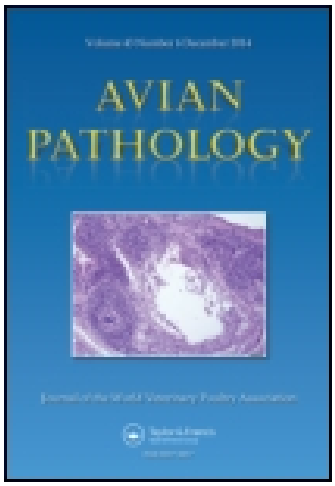

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Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage

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ORIGINAL ARTICLE

Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage

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Infectious bursal disease virus (IBDV) is one of the most concerning health problems for world poultry production. IBDVs comprise four well-defined evolutionary lineages known as classic (c), classic attenuated (ca), variant (va) and very virulent (vv) strains. Here, we characterized IBDVs from South America by the genetic analysis of both segments of the viral genome. Viruses belonging to c, ca and vv strains were unambiguously classified by the presence of molecular markers and phylogenetic analysis of the hypervariable region of the *vp2* gene. Notably, the majority of the characterized viruses (9 out of 15) could not be accurately assigned to any of the previously described strains and were then denoted as distinct (d) IBDVs. These dIBDVs constitute an independent evolutionary lineage that also comprises field IBDVs from America, Europe and Asia. The hypervariable VP2 sequence of dIBDVs has a unique and conserved molecular signature (272T, 289P, 290I and 296F) that is a diagnostic character for classification. A discriminant analysis of principal components (DAPC) also identified the dIBDVs as a cluster of genetically related viruses separated from the typical strains. DAPC and genetic distance estimation indicated that the dIBDVs are one of the most genetically divergent IBDV lineages. The *vp1* gene of the dIBDVs has non-vvIBDV markers and unique nucleotide and amino acid features that support their divergence in both genomic segments. The present study suggests that the dIBDVs comprise a neglected, highly divergent lineage that has been circulating in world poultry production since the early time of IBDV emergence.

Introduction

Infectious bursal disease is an acute, highly contagious viral disease that affects the immune system of young chickens and causes major economic losses to the poultry industry worldwide. The aetiologic agent is the infectious bursal disease virus (IBDV) belonging to the genus *Avibirnavirus* within the family *Birnaviridae*. IBDV is a non-enveloped icosahedral virus with a double-stranded RNA genome containing two segments (Müller *et al.*, 1979). Segment A (3.3 kb) has two partially overlapping open reading frames. Open reading frame A-1 encodes a polyprotein that is autocatalytically cleaved to yield the immature outer capsid protein pVP2, the viral protease VP4 and the ribonucleoprotein VP3 (Sánchez & Rodriguez, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002; Luque *et al.*, 2009). The pVP2 protein is further processed to yield the mature VP2, the immunodominant antigen of IBDV (Fahey *et al.*, 1991). Open reading frame A-2 encodes VP5, a non-structural protein involved in virion release from infected cells (Wu *et al.*, 2009). Genomic segment B (2.9 kb) encodes the RNA-dependent RNA polymerase VP1 (Spies *et al.*, 1987).

Two IBDV serotypes (1 and 2) are currently characterized by cross-neutralization assays. Serotype 2 has not been associated with disease in any avian species, while serotype 1 comprises all the known pathogenic IBDVs (McFerran *et al.*, 1980; Jackwood *et al.*, 1985). First outbreaks of the pathogenic serotype 1 occurred more than 50 years ago in Gumboro (Delaware, USA) (Cosgrove, 1962). The associated viruses, currently referred as classic (c) IBDVs, caused high morbidity and mortality in the affected broiler flocks. Vaccines against cIBDVs were rapidly developed and introduced in the routine vaccination schedules, reducing considerably the mortality rates and partially controlling the disease (Snedeker *et al.*, 1967; Edgar & Cho, 1976). This epidemiological scenario changed in the late 1980s with the emergence of the variant (va) and very virulent (vv) strains.

The vaIBDVs were characterized by an antigenic drift caused by point mutations affecting the neutralizing epitopes

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of VP2 (Ismail *et al.*, 1990; Vakharia *et al.*, 1994). These strains emerged in the North American continent and were characterized by causing B-lymphocyte depletion without eliciting an inflammatory response or clinical signs of disease (Rosales *et al.*, 1989; Sharma *et al.*, 1989). Although the vaIBDVs were clearly serotype 1 viruses, they had enough antigenic differences to evade the immunity induced by classic-type vaccines.

Almost simultaneously with the vaIBDV emergence, vvIBDVs emerged in Europe and caused a very severe form of the disease with unusual high mortality (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). These vvIBDVs were antigenically similar to the cIBDVs, but they were able to breakthrough the existing level of maternally derived protection (van den Berg *et al.*, 1996).

The three IBDV strains (c, va and vv) currently have a global distribution and occur in most countries with a developed poultry industry. Isolated IBDVs with different traits than the traditional strains have also been sporadically reported through the years in different parts of the world (Ikuta *et al.*, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007). These IBDVs have been generally considered atypical isolates or variants that have evolved in restricted geographic regions or during short periods of time under particular conditions.

An accurate identification of the field variants circulating in the poultry production has been essential to understand the epidemiology and control the disease (van den Berg, 2000). Genetic analysis has been the most widely used technique to characterize and classify IBDVs (Cao *et al.*, 1998; Banda & Villegas, 2004; Hernández *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Yamaguchi *et al.*, 2007; Tomás *et al.*, 2012). Studies have been mainly focused on the hypervariable domain of VP2 (hvVP2) because it codes for the most important immunogenic regions of the protein (Azad *et al.*, 1987; Bayliss *et al.*, 1990).

Virus classification can be performed by identifying nucleotide or amino acid residues specific for each IBDV strain. These strain markers are determined by molecular typing techniques (e.g., restriction fragment length polymorphisms, real-time polymerase chain reaction, PCR) or sequencing. A more comprehensive classification is achieved by phylogenetic analysis using mainly the hvVP2 sequence; viruses are classified according with their association with reference strains in a well-supported clade that share a single common ancestor, i.e., evolutionary lineage (Jackwood & Sommer-Wagner, 2007; Yamaguchi *et al.*, 2007; Kim *et al.*, 2010; Islam *et al.*, 2012). Phylogenetic trees using the hvVP2 region consistently recover the clades corresponding to the three IBDV strains (c, va and vv), and the clade composed of attenuated classic vaccine strains, usually denoted as "classic attenuated" (ca) IBDVs (Martin *et al.*, 2007; Wu *et al.*, 2007; Xia *et al.*, 2008; Kim *et al.*, 2010).

In addition to sequences of segment A, the analysis of the *vp1* gene of segment B is useful in providing complementary genetic information for more precise characterization. By studying both segments, it is possible to analyse their coevolution, to detect the occurrence of natural reassortants and to determine the role of VP1 in pathogenicity (Le Nouën *et al.*, 2006; Escaffre *et al.*, 2013). VP1 phylogeny resolves a lineage corresponding to the vvIBDV strain and a non-vvIBDV clade constituted by the remaining strains and serotype 2 viruses (Hon *et al.*, 2006).

The present study presents genetic comparative analyses of Uruguayan and Argentine field viruses with global IBDVs and the molecular characterization of a worldwide-spread genetic lineage divergent from the traditional IBDV strains.

Materials and Methods

Viruses. Bursal samples were collected during 2007–2012 from Uruguayan and Argentine broiler flocks. Samples came from chickens with clinical signs of immunodeficiency frequently associated with respiratory or urinary infections. Each sample was stored at –80°C until analysis. IBDV presence was confirmed in 15 samples (Table 1) with real-time PCR, as previously described (Tomás *et al.*, 2012).

PCR amplifications. PCR amplifications of a 643-bp fragment encompassing the hvVP2 region and a 594-bp fragment of the *vp1* gene were performed using previously described primers and conditions (Hernández *et al.*, 2006). An Applied Biosystems 2720 PCR Thermal Cycler was used for the amplification reaction. PCR products were assayed on 0.8% agarose gels stained with ethidium bromide, and purified using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK), and sequenced bi-directionally using an ABI prism 377-Perkin Elmer automated sequencer.

Phylogenetic inferences. Sequence homologies were first analysed using the nucleotide BLAST algorithm with GenBank database (http://www.ncbi.nlm.nih.gov). The Uruguayan and Argentine viruses were further compared with reference and representative IBDVs. Phylogenetic analyses included sequences encompassing 374 bp of hvVP2 coding region from position 795 to 1168, and a 534-bp fragment from nucleotide positions 322 to 855 of the *vp1* gene (genome numbering according to D6948 segment A and B, AF240686 and AF240687, respectively).

Sequences were aligned using MAFFT (Katoh *et al.*, 2002), and the best-fit model of nucleotide substitution was selected under the Akaike information criterion and Bayesian information criterion as implemented in jModelTest (Posada, 2008). Maximum-likelihood trees, with approximate likelihood ratio test for internal nodes support, were inferred using PhyML (Guindon & Gascuel, 2003). Phylogenetic trees were visualized and edited with TreeGraph2 (Stöver & Müller, 2010).

Discriminant analysis of principal components and between-group distance. The discriminant analysis of principal components (DAPC) multivariate method (Jombart *et al.*, 2010), used to infer the genetic structure of populations coupling classical principal components analysis and discriminant analysis, was performed with the VP2 nucleotide sequences used in the phylogenetic analysis and additional representatives of the different IBDV

Table 1. *Argentine and Uruguayan IBDV characterized in this study.*

Name	Origin	Type	Accession[a]
AR-A1	Argentina	vvIBDV	KM659881
AR-C7	Argentina	cIBDV	KM659882
UY-130D	Uruguay	caIBDV	KM659884
UY-131D	Uruguay	caIBDV	KM659885
UY-139D	Uruguay	caIBDV	KM659886
UY-01/09	Uruguay	caIBDV	KM659883
AR-C1	Argentina	dIBDV	KM659888
AR-C3	Argentina	dIBDV	KM659889
UY-42/07	Uruguay	dIBDV	KM659892
UY-04/09	Uruguay	dIBDV	KM659895
UY-06/10	Uruguay	dIBDV	KM659890
UY-07/10A	Uruguay	dIBDV	KM659891
UY-171101	Uruguay	dIBDV	KM659887
UY-421101	Uruguay	dIBDV	KM659893
UY-221201	Uruguay	dIBDV	KM659894

[a]Accession numbers correspond to VP2 coding sequences (hypervariable region). The vp1 sequences of samples UY-07/10A, UY-06/10; UY-221201 and UY-04/09 were deposited under the accession numbers KP274850-3, respectively.

strains. This method was applied using pre-defined groups and 75 principal components were retained and inputted to discriminant analysis. The analysis of the nucleotide sequences was implemented using *adegenet* package library of R statistical environment (R Development Core Team, 2013). Additionally, net between-groups mean distances were calculated as implemented in MEGA5.1 (Tamura *et al.*, 2011).

Accession numbers. The hvVP2 coding sequences and the partial sequences of the *vp1* gene were deposited under the GenBank accession numbers KM659881-95 and KP274850-3, respectively.

Results

An initial classification of the Argentine and Uruguayan IBDVs was performed by identifying amino acid markers of the hvVP2 coding region and comparing their nucleotide sequences with reference strains (c, ca, va and vv) (Figure 1). Six samples were straightforward to classify as belonging to traditional IBDV strains, but nine samples had singular genetic and amino acid sequences and were here denoted as distinct (d) IBDVs.

Very virulent infectious bursal disease viruses. Argentine AR-A1 has the vvIBDV signature 222A, 256I, 294I and 299S. This virus shows a maximum nucleotide similarity (99.8%) with the vv reference strain D6948, and high similarity with the Argentine hypervirulent isolates LD-847-04 and LD9569 (99.3% and 98.8%, respectively) and with previously reported Uruguayan vvIBDVs (98.8%).

Classic infectious bursal disease viruses. Argentine AR-C7 has the markers 263F and 312V observed in STC, one of the c reference isolates. Comparative nucleotide sequence analysis shows 100% nucleotide similarity with the cIBDVs HPR-2 and A-BH83 from the USA and Brazil, respectively, and has 99.8% of similarity with STC.

Classic attenuated infectious bursal disease viruses. Four Uruguayan viruses (UY-130D, UY-131D, UY-139D and UY-01/09) have the amino acid composition characteristic of caIBDVs. With the exception of UY-01/09, which shows the residue 253N, the remaining three isolates contain the signature 253H, 279N and 284T, typical of the classic vaccine viruses. The highest nucleotide similarity values of these viruses are with the attenuated isolates CEF94 and D78 (above 99%).

Distinct infectious bursal disease viruses. Seven Uruguayan (UY-42/07, UY-04/09, UY-06/10, UY-07/10, UY-171101, UY-421101 and UY-221201) and two Argentine (AR-C1 and AR-C3) viruses have a singular nucleotide and amino acidic composition. The VP2 hypervariable region consistently shows the 222S marker, and the signature 272T, 289P, 290I and 296F that in other viruses corresponds to 272V, 289L, 290M/L and 296I. These dIBDVs have similarity values above 99% with particular viruses from Argentina (e.g. 05A25, P30903), Brazil (e.g. B8), Colombia (01C5), Canada (e.g. 03–42857, 05-32115-B9), Puerto Rico (586), Hungary (e.g. P10, P3), Poland (78/GSz, 80GA), Russia (IBDVRF-8/96) and South Korea (K310), which have the same 272T, 289P, 290I and 296F signature.

Phylogenetic analysis using Argentine, Uruguayan and global IBDVs, including common reference strains and IBDVs from different years and regions, was performed to explore IBDV relationships based on the hvVP2 coding region. The phylogenetic analysis showed five well-supported clades corresponding to cIBDVs, caIBDVs, vaIBDVs, vvIBDVs and dIBDVs (Figure 2). The Argentine and Uruguayan IBDVs fall within the corresponding clusters according with the initial classification based on sequence comparison and amino acid markers.

Additional approaches were implemented to further analyse IBDV clustering. First, DAPC was carried out to infer the clustering pattern of IBDVs. Five clearly distinguishable groups were obtained and corresponded to the same clades identified in the phylogenetic cladogram (Figure 3A). When considering just the first discriminant function retained during the principal components analysis (which explained 70% of the genetic variance in the data-set), IBDV groups are still distinguishable (Figure 3B). Second, the net between groups mean distances were calculated, showing that vvIBDVs (median = 0.17) and dIBDVs (median = 0.14) are the most divergent groups (Figure 3C).

To gain insight into the variability of dIBDVs, we sequenced a region of the *vp1* gene in some of these viruses. VP1 has the non-vvIBDV markers 146E, 147G and 242D and a unique F243P change (Figure 4). The phylogeny recovers the vv lineage and a non-vvIBDV clade that includes the dIBDVs (Figure 5). The only available VP1 sequences for members of the dIBDV lineage were the Uruguayan IBDVs and the Poland isolates 78GsZ and 80GA (Figures 4 and 5). These dIBDVs constitute a closely related monophyletic group in the VP1 phylogeny (Figure 5).

Discussion

Three major events describe the evolutionary history of the infectious bursal disease; the detection of cIBDVs in the early 1960s, and the almost simultaneous emergence of va and vv strains about 25 years later. Genetic analyses revealed that these IBDV strains have nucleotide and amino acid signatures and constitute well-supported independent clades in phylogenetic trees (Boot *et al.*, 2000; van den Berg, 2000; Le Nouën *et al.*, 2005; Hon *et al.*, 2006). Besides the three lineages corresponding to c, va and vv strains, phylogenetic trees also resolve an independent lineage for the ca strains that comprise vaccine strains maintained in the laboratory and vaccine-like virus collected from the field (Kim *et al.*, 2010).

In order to map IBDV diversity in the southern poultry industry of South America, we characterized viruses from Argentina and Uruguay. Genetic analysis of the hvVP2 coding region revealed a great genetic diversity of the IBDVs circulating in the surveyed poultry production. With exception of the vaIBDVs, the remaining strains (c, vv and ca) are present in Argentina and Uruguay. Strain classification was unambiguously performed by the presence of molecular markers and sequence similarity with reference or previously classified IBDVs, and by phylogenetic analysis that clustered the viruses into well-defined lineages (Figures 1 and 2).

Remarkably, the majority of the characterized viruses (9 out of 15) have particular amino acid residues and grouped together in a well-supported clade separated from the pre-existing IBDV strains (Figures 1 and 2). Accordingly, the genetic analysis cannot accurately assign this group of viruses, here denoted as dIBDVs, to the typical c, ca, va and vv strains. These Uruguayan and Argentine dIBDVs are included in an independent evolutionary lineage with previously reported viruses from other countries in America (Jackwood & Sommer, 1997; Ikuta *et al.*, 2001; Banda *et al.*, 2003; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007), Europe (Domanska *et al.*, 2004) and Asia (Kwon *et al.*, 2000) (Figure 2). The hvVP2

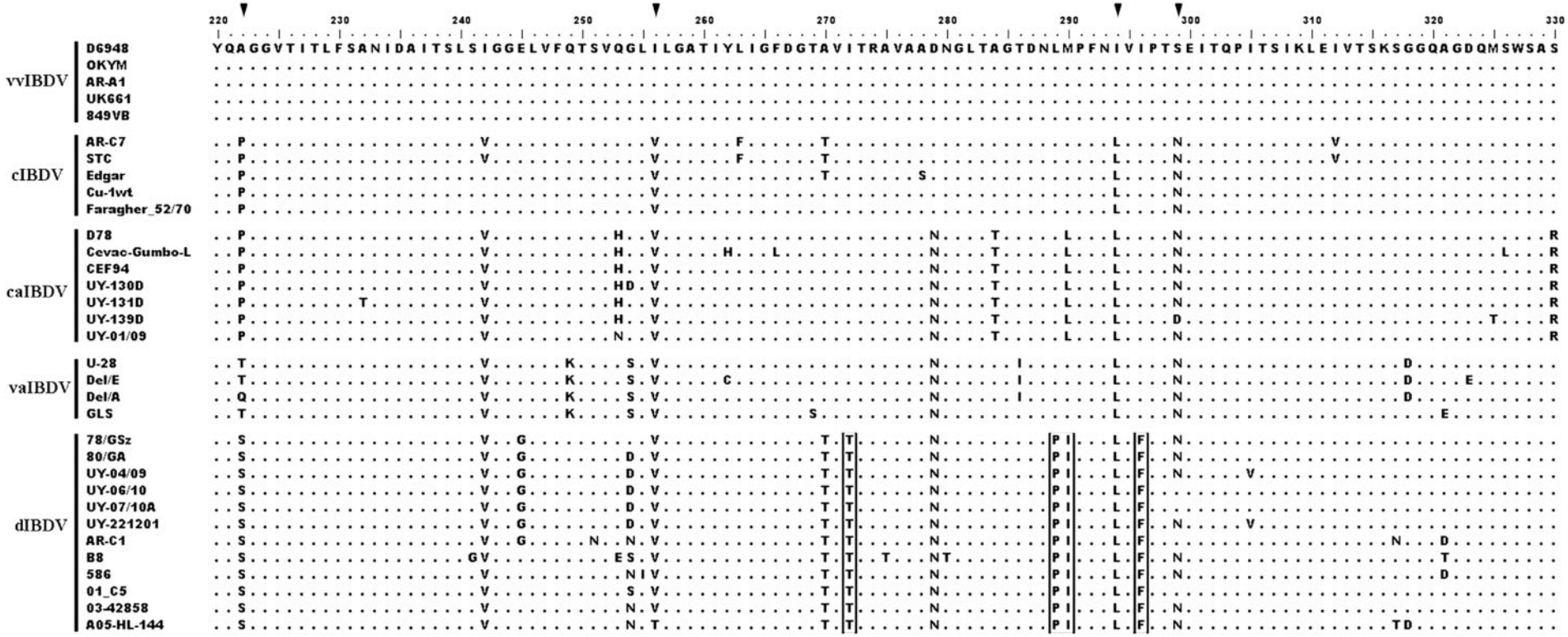

Figure 1. *Amino acid differences along the coding sequences of the hvVP2 region of very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs, and representatives of the distinct IBDVs (dIBDVs). Dots indicate position where the sequences are identical to the segment A of the hypervirulent D6948 strain (AF240686). Arrowheads indicate common amino acid markers used to distinguish hypervirulent from non-hypervirulent strains; the dIBDV group shares markers with both groups. The hvVP2 sequences of the dIBDV lineage have a highly conserved molecular signature: 272T, 289P, 290I and 296F (indicated by boxes). This signature may be considered a diagnostic character for the dIBDV lineage.*

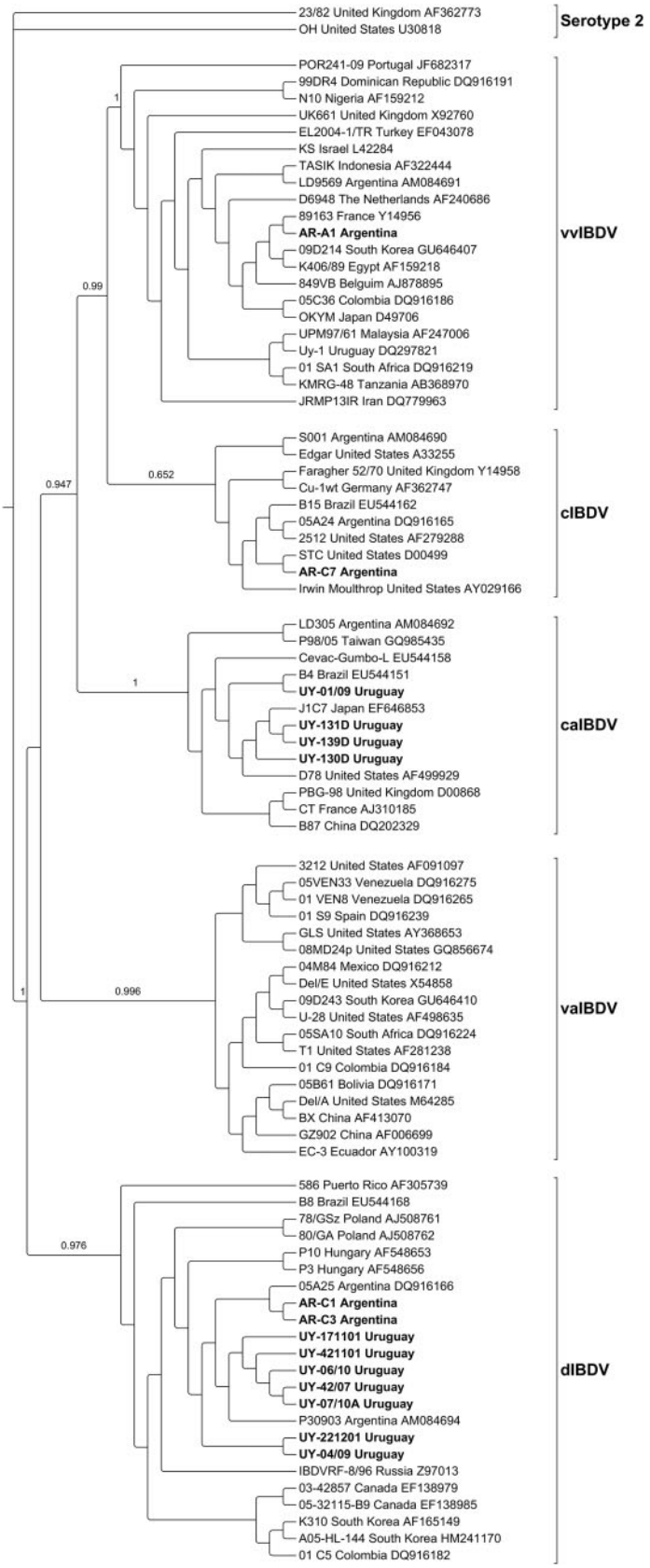

Figure 2. *Cladogram inferred by using the maximum-likelihood method with TIM2ef+I+G substitution model. Phylogenetic reconstruction was carried out using a 374-bp fragment (nucleotide positions 795–1168) that codes for the hvVP2 region. Uruguayan and Argentine IBDVs characterized in this study are indicated in bold. Name, origin and accession number of each sequence are denoted. Mapping uncertainties for interesting internal nodes are shown as approximate likelihood ratio test values. Besides serotype 2 strains, five well-supported clades are distinguishable: very virulent (vv), classic (c), classic attenuated (ca), variant (va) and distinct viruses (dIBDVs). Nine out of the 15 characterized IBDVs were grouped into the dIBDV clade. This lineage comprises viruses from America, Europe and Asia.*

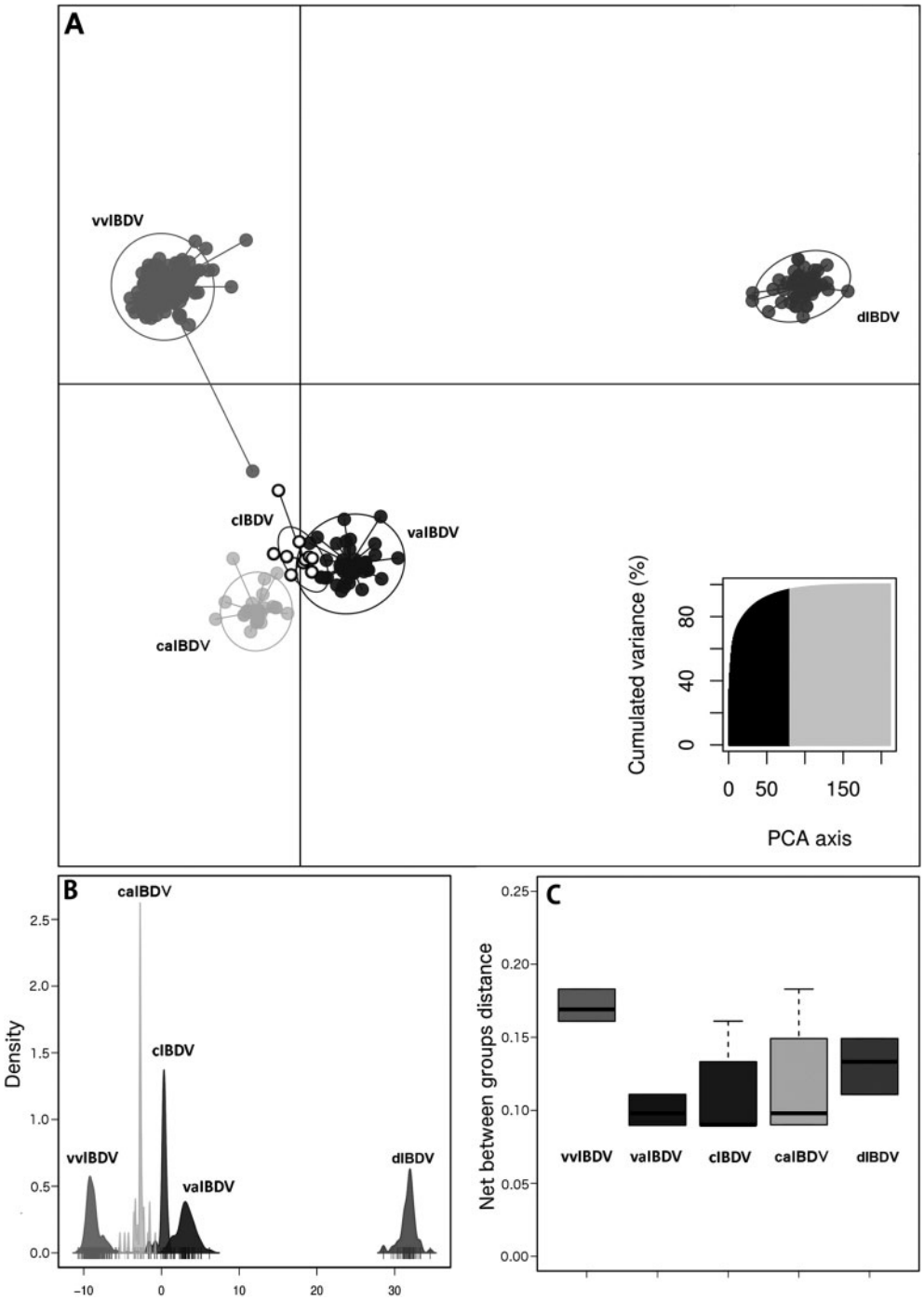

Figure 3. *(A) Discriminant analysis of principal components (DAPC). The data-set included the VP2 nucleotide sequences used in the phylogenetic analysis and additional representatives of the different IBDV strains (N=423). Groups are shown by different grey shades and inertia ellipses, while dots represent individual viruses. Five clusters are distinguished and correspond to the four traditional very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs and to the distinct IBDV group (dIBDV) described in the present study. The dIBDV and vvIBDV strains have the greatest between group variance, indicating that they are the most genetically divergent groups. (B) First simple discrimination function retained during the principal component analysis. This analysis explains 70% of the genetic variance in the data-set and correctly discriminates the five IBDV clusters. (C) Net between-groups mean distances. The vvIBDV (mean = 0.17) and dIBDV strains (mean = 0.14) show the highest divergence.*

sequence of the dIBDVs has a molecular signature (272T, 289P, 290I and 296F) that is unique, conserved and here suggested as a diagnostic character for the lineage (Figure 1). The dIBDVs share relevant amino acids markers (222S, 256V, 294L and 299N/S) with both vvIBDV and non-vvIBDV strains. The 222S residue was already reported in the classic Lukert strain, in few vvIBDVs, and in a Belgian isolate that was considered a European variant with a different antigenic profile produced by the P222S change (Rudd *et al.*, 2002; Jackwood & Sommer-Wagner, 2007; Letzel *et al.*, 2007). The 256V and 294L residues are characteristic of the non-vvIBDVs, while the dIBDVs have a 299N, like most non-vvIBDVs, or 299S, as most vvIBDVs. Notably, the Polish 78/GSz and 80/GA and Hungarian P3 and P10 isolates collected from outbreaks during 1975–1981 grouped within the dIBDVs clade in the VP2 phylogeny. These viruses have a singular antigenic profile and belong to a group of six isolates which were suggested to have

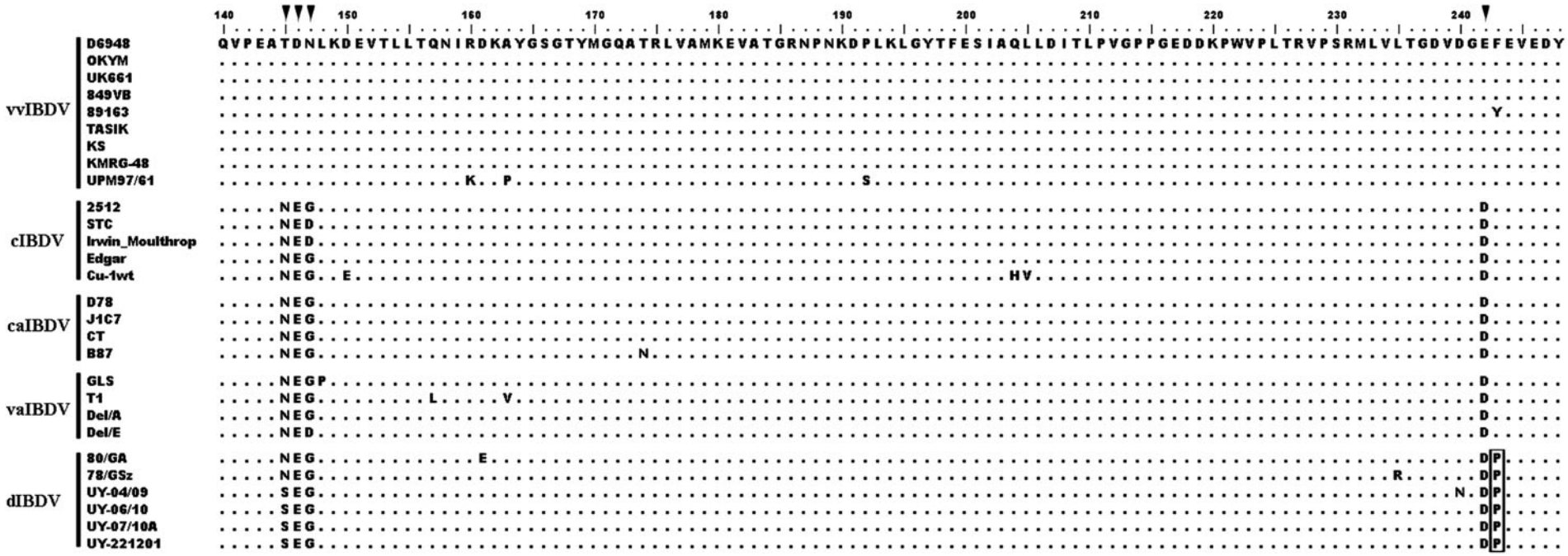

Figure 4. *Amino acid differences along partial VP1 coding region of very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs, and representatives of the distinct IBDV group (dIBDV). Dots indicate position where the sequences are identical to the segment B of the hypervirulent D6948 strain (AF240687). Arrowheads indicate common amino acid markers used to distinguish hypervirulent from non-hypervirulent strains; the dIBDV group shares markers with the non-hypervirulent group. The VP1 sequences of the dIBDV lineage have a highly conserved proline residue (indicated by a box). This residue may be considered a diagnostic character for the dIBDV lineage in the VP1 gene.*

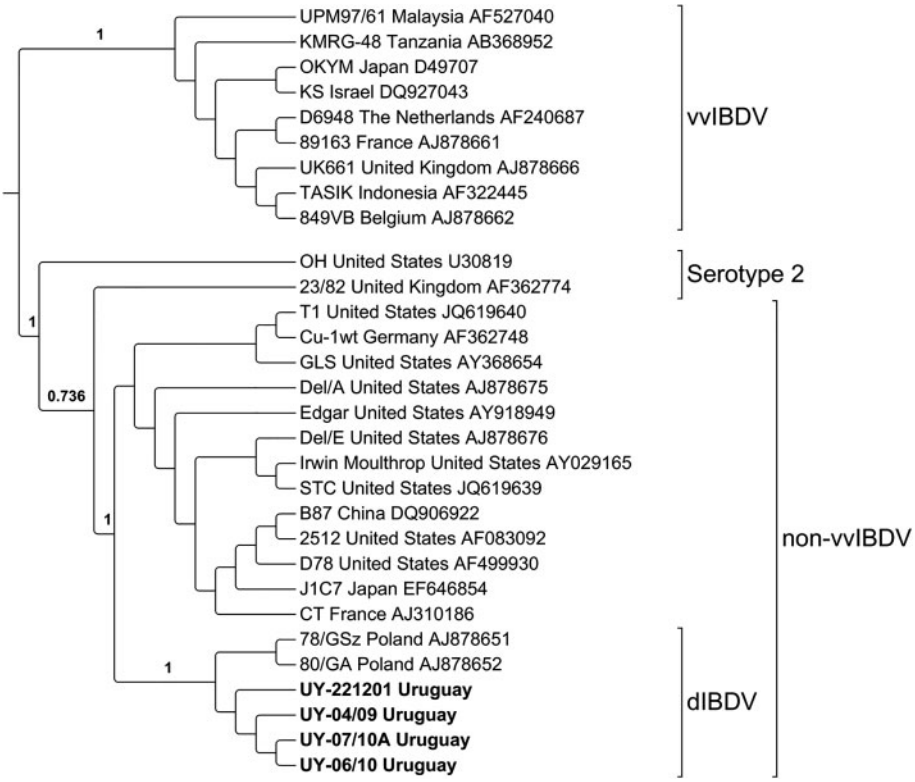

Figure 5. *Cladogram inferred by using the maximum-likelihood method with K80+I+G substitution model. Phylogenetic reconstruction was carried out using a 594-bp fragment (nucleotide positions 322–855 of the vp1 gene). Uruguayan and Argentine IBDVs characterized in this study are indicated in bold. Name, origin and accession number of each sequence are denoted. Mapping uncertainties for interesting internal nodes are shown as approximate likelihood ratio test values. The cladogram displays two major clades conformed by the vvIBDVs and non-vvIBDVs: classic attenuated, variant, classic IBDVs and the dIBDVs here defined.*

coexisted and being later displaced by "F52/70-like" viruses before the vvIBDV emergence in Europe (Domanska *et al.*, 2004). Rather than being extinct, our findings suggest that this lineage has been circulating worldwide for decades and is currently coexisting with traditional strains, reaching high prevalence in countries such as Argentina and Uruguay.

We explore the independence of this distinct lineage using DAPC, a novel-specific multivariate method that identifies and describes clusters of genetically related entities maximizing the separation between groups and minimizing within-group variation (Jombart *et al.*, 2010). This method has been extensively applied in viruses, bacteria, eukaryotic unicellular parasites and metazoans to reveal population structuring using different genetic markers (Jombart *et al.*, 2010; Ramírez *et al.*, 2012; Ogawa & Vallender, 2014; Vernière *et al.*, 2014). The analysis resolves five clusters corresponding to the four traditional IBDV strains plus the group of dIBDVs (Figure 3). The dIBDVs and vvIBDVs have the highest between-group variance (Figure 3A) and genetic distance (Figure 3C), indicating that both are the most genetically divergent groups of IBDVs. These results reinforce those obtained by the sequence and phylogenetic analyses and add evidence about the separation of the dIBDV lineage.

The divergence of the dIBDVs involves both genomic segments since these viruses also have unique markers (243P) and a particular phylogenetic clustering when using segment B (*vp1* gene) sequences (Figures 4 and 5). The dIBDVs have the markers 146E, 147G and 242D characteristic of the non-vv viruses (Hernández *et al.*, 2006). Non-vvIBDVs are usually associated with less pathogenicity than vvIBDVs (van den Berg, 2000), a feature that is consistent with the absence of notorious clinical manifestations of the Uruguayan and Argentine dIBDVs. Coincidentally, subclinical manifestations were reported for several of the viruses clustered within the dIBDV lineage, suggesting that low pathogenicity is a common feature in this group of viruses (Ikuta *et al.*, 2001; Smiley & Jackwood, 2001).

The isolates 78GsZ and 80GA from Poland appear closely related with the Uruguayan dIBDVs in the VP1 cladogram (Figure 5), despite being collected nearly four decades ago (Domanska *et al.*, 2004). This result reinforces the similarity between the Uruguayan dIBDVs and the Polish viruses, which had unique genetic and antigenic changes and low-pathogenicity.

Together, our findings reveal the existence of an IBDV evolutionary lineage highly divergent from the traditional strains that are defined by a unique amino acid signature. This lineage was detected with a high frequency co-circulating with typical strains in Argentina and Uruguay, but it spread in countries from other continents. Its wide geographic distribution, the existence of old European strains and the high divergence revealed by the DAPC analysis suggest that this lineage is ancient and has remained overlooked for a long time. Their sporadic detection and the lack of specific comparative studies (e.g. DAPC and up-to-date phylogenetic methods) would explain why these viruses were considered extinct or atypical isolates restricted to spatially confined regions, instead of being defined as a discrete evolutionary lineage with an ancient origin. It is possible that these dIBDVs were easily ignored in routine surveillances because of the lack of well known clinical signs.

The circulation of this divergent and widespread IBDV strain represents a very important finding that updates and

helps the understanding of the current epidemiology of the prevalent infectious bursal disease.

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References

Azad, A.A., Jagadish, M.N., Brown, M.A. & Hudson, P.J. (1987). Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. *Virology*, 161, 145–152.

Banda, A. & Villegas, P. (2004). Genetic characterization of very virulent infectious bursal disease viruses from Latin America. *Avian Diseases*, 48, 9–18.

Banda, A., Villegas, P. & El-Attrache, J. (2003). Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. *Avian Diseases*, 47, 87–95.

Bayliss, C.D., Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Müller, H. & Boursnell, M.E. (1990). A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *The Journal of General Virology*, 71, 1303–1312.

Boot, H.J., ter Huurne, A.A., Hoekman, A.J., Peeters, B.P. & Gielkens, A.L. (2000). Rescue of very virulent and mosaic infectious bursal disease virus from cloned cDNA: VP2 is not the sole determinant of the very virulent phenotype. *Journal of Virology*, 74, 6701–6711.

Cao, Y.C., Yeung, W.S., Law, M., Bi, Y.Z., Leung, F.C. & Lim, B.L. (1998). Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. *Avian Diseases*, 42, 340–351.

Chettle, N., Stuart, J.C. & Wyeth, P.J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. *The Veterinary Record*, 125, 271–272.

Cosgrove, A.S. (1962). An apparently new disease of chickens: avian nephrosis. *Avian Diseases*, 6, 385.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology*, 76, 2393–2402.

Domanska, K., Mato, T., Rivallan, G., Smietanka, K., Minta, Z., de Boisseson, C., Toquin, D., Lomniczi, B., Palya, V. & Eterradossi, N. (2004). Antigenic and genetic diversity of early European isolates of infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of infectious bursal disease virus revisited? *Archives of Virology*, 149, 465–480.

Edgar, S.A. & Cho, Y. (1976). The epizootiology of infectious bursal disease and prevention of it by immunization. *Developments in Biological Standardization*, 33, 349–356.

Escaffre, O., Le Nouën, C., Amelot, M., Ambroggio, X., Ogden, K.M., Guionie, O., Toquin, D., Müller, H., Islam, M.R. & Eterradossi, N. (2013). Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. *Journal of Virology*, 87, 80–93.

Fahey, K.J., Chapman, A.J., Macreadie, I.G., Vaughan, P.R., McKern, N.M., Skicko, J.I., Ward, C.W. & Azad, A.A. (1991). A recombinant subunit vaccine that protects progeny chickens from infectious bursal disease. *Avian Pathology*, 20, 447–460.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704.

Hernández, M., Banda, A., Hernández, D., Panzera, F. & Pérez, R. (2006). Detection of very virulent strains of infectious bursal disease virus (vvIBDV) in commercial broilers from Uruguay. *Avian Diseases*, 50, 624–631.

Hon, C.C., Lam, T.Y., Drummond, A., Rambaut, A., Lee, Y.F., Yip, C.W., Zeng, F., Lam, P.Y., Ng, P.T.W. & Leung, F.C.C. (2006). Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. *Journal of Virology*, 80, 8503–8509.

Ikuta, N., El-Attrache, J., Villegas, P., Garcia, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. & Marques, E.K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Diseases*, 45, 297–306.

Islam, M.A.T., Le, T.H. & Rahman, M.M. (2012). Molecular characterization of two Bangladeshi infectious bursal disease virus isolates using the hypervariable sequence of VP2 as a genetic marker. *Journal of Veterinary Science*, 13, 405–412.

Ismail, N.M., Saif, Y.M., Wigle, W.L., Havenstein, G.B. & Jackson, C. (1990). Infectious bursal disease virus variant from commercial Leghorn pullets. *Avian Diseases*, 34, 141–145.

Jackwood, D.J., Saif, Y.M. & Moorhead, P.D. (1985). Immunogenicity and antigenicity of infectious bursal disease virus serotypes I and II in chickens. *Avian Diseases*, 29, 1184–1194.

Jackwood, D.J. & Sommer, S.E. (1997). Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses. *Avian Diseases*, 41, 627–637.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal disease viruses from four continents. *Virology*, 365, 369–375.

Jombart, T., Devillard, S. & Balloux, F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11, 94.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30, 3059–3066.

Kim, H.R., Kwon, Y.K., Bae, Y.C., Oem, J.K. & Lee, O.S. (2010). Genetic characteristics of virion protein 2 genes of infectious bursal disease viruses isolated from commercial chickens with clinical disease in South Korea. *Poultry Science*, 89, 1642–1646.

Kwon, H.M., Kim, D.K., Hahn, T.W., Han, J.H. & Jackwood, D.J. (2000). Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. *Avian Diseases*, 44, 691–696.

Le Nouën, C., Rivallan, G., Toquin, D., Darlu, P., Morin, Y., Beven, V., de Boisseson, C., Cazaban, C., Comte, S., Gardin, Y. & Eterradossi, N. (2006). Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment-B-reassorted isolate. *The Journal of General Virology*, 87, 209–216.

Le Nouën, C., Rivallan, G., Toquin, D. & Eterradossi, N. (2005). Significance of the genetic relationships deduced from partial nucleotide sequencing of infectious bursal disease virus genome segments A or B. *Archives of Virology*, 150, 313–325.

Lejal, N., Costa, B.D., Huet, J.C., Delmas, B. & Da Costa, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology*, 81, 983–992.

Letzel, T., Coulibaly, F., Rey, F.A., Delmas, B., Jagt, E., van Loon, A.A.M. W. & Mundt, E. (2007). Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *Journal of Virology*, 81, 12827–12835.

Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodríguez, J.F. & Castón, J.R. (2009). Infectious bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *Journal of Molecular Biology*, 386, 891–901.

Martin, A.M., Fallacara, F., Barbieri, I., Tosi, G., Rivallan, G., Eterradossi, N., Ceruti, R. & Cordioli, P. (2007). Genetic and antigenic characterization of infectious bursal disease viruses isolated in Italy during the period 2002–2005. *Avian Diseases*, 51, 863–872.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. & Allan, G.M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. *Avian Pathology*, 9, 395–404.

Müller, H., Scholtissek, C. & Becht, H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. *Journal of Virology*, 31, 584–589.

Ogawa, L.M. & Vallender, E.J. (2014). Genetic substructure in cynomolgus macaques (*Macaca fascicularis*) on the island of Mauritius. *BMC Genomics*, 15, 748. London: BioMed Central.

Ojkic, D., Martin, E., Swinton, J., Binnington, B. & Brash, M. (2007). Genotyping of Canadian field strains of infectious bursal disease virus. *Avian Pathology*, 36, 427–433.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, 25, 1253–1256.

R Development Core Team. (2013). R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Ramírez, J.D., Guhl, F., Messenger, L.A., Lewis, M.D., Montilla, M., Cucunuba, Z., Miles, M.A. & Llewellyn, M.S. (2012). Contemporary cryptic sexuality in *Trypanosoma cruzi*. *Molecular Ecology*, 21, 4216–4226.

Remorini, P., Calderón, M.G., Aguirre, S., Periolo, O., La Torre, J. & Mattion, N. (2006). Characterization of infectious bursal disease viruses from Argentina. *Avian Diseases*, 50, 245–251.

Rosales, A.G., Villegas, P., Lukert, P.D., Fletcher, O.J. & Brown, J. (1989). Immunosuppressive potential and pathogenicity of a recent isolate of infectious bursal disease virus in commercial broiler chickens. *Avian Diseases*, 33, 724–728.

Rudd, M., Heine, H., Sapats, S., Parede, L. & Ignjatovic, J. (2002). Characterisation of an Indonesian very virulent strain of infectious bursal disease virus. *Archives of Virology*, 147, 1303–1322.

Sánchez, A.B. & Rodriguez, J.F. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology*, 262, 190–199.

Sharma, J.M., Dohms, J.E. & Metz, A.L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. *Avian Diseases*, 33, 112–124.

Smiley, J.R. & Jackwood, D.J. (2001). Genetic stability of the VP2 hypervariable region of four infectious bursal disease virus isolates after serial passage in specific-pathogen-free chicken embryos. *Avian Diseases*, 45, 1–8.

Snedeker, C., Wills, F.K. & Moulthrop, I.M. (1967). Some studies on the infectious bursal agent. *Avian Diseases*, 11, 519–528.

Spies, U., Müller, H. & Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Research*, 8, 127–140.

Stöver, B.C. & Müller, K.F. (2010). TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11, 7.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Tomás, G., Hernández, M., Marandino, A., Panzera, Y., Maya, L., Hernández, D., Pereda, A., Banda, A., Villegas, P., Aguirre, S. & Pérez, R. (2012). Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. *Journal of Virological Methods*, 185, 101–107.

Vakharia, V.N., He, J., Ahamed, B. & Snyder, D.B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. *Virus Research*, 31, 265–273.

Van den Berg, T.P. (2000). Acute infectious bursal disease in poultry: a review. *Avian Pathology*, 29, 175–194.

Van den Berg, T.P., Gonze, M. & Meulemans, G. (1991). Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. *Avian Pathology*, 20, 133–143.

Van den Berg, T.P., Gonze, M., Morales, D. & Meulemans, G. (1996). Acute infectious bursal disease in poultry: immunological and molecular basis of antigenicity of a highly virulent strain. *Avian Pathology*, 25, 751–768.

Vernière, C., Bui Thi Ngoc, L., Jarne, P., Ravigné, V., Guérin, F., Gagnevin, L., Le Mai, N., Chau, N.M. & Pruvost, O. (2014). Highly polymorphic markers reveal the establishment of an invasive lineage of the citrus bacterial pathogen *Xanthomonas citri* pv. *citri* in its area of origin. *Environmental Microbiology*, 16, 2226–2237.

Wu, C.C., Rubinelli, P. & Lin, T.L. (2007). Molecular detection and differentiation of infectious bursal disease virus. *Avian Diseases*, 51, 515–526.

Wu, Y., Hong, L., Ye, J., Huang, Z. & Zhou, J. (2009). The VP5 protein of infectious bursal disease virus promotes virion release from infected cells and is not involved in cell death. *Archives of Virology*, 154, 1873–1882.

Xia, R.X., Wang, H.Y., Huang, G.M. & Zhang, M.F. (2008). Sequence and phylogenetic analysis of a Chinese very virulent infectious bursal disease virus. *Archives of Virology*, 153, 1725–1729.

Yamaguchi, T., Kasanga, C.J., Terasaki, K., Maw, M.T., Ohya, K. & Fukushi, H. (2007). Nucleotide sequence analysis of VP2 hypervariable domain of infectious bursal disease virus detected in Japan from 1993 to 2004. *The Journal of Veterinary Medical Science*, 69, 733–738.