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Bornaviruses in naturally infected *Psittacus erithacus* in Portugal: insights of molecular epidemiology and ecology

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ABSTRACT

Background: The genus *Orthobornavirus* comprises non-segmented, negative-stranded RNA viruses able to infect humans, mammals, reptiles and various birds. Parrot bornavirus 1 to 8 (PaBV-1 to 8) causes neurological and/or gastrointestinal syndromes and death on psittacines. We aimed to identify and to produce epidemiologic knowledge about the etiologic agent associated with a death of two female *Psittacus erithacus* (grey parrot).

Methods and Results: Both parrots were submitted for a complete standardised necropsy. Tissue samples were analysed by PCR. The findings in necropsy were compatible with bornavirus infection. Analysis revealed PaBV-4 related with genotypes detected in captive and in wild birds. The N and X proteins of PaBV-4 were more related to avian bornaviruses, while phosphoprotein was more related to variegated squirrel bornavirus 1 (VSBV-1). Within the P gene/phosphoprotein a highly conserved region between and within bornavirus species was found.

Conclusions: Portugal is on the routes of the intensive world trade of psittacines. Broad screening studies are required to help understanding the role of wild birds in the emergence and spread of pathogenic bornaviruses. PaBV-4 phosphoprotein is closer to VSBV-1 associated with lethal encephalitis in humans than with some of the avian bornaviruses. The highly conserved P gene/phosphoprotein region is a good target for molecular diagnostics screenings.

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Introduction

Bornaviruses are enveloped, 80 to 100 nm in diameter with a non-segmented genome of single-stranded negative sense RNA of around 8900 nucleotides in length, belonging to the order *Mononegavirales* [1]. Bornaviruses replicate in the nucleus of the nerve cells of various organs and establish persistent, non-cytolytic infections by exploiting the cellular splicing mechanisms to efficiently use its genome, organized into six open reading frames (ORFs) [1] (Figure 1). Alternative transcription start and stop sites and splicing produce mRNAs that are translated to produce the viral-encoded proteins [1] (Figure 1). The first transcription unit contains an ORF for the nucleoprotein (N), the second transcription unit contains two overlapping ORFs for the phosphoprotein (P), and the X protein (X) [1] (Figure 1). The third transcription unit is spliced differently, and also has different transcription initiation and termination signals, enabling polymerase read-through during transcription, which results in expression of the matrix protein (M), the glycoprotein

(G), and the RNA-dependent RNA-polymerase (L) [1] (Figure 1). The P and X ORFs overlap; as well as, the M and G ORFs [1] (Figure 1). The immunogenicity of phosphoprotein [2], as well as, the degree of conservation of the phosphoprotein and its gene, within and between bornavirus species, make them good candidates as universal targets in laboratory diagnosis [3]. Once the family *Bornaviridae* is expanding speedily [4], producing knowledge about highly conserved regions within phosphoprotein will be useful for the development of sensitive laboratory diagnostic tools. So far, genus *Carbovirus*, *Cultervirus* and *Orthobornavirus* have been identified, which comprise 11 species [4]. From those, 10 species are associated with the development of severe neurological and/or gastrointestinal disease and death of its hosts [5–15] (Figure 2). The disease has been reported in humans, several species of pets, production and wild animals [5–15]. Namely, two species infect mammals (*Mammalian 1 to 2 orthobornavirus*), five infect birds (*Passeriform 1 to 2 orthobornavirus*, *Psittaciform 1 to 2 orthobornavirus* and *Waterbird 1 orthobornavirus*) and

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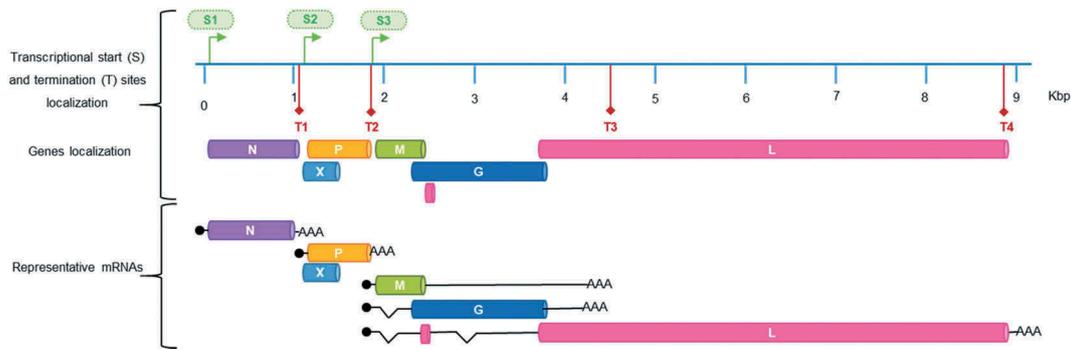


Figure 1. Borna disease virus 1 (BoDV-1) genomic map and protein-coding mRNA transcripts. BoDV uses alternative transcription strategies, like over-lapping open reading frames (ORFs) and usage of host cellular splicing mechanisms. Abbreviations: S1–S3 initiation sites of transcription; T1–T4 termination sites of transcription; (N) nucleoprotein gene; (X) X protein gene; (P) phosphoprotein gene; (M) matrix protein gene; (G) glycoprotein gene; (L) RNA-dependent RNA-polymerase gene [1]. The genomic map is similar for all bornaviruses, as far as known, except for *Queensland carbovirus* and the *Southwest carbovirus*, which have the gene order 3'-N-X-P-G-M-L-5', representing a transposition of the G and M genes [19].

three infect reptiles (*Queensland carbovirus*, *Southwest carbovirus* and *Elapid 1 orthobornavirus*) [4] (Figure 2). However, some *Mammalian 1 orthobornavirus* showed the ability to also infect farmed ostriches and wild birds (mallards and jackdaws) [10,16]. Wild birds are hosts of avian bornaviruses (e.g. strains of *Waterbird 1 orthobornavirus* and *Psittaciform 1 orthobornavirus*) [13–15,17] and mammalian bornaviruses (e.g. genotypes of *Mammalian 1 orthobornavirus*) [16]. Therefore, co-infection may play a role in the emergence of new pathogenic and zoonotic bornavirus species; once

X and P proteins of PaBV-4 look like to have different ancestors [3].

In *Psittaciformes*, parrot bornavirus 1 to 8 (PaBV-1 to 8) can cause proventricular dilatation disease (PDD), characterized by a flaccid and distended proventriculus impacted with feed, as a result of the inability of seeds' digestion (however throughout the gastrointestinal tract can occur variable distention) [18]. *Psittaciformes* can also show uncoordinated movements, postural disorders, apathy, blindness, and behavioural disorders, such as loss of appetite

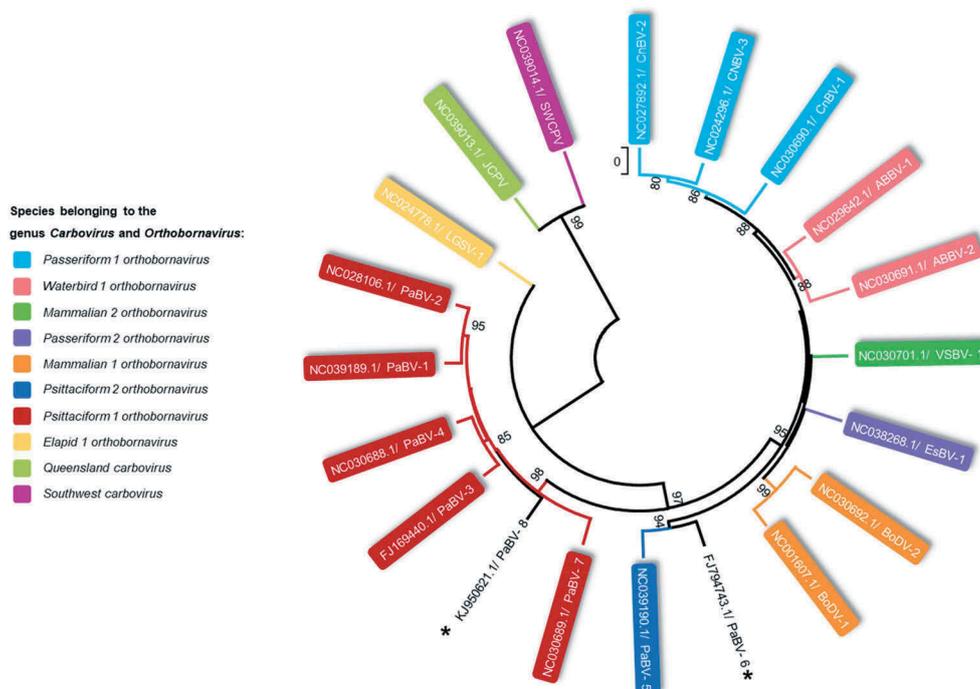


Figure 2. Phylogeny shows the species and strains belonging to the *Bornaviridae* family, which were associated with the development of neurological and/or gastrointestinal disease and/or death of its hosts. The phylogenetic relationship between species and strains was based on a segment of the M gene. Sequences identified by GenBank® accession numbers and abbreviated name of virus. Bornaviruses marked with asterisks are not classified following the currently accepted taxonomy [4]. ABBV-1 to 2 = aquatic bird bornavirus 1 to 2, BoDV-1 to 2 = Borna disease virus 1 to 2, CnBV-1 to 3 = canary bornavirus 1 to 3, EsBV-1 = estrilid finch bornavirus 1, JCPV = jungle carpet python virus, LGSV-1 = Loveridge's garter snake virus 1, SWCPV = southwest carpet python virus, PaBV-1 to 8 = parrot bornavirus 1 to 8, VSBV-1 = variegated squirrel bornavirus 1.

and self-mutilation (resulting from lesions in the central nervous system) [18]. Within captive birds, the virus has become relevant for *Psittaciformes* housed in reserves, in breeding projects of rare species, in private collections and zoos, because of the severe effects it may cause for bird welfare, economy, and biodiversity levels. Analyses of molecular epidemiology suggested that a world trade of psittacines without biosafety measures has been carried out [3]. There is, to the best of our knowledge, no publications identifying and characterizing the avian bornaviruses infecting pet parrots in Portugal. Moreover, there are still unresolved questions on the epidemiology of bornaviruses, such as the role of waterbirds in the emergence and dissemination of new pathogenic and zoonotic species, and the localization of highly conserved regions within P gene inter- and intra-species of bornaviruses.

The aim of this study was to identify and phylogenetically characterize the etiologic agent associated with clinical signs and necropsy findings consistent with avian bornavirus infection in two pet parrots in Portugal, as well as to produce molecular epidemiologic knowledge on bornaviruses.

Material and methods

Case histories and necropsy findings

Two female *Psittacus erithacus* (grey parrot) from different owners died with an interval of 2 months between them. One of the parrots (case number B240818) did not show clinical signs prior to sudden death. The other parrot (case number B261018) had a history of clinical signs compatible with bornavirus infection and had been examined (physical examination and X-ray) at the veterinary hospital prior to its death. The owners gave their informed consent for the necropsy, as well as for the collection of tissue samples and information on the clinical and life history of the parrots (by applying a structured questionnaire designed for the study). Both parrots were submitted for a complete standardised necropsy by a veterinarian specialized in exotic birds. Tissue samples from adrenal gland, brain, cecum, colon, crop, heart, ileum, jejunum, kidneys, liver, lung, ovary and proventriculus were collected and frozen at -80°C . The laboratory diagnosis was directed to search for avian bornavirus RNA based on 1) the clinical history of the captive flock (weight loss, sudden death, undigested seeds in faeces, diarrhoea, poor condition of plumage) from which the parrot B240818 originated; 2) the clinical signs, physical examination and X-ray of the parrot B261018; and 3) the macroscopic findings reported during the necropsy, for both parrots.

Bornavirus nucleic acid detection by polymerase chain reaction (PCR)

We used tissue samples from brain, liver, kidney, spleen, ventricle, lung, heart, duodenum and pancreas, to search for avian bornavirus RNA. Extraction of total RNA from the tissue samples was done as previously described [3]. Briefly, the TRIzol/chloroform method (TRIzol reagent, Life Technologies) was used followed by purification with the RNeasy[®] Kit (Qiagen, Hilden, Germany), as recommended by the manufacturer's instructions. Estimation of the total RNA concentration was achieved using NanoDrop[®] ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA), according to the manufacturer's instructions. Five hundred nanograms of total RNA were reverse transcribed into cDNA as previously described [3]. The cDNA was amplified by two conventional PCR protocols, one using a set of primers specific for a fragment of the N gene (the forward primer: 5'-CATGAGGCTATWGATTGGATTA-3' and the reverse primer 5'-TAGCCNGCCMKTGTWGGRTTYT-3') and the other was carried out with a set of primers specific for a fragment of the M gene (forward primer: 5'-CAAGGTAATYGTYCCTGGATGG-3' and the reverse primer: 5'-ACCAATGTTCCGAAGMCGAWAY-3'). In both protocols, the thermal cycling parameters and the reaction composition of PCR were carried out as previously described [3]. The expected length of the conventional products was 389 base pairs (bp) for the fragment of the N gene and 352 bp for the fragment of the M gene. From the positive samples, for both genes, selected tissues were used to conduct an additional PCR, combining the forward primer targeting the N gene and the reverse primer targeting the M gene, under previously reported experimental conditions [3]. The expected length of the PCR product was 1600 bp (covering a partial region of the N gene, a total region of the X and P genes and a partial region of the M gene). The visualization of the PCR products was in electrophoresis using a 2% agarose gel, staining with Gel Red[™] (Biotium Glowing Products for Science[™], Hayward, CA) at 0.002%. The PCR products from all tissue samples tested were purified using the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) and both strands of DNA were analysed by Sanger sequencing. The nucleotide sequences obtained in this study were submitted to GenBank[®] database under accessions numbers MK192982 and MK192983.

Virus identification, phylogenetic analysis and protein similarity within bornaviruses

The identification of bornaviruses was conducted by finding regions of similarity between the nucleotide sequences obtained (from all tissues tested) and sequences from the GenBank[®] database. Throughout the X and P protein, conserved regions between

bornavirus species were searched, comparing the amino acid sequences produced with the selected sequences (from GenBank® database we selected two amino acid sequences from each species of bornaviruses, when available). The identification of bornaviruses, as well as the search for conserved regions throughout the X and P proteins were conducted using the Basic Local Alignment Search Tool (BLAST®) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), the per cent identity (id) and its statistical significance (E-value) was collected.

The relationship between the genotypes detected in the present study and the parrot bornavirus 4 (PaBV-4) genotypes reported was evaluated by phylogenetic analysis, based on N gene segment. Therefore, some of the nucleotide sequences of PaBV-4 from each of the five previously identified genotype clusters [3] were included, as well as the new sequences deposited in GenBank® database. The output of the analysis was divided into clusters, the strains were classified to belong to the same cluster when the per cent identity between them were $\geq 98\%$. Since not all sequences identified by BLAST® search covered completely the PCR products produced, all sequences were trimmed to the length of the shortest sequence included in the respective phylogenetic analysis.

To evaluate the role of waterbirds in the emergence and dissemination of new pathogenic bornaviruses, the evolutionary relationship between the PaBV-4 and bornaviruses detected in wild birds (aquatic bird bornavirus 1 to 2 and Borna disease virus 1), as well as bornaviruses linked to infection in humans (Borna disease virus 1 and variegated squirrel bornavirus 1), was determined. Therefore, sequences representative of virus genotypes were selected from GenBank® database and included in the analysis. Phylogenetic analyses were conducted in MEGA7 software [20] using nucleotide sequences deposited in GenBank®.

Results

Case histories and necropsy findings

One of the parrots (case number B240818) had suddenly died without previous clinical history compatible with bornavirus infection. The parrot belonged to a private collection in which infection by avian bornaviruses was the cause of death of several parrots 3 years ago. Since the outbreak, the parrot B240818 was the only case of illness, reported by the owner. The parrot was born in captivity in Spain and lived in the northern region of Portugal until its death. The necropsy of the bird showed a severe cachexia, with splenomegaly and hepatomegaly. The other organs were grossly

normal, including a normal proventriculus without dilatation.

The other parrot (case number B261018) had a clinical history compatible with bornavirus infection, displaying regurgitation, seeds not digested in the faeces, cachexia and prostration. The X-ray revealed a severe dilatation of the proventriculus (Figure 3). The bird, at necropsy, showed a reduced muscle mass and the proventriculus showed a severe dilatation with a ruptured wall (Figure 4). The other organs were grossly normal. The parrot had always lived apparently healthy, as a pet in the central region of Portugal during 20 years. The owner reported that the parrot had been donated to him and was unaware of its origin.

The two parrots never had contact with each other during their lifetime.

Bornaviruses nucleic acid detection by polymerase chain reaction (PCR)

All tested tissue samples from the two *Psittacus erithacus* were positive for bornavirus RNA, with amplification of fragments of the N gene (with 389 bp) and of the M gene (with 352 bp). Additionally, from tissue samples of brain and lung of each *Psittacus erithacus*, nucleic acid fragments (with 1600 base pairs) comprising partially the N and M genes and totally the X and P genes were amplified.

Virus identification, phylogenetic analysis and protein similarity within bornaviruses

The bioinformatics analysis revealed that both parrots were infected with parrot bornavirus 4 (PaBV-4), based on the genetic segment that contained the partial N and M genes and the complete X and P genes (1543 base pairs for case number B240818 and 1537 base pairs for case number B261018). The highest per cent identity (id) found between PaBV-4 reported in the GenBank® database and the virus linked to B240818 was 99% (E-value = 0.0) and 98% (E-value = 0.0) to B261018. Additionally, the nucleotide composition of the two virus sequences in the present study, differed by 5% from each other (id = 95%, E-value = 0.0). When the analysis was restricted to the X and P genes, they differed from each other by 2% (id = 98%, E-value = $1e^{-129}$) and 8% (id = 92%, E-value = 0.0), respectively. The genetic differences found between the two PaBV-4 resulted into 5% and 2% of variation of amino acid composition of X protein (id = 95%, E-value = $1e^{-49}$) and P protein (id = 98%, E-value = $3e^{-42}$), respectively (Table 1).

Similar results were observed when comparing parrot bornavirus 4 (PaBV-4) genotypes detected in the present study with worldwide distributed bornaviruses (mammalian, reptile and avian bornaviruses) (Table 1). Specifically,

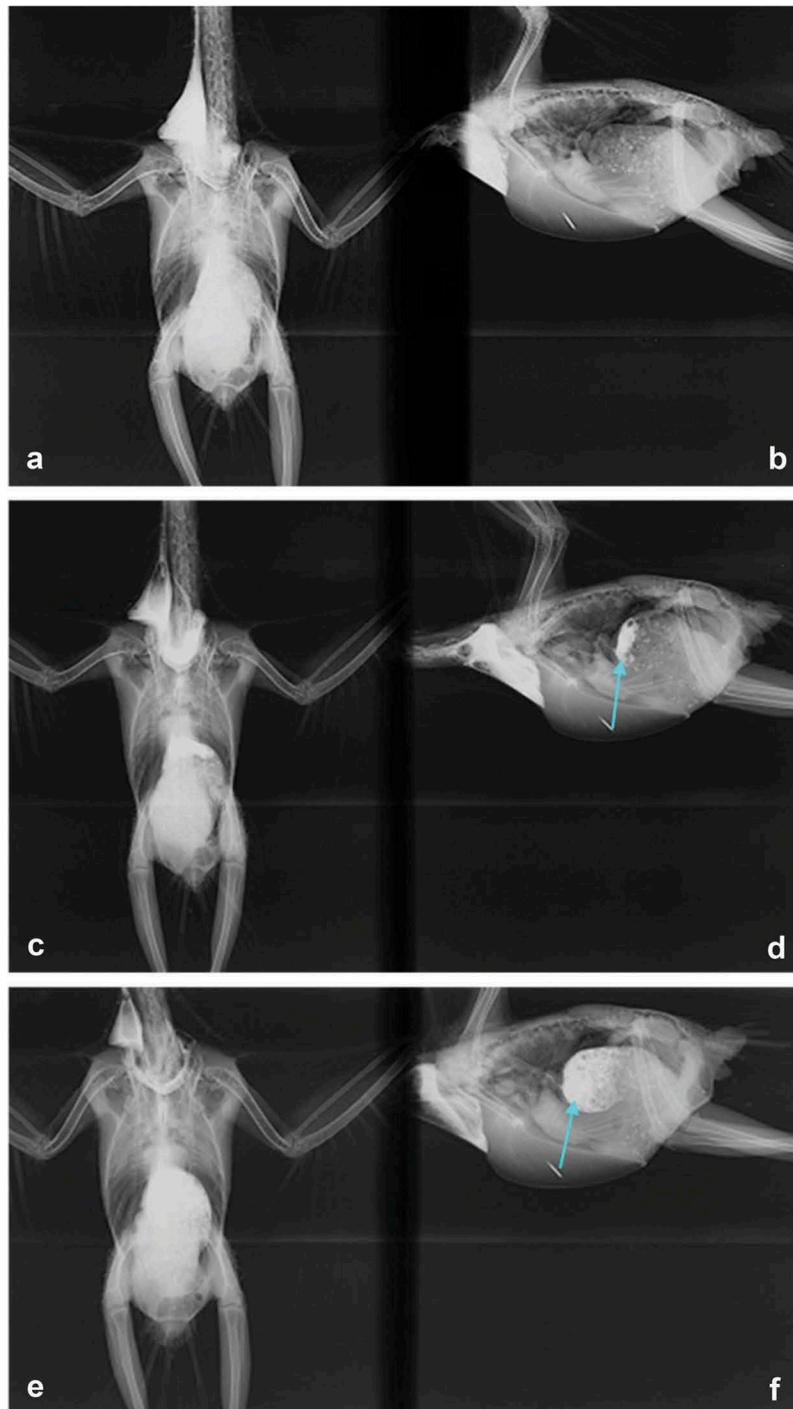


Figure 3. X-ray performed on the parrot case number B261018, using barium contrast. (a) Dorsal-ventral view and (b) side-to-side view after the introduction of barium contrast in the crop (marked with the blue arrow). (c) Dorsal-ventral view and (d) side-to-side view of the diffusion start of barium contrast in the proventriculus. (e) Dorsal-ventral view and (f) lateral-lateral view allowing the visualization of the diffusion of barium contrast throughout the proventriculus, highlighting its delineation, which revealed a severe dilatation (marked with the blue arrow).

the per cent identity found for X protein was lower (id = (36–95)%, E-value = $(4e^{-48} - 3e^{-32})$) than the identity found for P protein (id = (39–98)%; E-value = $(7e^{-41} - 1e^{-40})$), within viruses of the same species (within *Psittaciform 1 orthobornavirus*) and between viruses of different species (*Psittaciform 2 orthobornavirus*, *Passeriform 1 and 2 orthobornavirus*, *Mammalian 1 and 2 orthobornavirus* and *Elapid 1 orthobornavirus*) (Table 1). Within X protein, the higher variation of identity

occurred in the region shared with the P protein (id = (37–99)%, E-value = $(1e^{-40} - 9e^{-05})$) (Table 1). Moreover, in the non-shared region of X protein the identity between the two PaBV-4 genotypes and mammalian bornaviruses (*Mammalian 1 and 2 orthobornavirus* (id = 75%, E-value = $2e^{-12}$)) was the same for some avian bornaviruses (*Passeriform 1 orthobornavirus* (canary bornavirus 1 and 2 (E-value = $2e^{-12}$)) and *Water 1 orthobornavirus* (aquatic bird bornavirus 1 (E-value = $2e^{-12}$))) (Table 1).



Figure 4. The proventriculus of parrot case number B261018, at necropsy. The severe dilatation (asterisk) produced a small rupture in the proventriculus wall (arrow).

Concerning P protein, the amino acid sequence region non-shared with X protein revealed higher variation (id = (30–82)%, E-value = ($4e^{-53}$ – $1e^{-2.8}$)) than the shared region (id = (37–99)%, E-value = ($1e^{-40}$ – $9e^{-05}$)), within and between species of bornaviruses (Table 1). In the region shared between the two proteins, the identity found between the two PaBV-4 genotypes and members of *Mammalian 2 orthobornavirus* species was higher than the per cent identity found with some members of avian bornaviruses (Table 1). Namely, the case number B240818 showed a higher identity with *Mammalian 2 orthobornavirus* members (id = 74%, E-value = $5e^{-20}$) than with some members of *Waterbird 1 orthobornavirus* (Aquatic bird bornavirus 2, (id = 69%, E-value = $9e^{-18}$)) and *Passeriform 1 orthobornavirus* (Canary bornavirus 1, (id = 73%, E-value = $2e^{-20}$)) (Table 1). Also, the case number B261018 showed a higher per cent identity with *Mammalian 2 orthobornavirus* members (id = 69%, E-value = $2e^{-20}$) than with some members of *Waterbird 1 orthobornavirus* (Aquatic bird bornavirus 2, (id = 64%, E-value = $4e^{-18}$)) (Table 1). Additionally, only in the region shared by both proteins, a statistically significant similarity was found between the two PaBV-4 genotypes reported and members of reptilian bornaviruses (id = (37–45)%, E-value = ($5e^{-99}$ – $9e^{-05}$)), (Table 1).

The results obtained for X and P proteins, described above, were complemented by the phylogenetic analysis based on the segment of N gene, since the reported genotypes, in the present study, showed to be phylogenetically adjacent to PaBV-4 genotypes worldwide distributed (Figure 5). Namely, they were mostly related with PaBV-4 genotypes detected in several species of pet psittacines, in countries of Asia (Israel and Japan), Europe (Austria, Germany, Hungary, Sweden and Switzerland) and North America (Canada and USA) and South America (Brazil) (Figure 5). Moreover, the analysis of the N segment revealed that the detected PaBV-4

genotypes, in pet parrots sampled in Portugal, were also phylogenetically adjacent to those found in wild birds in Japan (such as *Anas* spp., *Emberiza* spp. and *Grus* spp.) (Figure 5). Besides, the analysis showed that PaBV-4 genotypes circulating in Portugal were closely related to genotypes which have been circulating in the last 19 years, all over the world (according to the earliest sampling date reported in GenBank® for PaBV-4) (Figure 5).

The second phylogenetic approach, based on the N gene segment, confirmed the results obtained for the relationship between the PaBV-4 genotypes and bornaviruses (of avian and mammalian) in bioinformatics analysis of X protein (Table 1); moreover, it added information about their evolutionary relationship (Figure 6 A). Namely, phylogenetic analysis revealed that PaBV-4 genotypes, detected in the present study, showed to be more closely related to avian bornaviruses than to mammalian bornaviruses (Figure 6). Within *Waterbird 1 orthobornavirus* species, the aquatic bird bornavirus 1 (ABBV-1) genotypes are phylogenetically closer to PaBV-4 than to aquatic bird bornavirus 2 (ABBV-2) genotypes (Figure 6(a)). Regarding mammalian bornaviruses, the *Mammalian 2 orthobornavirus* members were more related to PaBV-4 than to *Mammalian 1 orthobornavirus* members (Figure 6(a)). Specifically, the variegated squirrel bornavirus 1 (VSBV-1) genotypes detected in squirrels (from zoos and breeding collections) and in humans (with a fatal limbic encephalitis) are phylogenetically more close related to PaBV-4 than to Borna disease virus 1 (BoDV-1) detected in humans (associated with a lethal encephalitis) and in wild birds (Figure 6(a)). When the phylogenetic analysis was conducted based on the amino acids encoded by the segment of N gene, the main evolutionary relationship persisted between parrot bornavirus (PaBV-4), aquatic bornavirus (ABBV-1, ABBV-2), variegated squirrel bornavirus (VSBV-1) and Borna disease virus (BoDV-1) (Figure 6(b)).

The third phylogenetic approach, based on the P gene segment, revealed that members of *Mammalian 2 orthobornavirus* species are the most related to PaBV-4 genotypes than of all bornaviruses detected and reported in wild birds, other than parrots (Figure 7(a)). That is, the PaBV-4 genotypes are evolutionarily closer to the VSBV-1 genotypes, linked to the infection of squirrels (from zoos and breeding collections) and humans than members of *Waterbird 1 orthobornavirus* (ABBV-1 and ABBV-2) and of *Mammalian 1 orthobornavirus* (BoDV-1), detected in samples of wild birds (Figure 7). Namely, within genotypes detected in wild-bird samples, Borna disease virus (BoDV-1) is the most evolutionarily distant from parrot bornavirus (PaBV-4) (Figure 7(a)). However, within Borna disease virus (BoDV-1) the genotype associated with lethal encephalitis in humans (accession n° MH190827.1) is evolutionarily more distant from PaBV-4 than the variants detected in samples

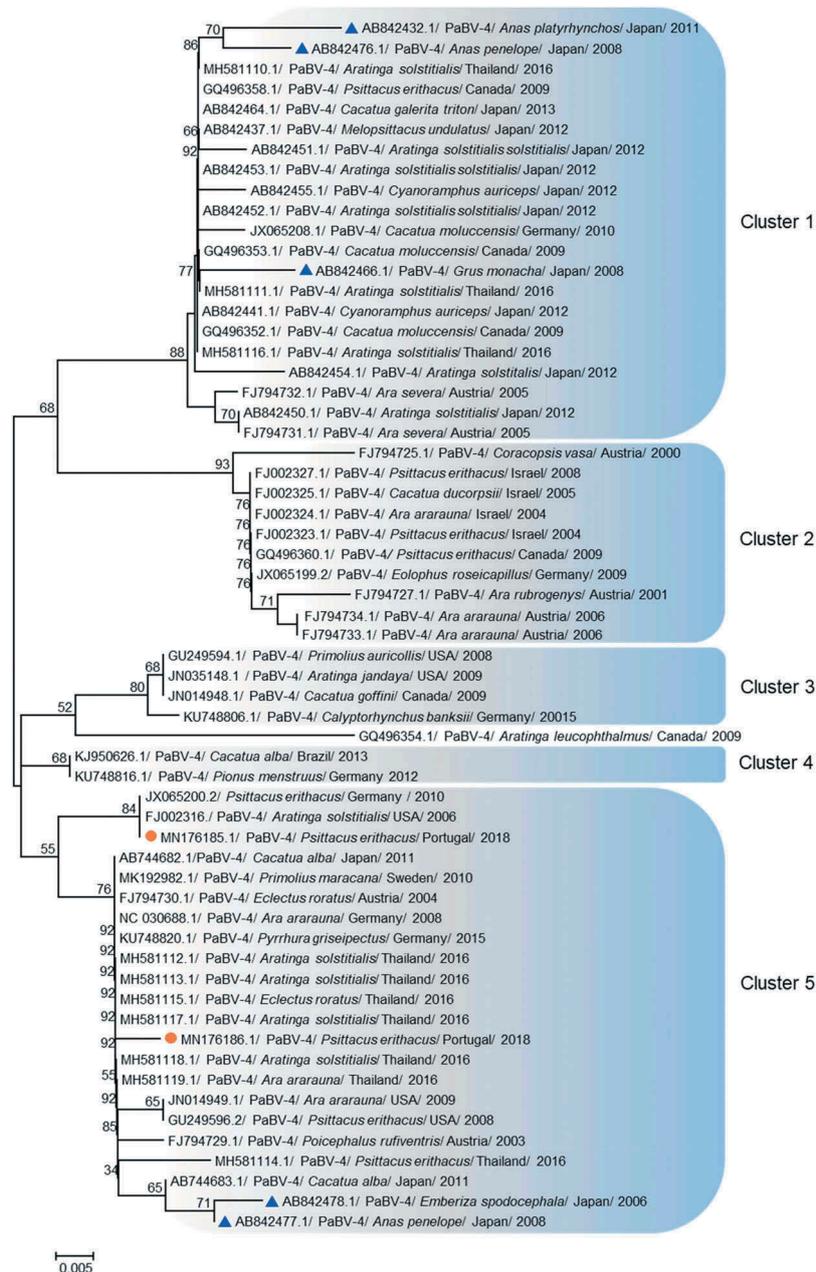


Figure 5. Phylogenetic relationships between PaBV-4 genotypes identified in the present study and PaBV-4 detected in wild birds as in pet parrots, regarding N gene. The evolutionary history was inferred using the Neighbor-Joining method [21]. The confidence probability was estimated using the bootstrap test (1000 replicates) and is shown above the branches [22,23]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method [24] and are in the units of the number of base substitutions per site. The analysis involved 60 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 163 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [20]. Sequences identified by GenBank® accession numbers, abbreviation name of virus and its hosts, year and geographic origin of sampling. PaBV-4 = parrot bornavirus 4. The sequences marked with a circle were produced during this study and triangles marked nucleotide sequences identified from wild birds.

of wild *Anas spp.* (Figure 7(a)). When the phylogenetic analysis was conducted based on the amino acids encoded by the segment of the P gene, the main evolutionary relationship remained between bornaviruses (Figure 7(b)).

The findings obtained through the several analyses conducted suggest that within parrot bornavirus (PaBV-4) genotypes the N and P gene had different ancestors. Since the N gene of PaBV-4 is

evolutionarily more close to the N gene of avian bornaviruses (Figure 6(a,b)), while P gene is more related to mammalian bornaviruses, specifically with the zoonotic variegated squirrel bornavirus (VSBV-1) genotypes (Figure 7(a,b)). Additionally, the findings showed that the Borna disease virus (BoDV-1) detected in samples of wild birds (accession n° AF233071.1, AF232702.1 and AF232703.1) are intermediate variants between the two BoDV-1 genotypes

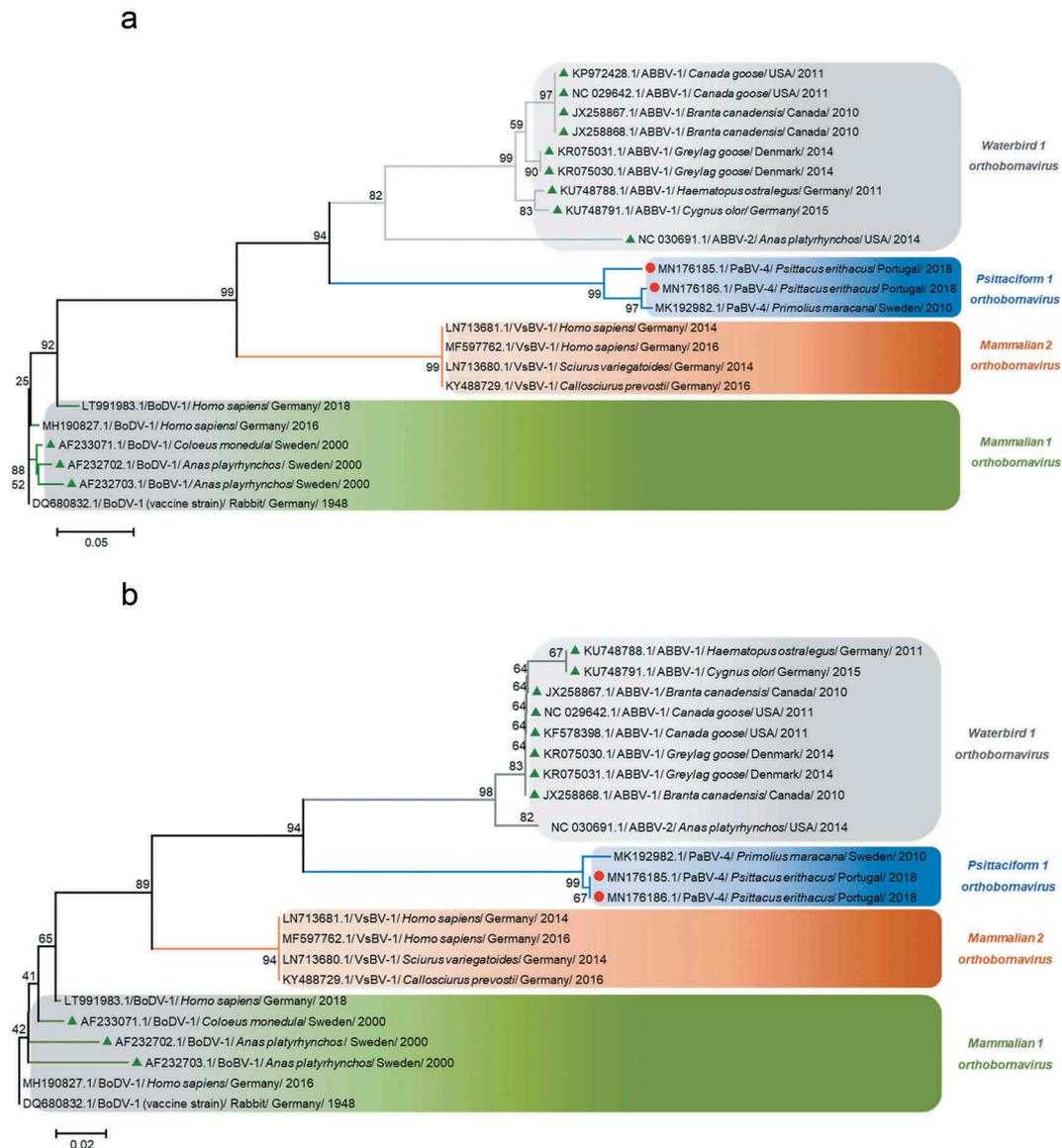


Figure 6. Phylogenetic relationships between PaBV-4 genotypes identified in the present study and bornaviruses detected in mammalian and in wild birds, based on N gene (a) and on N protein (b). The evolutionary history was inferred using the Neighbor-Joining method [21]. The confidence probability (multiplied by 100) was estimated using the bootstrap test (1000 replicates) and is shown next to the branches [22,23]. Both trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Both trees were rooted with a vaccine strain (accession n° DQ680832.1). Evolutionary analyses were conducted in MEGA7 [20]. (a) The evolutionary distances were computed using the Kimura two-parameter method [24] and are in the units of the number of base substitutions per site. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 215 positions in the final dataset. (b) The evolutionary distances were computed using the Poisson correction method [25] and are in the units of the number of amino acid substitutions per site. The analysis involved 23 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset. Sequences identified by GenBank® accession numbers, abbreviation name of virus and its hosts, year and geographic origin of sampling. ABBV-1 = aquatic bird bornavirus 1, ABBV-2 = aquatic bird bornavirus 2, BoDV-1 = Borna disease virus 1, PaBV-4 = parrot bornavirus 4, VSBV-1 = variegated squirrel bornavirus 1. The sequences marked with a circle were produced during this study and triangles marked nucleotide sequences identified from samples of wild birds.

(accession n° LT991983.1 and MH190827.1) linked with lethal encephalitis of humans, on the evolutionary line (Figures 6(b) and 7(a,b)).

Discussion

Although parrot bornavirus 1 to 8 (PaBV-1 to 8) are recognized as the etiologic agent of Proventricular

Dilatation Disease (PDD) in psittacines, there are still unanswered questions regarding its epidemiology. This is the first study that characterizes, at genetic and protein level, genotypes of *Psittaciform 1 orthobornavirus*, circulating in Portugal. The sequencing of more than 1000 base pairs of the viral genome allowed the identification of parrot bornavirus 4 (PaBV-4) with statistically significant probability (percent identity (id) = (98.31–98.51)%,

parrot B261018, the source of infection is unknown; however, it is probable that virus entered through oral route and spread from proventriculus to the central nervous system, as postulated in the literature [18], based on the clinical picture characterized by the development of gastrointestinal signs followed by neurological signs at the final stage of disease.

In the present study, we produced additional and detailed information to the previous reported insights about the degree of conservation of X and P protein within and between species of bornaviruses [3]. As previously reported, the P protein (phosphoprotein) showed to be more conserved between and within species of bornaviruses than the X protein [3]. However, the present study showed that, within and between bornaviruses, the degree of conservation throughout the P protein was not constant (Table 1). Namely, intra- and inter-species of bornaviruses, the identity percent of the first 68 amino acid was higher than the following 133 amino acid of P protein (Table 1). The region showing a higher identity percentage is a shared amino acid sequence with X protein. In contrast, throughout the X protein, the lower identity percent (intra- and inter-species of bornaviruses) comes from the region shared with P protein (Table 1). Therefore, the first 18 amino acid of the sequence revealed to be more conserved within X protein (Table 1). Nevertheless, when comparing both conserved regions of the two proteins, the X protein showed a smaller variability range of the amino acid sequence (Table 1). However, its most conserved region represents about 20.9% of the X protein, which corresponds approximately to 54 nucleotides of the gene, making it less advantageous as a target region (in the design of tools) for molecular diagnostics, compared to the more conserved region of the P protein. In the case of P protein, the most conserved region corresponds approximately to 68 nucleotides, among species of family *Bornaviridae*, allowing more options in the design of molecular tools, aiming to decrease the occurrence of false negatives. In some cases, despite all findings of clinical diagnosis, the inability to amplify the viral nucleic acid might be a consequence of the presence of unknown species of bornaviruses in samples [7]. Thus, a decrease in the number of false negatives is expected, since the highly conserved regions in evolutionarily more distant viruses will be more identical to the bornaviruses described so far. Therefore, the highly conserved region is a good candidate as a target in laboratory diagnostics for screening purposes of potential reservoirs or asymptomatic carriers, as well as to confirm the clinical diagnosis. The information produced about the highly conserved region is thus useful, since the diversity of members of the family *Bornaviridae* is expected to be higher than reported, considering the evolution of

the scientific knowledge produced. In fact, between the end of twentieth century and the beginning of twenty-first century, the family *Bornaviridae* comprised only the *Mammalian 1 orthobornavirus* species [26]. From then to the present, the family *Bornaviridae* includes 11 species and 19 strains [4]. Moreover, within each of the 19 strains, there are several genotypes described, as, for example, the taxonomic group PaBV-4 that integrates more than 30 genotypes [3].

The evidence produced by the phylogenetic analysis restricted to the parrot bornavirus (PaBV-4) genotypes suggests that Portugal is on the trade route of psittacines; because both genotypes cluster together with genotypes worldwide distributed and detected from captive parrots [3]. Over the years, the international trade, without biosafety measures [3], is expected to have had negative implications on biodiversity. Whereas, in the last 19 years (based on the oldest data collection (Figure 5)), bornavirus infection has contributed to the reduction of endangered specimens [3,27]. The *Psittacus erithacus* is one of the endangered species (according to the International Union for Conservation of Nature (IUCN)) [27] included in the list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [28]. In Portugal, the lawful trade of endangered species of parrots is supervised by several national entities working with CITES [29].

The present study further considered parrot bornavirus 4 (PaBV-4) genotypes detected in wild birds (*Anas* spp., *Emberiza* spp. and *Grus* spp.) and in pet psittacines of Japan and Thailand (Figure 5), more inclusive and updated than in the previous study [3], resulting in a broader view of the geographic dimension of the psittacines trade. Also, it suggests there are at least two factors (the wild birds and the world trade of psittacines) that, simultaneously or singularly, might be a factor for the dissemination of the PaBV-4 infection. Some of the PaBV-4 genotypes detected in pet parrots are phylogenetically adjacent to some genotypes detected in wild bird samples (Figure 5). In our analytical approach, it was not possible to include the genotypes of PaBV-4 detected in wild parrots from Brazil [30], because the nucleotide sequences were not available in the GenBank® database.

Over the last years, the evidence that wild birds could be reservoirs of bornaviruses has been accumulated [13–17]. In addition to the parrot bornavirus 4, cases of infection in wild birds with several genotypes of parrot bornavirus 2 (such as *Anas* spp., *Grus* spp., *Haliaeetus* spp., *Larus* spp., *Galidris* spp., *Calidris* spp. and *Emberiza* spp.), Borna disease virus 1 (such as *Anas* spp., and *Coloeus* spp.) and aquatic bird bornavirus 1 to 2 (such as *Cygnus* spp., *Greylag* spp., *Canda* spp. and *Anas* spp.) have been reported [13–17]. The co-infection was reported to occur in wild birds by different strains and genotypes belonging to *Psittaciform 1*

orthobornavirus [17]. It could then be expected that all members of the family *Bornaviridae* could be involved in co-infection events, since there are species of wild birds that may be hosting a wide range of genotypes from several species of bornaviruses (such as *Waterbird 1 orthobornavirus* [13–15], *Psittaciform 1 orthobornavirus* [17] and *Mammalian 1 orthobornavirus* [16]). Therefore, co-infected reservoirs can play a role in the emergence of new virus variants, providing conditions for the occurrence of changes in virulence and in host range by two major ways: through recombination exchange between virus strains and through accumulation of amino acid changes that are under pressure by the immune system (antigenic drift event). Both events have been reported to occur within viruses belonging to the order *Mononegavirales* [31–35]. A previous study reported findings of Borna disease virus 1 (BoDV-1) genome segments in samples from wild birds (such as *Anas* spp. and *Coloelus* spp.) in Uppsala, Sweden, which showed an intra-species divergence [16]. Namely, they revealed to be divergent from each other, as they were divergent from the Borna disease virus (BoDV-1) genotypes detected from naturally infected cat, lynx and horse in the same area [12]. In the present study, the bioinformatics approach suggests that the N and P genes of parrot bornavirus 4 (PaBV-4) have had different ancestors (Figure 6(a,b); and 7(a,b)). Namely, within PaBV-4 genotypes, the N gene showed to be evolutionarily close to avian bornaviruses (members of *Waterbird 1 orthobornavirus*) and P gene revealed to be closer to mammalian bornaviruses (members of *Mammalian 2 orthobornavirus*) (Figures 6 and 7). Moreover, the phylogeny conducted based on the P gene segment revealed that the *Mammalian 2 orthobornavirus* members (VSBV-1 (accession n° LN713681.1 and MF597762.1)) associated with lethal encephalitis of humans in Germany are evolutionarily closest related to PaBV-4 (Figure 7(a)). On the other hand, the *Mammalian 1 orthobornavirus* genotypes associated with death of humans in Germany, (accession n° MH190827.1 and LT991983.1), are evolutionarily closer to bornaviruses detected in wild birds (such as ABBV-1, ABBV-2 and BoDV-1 in Denmark, Germany, Sweden and USA) (Figure 7(a)). Furthermore, the Borna disease virus 1 (BoDV-1) variants detected in samples of wild *Anas* spp. (accession n° AF232700.1 and AF232701.1) are phylogenetically adjacent to BoDV-1 genotypes associated with lethal outcomes in humans (accession n° LT991983.1 and MH190827.1), as well as to the vaccine strain (accession n° DQ680832.1/rabbit, Germany, 1948) (Figure 7(a)). In fact, the Borna disease virus 1 (BoDV-1) detected in samples of wild *Anas* spp. are intermediate genotypes, positioned in the evolutionary line, between the two genotypes that were the cause of death of humans (accession n° MH190827.1 and LT991983.1) (Figure 7(a)). When the analysis was conducted based on the amino acid sequence encoded by

the P gene segment, the main phylogenetic relationship remained between aquatic bird bornavirus (ABBV-1 to 2), parrot bornavirus (PaBV-4), variegated squirrel bornavirus (VSBV-1) and Borna disease virus (BoDV-1) genotypes (Figure 7(b)). However, their evolutionary relationship changed when the phylogenetic analysis was based on the segment of the N gene (Figure 6(a)). The findings revealed that the N gene of PaBV-4 genotypes shared their ancestor with avian bornavirus (ABBV-1 and ABBV-2) detected in wild birds (Figure 6(a)). Furthermore, the parrot bornavirus (PaBV-4) and variegated squirrel bornavirus (VSBV-1) genotypes diverged earlier in the evolutionary line, regarding N gene (Figure 6(a)). However, parrot bornavirus (PaBV-4) remains evolutionarily closer to variegated squirrel bornavirus (VSBV-1) than to the Borna disease virus (BoDV-1) detected in samples of wild birds (accession n° AF233071.1, AF232702.2 and AF232703.1) and in human tissues (accession n° MH190827.1 and LT991983.1) (Figure 6(a)). Interestingly, from an evolutionary point of view, the Borna disease virus (BoDV-1) detected in wild birds remains as intermediate variants between the two genotypes linked to the death of humans, based on their nucleoprotein profile (Figure 6(b)).

In addition, wild birds can also disseminate viruses along their migration routes [36,37]. According to published studies, the flow of migratory birds occurs within and between the four main migratory routes (Pacific North of America/Atlantic North of America, East Asian/Australian, Central Asia and Black Sea/Mediterranean) [36,37], covering the geographical regions where the species *Waterbird 1 orthobornavirus* (e.g. ABBV-2), *Mammalian 1 orthobornavirus* (e.g. BoDV-1 strains) and *Psittaciform 1 orthobornavirus* (e.g. PaBV-2 and PaBV-4 strains) were detected in samples of *Anas* spp [14,16,17]. Particularly the *Anas* spp. are considered long-distance transporters of virus, within and between continents [36,37]. Therefore, more screening studies of some species of wild birds may be promising to understand the contribution of these species in the epidemiology of bornaviruses.

In conclusion, this is the first study characterizing at genetic and protein level that parrot bornavirus 4 (PaBV-4) is circulating in Portugal. The characterized viruses were phylogenetically related with PaBV-4 worldwide distributed in captive psittacines, suggesting that Portugal is on the route of the international trading, which occurs without biosafety measures. Moreover, the PaBV-4 genotypes detected in Portugal are evolutionarily closer to some genotypes found in wild birds in Japan, than with some of those found in pet psittacines in Europe. More studies are needed to clarify the role of wild birds in the introduction of bornaviruses to captive parrots. The N and X proteins of PaBV-4 are more related with avian bornaviruses detected in samples of wild migratory waterbirds, while phosphoprotein is

phylogenetically closer to mammalian bornaviruses, as the variegated squirrel bornavirus (VSBV-1) genotypes linked to lethal encephalitis of humans. Wild migratory birds may be a key element in understanding the intra- and inter-continental emergence and dissemination of pathogenic variants of mammalian and bird bornaviruses. The role of co-infected wild reservoirs in the emergence of new virulent variants and in the host range modification should be addressed in future studies. For screening purposes, the highly conserved P gene/protein region is a good candidate as universal target in laboratory diagnostics of bornaviruses.

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