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# Evolution of mammalian and avian bornaviruses

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ABSTRACT

Recently, *Avian Bornavirus* (ABV) was identified to be a new member of the *Bornaviridae* family consisting solely of the mammal-infecting *Borna disease virus* (BDV). Here, to gain more insights into the evolution of these bornaviruses, the time-stamped *N* gene sequences of BDV genotype 1 (BDV1) and ABV were subjected to Bayesian coalescent analyses. The nucleotide substitution rates and the divergence times were estimated. Age calculations suggested that the first diversification event of the analyzed BDV1 isolates might have taken place about 300 years ago, and revealed that ABV was an old virus newly recognized. Great differences were observed in the rate of nucleotide substitution and the pattern of codon usage bias between BDV1 and ABV. Moreover, the analyzed bornaviruses might be descended from an AT-rich ancestor.



## 1. Introduction

Borna disease (BD) is an infectious neurologic syndrome of warm-blooded animals that may lead to profound behavioral abnormalities and even fatalities (Lipkin et al., 2011; Lipkin et al., 1990). It is named after the devastating epidemic that killed a large number of horses during the 1890s in Borna, a town in Saxony, Germany. However, its history can even be dated back to the early 18th century (Kolodziejek et al., 2005; Lipkin et al., 2011). To date, it is still endemic yet sporadic in Central Europe predominantly afflicting horses and sheep as nonpurulent meningoencephalitis (Dürrwald et al., 2006). Due to the restricted pattern, BD did not draw international attention until the 1980s when the zoonotic potential and a global distribution were suggested (Dürrwald et al., 2006; Rott et al., 1985). This prompted the identification and characterization of the etiologic agent *Borna disease virus* (BDV) in the 1990s (Briese et al., 1994; Cubitt et al., 1994; Lipkin et al., 1990), ~70 years after the viral nature was proven (Zwick et al., 1926, 1929).

Now, it is well acknowledged that BDV is an enveloped RNA virus with its non-segmented, negative-sense, single-stranded genome of ~8.9 kb packaged in a spherical virion (Lipkin et al., 2011; Richt and Rott, 2001). By virtue of alternative splicing and overlapping translation, six open reading frames (ORFs) are skillfully compacted in the small genetic material (de la Torre, 1994; Schneemann et al., 1995). In addition to the five proteins common in the order *Mononegavirales*: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase (L) (Lipkin et al., 2011), a unique non-structural protein (X) is encoded by a short ORF upstream and overlapping that of P (Wehner et al., 1997). Another unusual feature is that the virus accomplishes replication and transcription in the nuclei of the host cells (Briese et al., 1994). Therefore, BDV is solely endowed with a new family *Bornaviridae* (Pringle, 1996), in special relation to the family *Rhabdoviridae* (Cubitt et al., 1994).

Surprisingly, despite the inherent error-prone RNA polymerase, all but one BDV isolates from various hosts over several decades so far exhibit remarkable sequence homology, composing a large group designated genotype 1 (BDV1) (Kolodziejek et al., 2005). No/98, the single BDV2-type virus isolated from an Austrian pony, differs from all others by over 15% at the nucleotide level (Nowotny et al., 2000). Due to such great conservation leading to suspicion of contamination, as well as several unrepeatable serological tests, the involvement of BDV in human neuropsychiatric disorders remains controversial (Dürrwald et al., 2007; Lipkin et al., 2011). In fact, our genomes, in which BDV-like segments (EBLNs) are endogenized (Belyi et al., 2010; Horie et al., 2010), have witnessed that ancient bornaviruses did infect us human beings once upon a time.

BDV was long thought to be the singular member of the family until 2008 when *Avian Bornavirus* (ABV) was isolated during the investigation on Proventricular Dilatation Disease (PDD), a fatal neurologic condition of pet parrots (Honkavuori et al., 2008; Kistler et al., 2008). Unlike the mammalian relative, the psittacine bornavirus (ABV-P) is clearly widespread and has had seven genotypes (1–7) identified (Payne et al., 2012). Later, distinct ABV strains were detected in captive finches and wild waterfowl, including ABV-C in canaries and Bengalese finches (Rubbenstroth

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et al., 2013; Weissenböck et al., 2009), ABV-EF in Estrildid finches (Rubbenstroth et al., 2014), and ABV-CG in Canada geese and swans (Delnatte et al., 2013; Guo et al., 2012; Payne et al., 2011). Recently, a distantly related *Reptile Bornavirus* (RBV) was reported from a Gaboon viper venom gland (Fujino et al., 2012).

Here, to gain more insights into the evolution of the seemingly emerging bornaviruses, Bayesian coalescent method was applied to the dated complete sequences of the *N* gene, with emphasis on the divergence scenario. Moreover, to better understand the processes governing their evolution, selection on the *N* gene and codon usage biases of the six genes were analyzed.

## 2. Materials and methods

Full-length ORF sequences of the six bornaviral genes were retrieved from GenBank and aligned with CLUSTAL W (Thompson et al., 1997). Dataset compilation, Bayesian estimates, selection analyses, and surveys of codon usage bias were performed as previously described (He et al., 2013). In Bayesian analyses, panels of the *N* gene sequences of BDV1 and ABV (No/98 included) were separately compiled with only time-stamped field isolates.

To estimate the nucleotide substitution rates and the times to the most recent common ancestor (TMRCAs), the Bayesian Markov chain Monte Carlo (MCMC) method (Drummond et al., 2012) was employed. For each dataset, the 20 kinds of combinations of 4 clock models (strict, exponential, lognormal and random local) and 5 demographic models (constant, exponential, expansion, logistic and Bayesian skyline plot) were compared, and the better one (Table 1) was chosen according to convergence and performance. Independent analyses for 10–15 million MCMC iterations (with 10% burn-in) were combined. Isolate information (name/year/country/host/accession) was given in each maximum clade credibility (MCC) tree.

Moreover, alignments of representative partial *N* (382 nt, referred to nucleotide positions 632-1013 of strain V (Accession No. U04068)), entire *X–P* and partial *M* (308 nt, 1950-2257 of strain V) sequences were respectively created for phylogenetic analyses. Each Maximum Likelihood (ML) tree was drawn by MEGA 5.1 (Tamura et al., 2011) with 1000 bootstrap replicates under the best-fit nucleotide substitution model (GTR + G + I for *N* and *M*; GTR + G for *X–P*) determined by MODELTEST in HyPhy (Pond et al., 2005).

## 3. Results and discussion

### 3.1. Great difference in the nucleotide substitution rate of the N gene between BDV1 and ABV

As with the strong dissimilarity in genetic diversity, great difference in nucleotide substitution rate between BDV1 and ABV was observed (Table 1). When ORF sequences of the *N* gene from 56 natural BDV1 isolates spanning 27 years were subjected to Bayesian analysis, the average rate was calculated to be $1.06 \times 10^{-4}$ subs/site/year, with the 95% highest probability density (HPD) values ranging from $4.20 \times 10^{-5}$ to $1.78 \times 10^{-4}$. It was only about one seventeenth of the mean rate at $1.79 \times 10^{-3}$ ($4.87 \times 10^{-4}$ to $3.16 \times 10^{-3}$) subs/site/year estimated for the *N* gene conducted on 32 field isolates consisting of one BDV2 isolate in 1998 and 31 ABV isolates spanning 7 years. Notably, there was no intersection between their HPD values.

Such evolutionary difference may be related to host difference. Each of the two relatives possesses a wide host range, with various kinds of mammals for BDV and diverse bird species for ABV; however, BDV may employ a special species, the bicolored white-toothed shrew (*Crocidura leucodon*), as an indigenous viral reservoir and other animals including horses and sheep as spill-over hosts (Dürrwald et al., 2014). This may impose more constraints on virus evolution and thus result in a remarkably homogenous virus group.

**Table 1**
Details of datasets and estimates of bornaviruses based on the *N* gene.

| Parameter[a] | BDV1 | ABV[b] |
|---|---|---|
| No. of sequences | 56 | 32 |
| Time span | 1985–2012 | 1998, 2006–2013 |
| Substitution model | GTR + G | GTR + G + I |
| Molecular clock | Lognormal | Random local |
| Demographic model | Exponential | Logistic |
| Mean substitution rate | $1.06 \times 10^{-4}$ | $1.79 \times 10^{-3}$ |
| 95% HPD rate | $4.20 \times 10^{-5}$–$1.78 \times 10^{-4}$ | $4.87 \times 10^{-4}$–$3.16 \times 10^{-3}$ |
| Mean TMRCA | 302 | 772 |
| 95% HPD year | 132–525 | 262–1593 |
| $d_N/d_S$ | 0.034 | 0.046 |

[a] HPD: highest probability density; TMRCA: time to the most recent common ancestor; $d_N/d_S$ ratio: mean ratio of nonsynonymous to synonymous substitution per site.
[b] No/98 (BDV2 isolate in 1998) included.

### 3.2. Purifying selection on the bornaviral N gene

However, their nucleotide substitution patterns were much similar. As revealed by selection analyses using the ML-based single likelihood ancestor counting (SLAC) method implemented in HyPhy (Pond et al., 2005), the synonymous substitution was predominant over the nonsynonymous one in the evolution of the *N* gene ($d_N/d_S < 0.05$, Table 1), reflecting intense purifying selection on bornaviruses. Actually, isolate No/98 was a good example. It shared 93–98% similarity (except X sacrificing for conservation of P) to BDV1 isolates at the amino acid level in contrast to the over 15% variability at the nucleotide level (Nowotny et al., 2000).

### 3.3. Divergence of BDV1 based on the N gene

Due to the uncommon evolutionary mode of BDV1, the divergence times of BDV1 and ABV were separately estimated. Based on the *N* gene, the mean TMRCA calculated for BDV1 was 302 (95% HPD: 132-525) years before 2012 (Table 1), that is, the first diversification event of the analyzed BDV1 isolates might have taken place in the early 18th century, around the time the meningoencephalitis of German horses was observed (Lipkin et al., 2011).

The MCC tree of BDV1 (Fig. 1A) confirmed the finding of Kolodziejek et al. (2005) that there were five different clusters corresponding to the geographical origins: a Saxony-Anhalt and bordering northern Saxony group (termed G1 here), a mixed group mainly from Thuringia and Lower Saxony (G2), a Bavaria I group (G3), a Baden-Wurttemberg and Bavaria II group (G4), as well as a Swiss and Liechtenstein group (SL). The former three were ancestral to G4 and SL, the geographically adjacent sister groups diverging from each other ~200 years ago, which could be inferred from the MCC tree of the *X–P* gene (Fig. S1). In addition, according to the fine regional correlation, the etiologic agent of the epidemic in Borna of Saxony, after which the disease was named, was most likely to be a G1-type virus.

Based on the first reliable description of BD in the Swabian Alb in the 1820s, Dürrwald et al. (2014) speculated that BDV1 might have occurred first in Southern Germany (G3 and G4 region) and spread from there northward to Central Germany (G1 and G2) and southward to Switzerland and Liechtenstein (SL). However, tip calibration demonstrated that BDV1 indeed had emerged to be the cause of horse meningoencephalitis in the early 1700s. In fact, judging from the time-scaled MCC trees (Fig. 1A and S1), either Southern Germany (G3) or Central Germany (G1) could be the origin of BDV1. Here, considering that a G1-type virus population has been established in the natural viral reservoir *C. leucodon*

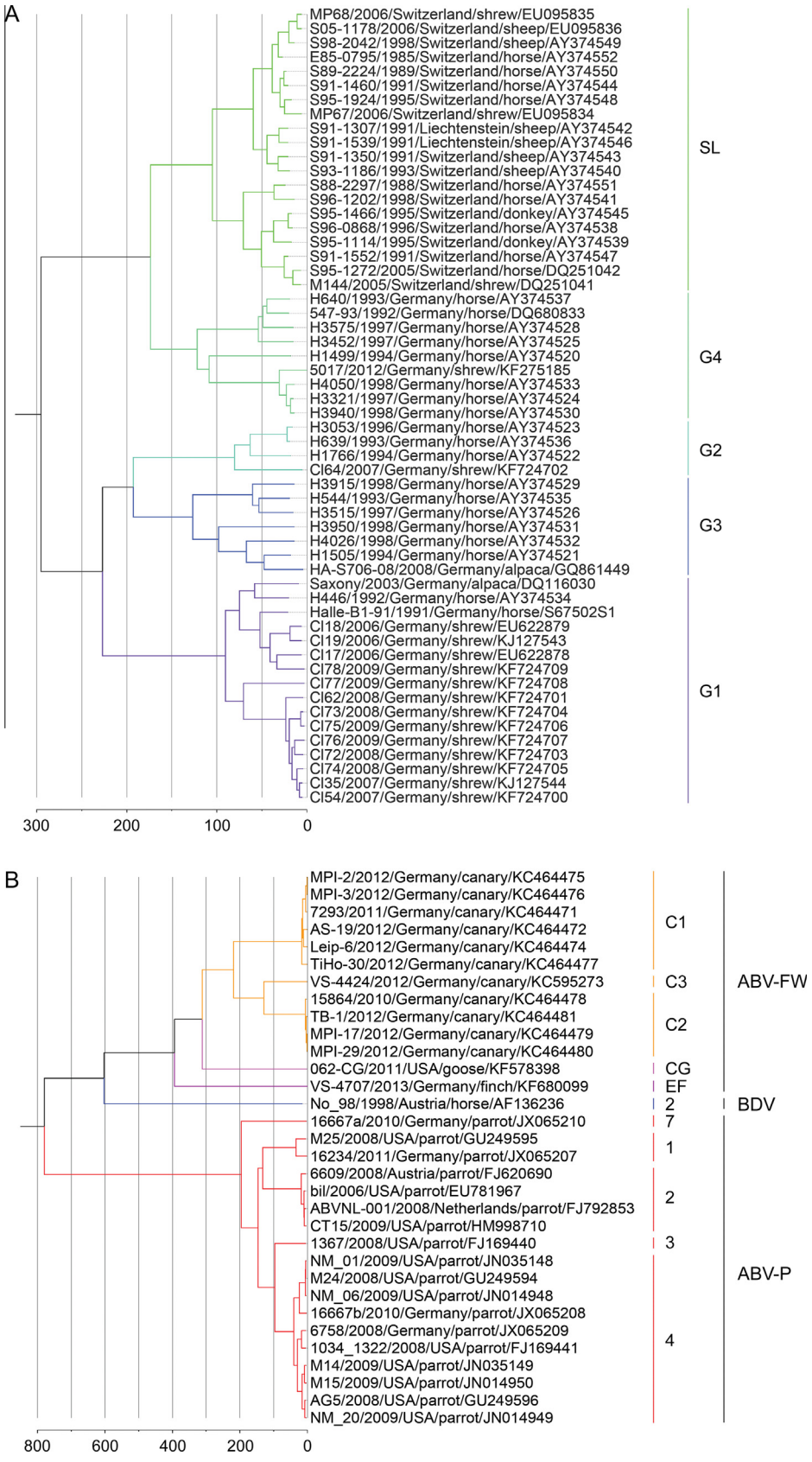


**Fig. 1.** Maximum clade credibility (MCC) phylogenies of bornaviruses. Based on the *N* gene, MCC trees were calculated for *Borna disease virus* genotype 1 (BDV1, A) and *Avian Bornavirus* (ABV, B), respectively. The single BDV2 isolate No/98 was included in the ABV group. Each tree was scaled to time generated under the better molecular clock and demographic models (Table 1). Nodes corresponded to mean TMRCAs. Isolate information (name/year/country/host/accession) and strain/genotype classification were shown on the right. A) G1: Saxony-Anhalt and bordering northern Saxony group; G2: Central German group mainly from Thuringia and Lower Saxony, G3: Bavaria I group; G4: Baden-Wurttemberg and Bavaria II group; SL: Swiss and Liechtenstein group. B) ABV-FW: finch and waterfowl bornavirus; ABV-P: psittacine bornavirus.

shrews (Fig. 1A and S1), we proposed that BDV1 originated in Saxony-Anhalt and Saxony, the north part of Central Germany.

Currently, as No/98 was the only exception to the highly conservative BDV group, it was difficult to date the divergence between BDV1 and BDV2, namely the primary bifurcation of BDV. However, the emergence time of BDV could be estimated when No/98 was included in the ABV dataset, since it was clear in the phylogenies (Fig. 1 and 2) that BDV shared a common ancestor with the three ABV strains collected from finches and waterfowl (designated ABV-FW here) and its divergence was a secondary event. According to the age calculation, the mammal-infecting lineage might diverge out ~600 years ago (Fig. 1B), i.e., in the early 15th century.

### 3.4. Divergence of ABV based on the N gene

When it came to ABV, the average TMRCA calculated for the analyzed isolates was 772 (262, 1593) years before 2013 (Table 1). It should be pointed out that the accuracy of the current age estimate on ABV was influenced by many factors including the limited amount of complete sequence data, the short time span (only 7 years) due to recent discovery of ABV, and the strong purifying selection ($d_N/d_S < 0.05$, Table 1) that can lead to underestimation of the lengths of long branches (Wertheim et al., 2013). However, although some variation or branch length expansion could be expected, there was no doubt that ABV was an old virus newly recognized.

In fact, all the four ABV strains have existed for a long time. As was evident in the MCC phylogeny of ABV (Fig. 1B), the primary bifurcation event took place between the ancestor of the five analyzed ABV-P genotypes (1–4 and 7) and that shared by BDV and the other three ABV strains. Although the divergence of the five ABV-P genotypes initiated much later, their most recent common ancestor (MRCA) has been ~200 years old, which was also supported by the age estimates conducted on the *X–P* and *M–G* gene datasets (data not shown). Notably, even the two youngest sister genotypes, P3 and P4, have emerged nearly one century ago.

On the other branch (Fig. 1B), the divergence of ABV-FW, the finch and waterfowl bornavirus group, commenced ~200 years after the separation from BDV. This was marked by the emergence of ABV-EF. In succession, ABV-CG diverged out ~300 years ago from ABV-C. Among the three analyzed genotypes of ABV-C, C1 was the first one to branch off, nearly one century earlier than the two sisters, C2 and C3. Coincidentally, the MRCA of the three ABV-C genotypes was contemporary with that of the five ABV-P genotypes.

### 3.5. Incongruent phylogenies of ABV-P genotypes 5 and 6

As no dated entire gene sequence was available for ABV-P genotypes 5 and 6 thus far, ML phylogenies were constructed to locate their taxonomic positions. Surprisingly, P5 was clustered with the other five ABV-P genotypes (1–4 and 7) based on the *N* fragment (Fig. 2A), but fell into the branch of BDV and ABV-FW according to both complete *X–P* (Fig. 2B) and partial *M* sequences (Fig. 2C), while P6 was closely related to P5 in the *M* tree (Fig. 2C). Such inconsistent topologies reflected the intergradation role of the two older parrot genotypes, which might be the vestiges of an old recombination event that is not statistically supported nowadays by the detection approaches as applied previously (He et al., 2012). Indeed, natural co-infections with different ABV strains (P4 and P7; P2 and P4; P2 and P6) have been observed (Nedorost et al., 2012; Rubbenstroth et al., 2012), which meets the premise of recombination.

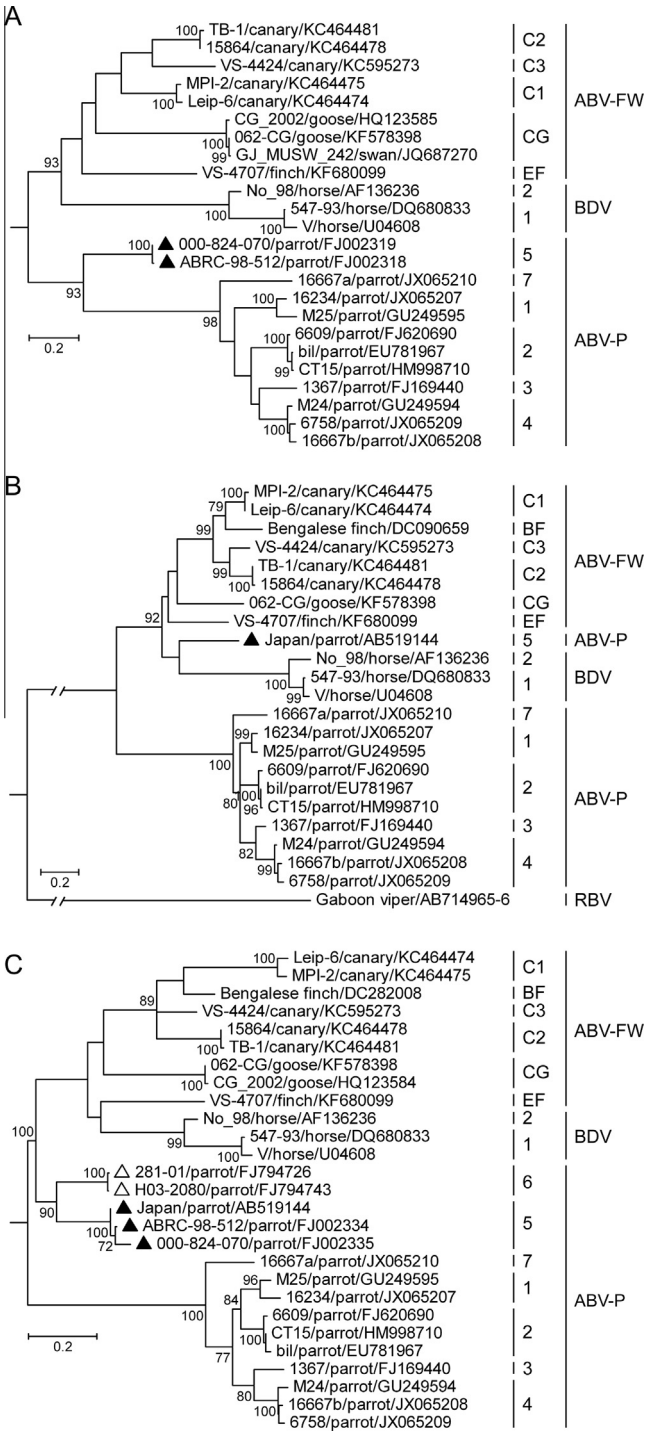


**Fig. 2.** Incongruent phylogenies of ABV-P genotypes 5 and 6. Based on the partial *N* (A), entire *X–P* (B), and partial *M* (C) sequences, Maximum Likelihood (ML) phylograms were constructed under the best-fit nucleotide substitution models, respectively. Branches supported by >70% bootstrap value (1000 replicates) were shown. Information about each isolate used was given as name/host/accession, if available. ABV-P genotypes 5 and 6 with incongruent topologies were marked with "▲" and "△", respectively. ABV-FW: finch and waterfowl bornavirus; BDV: Borna disease virus; ABV-P: psittacine bornavirus; RBV: reptile bornavirus.

### 3.6. Codon usage biases of the bornaviral genes

Furthermore, to better understand the processes that govern the evolution of these bornaviruses, CodonW 1.4.4 (http://codonw.sourceforge.net) was run to measure codon usage bias in each bornaviral gene represented by the effective number of

codons used by a gene (*Nc*) and the frequency of G + C at the synonymous 3rd codon position ($GC_{3S}$) (He et al., 2014).

Another contrast between BDV and ABV was then observed in $GC_{3S}$, an index of the extent of mutational bias. As listed in Table 2, each BDV gene, especially *P*, had a significantly higher $GC_{3S}$ than the ABV counterpart (Student's t-Test, $P < 0.001$). This was plain in the plots of *Nc* versus $GC_{3S}$ (Fig. 3). Possibly due to the priority of the overlapping P codons, difference in the *X* gene was minute. Then, *X* was excluded in calculating the overall relative synonymous codon usage (RSCU) values (Table S1), from which a preference of U over C in most of the synonymous 3rd positions was observed for both ABV-P and ABV-FW, whereas BDV1 exhibited an opposite pattern in many codons.

Despite the distinct GC favor, their *Nc* values were all higher than 40 (Table 2), which demonstrated slight codon usage biases in that *Nc* can take values from 20 for top bias when each amino acid only adopts one codon, to 61 for no bias when the synonymous codons are randomly used (Wright, 1990). However, as was apparent in the *Nc*-plots (Fig. 3), the points of the *P* and *M* genes were much more dispersed than those of the *N*, *G* and *L* genes, suggesting different selective pressure on these genes.

Moreover, the distributions of most points away from the curve of the expected values under the assumption of no selection other than GC composition indicated that codon usage biases of the five genes were influenced by factors aside from mutational bias (He et al., 2014). For example, the *Nc* values of the five BDV genes were in significantly positive correlation with protein hydrophobicity ($R = 0.78$, $P < 0.01$). However, GC composition was still the major one as suggested by the correspondence analysis in which the first axis was significantly positively correlated with $GC_{3S}$ ($R = 0.55$, $P < 0.01$).

Notably, the codon choice of the *N* gene was more dictated by GC content as its points closely surrounded the expected curve (Fig. 3A). An intriguing variation in $GC_{3S}$ of the *N* gene was observed for the five ABV-P genotypes: the values were gradually increasing along with the divergence and distance from the ancestral node (from 0.353 of P7 to 0.456 of P4, Fig. S2). Taking into consideration the 3rd letter GC contents available for horse (*Equus caballus*, 0.624), canary (*Serinus canaria*, 0.681) and mallard (*Anas platyrhynchos*, 0.613) (http://www.kazusa.or.jp/codon/), such process might have been undergone by BDV (Fig. S2). Thus, the current difference in $GC_{3S}$ between BDV and ABV was likely to result from host adaptation.

In addition, such codon usage information might be of practical value in viral gene regulation and vaccine design. For instance, optimizing the codon choice of ABV to the favor of the avian hosts might be able to increase gene expression and thus obtain sufficient viral proteins for generating immunity (Haas et al., 1996).

### 3.7. Speculations on the evolution of modern bornaviruses

Based on the changes in GC favor, it could be speculated that the analyzed bornaviruses were descended from an AT-rich ancestor.

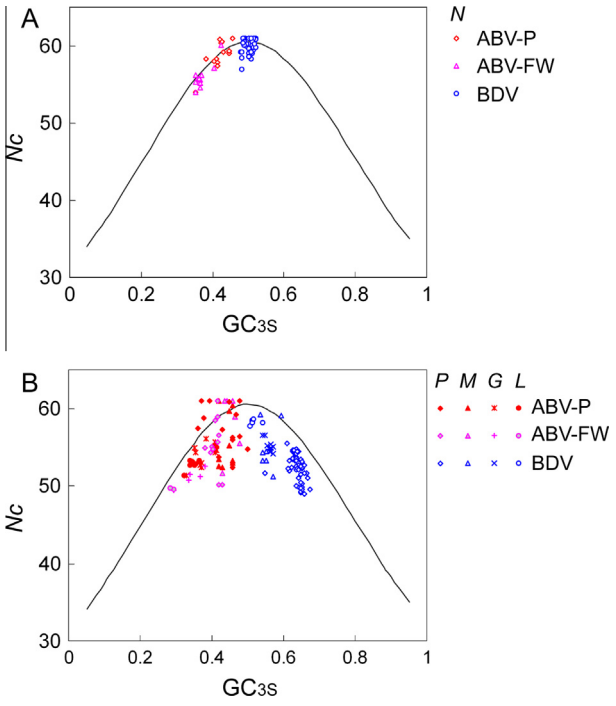


**Fig. 3.** *Nc*-plots of the five structural genes of bornaviruses. *Nc* (the effective number of codons) vs. $GC_{3S}$ (the GC content at the synonymous third codon position) was plotted for the *N* (A), *P*, *M*, *G*, and *L* (B) genes of each isolate. Points were shown in different forms and colors according to phylogenetic groups as indicated on the right. The continuous curve represented the expected *Nc* values under the assumption of no selection other than GC composition. ABV-FW: finch and waterfowl bornavirus; BDV: Borna disease virus; ABV-P: psittacine bornavirus.

Notably, its similarity to the closely related arthropod-borne rhabdoviruses with nuclear localization, such as *Lettuce necrotic yellows virus* ($GC_{3rd}$ 0.454) and *Sonchus yellow net virus* ($GC_{3rd}$ 0.437) (http://www.kazusa.or.jp/codon/), rendered a possibility that this ancestor might be an insect virus before infecting birds and mammals. Coincidentally, the bicolored white-toothed shrew *C. leucodon* that has been proven to harbor BDV is insectivore (Bourg et al., 2013; Dürrwald et al., 2014; Hilbe et al., 2006).

Notably, as BDV is clustered within the ABV group, the likelihood that certain avian species serve as the vectors for modern BDV cannot be excluded, although there is no definite evidence for this link thus far. BDV1 sequences have been detected from wet droppings, which may contaminate the environment, of a mallard (*A. platyrhyncos*) and a jackdaw (*Corvus monedula*) in Sweden (Berg et al., 2001). However, this single event has not been further verified yet. Thus, it is uncertain whether there is a waterfowl bornavirus lineage in Europe. Moreover, it is interesting that shrew *C. leucodon* trapped in Güterglück, Saxony-Anhalt, might be attracted by poor hygienic conditions under which chickens and ducks were kept (Dürrwald et al., 2014). Whether the two domestic fowl species are involved in the transmission cycle deserves further investigation.

**Table 2**
Codon usage bias of each bornaviral gene.

| Parameter[a] | Viruses | *N* | *X* | *P* | *M* | *G* | *L* |
|---|---|---|---|---|---|---|---|
| *Nc* | All | 53.96–61.00 | 40.97–61.00 | 48.94–61.00 | 51.21–61.00 | 49.36–56.56 | 49.64–58.64 |
| $GC_{3S}$ | ABV-P | 0.353–0.456 | 0.435–0.518 | 0.360–0.479 | 0.420–0.457 | 0.330–0.413 | 0.322–0.365 |
| | ABV-C | 0.354–0.406 | 0.453–0.477 | 0.382–0.429 | 0.435–0.478 | 0.408–0.422 | 0.294 |
| | ABV-CG | 0.369 | 0.502–0.510 | 0.397 | 0.457 | 0.402 | 0.283 |
| | ABV-EF | 0.423 | 0.465 | 0.416 | 0.428 | 0.382 | n.a. |
| | BDV2 | 0.483 | 0.512 | 0.547 | 0.594 | 0.511 | 0.542 |
| | BDV1 | 0.479–0.521 | 0.494–0.523 | 0.611–0.668 | 0.536–0.572 | 0.500–0.510 | 0.508–0.516 |

[a] *Nc*: the effective number of codons used by a gene; $GC_{3S}$: the GC content at the synonymous third codon position.

Furthermore, since both the daughter ABV-P (1–4 and 7) and the sister RBV (Fig. 2B) lacked the characteristic short segment (Fig. S3) in the intergenic region between the *N* and *X* ORFs of BDV, ABV-FW and ABV-P5 (Fujino et al., 2012; Payne et al., 2011; Rubbenstroth et al., 2013), this ancestor should not possess the insertion as well. Thus, the one much closer to their ancestor was the progenitor of ABV-P (1–4 and 7) but not that of ABV-FW. Then, taking into account the pure blood of ABV-P (1–4 and 7) so far (Fig. 1B and 2), there was little chance that waterfowl served as the viral reservoir of psittacine birds.

On the background that PDD was first observed in American captive parrots in the 1970s (Mannl et al., 1987) when many captured birds were intensively cultured in facilities where cross species contact was not prevented while being tested for Newcastle disease before imported into the USA, Payne et al. (2012) hypothesized that macaws were infected by contact with ABV-infected waterfowl during quarantine. Here, our finding suggested that PDD was most likely to be directly introduced into the USA via carrier macaws, but not waterfowl-transmitted macaws during quarantine.

In addition, the identification of EBLNs in the genomes of various mammals but not chickens, zebra finch and scarlet macaw (Payne et al., 2012) suggested that ancient bornavirus might rarely infect birds. It was thus intriguing that why the modern one had changed the favor. By this token, more avian species would be found infected by bornavirus in the future.

In conclusion, divergence of the recognized mammalian and avian bornaviruses was dated by Bayesian relaxed-clock analyses applied to the time-stamped *N* gene sequences, which revealed that ABV was an old virus. Moreover, great differences in the rate of nucleotide substitution and the pattern of codon usage bias were observed between BDV and ABV, progenies of the same AT-rich ancestor.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.07.006.

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