

# Investigation and sequence analysis of avian polyomavirus and psittacine beak and feather disease virus from companion birds in eastern Turkey

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## Abstract

**Introduction:** Avian polyomavirus (APV) and psittacine beak and feather disease virus (PBFDV) induce contagious and persistent diseases that affect the beaks, feathers, and immune systems of companion birds. APV causes hepatitis, ascites, hydropericardium, depression, feather disorders, abdominal distension, and potentially death. PBFDV can induce progressive beak deformity, feather dystrophy, and plumage loss. We conducted the first prevalence survey of both APV and PBFDV infections in companion birds in eastern Turkey. **Material and Methods:** A total of 113 fresh dropping samples from apparently healthy companion birds were collected in a random selection. The dropping samples were analysed for PBFDV and APV by PCR. Positive samples were sequenced with the Sanger method. The sequence was confirmed through alignment and the phylogenetic tree generated through the maximum likelihood method computationally. **Results:** PBFDV and APV were detected in a respective 48.7% and 23.0% of samples. Coinfection was found in 12.4% of the samples, these all being from budgerigars (*Melopsittacus undulatus*). APV and PBFDV were detected in budgerigar and cockatiel (*Nymphicus hollandicus*) samples. **Conclusion:** This report provides a foundation for future studies on the influence of these viruses on the health of companion birds. These high positive rates for both pathogens emphasise that healthy *M. undulatus* and *N. hollandicus* in eastern Turkey may be prone to the emergence and spread of APV and PBFDV with subclinical potential.

**Keywords:** companion birds, avian polyomavirus, psittacine beak and feather disease virus, dropping samples, phylogenetic analysis.

## Introduction

Avian polyomavirus (APV) and psittacine beak and feather disease virus (PBFDV) are aetiological agents of diseases that adversely affect the skin and feathers of companion birds (15). They are responsible for the most common clinical problems in companion birds such as sudden death, difficulties in treatment, subclinical course, and cross-transmission between bird species, and also inflict severe economic losses on breeders (13, 21).

APV infection, also known as budgerigar fledgling polyomavirus (BFPyV) disease and psittacine polyomavirus was reported both in budgerigars (*Melopsittacus undulatus*) (15) and cockatoos (*Cacatuidae*) (1). APV is assigned to the *Polyomaviridae* family and

*Avipolyomavirus* genus and classified as a non-enveloped virus with an icosahedral viral capsid containing double-stranded DNA. The major viral protein of the virus was designated viral protein one (VP1) and the minor viral proteins are viral protein two (VP2), viral protein three (VP3), and viral protein four (VP4) (16). The subclinical form of the disease occurs in many companion birds including budgerigars and parrots. APV infection has a high mortality rate in young birds and can rarely be fatal to adults. The clinical signs of the disease are generally feather losses, paleness, loss of appetite, and subcutaneous bleeding (7). In addition, embryo deaths happen *via* germinal transmission during the hatching period (4, 10, 18).

PBFDV was first detected in 1984 in Australia in a wild cockatoo, and later found in many species

including canaries, ostriches, pigeons, ducks, geese, finches, gulls, ravens, pheasants, jays, and starlings (17, 24). The *Circoviridae* family of icosahedrally structured DNA viruses include two genera: *Cyclovirus* and *Circovirus*. A circovirus is the major causative agent of the PBFVDV infection in companion birds, which has the most severe course in young birds (20). It is transmitted both horizontally and vertically and inflicts high morbidity but low mortality (24). The mortality of the infection relates to age, breed, and the occurrence of secondary infections such as peritonitis, mycotic ventriculitis, and chlamydiosis (5, 25). Transmission of the infection occurs *via* direct contact with infected birds or through gastrointestinal or respiratory intake of infected faeces or feathers (10). The typical clinical findings include immunosuppression, feather loss, bleeding of feather follicles, abnormal feather growth, and beak anomalies such as being shiny, enlarged, or broken. Feather and beak lesions may occur together or separately (4, 20).

PBFVDV and APV infections are of increasing economic importance due to widespread bird die-offs and risks to breeding potential because of immunosuppression and vertical and digestive tract transmission. This study, which is the first in eastern Turkey, was designed to investigate PBFVDV and APV in the dropping swab samples of companion birds through PCR and phylogenetic analysis.

## Material and Methods

**Sampling.** A total of 113 fresh dropping swab samples (one from *Ara ararauna*, three from *Agapornis* sp., two from *Nymphicus hollandicus*, one from *Psittacus erithacus*, and 106 from *Melopsittacus undulatus*) from apparently healthy companion birds were collected in a random manner from thirteen bird sellers (pet shops) in January–May 2016 and August 2020 in Erzurum, Turkey (Table 1). The age range of the animals was 2–24 months. Birds were observed in their cages for excretion and a swab was taken from the topmost layer of fresh droppings. The fresh dropping swab samples were stored at 4°C until shipping to the laboratory. All samples were stored at –20°C in the laboratory until further use.

**DNA extraction.** Viral DNA was extracted from the dropping swabs using a PureLink Genomic DNA Kit (Invitrogen, USA) as instructed by the manufacturer.

DNA samples were maintained at –20°C until molecular testing was initiated (1). DNA concentration was then measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The DNA quantity and A260/A280 optical density value as indicative of DNA purity were noted for each sample.

**PCR.** The PCR methods to amplify PBFVDV and APV were performed as previously described respectively by Ritchie *et al.* (21) and Altan *et al.* (1) (Table 2). The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C (PBFVDV) or 60°C (APV) for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. The amplified PCR products were viewed on 1.5% agarose gel under UV light.

**Sequencing and phylogenetic analysis.** Six PCR-positive samples (one from *N. hollandicus* and one from *M. undulatus* for APV and three from *M. undulatus* and one from *N. hollandicus* for PBFVDV) tested in this study were chosen for Sanger sequencing. The selection criteria of samples for sequence analysis included the necessity that samples represented each different positive species. The reference sequencing data from the GenBank database for phylogenetic trees were selected to correspond to the countries where the birds are indigenous, the countries importing these birds, and the neighbouring countries of Turkey. Sequence results were compared to the reference strains using ClustalW multiple sequence alignments (23) and manually edited using BioEdit version 7.0.5 software (8). A phylogenetic analysis was then generated based on the sequences of VP1 and the *rep* gene for APV and PBFVDV, respectively, using the MEGA v X programme (14, 22). All results were analysed by bootstrap analysis (1,000 replicates) according to a maximum likelihood method phylogenetic tree (the Kimura 2-parameter method). Afterward, the partial sequence results were deposited in GenBank.

## Results

In total, 26 traces of BFDV DNA and 55 of APV DNA were detected from the 113 fresh dropping swab samples collected from companion bird species in Erzurum province, eastern Turkey in January–May 2016 and August 2020 (Table 3). Eleven bird sellers' stock was positive for APV, whereas eight sellers' companion birds were positive for PBFVDV.

**Table 1.** Species distribution of fresh dropping samples and PCR results

Companion birds (n)	Age (months)	Number of samples by bird seller												
		A*	B	C	D	E	F	G	H	I	J	K	L	M
<i>Agapornis</i> sp. (3)	60				3									
<i>A. ararauna</i> (1)	48				1									
<i>M. undulatus</i> (106)	2–12	11	5	5	10	2	7	10	15	10	8	8	7	8
<i>N. hollandicus</i> (2)	24							2						
<i>P. erithacus</i> (1)	36	1												
Total (113)		12	5	5	14	2	9	10	15	10	8	8	7	8

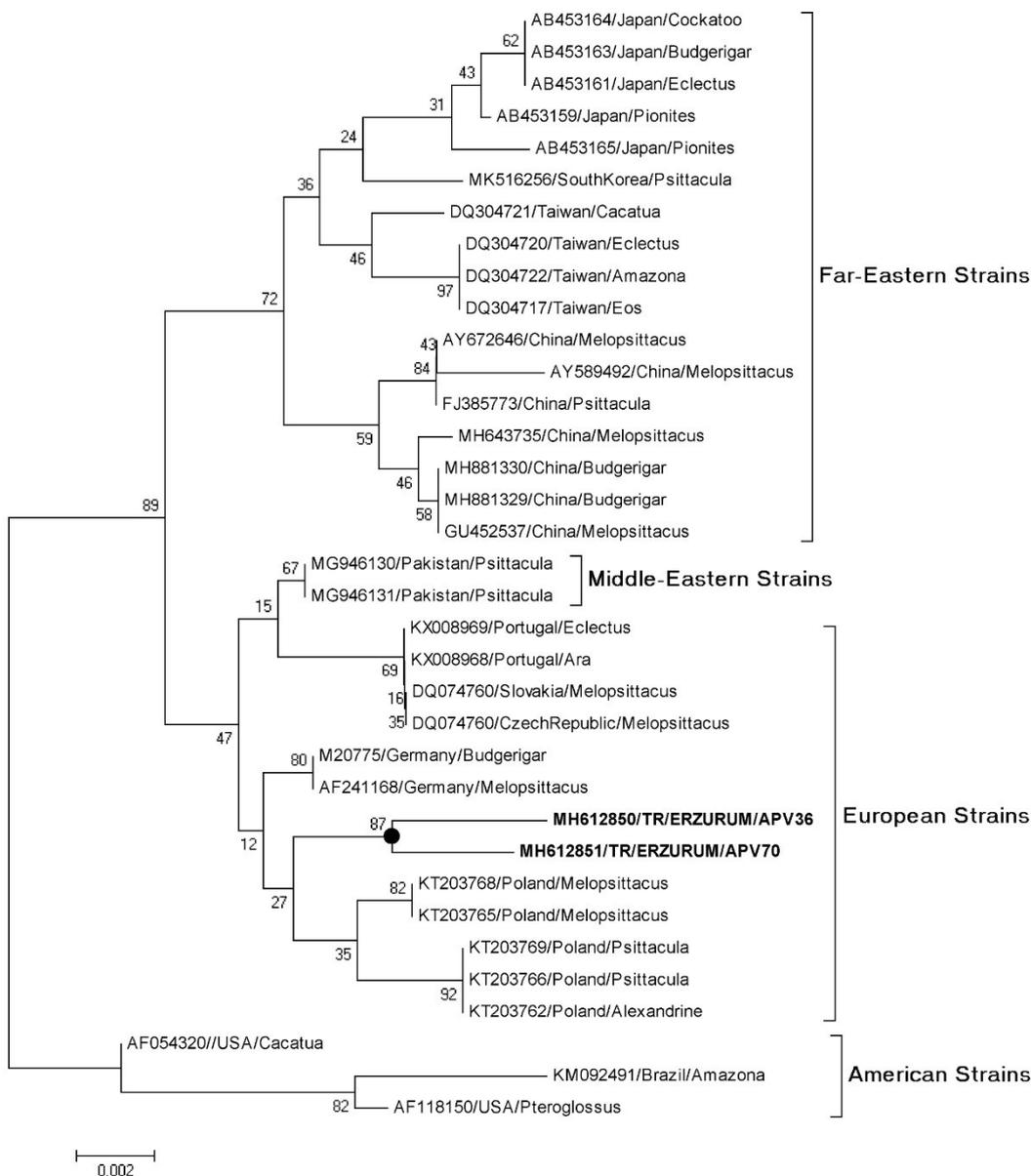
\*A to M indicate thirteen different bird sellers

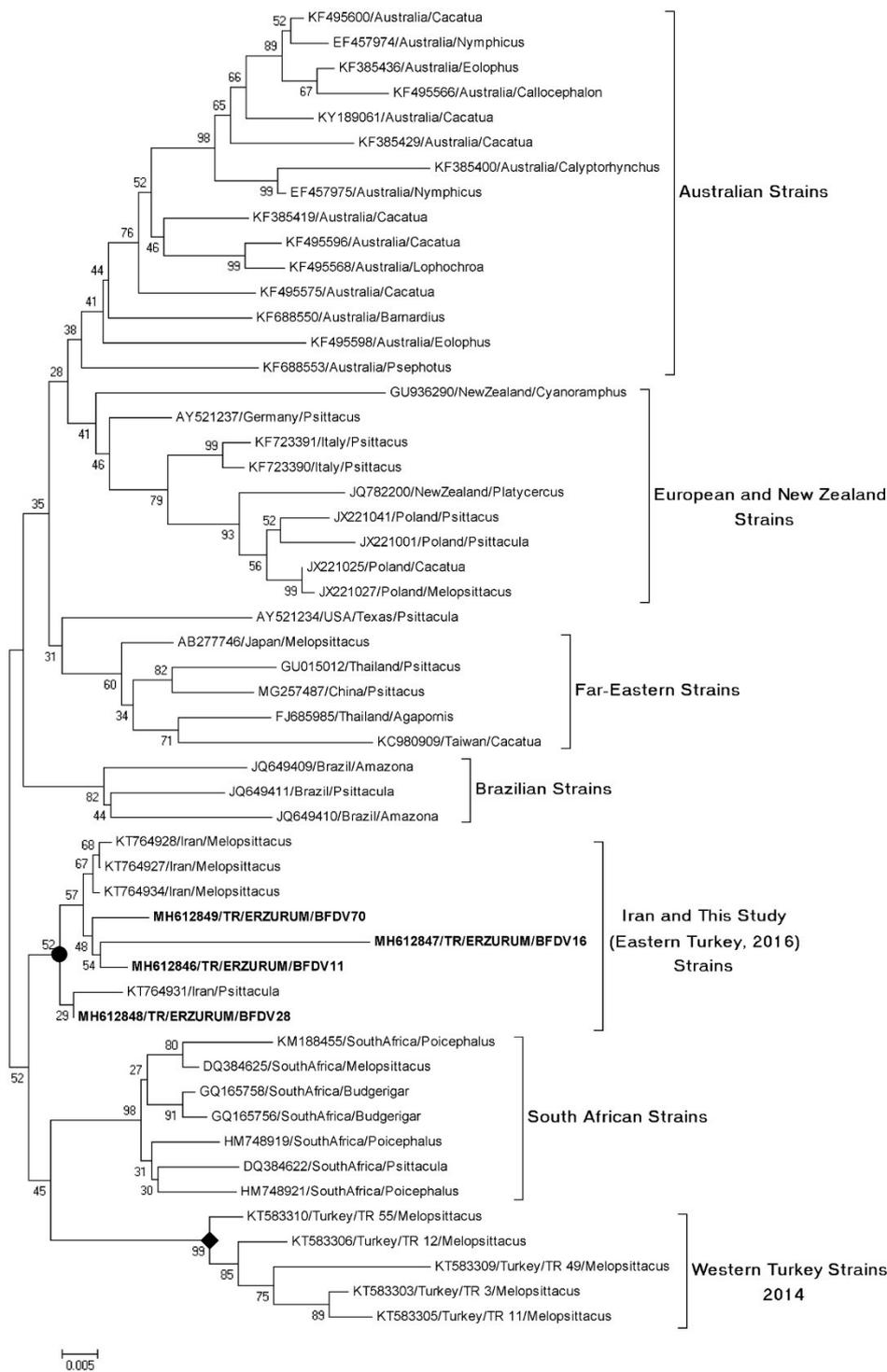
**Table 2.** Primers used in the study, target region, and amplicon lengths

Primer names	Sequence (5'-3')	Target region	Size (bp)	References
BFDV-seq-F	TTAACAACCCTACAGACGGCGA	replication associated protein (rep) gene	605	(21)
BFDV-seq-R	GGCGGAGCATCTCGCAATAAG			
APV-Ot-2,105-F	CAGCACAGAGGTACCGTGT	VP1 gene	831	(1)
APV-Ot-2,846-R	ATCAGAGCCCTGCATGCTTT			

**Table 3.** Species distribution of dropping swab samples positive for APV, PBFVDV, and APV & BFDV by PCR

Companion bird	Only APV Positive/total examined (%)	Only BFDV Positive/total examined (%)	APV & BFDV Positive/total examined (%)
<i>Agapornis</i> sp.	0/3 (0)	0/3 (0)	0/3 (0)
<i>A. ararauna</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>M. undulatus</i>	40/106 (37.7)	11/106 (10.4)	14/106 (13.2)
<i>N. hollandicus</i>	1/2 (50)	1/2 (50)	0/2 (0)
<i>P. erithacus</i>	0/1 (0)	0/1 (0)	0/1 (0)
Total	41/113 (36.3)	12/113 (10.6)	14/113 (12.4)

**Fig. 1.** Phylogenetic tree of different avian *Polyomavirus* (APV) strains generated using the maximum likelihood method in MEGA v X. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum likelihood method and are in the units of base substitutions per site. Codon positions included were 1<sup>st</sup> + 2<sup>nd</sup> + 3<sup>rd</sup> + noncoding. The analysis involved 33 nucleotide sequences



**Fig. 2.** Phylogenetic tree of different *Circovirus* strains generated using the maximum likelihood methods in MEGA v X. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of base substitutions per site. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. Codon positions included were 1<sup>st</sup> + 2<sup>nd</sup> + 3<sup>rd</sup> + noncoding

The APV positivity rate was 48.7% for all bird sellers together. *M. undulatus* was the species of origin of 54 positive samples and *N. hollandicus* gave one positive sample. The VP1 gene as it appears in the phylogenetic tree for APV shows two main clades (Fig. 1). The tree indicates that the *Polyomavirus* sequences in

this study, which are one designated TR/Erzurum/APV36 from *N. hollandicus* under GenBank accession number MH612850 and one designated TR/Erzurum/APV70 from *M. undulatus* under GenBank accession number MH612851, were genetically distinct from the GenBank database Far Eastern and American strains submitted

from those regions, whereas they were more closely related to *M. undulatus*-derived strains from Poland. An observation of note is that the TR/Erzurum/APV36 strain (isolated from *N. hollandicus*) formed a separate small cluster with the TR/Erzurum/APV70 strain (isolated from *M. undulatus*), indicating very high similarity. The three Polish strains that were reported in 2015 were grouped with the European strains, whereas the German strains that were reported in 1988 were placed in a separate cluster.

The proportion of PBFVDV positive samples was 23.0%, *M. undulatus* yielding 25 isolates and *N. hollandicus* one and the aggregate positive sample total for both viruses being 113. The presentation of the *rep* gene in the phylogenetic tree shows two main clades (Fig. 2). The tree makes apparent that the PBFVDV-positive sequences in this study, which are the three of TR/Erzurum/BFDV11, TR/Erzurum/BFDV16, and TR/Erzurum/BFDV70 from *M. undulatus* with the respective GenBank accession numbers MH612846, MH612847, and MH612849 and the single TR/Erzurum/BFDV28 from *N. hollandicus* with the GenBank accession number MH612848, were genetically distinct from the five other PBFVDV-positive strains previously reported from Turkey. However, they were more closely related to *Psittacula krameri* and *M. undulatus* sequences from Iran. Two interesting outcomes are that the TR/Erzurum/BFDV28 strain (isolated from *N. hollandicus*) formed a separate small cluster with an Iranian *P. krameri* sequence, and TR/Erzurum/BFDV11, BFDV16, and BFDV70 (isolated from *M. undulatus*) comprised a different cluster with three Iranian *M. undulatus* sequences. The seven South African strains that were submitted between 2006 and 2014 to the GenBank database were closely placed in a separate cluster (Fig. 2).

In this study, 14 out of 113 (12.4%) samples were positive both for APV and PBFVDV. All coinfecting birds were *M. undulatus*, and these birds were between 2 and 12 months of age. The birds tested in this study for PBFVDV and APV were between 2 and 24 months of age. The single *N. hollandicus* with a dropping sample positive for PBFVDV and its companion with a sample positive for APV were birds of 24 months old. No age and positivity correlation were detected in the other bird species tested in this study.

## Discussion

In this study, we first investigated the occurrence of APV and PBFVDV in 113 fresh dropping samples from different companion bird species in January–May 2016 and August 2020 in eastern Turkey. The total of 55 APV- and 26 PBFVDV-positive samples which were found all had their origin in *M. undulatus* and *N. hollandicus*. Further characterisation of the isolates from these positive samples by APV-VP1 and PBFVDV-*rep* gene-based phylogenetic analysis showed their close relatedness to other positive sequences from various

countries including Poland in respect of APV sequences and Iran in respect of PBFVDV, suggesting the widespread occurrence of these infections.

APV has been detected in Australia (10), Costa Rica (4), Poland (18), Italy (2), and Taiwan (9), with prevalences of 13%, 4.8%, 22.2%, 0.8%, and 15.2%, respectively. PBFVDV has been reported in Australia (10), Costa Rica (4), Poland (18), Germany (19), Italy (2), and Taiwan (9), in 31%, 19.7%, 25.3%, 39.2%, 8.05%, and 41.2% of tested samples, respectively. Although the APV prevalence (0.8%–22.2%) was relatively low compared to PBFVDV (8.0%–41.2%) worldwide (13), the APV rate was higher than PBFVDV in this study. In a study conducted in Turkey, APV (14.5% overall detection rate in *M. undulatus*, *P. erithacus*, and *Psephotus haematonotus*) and PBFVDV (19.3% overall detection rate in *M. undulatus* and *P. erithacus*) nucleic acids were detected in feather samples taken from clinically sick birds (1). In the current study, the APV rate (48.7%) was found to be considerably higher than in other reported studies (2, 6, 17), whereas the PBFVDV rate (23.0%) was only slightly different. This finding indicates that APV has wide dissemination in asymptomatic *M. undulatus* and *N. hollandicus* individuals around eastern Turkey.

The comparison of the sequence analyses revealed that the VP1 region of avian polyomavirus was conserved between isolates with 99.0% to 100% similarity (25). The TR/Erzurum/APV36 and TR/Erzurum/APV70 sequences in the phylogenetic tree obtained by sequencing of the VP1 region were in the same cluster as the sequences reported in Poland (3). The detection of avian polyomavirus in different bird species may be due to the fact that it is unrelated to the origin and genus of birds.

Previously, Altan *et al.* (1) reported that PBFVDV was detectable in feather samples of *M. undulatus* and *P. erithacus* in western Turkey. The sequences from that study were genetically distinct from the TR/Erzurum/BFDV11, BFDV16, BFDV28, and BFDV70 sequences as determined in the phylogenetic tree based on the *rep* gene (Fig. 2). The five PBFVDV sequences from the previous Turkish study (1) shared two main clusters with South African strains. In contrast, Erzurum/BFDV/28 sequences (isolated from *N. hollandicus*) were in the same clusters as a *Psittacula krameri* PBFVDV sequence in Iran and other sequences were also close matches to several *M. undulatus* sequences of PBFVDV in Iran. A study in Poland reported that PBFVDV strains were highly recombinant, hence, it showed that sequences from different countries could be in the same node (12). It was also reported that the mutation in circoviruses could happen in their core genomes (7, 11) and tended to be species- and region-specific (21).

PBFVDV and APV were detected in blood, feather, faeces, and visceral organs by molecular and serological methods (20, 25). It has been suggested that periodic sampling of faecal specimens to elucidate the shedding of APV and PBFVDV would contribute to the detection

of these infections (10). Faecal specimens, which are easier to collect than the other sample types especially during the breeding season, may easily be used for uncovering infections. Thus, fresh dropping samples were used to investigate both viruses in this study.

PBFDV and APV detection based on serology and PCR is costly when applied correctly together with quarantine and hygiene measures. Recent studies have shown that these precautions are not enough to remove the long-term threat of PBFD. The infective nature and progressive morbidity of the disease can result in the culling of infected birds, but the culling option is nonsensical for animals threatened with extinction (20, 24). Adult and fledgling birds alike are at significant risk, as adult birds can carry their infections without any clinical signs (15). While researchers have developed a vaccine against APV infection (16), an effective vaccine against PBFDV infection has not yet been created. The APV vaccine is used in relatively limited territories (16). It is recommended that a comprehensive hygiene strategy, screening protocols, vaccination, and long quarantine measures be implemented in hatcheries to prevent APV disease and PBFD.

In this study, we report for the first time the high rate of APV and PBFDV from *M. undulatus* and *N. hollandicus* bird dropping swab samples in eastern Turkey. Phylogenetic analysis indicated that the APV was closely related to Polish sequences, whereas the PBFDV was placed with the Iranian sequences. The five Turkey PBFDV sequences reported in 2016 constituted a separate cluster in the phylogenetic tree. The phylogenetic analysis indicates that these diseases can be transmitted *via* imported birds. Hence, early diagnosis, especially by PCR, is very important to protect healthy birds. Also, measures such as routine testing, elimination of positive birds, and application of strict hygiene rules should be undertaken in the import process to control the spread of the diseases with APV and PBFDV agents that may cause economic losses.

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