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Snapshot of the Phylogenetic Relationships among Avian Pox-Viruses Circulating in Portugal between 2017 and 2023

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Posted Date: 29 September 2023

doi: 10.20944/preprints202309.2043.v1

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Snapshot of the Phylogenetic Relationships among Avian Pox-Viruses Circulating in Portugal between 2017 and 2023

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Simple Summary: Avipoxvirus is the causative agent of the avian pox disease and is able to infect more than 278 bird species. It causes multiple negative consequences for both the economy and the ecosystem. This occurs because there is a decrease in egg production, a decrease in mating success, retarded growth, and death can also happen. There are several diagnostic methods, and prevention is done through the implementation of sanitary measures and vaccination. To evaluate and characterize the avipoxviruses circulating in Portugal since 2017, ten positive samples for this virus were analyzed. Subsequently, they were compared to other isolates from other hosts, countries, and years. As a result, it was possible to understand that certain variants of the virus continued to circulate over the years, but there were also new viral introductions.

Abstract: Avipoxvirus (APV), a linear dsDNA virus that belongs to the subfamily *Chordopoxvirinae* of the family *Poxviridae*, affects more than 278 species of domestic and wild birds. It is responsible for causing the avian pox disease, characterized by its cutaneous and diphtheric forms. With a high capacity for transmission, it can lead to high economic losses and damage to the ecosystem. There are several methods of diagnosis, and bird vaccination can be an effective preventive measure. In order to update the molecular characterization and phylogenetic analysis of viruses isolated in Portugal since 2017, ten APV positive samples were analyzed. A P4b gene fragment was amplified by PCR and the nucleotide sequence of the amplicons was determined by Sanger sequencing. The sequences obtained were aligned by ClustalW, and a phylogenetic tree based on the maximum likelihood method was generated. With this study, it was possible to verify that the analyzed sequences are distributed through subclades A1, A2, B1, and B3. As some of them are quite similar to others from different countries and years, it is possible to infer that there were several viral introductions in Portugal. Finally, it was possible to update with success the data about avipoxvirus in Portugal.

Keywords: Avipoxvirus; molecular characterization; phylogenetic analysis; viral introduction; Portugal

1. Introduction

The family *Poxviridae* is divided into subfamilies *Chordopoxvirinae* and *Entomopoxvirinae*, responsible for infecting vertebrates and insects, respectively [1]. The genus Avipoxvirus is included within the subfamily *Chordopoxvirinae*, together with other seventeen genera [2]. Considering the different avian species that the virus infects, there are twelve species of avipoxviruses (APVs) [3].

According to Manarolla et al. [4], the nomenclature given to APVs is based on the type of species present in each clade, having already been identified five clades, A to E. Clade A is associated with fowlpox, clade B with canarypox, and clade C with psittacinepox viruses. To date, seven subclades within clade A and four subclades within clade B were described. Clade D includes a unique strain, QP-241, which was isolated in Italy from a quail [4]. Clade E contains sequences isolated from APVs from chickens in Brazil [5] and Mozambique [6], and also from a turkey in Hungary [7].

The avipoxviruses are very large viruses [8] that can be either oval or brick-shaped [9]. The dsDNA genome is in a linear configuration [9] and has a very low GC content [10]. Among all the poxviruses, avipoxviruses have the largest genome, with about 180 kbp [11].

 doi:10.20944/preprints202309.2043.v1

APV infection can cause the development of avian pox disease, which can be presented by its cutaneous or diphtheric forms. These are caused by different routes of infection. The cutaneous form is originated by mechanical trauma [12], causes nodular lesions in feather-free areas and is associated with very low mortality rates [9]. The diphtheric form, on the other hand, occurs after inhalation or ingestion of the virus [12] and is characterized by the formation of proliferative nodular lesions in the mucous membranes of the digestive and respiratory tracts [9]. Since the nodular lesions can cause difficulty in breathing and eating, the mortality rate of this form of the disease is higher [13]. Transmission may occur through mechanical vectors like arthropods, aerosols released by sick birds, direct contact with lesions, or ingestion of contaminated food or drink [14]. Wild birds' behavior, such as bird migration, introduction of new species, and habitat change, also lead to transmission [3]. However, the disease can be prevented by implementing sanitary measures to avoid mechanical vectors and contaminated sources [5] or through birds' vaccination [15].

Infection leads to a variety of negative consequences, both economical and in the ecosystem. A decrease in egg production and immunity is commonly seen, resulting in a decreased ability to survive to secondary infections. Reduced mating success, retarded growth, blindness, feeding difficulties, and death can also occur [3,16]. Therefore, poultry farmers suffer significant economic losses due to the need to replace livestock, sales losses, and sanitation costs [5]. At the ecosystem level, the birds are more vulnerable, leading to increased predation, and a decrease in mating, thus resulting in a decline in population [17].

In this paper, molecular characterization and phylogenetic analysis of avipoxviruses isolated from ten samples detected in Portugal since 2017 are performed, in order to infer about the possible origin of the viruses circulating in the country.

2. Materials and Methods

2.1. Samples

Ten avipoxvirus positive samples were detected at INIAV since 2017. Those positive samples were preserved at -80°C.

Table 1. Avipoxvirus positive samples diagnosed at INIAV since 2017 and used in this study.

Host		Sequence ID	Accesion number	Country	Collection date	Isolation source
Common name	Scientific name					
Flamingo	*Phoenicopterus ruber*	24569-17	OQ615872	Portugal: Lisboa	26/Sep/17	Pool of organs
Chicken	*Gallus gallus*	23049-18	OQ615873	Portugal: Porto Santo, Madeira	24/Jul/18	Pool of organs
Puffin	*Fratercula*	11612-19	OQ615874	Portugal: Lisboa	16/Apr/19	Cutaneous lesion
Canary	*Serinus canaria*	37026-19	OQ615875	Portugal: Freixianda, Ourém	22/Nov/19	Pool of organs
Canary	*Serinus canaria*	03779-20	OQ615876	Portugal	04/Feb/20	Pool of organs
Chicken	*Gallus gallus*	04482-20	OQ615877	Portugal	11/Feb/20	Cutaneous lesion
Blackbird	*Turdus merula*	16735-20	OQ615878	Portugal	09/Jun/20	Cutaneous lesion
Chicken	*Gallus gallus*	P-08508-21	OQ615879	Portugal: Maia, Porto	22/Sep/21	Pool of organs
Penguin	*Spheniscidae*	P-09292-22	OQ615880	Portugal: Avintes, Porto	17/Oct/22	Pool of organs
Chicken	*Gallus gallus*	00917-23	OQ615881	Portugal: Évora	16/Jan/23	Pool of organs

2.2. Nucleic acids extraction

The viral DNA extraction was performed in a nucleic acid extraction workstation Kingfisher Flex (Thermo Fisher Scientific, Massachusetts, USA) using IndiMag Kit (Indical Bioscience, Germany), following the manufacturer's instructions. After extraction, the DNA was stored at 4°C.

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2.3. PCR amplification of P4b gene fragment

The amplification of the P4b gene fragment was performed by conventional PCR with primers described by Huw Lee and Hwa Lee [18], using NZYTaq II 2x Green Master Mix (0.2 U/µL) (NZYTech, Portugal). The reaction contained 0.5 µL of each primer (50 pmol/µL) (Table 2) and 5 µL of DNA, in a total of 25 µL. The PCR program was executed in an UNO II thermocycler thermoblock (Biometra, Germany) and consisted of an initial denaturation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 40 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR product was separated by a 1% agarose gel electrophoresis stained with GreenSafe Premium (NZYTech, Portugal). The expected fragments of 578 bp, corresponding to the P4b gene amplified fragment were excised and purified using NZYGelpure Kit (NZYTech, Portugal), according to the manufacturer's instructions.

Table 2. Sequences of P4b primers used in PCR reactions, described by Huw Lee and Hwa Lee [18].

Primer	Sequence	bp	Tm (°C)	% GC
Pox-VP1	5′ – CAGCAGGTGCTAAACAACAA – 3′	20	62	45
Pox-VP2	5′ – CGGTAGCTTAACGCCGAATA – 3′	20	64	50

2.4. P4b gene fragment Sanger sequencing

The P4b gene fragments were sequenced by the Sanger method, using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, USA), following the manufacturer's guidelines. The primers used in the sequencing reaction are described in Table 2. In order to improve the sequencing quality, different volumes of sample were used in each reaction depending on the DNA concentration. The samples were sequenced in a 3130 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). The sequence alignment was performed using SeqScape Software from Applied Biosystems. With the use of Clustal Omega by EMBL-EBI, percent identity matrices were also created between the sequences of subclades A1, A2, B1, and B3 (data not shown) and the sequences isolated and sequenced in this work. The GenBank accession numbers given to sequences obtained in this study are represented in Table 1.

2.5. Phylogenetic analysis

To study the phylogenetic origin of the avipoxviruses detected and their phylogenetic relationship with the sequences published in GenBank (Table 3), a multiple alignment between them was performed using ClustalW method. Sequences from different countries, years, and clades were chosen, in order to get a reliable analysis. To generate the phylogenetic trees, maximum likelihood, neighbor-joining, and bayesian methods were used. Each tree was produced using MEGA11 with the Tamura 3-parameter G+I, determined as the best fit model also by MEGA11, and 1000 bootstrap replicates.

Table 3. Details of APVs sequences published in GenBank and used in this study.

Host		Accesion number	Country	Collection date	Clade
Common name	Scientific name				
Turkey	*Meleagris gallopavo*	AY530304	Germany	2001	A1
Silver pheasant	*Lophura nycthemera*	HM481406	India	2008	A1
Chicken	*Gallus gallus*	KF722860	Tanzania	2012	A1
Chicken	*Gallus gallus*	KM974727	Portugal	2013	A1
Chicken	*Gallus gallus*	KP987214	Nigeria	2013	A1

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Backyard turkey	*Meleagris gallopavo*	KU522210	Iran	2015	A1
Quail	*Coturnix coturnix*	DQ873809	India	-	A2
Indian little brown dove	*Spilopelia senegalensis*	HM481408	India	2009	A2
Eurasian stone-curlew	*Burhinus oedicnemus*	HM627224	Spain	1980	A2
Rock dove	*Columba livia*	KC017966	USA	1995	A2
Indian peafowl	*Pavo cristatus*	KC017975	Hungary	2003	A2
Booted eagle	*Hieraaetus pennatus*	KC017976	Spain	2000	A2
Pigeon	*Columbidae*	KJ913659	Tanzania	2013	A2
Wood-pigeon	*Columba palumbus*	EU798994	Czech Republic	2008	A3
Pelagic cormorant	*Phalacrocorax pelagicus*	KC017982	USA	1989	A3
Eurasian eagle owl	*Bubo bubo*	KC017983	South Korea	-	A3
Common murre	*Uria aalge*	KC017985	USA	1991	A3
Laysan albatross	*Phoebastria immutabilis*	KC017986	USA	1983	A3
Magellanic penguin	*Spheniscus magellanicus*	KC017987	Argentina	2007	A3
Falcon	*Falco sp.*	AY530306	United Arab Emirates	2002	A4
Red-footed falcon	*Falco vespertinus*	KC017989	Hungary	2007	A4
Trumpeter swan	*Cygnus buccinator*	KC017990	USA	1991	A5
Mottled duck	*Anas fulvigula*	KC017991	USA	2005	A5
Redhead duck	*Aythya americana*	KC017993	USA	1991	A5
Trumpeter swan	*Cygnus buccinator*	KC017995	USA	1989	A5
Wood duck	*Aix sponsa*	KC017996	USA	1991	A5
Domestic mallard duck	*Anas platyrhynchos*	KJ192189	China	2013	A5
Mourning dove	*Zenaida macroura*	KC018000	USA	1987	A6
Canada goose	*Branta canadensis*	KC018002	USA	1992	A6
Common buzzard	*Buteo buteo*	KC018009	Hungary	2000	A7
Stone curlew	*Burhinidae*	AY530310	United Arab Emirates	1998	B1
Palila	*Loxioides bailleui*	EF568381	USA	-	B1
Amakihi	*Hemignathus virens*	EF568401	USA	-	B1
Blue jay	*Cyanocitta cristata*	GQ487567	Canada	1998	B1
Canary	*Serinus canaria*	GU108510	Austria	2009	B1
Red crossbill	*Loxia curvirostra*	HM627227	Spain	1930	B1
Golden eagle	*Aquila chrysaetos*	KC018058	Spain	2000	B1
House sparrow	*Passer domesticus*	HM627220	Marocco	2009	B2
Flamingo	*Phoenicopterus ruber*	HQ875129	Portugal	2010	B2
Great bustard	*Otis tarda*	KC018066	Hungary	2005	B2
American crow	*Corvus brachyrhynchos*	DQ131891	USA	2003	B3
House finch	*Haemorhous mexicanus*	DQ131896	USA	2003	B3
Great blue heron	*Ardea herodias*	DQ131898	USA	2004	B3
Northern cardinal	*Cardinalis cardinalis*	DQ131899	USA	2003	B3
Red-tailed hawk	*Buteo jamaicensis*	DQ131901	USA	2003	B3
Chicken	*Gallus gallus*	AM050382	United Kingdom	1986	C
Parrot	*Psittaciformes*	AM050383	United Kingdom	1989	C
Lovebird	*Agapornis*	AY530311	Germany	-	C

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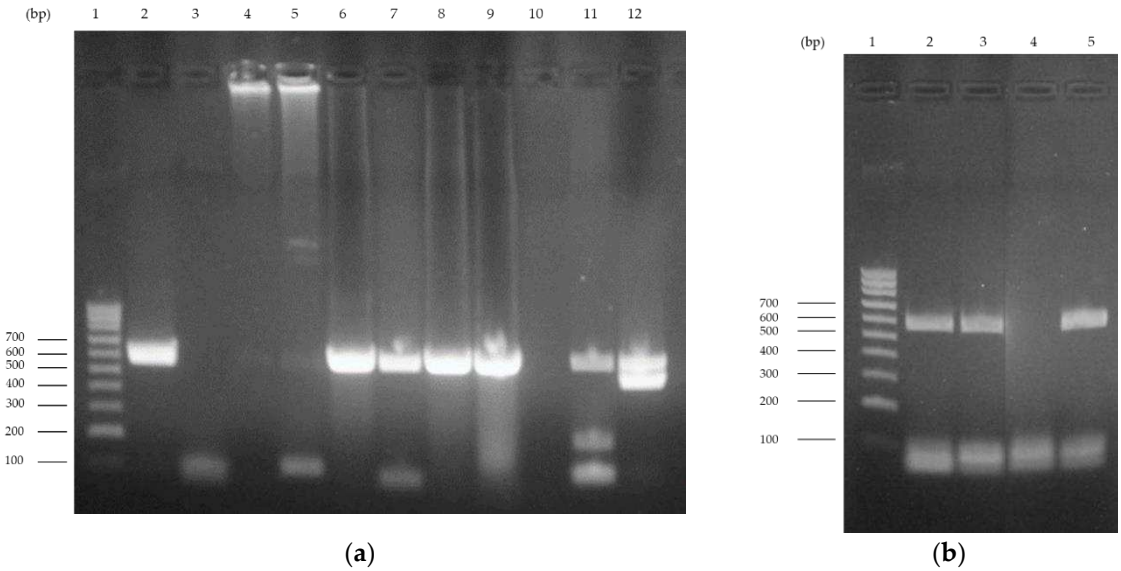
Yellow-crowned amazon	*Amazona ochrocephala*	KC018069	USA	1980	C
Quail	*Coturnix coturnix*	GQ180200	Italy	-	D
Chicken	*Gallus gallus*	MW349699	Brazil	2019	E
Chicken	*Gallus gallus*	MW349701	Brazil	2019	E
Domestic mallard duck	*Anas platyrhynchos*	KJ192189	China	2013	A5
Mourning dove	*Zenaida macroura*	KC018000	USA	1987	A6

3. Results and Discussion

3.1. PCR amplification of P4b gene fragment

The amplification of the 578 bp P4b gene fragment was confirmed by 1% agarose gel electrophoresis. The results obtained are shown in Figure 1. It is possible to observe the presence of the expected fragment in all lanes except lanes 3, 4, and 5, which correspond to samples 11612-19 (puffin), 37026-19 (canary), and 03779-20 (canary), respectively. Amplification of DNA from samples 37026-19 and 03779-20 obtained in a previous extraction process was achieved (lanes 11 and 12) (Figure 1a). A new PCR reaction led to the amplification of the sample 11612-19 (Figure 1b).



(**a**) (**b**)

Figure 1. 1% agarose gel electrophoresis of the 578 bp P4b gene fragment PCR amplification. (**a**) Lane 1 corresponds to the molecular weight marker, NZYDNA Ladder V (NZYTech, Portugal). Lanes 2 to 9 correspond to the amplification of samples 24569-17 (flamingo), 11612-19 (puffin), 37026-19 (canary), 03779-20 (canary), 04482-20 (chicken), 16735-20 (blackbird), P-08508-21 (chicken), and 23049-18 (chicken), respectively. Lane 10 is the negative control. Lanes 11 and 12 correspond to samples 37026-19 (canary) and 03779-20 (canary) obtained in a previous extraction. (**b**) Lane 1 corresponds to the molecular weight marker, NZYDNA Ladder V. Lanes 2 and 3 correspond to sample 11612-19 (puffin) from different extraction reactions. Lanes 4 and 5 are the negative and positive controls, respectively.

3.2. P4b gene fragment sequencing

The alignment of forward and reverse nucleotide sequences obtained, originated a sequence of 538 bp for all samples, except for sample 03779-20 (canary), after removing the primers sequences. Due to incomplete sequencing of the 3' end, only 513 bp were obtained for this sample. This result can be explained by DNA damage, incorrect amplification, or inadequate purification.

The representation of the alignment between the studied sequences is present in Figure 2, and its analysis is done in the following section, together with the phylogenetic analysis.

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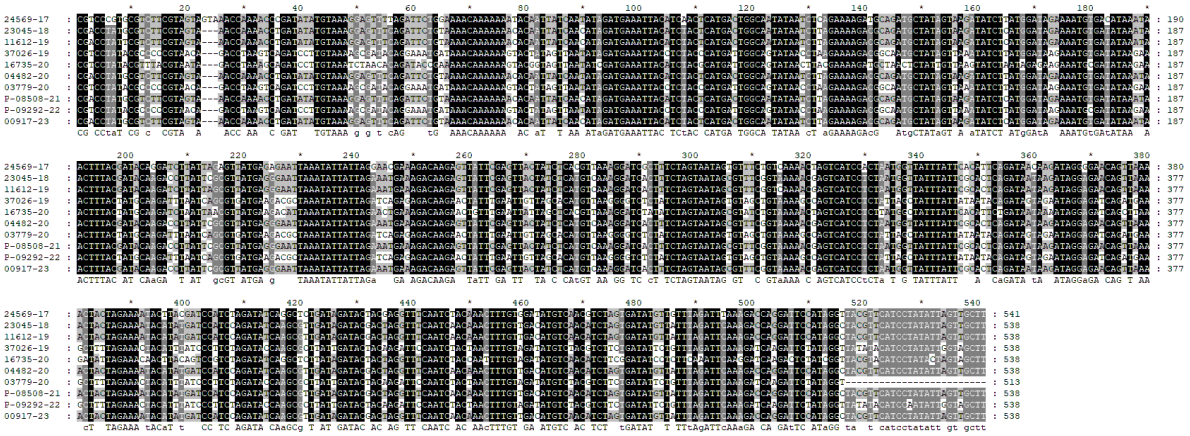

Figure 2. Alignment of the studied sequences.

3.3. Phylogenetic analysis

The avipoxvirus nucleotide sequences obtained were phylogenetically analyzed using different algorithms, specifically maximum likelihood, neighbor-joining, and bayesian analysis. The three algorithms originated phylogenetic trees with similar topologies. However, the maximum likelihood tree originated a better resolution of the sequences, and therefore this was the chosen method (Figure 3). The phylogenetic tree obtained, including representatives from all subclades (except B4), shows that clades C and D have a common most recent ancestor, as observed in previous studies [5,14].

The phylogenetic analysis reveals that the samples from Portugal are quite distributed, with five samples belonging to subclade A1, one to subclade A2, three to subclade B1, and one to subclade B3. Moreover, their distribution is quite consistent with the nomenclature given, since samples from *Gallus gallus* belong to the fowlpox clade (clade A), while samples from *Turdus merula* and *Serinus canaria*, both passeriformes, belong to the canarypox clade (clade B). *Phoenicopterus ruber*, *Fratercula*, and *Spheniscidae* are not fowl or Passeriformes representatives. The viruses detected in the first two hosts were included in the fowlpox clade, while the penguinpox virus belongs to the canarypox clade.

When comparing the alignment (Figure 2) and the percent identity matrix of the sequences obtained in this study (Table 4), several conclusions can be reached. Sample 16735-20 (blackbird) is the most distinct of all, as it has several different nucleotides along the entire sequence. Therefore, it has a very low identity percentage relative to all the other sequences, with the highest identity percentage value being only 81.60%. The nucleotide sequence of sample 24569-17 (flamingo) is the second most distinct sequence, showing a maximum identity percentage of 90.52%. In addition, it contains an extra valine codon at position 23.

By comparing the alignment of all the sequences present in the phylogenetic tree, it is observable that sample 16735-20 (blackbird) has two unique mutations. It contains a cytosine in position nt 63 instead of a thymine, and a thymine at position nt 148, instead of a guanine or an adenine.

Interestingly, when analyzing the percentage identity matrix between the sequences of the A1 subclade, it can be observed that the puffin (*Fratercula*) isolate sequence is not completely identical with any other sequence, but the identity percentages are quite high, showing that the sequences are very similar.

On the other hand, all the chicken (*Gallus gallus*) isolates have an identity percentage of 100%, except sample sequence 04482-20. Yet the identity percentage is still very high, 99.63%, with only two nucleotides differing. It can also be observed that the sequences of the samples isolated from turkey (*Meleagris*, Germany, 2001) and wild turkey (*Meleagris gallopavo*, Iran, 2015) are also similar to those isolated from chickens. By analyzing the position of the remaining sequences in the phylogenetic tree, it is possible to verify the presence of samples isolated from chickens also in subclades C and E. When comparing those of subclade A1 with these, it is found that the percentage of identity is quite low, approximately 74%, confirming the classification in different clades.

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Regarding the sequences of A2 subclade, it is possible to observe that the sequence of the sample isolated from the flamingo (*Phoenicopterus ruber*) is different from all the other sequences. However, the percentages are quite high, varying between 92.15% (pigeon) (*Columbia livia*, USA, 1995) and 99.63% (pigeon) (*Columbidae*, Tanzania, 2013). Additionally, it can be seen that both APVs isolated from flamingos in Portugal belong to different clades, the one from 2010 belongs to B2 and the one from 2017 to A2. Indeed, the identity percentage between them is only 75.84%. When they are aligned, it can be noticed that the 2010 sequence has an extra isoleucine codon at position nt 24, while the 2017 has an extra phenylalanine codon at location nt 60.

The analysis of the sequences of subclade B1 shows that both isolates of canaries (*Serinus canaria*) from Portugal share the same nucleotide sequence. The canary's isolate from Austria is not 100% identical with these canaries' sequences, however it is very similar, having an identity percentage of 99.81%, with only one nucleotide differing. The sequence from the penguin (*Spheniscidae*) isolate is 100% identical with the sequences from three other isolates, the golden eagle isolate (*Aquila chrysaetos*, Spain, 2000), the stone-curlew isolate (*Burhinidae*, United Arab Emirates, 1998) and the crossbill isolate (*Loxia curvirostra*, Spain, 1930). Comparing the sequence of the avipoxvirus isolated from the penguin in Portugal with the one from Argentina, it is found that they are classified in distinct subclades, B1 and A3, respectively. These two sequences show several mutations between them, showing a percentage of identity of only 72.68%.

Comparing the sequences of the B3 subclade, it is possible to verify that the blackbird (*Turdus merula*) isolate sequence is not completely identical with any other sequence. However, the percentage of identity is quite high among all of the sequences (97.03% to 97.58%).

The results of this study indicate that the APVs circulating in Portugal in chickens and canaries are quite similar, since the nucleotide sequences of the isolates did not undergo considerable changes. However, the isolates from flamingos show the opposite situation, since the virus that circulated in 2010 is very different from that circulating in 2017. This suggests the occurrence of a second viral introduction in Portugal. Finally, as the nucleotide sequence of the isolate from penguin is very similar to that isolated from golden eagle, stone-curlew, and crossbill, it is possible to hypothesize that there was a third viral introduction in Portugal.

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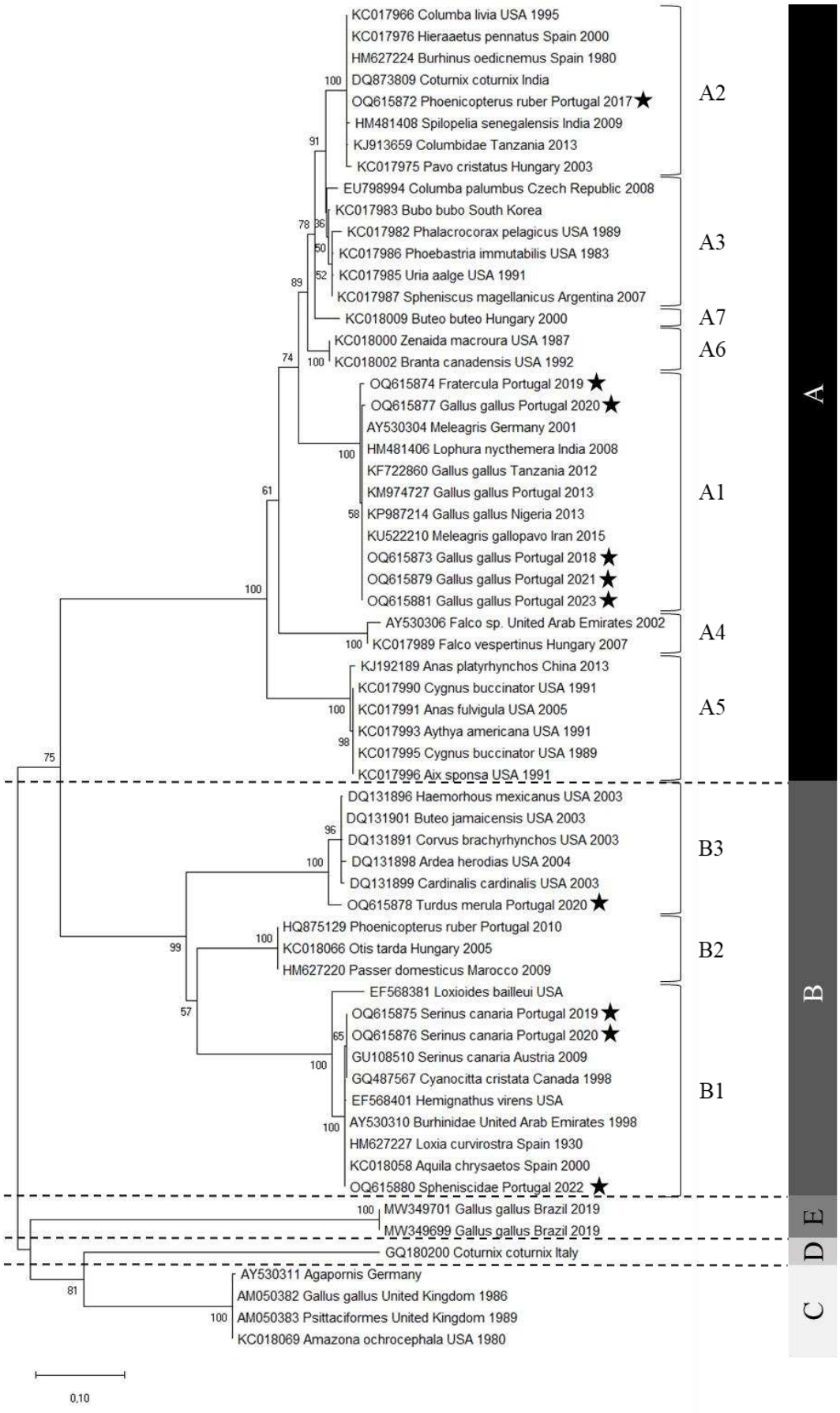

Figure 3. Evolutionary analysis by Maximum Likelihood method. The evolutionary history was

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inferred by using the Maximum Likelihood method and Tamura 3-parameter model [19]. The tree with the highest log likelihood (-3505.45) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.4400)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.73% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 62 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 485 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [20].

Table 4. Percent identity matrix between APVs sequences isolated in this study.

		1	2	3	4	5	6	7	8	9	10
1	*Phoenicopterus ruber* (24569-17)		90.52	89.78	89.78	89.78	90.15	75.46	72.68	73.05	72.71
2	*Fratercula* (11612-19)	90.52		99.26	99.26	99.26	98.88	76.39	74.54	74.91	74.66
3	*Gallus gallus* (23049-18)	89.78	99.26		100.00	100.00	99.63	76.21	74.35	74.72	74.46
4	*Gallus gallus* (P-08508-21)	89.78	99.26	100.00		100.00	99.63	76.21	74.35	74.72	74.46
5	*Gallus gallus* (00917-23)	89.78	99.26	100.00	100.00		99.63	76.21	74.35	74.72	74.46
6	*Gallus gallus* (04482-20)	90.15	98.88	99.63	99.63	99.63		76.39	74.72	75.09	74.85
7	*Turdus merula* (16735-20)	75.46	76.39	76.21	76.21	76.21	76.39		81.60	81.60	81.48
8	*Spheniscidae* (P-09292-22)	72.68	74.54	74.35	74.35	74.35	74.72	81.60		99.63	99.81
9	*Serinus canaria* (37026-19)	73.05	74.91	74.72	74.72	74.72	75.09	81.60	99.63		100.00
10	*Serinus canaria* (03779-20)	72.71	74.66	74.46	74.46	74.46	74.85	81.48	99.81	100.00	

4. Conclusions

In conclusion, it was possible to verify that the samples studied are widely distributed in the phylogenetic tree, with representatives in subclades A1, A2, B1, and B3. In addition, it was possible to observe that some of the isolates, such as those from chickens and canaries, suggest that the APV in circulation in Portugal originated from the same virus. Other isolates, such as flamingo and penguin isolates, suggest new viral introductions, since they are similar to other strains from different years and geographic origins. These data suggest that the viruses circulating in Portugal have distinct origins. Interestingly, the sequence from the blackbird isolate presents two unique mutations, when compared with all the sequences used in the phylogenetic analysis. With this study, it was possible to update the molecular and taxonomic data of avipoxviruses in Portugal.

Acknowledgments: The authors thank Maria João Teixeira, Fátima Cordeiro, Rosário Ferreira and Sandra Nunes for technical assistance.

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