be able to reduce viral replication both in vitro and in vivo.

## Segment B

The coding region contained 2,634 nucleotides, which encoded the VP1 protein (878 AA). The VP1 AA sequence showed that IBDV/Italy/1829/2011 shared 6 AA residues with the non-vvIBDV strains (Ile 4, Val 61, Thr 287, Arg 508, Gly 646, and Ser 687) and 2 AA residues with the vvIBDV strains (Thr 145 and Ser 511). In addition, 3 peculiar AA residues, Phe 413, His 561, and Arg 756, were found as compared to Tyr 413, Gln 561, and Lys 756, which were detected in both the vv and the non-vvIBDV strains (Supplementary Figures 9–12). It has been reported that specific VP1 AA contributed to enhancing viral replication, pathogenicity, and virulence (Van den Berg, 2000; Yu et al., 2013; Gao et al. 2014). In particular, the AA at position 4 and the 145/146/147 AA triplet are located in the N-terminal domain of VP1, which is responsible for the protein priming activity of IBDV, potentially affecting the polymerase activity function (Yu et al., 2013). The VP1 protein of IBDV/Italy/1829/2011 showed Ile 4 as a non-vvIBDV strain and a Thr-Glu-Gly triplet at positions 145/146/147 as vvIBDV strains. Using a reverse-genetics system, it has been reported that the 4 Val > Ile substitution reduces viral replication and pathogenicity in SPF chickens (Yu et al., 2013). Moreover, Gao et al. (2014) demonstrated that the Thr-Glu-Gly triplet was able to enhance viral replication in chickens as compared to Asn-Glu-Gly, the typical triplet of the non-vvIBDV strains.

With respect to the recombinations and phylogentic analysis, the IBDV/Italy/1829/2011 strain displayed a genetic distance between 0.044 and 0.168, 0.040 and 0.120, and 0.043 and 0.124 for segment A, segment B, and both segments, respectively. The presence of several reticulations in the phylogenetic network and Phi test results suggested frequent recombination events among IBDV strains. Nevertheless, these did not seem to affect the IBDV/Italy/1829/2011 strain (Figure 1). The RDP and the Genetic Algorithm Recombination Detection analyses provided concordant results that confirmed this evidence. Reassortment and recombination were proven to be frequent. More specifically, reassortment was detected in 25 strains out of 46, while recombination was detected only within segment A where 21 strains out of 46 displayed a recombinant insert, approximately between positions 1169 and 1456. The only 2 exceptions were represented by the EU595670 strain (recombination breakpoints 1 to 1351) within segment A and the KC109816 strain (recombination breakpoints 437 to 2087) within segment B. Nevertheless, although suggestive, the limited number of complete genome sequences available requires caution in generalizing these results and defining actual recombination hot spots. No evidence of recombination was found for the IBDV/Italy/1829/2011 genotype. The phylogenic tree confirmed the absence of a close relationship between IBDV/Italy/1829/2011 and other representative strains (Figure 2). Analysis of the phylogenetic tree based on segment A, in which IBDV serotypes 1 and 2 are most divergent, demonstrated that the current isolate is the one most related to serotype 2 strains, and it could therefore represent the descent of a group that originated in the distant past (Figure 2). These results were also confirmed when a broad collection of VP2 partial sequences (date range of the origin of the strains was from 1995 to 2015) was used for phylogenetic inference (Supplementary Figure 13).

In this study, the authors reported the results of the genome sequencing of the IBDV/Italy/1829/2011 strain, confirming that it was a genetically distinctive IBDV strain. Interestingly, multiple AA sequence alignments revealed that IBDV/Italy/1829/2011 shared several AA residues with vvIBDV strains, and some virulence markers, especially in the VP1 protein, were detected. Nevertheless, detailed analysis of the predicted AA sequences pointed out the presence of some residues typical of IBDV strains at a low degree of virulence. The main mechanisms of evolution of RNA viruses is their high substitution rate, which might cause the

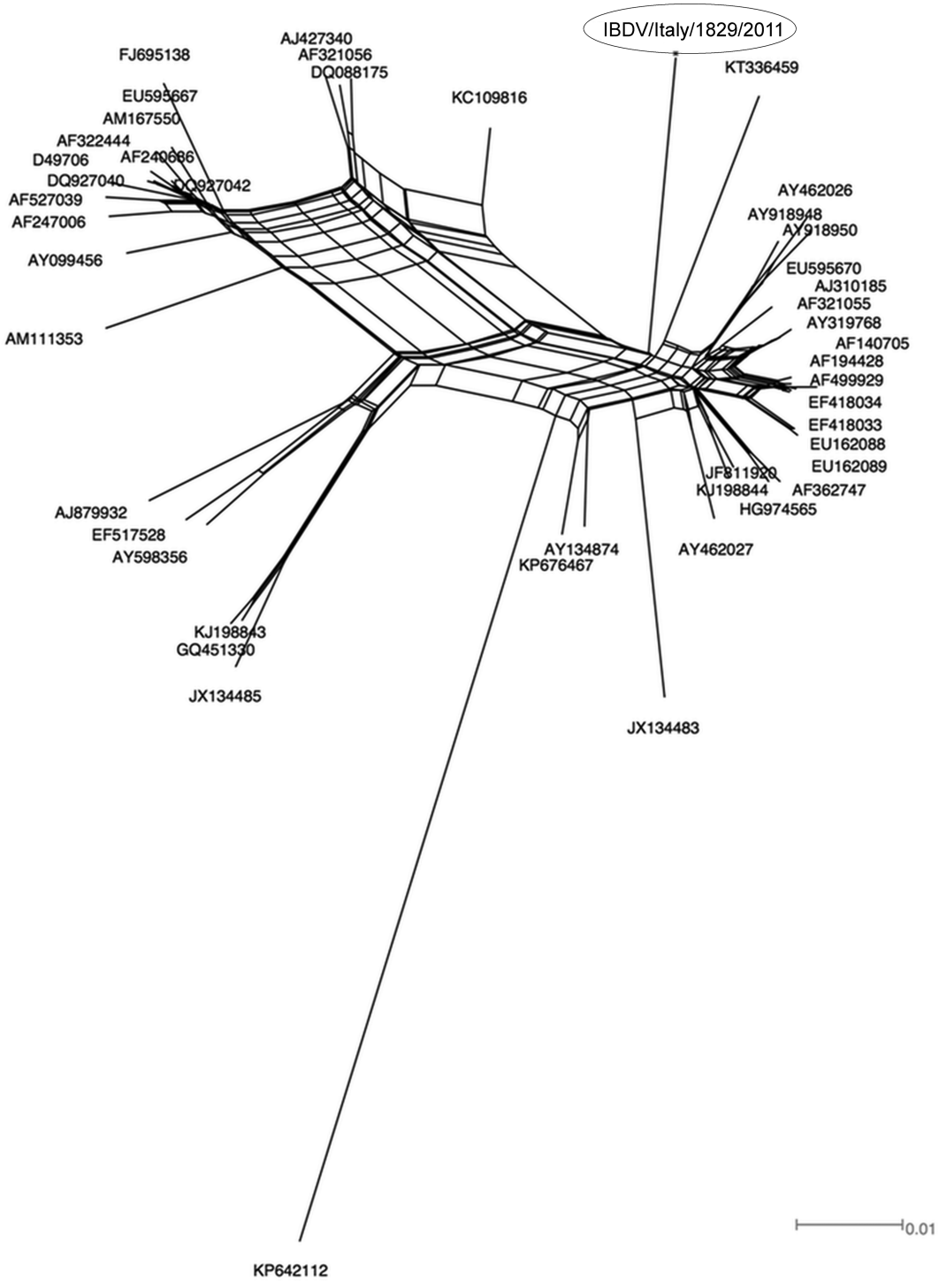


**Figure 1.** Phylogenetic network based on the NeighborNet method, including 47 IBDV genomes (concatenation of segments A and B). The IBDV/Italy/1829/2011 strain is circled.

emergence of new IBDV strains able to overcome vaccine immunity (Eterradossi and Saif, 2013), as has been reported for other avian RNA viruses (Catelli et al., 2010; Cecchinato et al., 2010). Moreover, homologous recombination (He et al., 2009; Jackwood, 2012) and reassortant phenomena may naturally occur between different IBDV strains (Wei et al., 2006; Cui et al., 2013; Kasanga et al., 2013). Interestingly, no evidence of recombination or reassortment was found in IBDV/Italy/1829/2011, whereas reassortment and recombination events were detected in 25 and 21 out of 46 IBDV genomes analyzed, respectively.

The IBDV/Italy/1829/2011 strain had been isolated from IBD live-vaccinated broilers that did not show clinical signs of IBD. Multiple AA sequence alignments revealed that it shared AA residues with both

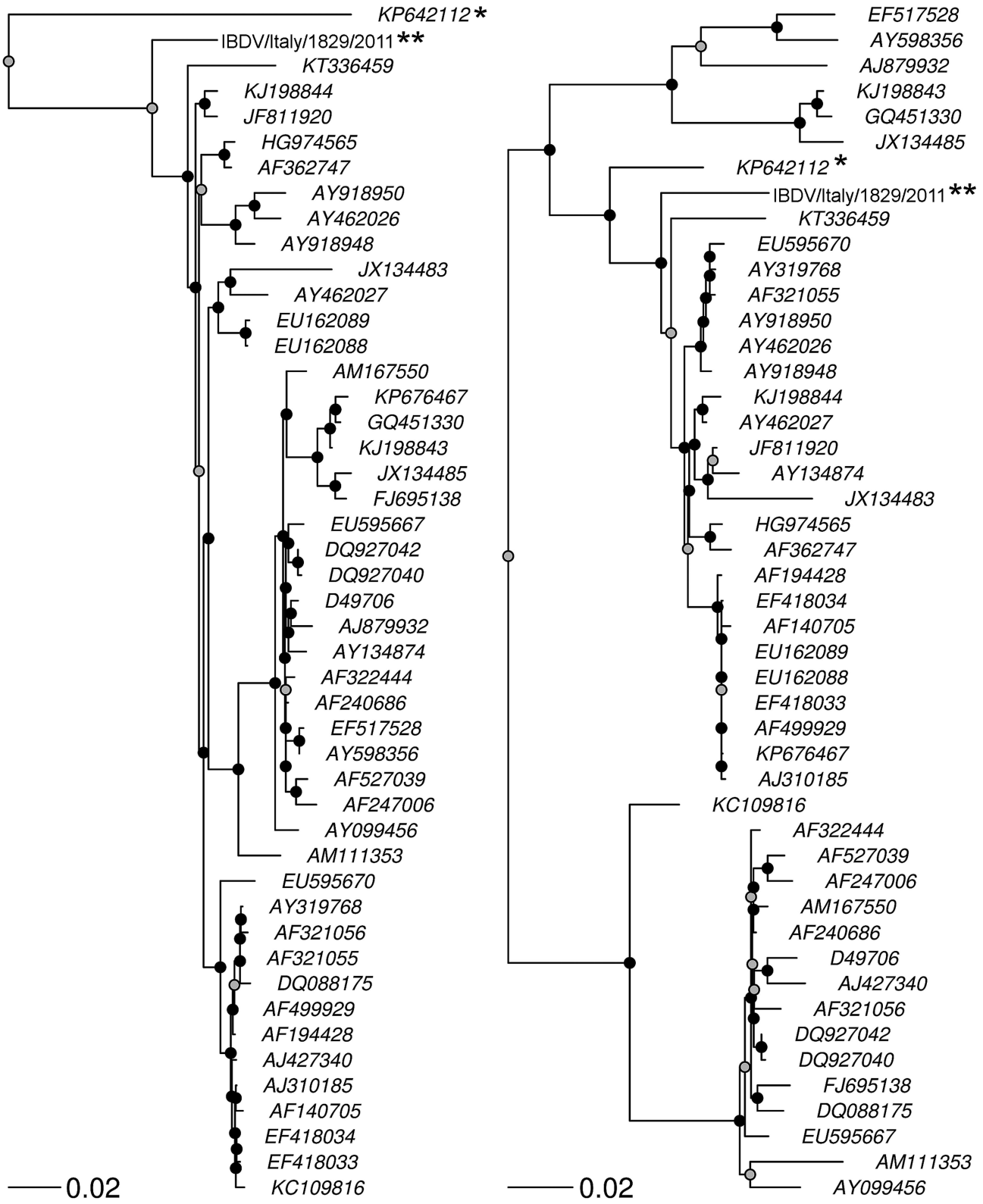


**Figure 2.** Midpoint-rooted maximum likelihood (**ML**) phylogenetic tree reconstructed using the segment A (left) and segment B (right) alignments. Branch support estimated with the Shimodaira–Hasegawa [SH]-aLRT model is reported as black (>0.7) or gray (>0.5) circles near the corresponding node. IBDV/Italy/1829/2011 strain (double asterisk). IBDV serotype 2 (asterisk).

vvIBDV and IBDV strains at low degrees of virulence, but homologous recombination and reassortant phenomena were not found in its genome, confirming IBDV/Italy/1829/2011 to be a genetically peculiar IBDV strain. In order to elucidate the relationship between the genetic profile and the virulence of the IBDV/Italy/1829/2011 strain, additional in vivo pathogenicity studies are needed.

## SUPPLEMENTARY DATA

Supplementary data are available at *PSCIEN* online.

**Supplementary Figure 1.** Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with VP5 sequences of the IBDV reference strains representing the attenuated strains.

**Supplementary Figure 2.** Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV reference strains representing the classic strains.

**Supplementary Figure 3.** Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV reference strains representing the variant strains.

**Supplementary Figure 4.** Alignment of the VP5 IBDV/Italy/1829/2011-deduced AA sequence with the VP5 sequences of the IBDV references strains representing the vvIBDV strains.

**Supplementary Figure 5.** Alignment of the deduced amino acid sequences of VP2 (1 to 512 amino acids of polyprotein), VP4 (513 to 755 amino acids of polyprotein), and VP3 (756 to 1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the attenuated strains.

**Supplementary Figure 6.** Alignment of the deduced amino acid sequences of VP2 (1 to 512 amino acids of polyprotein), VP4 (513 to 755 amino acids of polyprotein), and VP3 (756 to 1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the classic strains.

**Supplementary Figure 7.** Alignment of the deduced amino acid sequences of VP2 (1 to 512 amino acids of polyprotein), VP4 (513 to 755 amino acids of polyprotein), and VP3 (756 to 1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the variant strains.

**Supplementary Figure 8.** Alignment of the deduced amino acid sequences of VP2 (1 to 512 amino acids of polyprotein), VP4 (513 to 755 amino acids of polyprotein), and VP3 (756 to 1012 amino acids of polyprotein) of the IBDV/Italy/1829/2011 strain with the corresponding sequences of the IBDV reference strains representing the vvIBDV strains.

**Supplementary Figure 9.** Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the attenuated strains.

**Supplementary Figure 10.** Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the classic strains.

**Supplementary Figure 11.** Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the variant strains.

**Supplementary Figure 12.** Alignment of the VP1 IBDV/Italy/1829/2011-deduced AA sequence with the VP1 IBDV reference strains representing the vvIBDV strains.

**Supplementary Figure 13**. Maximum likelihood (**ML**) phylogenetic tree reconstructed using partial VP2 alignment (399 nucleotides) after removal of the identical sequences. Clusters have been defined (renamed and color-coded) on the basis of a maximum genetic distance (i.e., $P$ distance among sequences $<0.01$) and branch support (i.e., support $>0.8$) thresholds.

IBDV/Italy/1829/2011 (double asterisk).

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