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Article

Discovery of Avian Paramyxoviruses APMV-1 and APMV-6 in Shorebirds and Waterfowl in Southern Ukraine

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Abstract: Emerging RNA virus infections are a growing concern among domestic bird and poultry industries due to the severe impact it can have on the flock health and economic livelihoods. Avian paramyxoviruses (APMV) are pathogenic, negative sense RNA viruses that cause serious infections in the respiratory and central nervous system. APMV was detected in multiple avian species during the 2017 migration season in Ukraine, and studied using PCR, virus isolation, and sequencing. Of the 4090 wild bird samples, eleven swabs were isolated in chicken embryos and identified for APMV serotype by hemagglutinin inhibition test: APMV-1, APMV-4, APMV-6, APMV-7. At a variety of sites in Ukraine we characterized the virulence of the virus and further analyzed and predicted the potential risks of spillover to immunologically naïve populations. RNA was extracted and amplified using a multiplex-tiling primer approach to encompass full APMV genomes. Full-length APMV-1 (n=5) and APMV-6 (n=2) genomes were sequenced on an Oxford Nanopore MinION device in Ukraine. All APMV-1 and APMV-6 fusion (F) proteins possessed a monobasic cleavage site, suggesting these APMV were likely low virulence, annually circulating strains. Utilization of this low-cost method will identify gaps in viral evolution and circulation in this understudied but important critical region for Eurasia.

Keywords: Viral Ecology; Surveillance of Avian Paramyxoviruses; APMV; wild birds; next generation sequencing; MinION; Azov-Black Sea region in Ukraine

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1. Introduction

Avian paramyxoviruses (family Paramyxoviridae; also known as avian avulavirus) are a highly diverse group of zoonotic, negative sensed, single stranded RNA viruses detected in a variety of domestic and wildlife species. Avian paramyxoviruses (APMV) range in genomes sizes from 13-17 kilobases (kb) long comprising 20 unique subtypes or serotypes (1-20) classified into three distinct genera according to the International Committee on Taxonomy of Viruses: metaavulavirus (serotypes – 2, 5, 6, 7, 8, 10, 11, 14, 15, 20); orthoavulavirus (serotypes – 1, 9, 12, 13, 16, 17, 18, 19); and paraavulavirus (serotypes – 3, 4). Among these species of viruses is avian orthoavulavirus serotype 1 (APMV-1) that commonly induces Newcastle disease [1]. In previously published research, APMV-1 is commonly referred to as Newcastle disease virus (NDV), particularly when found in domestic poultry, while APMV-1 was commonly characterized in wild birds [1]. The pathogenicity in avian paramyxoviruses ranges from velogenic (high), mesogenic (mild), to lentogenic (low) depending on the strain, the serotype, infected host, and specific molecular characteristics of the proteolytic cleavage site located in the fusion protein [2]. Severe avulaviruses infections (mainly for APMV-1/NDV) cause symptoms including depression, conjunctiva, mucosal excretions from the crop, green diarrhea, hemorrhages, necrosis and misshapen egg production [3]. APMV-1 (Newcastle disease) is an emergent disease and is very important for poultry industry, which typically induces neurotropic symptoms associated with velogenic Newcastle disease (ND) including lethargy, muscle tremors, paralysis and ultimately death [3]. APMV-1 has shown to elicit severe symptoms and is of highest concern because of the deleterious effects it can have on the poultry industry [3,4]. APMV is found worldwide among a variety of species; however, each serotype will elicit a suite of symptoms depending on the species infected.

APMV of different serotypes is commonly isolated from poultry and other domesticated birds, however, other wildlife hosts have been described [5–11]. APMV-1 is the most diverse of the serotypes and is classified into two distinct classes (class I or class II) and further characterized into either 1 genotype (class I) or 15 genotypes (class II) [8]. Class I viruses is solely isolated from wild birds (e.g. non-farmed birds), while class II viruses encompasses animals from poultry, domesticated, and wild captive populations [8,12]. Little is known about the effects of other APMV serotypes in wildlife vectors due to the limited amount of data collected.

Aside from APMV-1, other serotypes of avulavirus are less prevalent in domestic birds and poultry, however, other serotypes are typically isolated from wild birds. Among these, avian metaavualavirus 6 (APMV-6) has been described to cause mild respiratory infection as well as reproductive implications in poultry and turkeys [3,13,14]. The first description of APMV-6 was isolated from a domestic duck in Hong Kong in 1977 [13]. It was suggested that APMV-6 can be further characterized into two distinct subgroups based on both the antigenic and genomic make-up of the viruses found within the serotype [13]. APMV-6 is genetically different from other APMV serotypes because it encodes a small hydrophobic (SH) protein. The SH protein has been identified in other paramyxoviruses, with an unique function for the associated virion [13]. The function of the SH protein in APMV-6 is still unknown, however, it was hypothesized to be the oldest ancestor of all avian paramyxoviruses due to the similarity of the SH protein as seen with other viruses in *Paramyxoviridae* family. The diversity of avian paramyxoviruses is widespread among hosts, however, there is a large gap in knowledge still in understanding the movement of these viruses within wild populations. A large portion of the data collected is dependent on existing bird sampling efforts for other viruses (e.g. avian influenza viruses, or AIV) with consequent bias towards geographical regions.

Ukraine is a hot spot for wild bird populations because of the geographical region spanning three major flyways: East Africa – West Africa; Central Asia; and the Black Sea – Mediterranean. Similar to other RNA viruses (e.g. AIV) the introduction of APMV to naïve populations is thought to be transmitted from wild migratory birds [6,7,8,10]. Horizontal transmission occurs through oropharyngeal and fecal secretions thus resulting in

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an easy route for species to contract infection. Birds are highly gregarious and inhabit similar physical locations, so the potential for transmitting viral pathogens and commensal microbes is high [15]. APMV and especially APMV-1 are important pathogens to control in the Ukrainian poultry industry to maintain a healthy and thriving poultry industry. Importantly, APMV-1 outbreaks in poultry occur worldwide, so understanding of APMV-1 subtypes and pathotypes has potential to contribute to deeper understanding of APMV distribution and evolution worldwide.

Broad wild bird surveillance for AIV and APMV was conducted from 2006 to 2016 in Ukraine, in regions observed to be on intercontinental flyways for wild birds in Eurasia (North-South and East-West flyways). A total of 21,511 samples were collected from 105 species of wild birds representing 27 families and 11 orders. Eighty-two low pathogenic avian influenza (LPAI) viruses were isolated from wild birds with a total of 23 antigenic hemagglutinin (HA) and neuraminidase (NA) combinations [16]. Fifteen of 16 known avian influenza HA subtypes were isolated. Five H5N8 highly pathogenic avian influenza (HPAI) viruses and two H5N2 LPAI viruses were isolated from live wild birds and environmental samples (fresh bird feces) from samples collected for surveillance to understand risks of AIV and APMV outbreaks in poultry [17,18].

Within Ukraine over 154 RNA viruses have been isolated including 14 AIV hemagglutinin subtypes detected among 66 wild bird species [16,17,18]. In previous Ukrainian bird surveillance efforts APMV-1, APMV-4, APMV-6, and APMV-7 were detected by serological analyses of APMV isolated from wild bird samples; however, the genetic sequences and severity of associated diseases have been poorly understood. Twenty APMV viruses were isolated and subsequently identified as APMV-1 (n= 9), APMV-4 (n=4), APMV-6 (n=3), and APMV-7 (n=4) in Ukraine from wild birds in 2006-2011 [11]. Currently in Ukraine, diagnosis of APMV and NDV has relied on PCR, virology and serological analysis, however, it can be difficult to distinguish between velogenic and lentogenic strains of APMV without the whole genome sequence [3]. Sequencing APMV genomes can alleviate problems with ambiguity of serological assays, and identify genetic characteristics important for pathotyping [8]. To build genomic sequencing capacity for avian pathogens, we developed protocols for, and deployed the Oxford Nanopore Technologies (ONT) MinION portable nanopore sequencing platform in sentinel veterinary health labs in Ukraine. MinION has revolutionized avian disease surveillance efforts in low-resource regions that previously relied on sending samples to high throughput sequencing labs at an expensive cost [19].

Ukraine relies on the agricultural industry with an important poultry component (235-250 million birds) divided among industrial poultry and backyard flocks. The impacts of Newcastle disease outbreaks on the agricultural industry are large and can affect the economy [20]. For this reason, surveillance, isolation, and sequencing of APMV of different subtypes is very important for the persistence of Ukraine and Europe's agricultural industry. Thus, we isolated and sequenced novel APMV-1 and APMV-6 from wild birds in Ukraine, and analyzed the phylogenetic relation of these pathogens with known isolates of APMV from Eurasia.

2. Materials and Methods

2.1. Sample collection

Wild bird surveillance was conducted from December 2016 to December 2017 in northern and southern regions of Ukraine (**Figure 1**). Research was conducted under an approved IACUC protocol (NSC IECVM UP-4 IACUC and ethical review, protocols approved: 18 Jan 2017; #1-19, 20.02.2019; #2-19, 22.02.2019). In cooperation with ornithologists who helped identify bird species, 4,790 fecal (environmental) samples were collected from 40 species of wild birds (**Table S1**). Sampling from wild birds was carried out according to standard operating procedures (SOP) describing biosafety measures for collectors, sample collection and preservation, and safe cryopreservation (liquid nitrogen) and overland transport. Sample collection was dependent on flock size; at least 25 samples

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per 500 birds in the flock and at least 50 samples per 1,000 birds in the flock. Feces were collected only if the origin and type of bird had been established. Samples of feces were taken in a checkerboard pattern at a distance of at least 1.5-2 m from each other, to avoid selecting feces from the same bird. 4315 fecal samples were collected from the Azov-Black Sea region of Southern Ukraine (Kherson, Odesa, Mykolaiv, Zaporizhzhya Oblasts), one of the most important regions in Eastern Europe, in regard to wild birds of different ecological groups. 475 fecal samples were obtained from wild birds in North of Ukraine (Chernihiv Oblast).

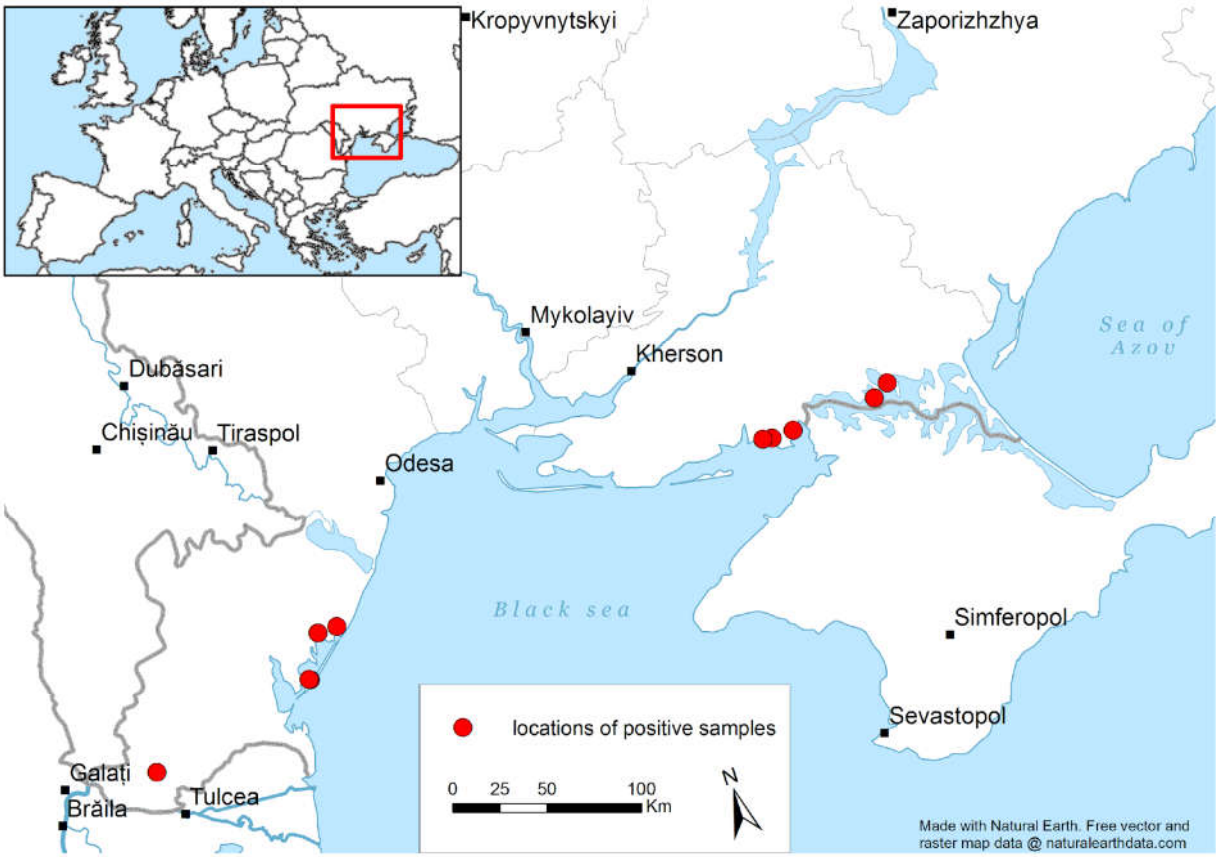

Figure 1. Map of locations of samples that were positive for avian paramyxoviruses in southern Ukraine. Environmental (faeces) samples from wild birds were collected in southern Ukraine (2016-2017) in Kherson, Odesa, Mykolaiv, and Zaporizhzhya *Oblasts*. Locations where samples tested positive for avian paramyxoviruses by diagnostic RT-PCR, with C_t value < 45, are indicated (•). Positive wild bird samples were found in proximity to brackish or saltwater *liman* and Sivash (bays) in Kherson *Oblast* near Crimea, and in proximity to Sasyk Lagoons and the Danube River Delta region in southern Odesa *Oblast*. .

Samples were collected in cryotubes containing 1.0 ml of viral transport media (BHI, Brain Heart Infusion broth, Sigma-Aldrich, #53286-100G) with antibiotics (penicillin 10,000 U/ml, streptomycin 10 mg/ml, gentamicin 250 µg/ml and nystatin 5,000 U/ml). Commercially available powdered concentrates were prepared and sterilized immediately prior to use [21]. Fecal samples were stored at –196° C in liquid nitrogen, where they were kept until processing.

2.2. Virus isolation

Virus isolation from the fecal samples was conducted in accordance with typical OIE procedures [22,23]. Fecal samples were inoculated into the allantoic cavity of 9-10 day old embryonated chicken eggs and passaged three times *in ovo*. The presence of hemagglutinating (HA) viruses in allantoic fluid was determined by a hemagglutinin inhibition (HI)

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test with a 1% suspension of chicken red blood cells [22,23]. Samples from PCR positive pools were retested individually to identify positive sample(s).

2.3. Virus identification

The HA virus subtype was determined by HI tests (previously described) [22–24]. For these studies, the following antisera were used: H1N1, H2N3, H3N8, H4N6, H5N1, H6N8, H7N1, H8N4, H9N2, H10N7, H10N9, H11N6, H12N5, H13N6, H14N6, H15N9, H16N3, APMV-1, APMV-2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by Veterinary Laboratories Agency (Animal and Plant Health Agency, Weybridge, UK); and antisera to H1N1, H2N3, H3N8, H4N8, H5N3, H6N2, H7N3, H8N4, H9N7, H10N1, H11N9, H12N5, H13N6, H14N5, H15N9, H16N3, APMV-1, APMV -2, APMV-3, APMV-4, APMV-6, APMV-7, APMV-8, and APMV-9 produced by the Instituto Zooprofilattio Sperimentale delle Venezie, Padova, Italy. Positive sera produced at NSC IECVM (Kharkiv, Ukraine) against HPAI virus A/chicken/Syvash/02/2005 (H5N1) and LPAI virus A/teal/Djankoy/4-17-11/2010 (H5N2) were also used.

2.4. RNA extraction and diagnostic RT-PCR for APMV

Pooled samples were analyzed by real-time reverse transcription PCR (RT-PCR). RNA extraction was performed using QIAamp Viral RNA Mini Kit (QIAGEN). Extracted RNA was stored at -70^{o}C and used for amplification and detection of APMV-1 in one-step RT-PCR using AgPath-ID™ one-step RT-PCR Reagents (Applied Biosystems). The RT-PCR analysis was qualitative by analysis of cycle threshold (C_t) values. Detection of APMV-1 by qRT-PCR was conducted using AgPath-ID™ One-Step RT-PCR reagents and specific primers according to Czegledi et al. (2006) [25]. The following primers and probe was employed for qRT-PCR [26]: Forward primer M+4100: 5'-AGTGATGTGCTCGGAC-CTTC-3'; reverse primer M-4220: 5'-CCTGAGGAGAGGCATTTGCTA-3'; Probe M+4169: 5'-[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3' All samples from APMV-1 positive pools were tested by the same method.

2.5. Dataset development for genome assembly

Datasets were created to assemble sequencing reads as well as to reconstruct phylogenetic relationships. Sequences were downloaded from the Virus Pathogen Resource (ViPR, at https://www.viprbrc.org/) or NCBI GenBank (https://www.ncbi.nlm.nih.gov/) and were specified to include full length viral genomes. Vaccine strains and duplicated sequences were removed from the dataset. Metadata was collected for each sequence from GenBank or ViPR including the following fields: host (common name), sample location, and sample collection date. Representative sequences from each serotype were compiled for preliminary analysis and genome assembly as described above.

2.6. Development of tiling primers for full genome amplification

Due to variable viral load in each sample, RNA was amplified to maximize sequencing efficiency. Viral RNA was amplified in either 1000 nucleotides (nt) or 1500 nt regions generated spanning the viral genome with a 50 nt or 200 nt overlap, using the Primal Scheme program (http://primal.zibraproject.org/) [27]. Sequences presented here utilized both types of schemes (1000 nt region with 50 nt overlap; 1500 nt region with 200 nt overlap), however, schemes with a 1500 nt region and a 200 nt overlap were more efficient and cost effective for amplification (**Table S5**). Primers (reverse and forward) were pooled into four pools according to primal scheme output.

2.7. Amplicon synthesis, cDNA library preparation, and bioinformatics

APMV genome sequencing allowed simultaneous identification of serotype (subtype) and evaluation of pathotype of the virus. Previous sequencing of virus genomes has relied on resource-intensive Illumina or Sanger sequencing. However, with the development of Oxford Nanopore Technologies (ONT) third generation sequencer, MinION,

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sequencing is portable, inexpensive, and has the ability to produce real time data within hours [28].

RNA was amplified using Superscript III One Step protocol (Invitrogen) following the manufacturer instructions, with the tiling primers described above, and the following thermocycling conditions: 2 min at 55º C, 60 min at 42 º C, 2 min at 94 º C, 39 cycles of 30 sec at 94 º C, 35 sec at 55 º C., 2 min at 68 ºC. Final elongation was set for 5 min at 68 ºC. SPRI bead clean-up using Agencourt AMPure XP beads (1:1 sample to beads) removed impurities from the PCR amplicon preparations for subsequent sequencing steps. All samples were barcoded using ONT Native Barcoding kit (EXP-NBD 104) in equimolar concentrations according to the manufacturer's protocol. The cDNA library was prepared using ONT genomic sequencing ligation kit (SQK-LSK 109) and sequenced for 48 hours on a FLO-MIN 106 flow cell (R9.4.1) using a MinION Mk1B device.

All reads were basecalled using Guppy v3.4.4 (ONT) default parameters. Reads were demultiplexed and barcodes were trimmed using Guppy v3.4.4 (ONT; guppy_barcode) default parameters. Reads were filtered for quality with a q score ≥ 10 (min_mean_q 90) and a length ≥ 200 bp (min_length 200) using Filtlong v.3.0 (https://github.com/rrwick/Filtlong). Samples with more than 800,000 reads were downsampled to facilitate computational analysis by increasing the min_length value based on the N50 score. AMPV subtype was confirmed by mapping reads to a reference database containing representative sequences from each APMV subtype (**Table S6**) using Minimap2 v2.17 [29]. Following initial read-based subtyping, reads were subsequently mapped to a subtype specific database (e.g. all published APMV-1 or APMV-6 genomes respectively as described above in section 2.5) using Minimap2 (default parameters) to identify the closest reference genome match [29]. Reads were re-mapped to the APMV reference genome with the most hits using Minimap2, and further analyzed with bedtools v2.29.2 to identify the coverage of reads across the genome of each segment [29,30]. Medaka (model r941_min_high) v0.11.5 was used to generate a consensus sequence, filtered reads were used as the input and the reference sequence specified earlier was used as the reference scaffold.

Genomes were assembled by aligning the consensus sequence to the NCBI reference sequence GenBank file (containing protein features) using Muscle pairwise aligner v.3.8.425 with default parameters through Geneious v.11.0.3 program [31]. Genome assemblies indicate similar sizes typical for APMV-1 and APMV-6 respectively (**Figure S1-S2; Table S7**). Homopolymers due to sequencing errors were identified in each sample and were manually deleted: APMV-1/Mallard/Myt Kherson/1-4/4-09/17 had 1 manual deletion; APMV-1/Mallard/AN Kherson/TM434778/2002 had 4 manual deletions; APMV-6/Environmental/ND Kherson/41-45/7-08/17 had 2 manual deletions; APMV-6/Mallard/ND Kherson/11-15/4-09/17 had 1 manual deletions; APMV-1/mallard/Dr Kherson /1-3/5-09/17 had 3 manual deletions; APMV-1/Grey goose/Myt/1-4/4-09/17 had 2 manual deletions; and APMV-1/Shelduck/Chur/1-5/2-11/17 had 4 manual deletions (**Tables S8-S14**). We tested primer amplification, nanopore MinION sequencing, and bioinformatics assembly protocols using a lab-grown vaccine strain of Newcastle disease virus (NDV/APMV-1) La Sota (a kind gift of Dr. Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai, NY), and captured >99% identity to the NDV reference strain (AF077761.1).

2.8. Phylogenetic analysis

Phylogenetic analysis was used to analyze the evolutionary relationships within and between the APMV-1 and APMV-6 genomes. Sequences were stratified according to APMV group, host species, year and location (lowest administrative unit) based on information contained in the strain name. Downsampling of the published APMV genomes was performed to allow a maximum of one sequence for each combination of APMV group-host-year-location, resulting in a final dataset of 322 sequences representing genomes from all 14 subtypes. The sequences were aligned using MAFFT v.7.450-1 with

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auto alignment methods and default parameters [32–34]. Alignment was manually edited to eliminate exogenous features altering biological integrity of the alignment using Jalview v2.11.0 [35]. RAxML (Randomized Axelerated Maximum Likelihood) v.8.2.4 with high performance computing (HPC) was used to reconstruct maximum likelihood trees using a generalized time reversible (GTR) substitution model with a gamma rate of heterogeneity and a 99 random seed parsimony [36]. Significance of trees were tested by bootstrap support analysis (100 iterations). The bestTree output with highest likelihood support was visualized using FigTree v.1.4.4 specifying a midpoint tree root and a decreasing node order.

In addition, evolutionary relationships among avian avulavirus sequences were inferred using the time-scaled Bayesian approach using BEAST v1.10.4 [37]. Evolutionary relationships were constructed using a HKY nucleotide substitution model, a constant coalescent, and an uncorrelated relaxed clock [38,39]. Four independent Markov Chain Monte Carlo runs were performed each having 300,000,000 state, sampling every 30,000 states, to generate trees and posterior probabilities of nodes.

3. Results

3.1. Avian avulavirus detection in wild birds in Ukraine

From December 2016 to December 2017, 4790 fresh fecal specimens were collected from the environment in proximity to wild birds, in conjunction with ornithological surveys of avian populations, ecology and disease surveillance. Samples were stored immediately in liquid nitrogen and shipped to veterinary laboratories for screening of avian influenza and paramyxoviruses by diagnostic RT-PCR and virus isolation *in ovo*. To uncover host species diversity of AIV and APMV in Ukraine, a broad selection of samples was collected from 40 avian species from seven different orders: Anseriformes, Charadriiforms, Podicipediformes, Gruiformes, Ciconiiformes, Pelecaniformes, and Falconiformes (**Table S1**). Samples were collected across avian migratory periods in the Azov-Black Sea region of Ukraine, and in the north, during the fall migration (August-October), wintering (November-February), spring migration (March-May), and during the localized movement in June-July that typically occurs after the nesting period.

Eleven samples collected from Odesa (n=5) and Kherson (n=6) in southern Ukraine tested positive for APMV-1 by diagnostic RT-PCR (**Figure 1**). APMV-1 infection rate (by RT-PCR testing) in wild birds was 0.23%, varying from 0.09% to 2.43% (**Table S2**). A majority of samples that were PCR-positive for APMV-1 were obtained from waterfowl, including white-fronted goose (*Anser albifrons*) (n=1), mallard duck (*Anas platyrhynchos*) (n=4), whooper swan (*Cygnus cygnus*) (n=1), common shelduck (*Tadorna tadorna*) (n=2), white pelican (*Pelecanus onocrotalus,* n=1); and shorebirds including a Mediterranean gull (*Larus melanocephalus*; n=1), and a snipe (*Gallinago gallinago*; n=1) (**Table S2**). No samples tested positive in northern Ukraine (Chernihiv Oblast).

Among the RT-PCR positive samples, 40 hemagglutinating isolates were detected from wild bird fecal samples amplified by virus isolation *in ovo*, and serotyped by hemagglutination inhibition (HI) assay. HI positives included avian influenza (n=19) and avian paramyxovirus (n=11), including those also identified by RT-PCR diagnostic assays. In 2017 the isolation rate by virus inoculation *in ovo* of APMV among the screened samples varied from 0.26% to 1.94% with the highest rate of isolation from ruddy shelduck (*Tadorna ferruginea*) (**Table S3**). Among the 11 avulavirus isolates, APMV-1 (n=6), APMV-4 (n=2), APMV-6 (n=2), and APMV-7 (n=1) were serotyped by HI assays (**Table S4**). One sample showed evidence of both APMV-1 and APMV-7 serotypes, but we were unable to obtain sequence data to confirm mixed infection. A portion of positively hemagglutinating samples were unidentifiable in our serological assays (n=10: **Table S4**).

3.2. Sequencing and phylogenetic analysis

Genomes of 5 new APMV-1 and 2 new APMV-6 isolates were constructed by reference-based assembly with a range in sizes from 14,098 nt to 16,235 nt consistent with the

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average sizes of both APMV-1 and APMV-6 (**Table 1**; **Table S7**). In general, high quality complete genomes were obtained by nanopore sequencing on a MinION device in veterinary labs in Ukraine. APMV-1/Mallard/Myt Kherson/1-4/4-09/17 had no coverage until position 885 along the genome and had adequate coverage (>25x) until position 14,530 where the coverage dropped to 0x and a small region between 13,680 -13,998 having an average 20x coverage; the remaining samples had adequate >25x coverage across the genome except for the ends in the non-coding regions (**Figure 2**; **Figure S1-S2**; **Table S7**). Genome assemblies indicate similar sizes typical for APMV-1 and APMV-6 respectively (**Figure S1-S2**; **Table S7**).

The APMV-1 and APMV-6 genomes sequenced in this study were compared to 315 full length avian avulaviruses with representatives from each serotype (except APMV-17, APMV-18, and APMV-19 due to lack of publicly available full-length sequence data), by construction of a maximum likelihood (ML) phylogenetic tree (**Figure 3**). Bayesian analysis of the same 322 sequenced had similar topology to the maximum likelihood analysis (**Figure 4**). Interpreting the phylogenies, the Ukrainian APMV-1 and APMV-6 sequences showed the highest similarity to the reference sequences used in the genome assembly. The APMV-1 viruses isolated from Ukraine clustered with duck and other wild Anseriformes viruses detected across Eurasia, with a subclade in Ukraine and Russia (ML bootstrap value, 98/100, 100 bootstraps; Bayesian posterior, 0.77), and two distinct sublineage branches (ML bootstrap value, 98/100, 100 bootstraps; Bayesian posterior, 0.71) that suggest at least two separate infection events (**Figure 3, Figure 4**). Similarly, the APMV-6 viruses formed subclades that appeared to be the result of multiple distinct infection events (bootstrap value, 100; Bayesian posterior, 0.92) among ducks and wild Anseriformes originating in a broad group of APMV-6 sequences spanning Europe and Asia (**Figure 4**). These results suggest ecologically diverse APMV-1 and APMV-6 reservoirs in Eurasia, with multiple host species and broad geographic ranges.

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Table 1. APMV viruses isolated from Ukraine during the 2016 - 2017 field season. Both APMV-1 and APMV-6 were predominately isolated from the southern Oblasts of Ukraine. HI = hemagglutinin assay. .

Sample name	HI Test Results	Sample Description
APMV1|Mallard|Myt_Kherson|1-4-4-09|2017	APMV1	Genetic material, RNA. The material was collected in September 2017 in Kherson Oblast from clinically healthy wild mallard.
APMV1|Mallard|AN_Kherson|TM434778|2002	APMV1	Genetic material, RNA. The material was collected in December 2002 in Kherson Oblast from clinically healthy wild mallard.
APMV-6|Environmental|ND Kherson|41-45|7-08|2017	APMV6	Genetic material, RNA. The material was collected in August 2017 in Kherson Oblast from environmental.
APMV-6|Mallard|ND Kherson|11-15|4-09|2017	APMV6	Genetic material, RNA. The material was collected in September 2017 in Kherson Oblast from clinically healthy wild mallard.
APMV-1|Mallard|Dr_Kherson|1-3|5-09|2017	APMV1	Genetic material, RNA. The material was collected in September 2017 in Kherson Oblast from clinically healthy wild mallard.
APMV-1|Grey_Goose|Myt_Kherson|1-4|4-09|2017	APMV1	Genetic material (RNA) collected in September 2017 in Kherson Oblast from clinically healthy wild grey goose.
APMV-1|Shelduck|Chur_Kherson|1-5|2-11|2017	APMV1	Genetic material (RNA) collected in November 2017 in Kherson Oblast from clinically healthy wild shelduck.

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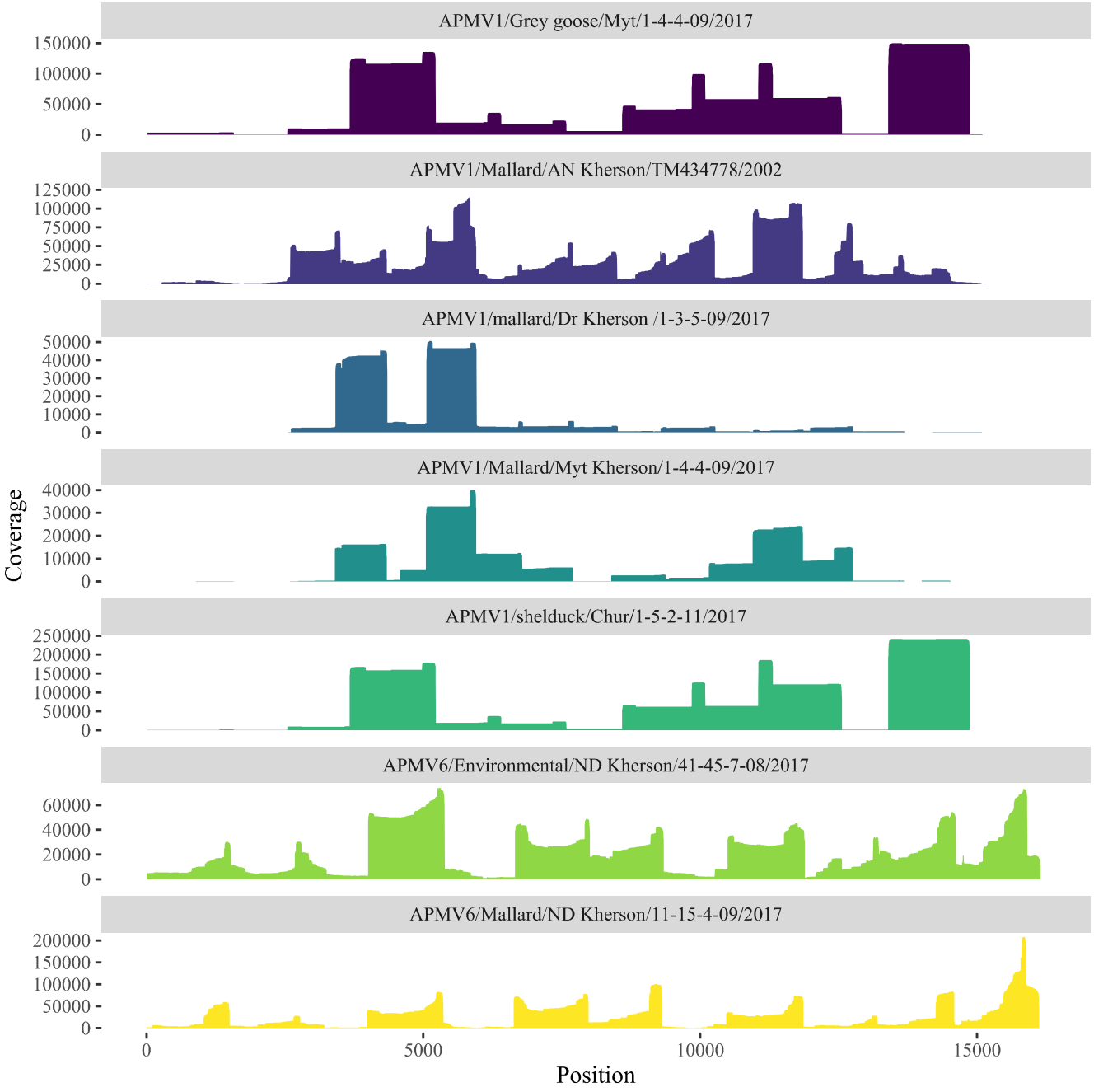

Figure 2. Sequencing coverage depth across full-length genomes for the seven APMV samples sequenced in this study. Viral RNA was amplified by RT-PCR with a panel of tiling primers covering the whole genome, and cDNA amplicons were sequenced using MinION. Reads were mapped to a reference and coverage depth was calculated with the bedtools genomic arithmetic package.

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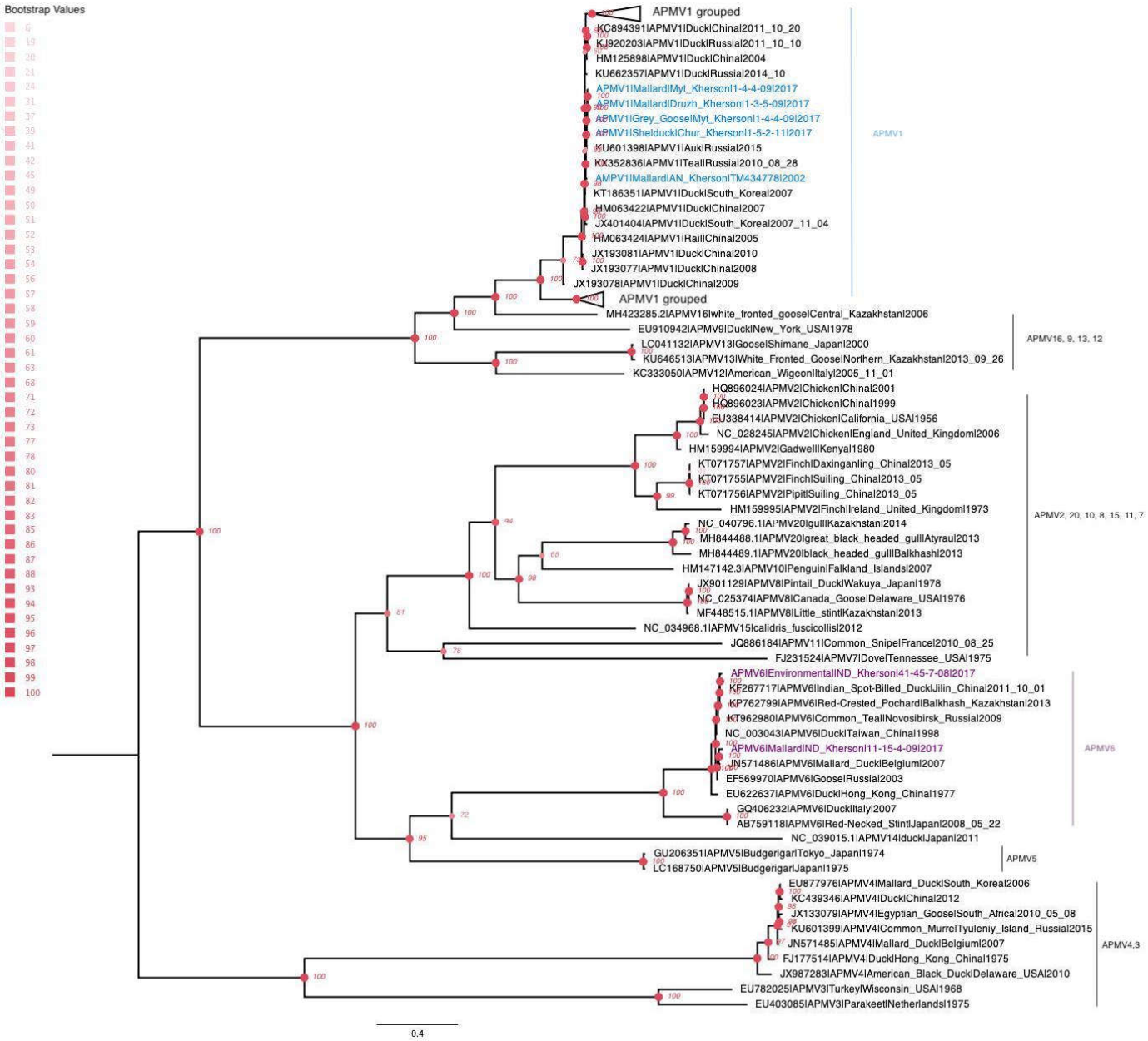

Figure 3. Global phylogenetic analysis comparing APMV-6 and APMV-1 to all avian avulavirus serotypes. Maximum likelihood tree showing evolutionary relationships among 322 full length APMV sequences, including the 7 sequenced in this study, and the phylogenetic position of Ukraine APMV-6 and APMV-1 sequences within their serotypes. APMV-1 strains sequenced in this study are highlighted in blue and APMV-6 sequenced from this study are highlighted in purple. 89 full length segments are displayed, and the remaining 233 sequences are collapsed in at the top of the tree. The collapsed sequences are all APMV-1 serotypes. Nodes indicate maximum likelihood bootstrap support under using bestTree and visualized in FigTree v.1.4.4 with a midpoint tree root.

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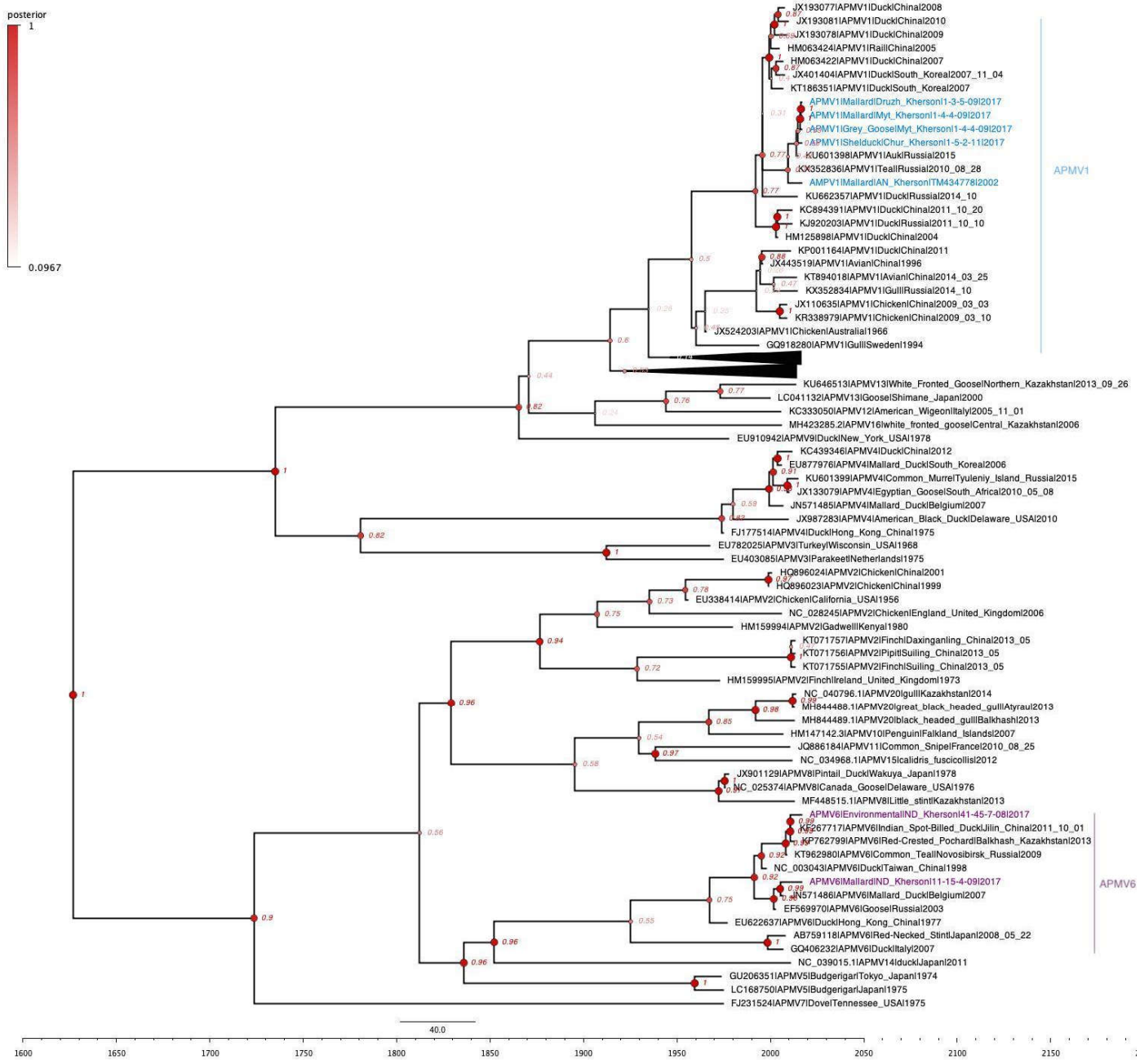

Figure 4. Phylogenetic analysis comparison of all APMV-1 and APMV-6 to APMV strains by a time-scale Bayesian maximum clade credibility tree of 315 APMV reference strains and seven isolated in Ukraine. APMV-1 strains sequenced in this study are highlighted in blue and APMV-6 are highlighted in purple. Collapsed sequences are APMV-1 serotypes. Nodes indicate posterior probability support with a midpoint tree root.

4. Discussion

Avian influenza surveillance in the Azov-Black Sea region in Ukraine has opened the door for detection of other avian pathogens such as APMV. 4,790 fecal samples from a variety of birds predominately from wild waterfowl and shorebirds (the Anseriformes and Charadiformes orders) were screened for AIV and APMV. In Ukraine, the mean paramyxovirus isolation rate ranged from 0.26% to 1.94% in a variety of Anseriformes species (Greylag Goose, Mallard, Common Shelduck, and Ruddy Shelduck; Table S2) which is low in comparison to the 2006 -2011 sampling seasons [11]. Muzyka et al. (2014) detected a seasonality effect in isolation rate, so further analysis will identify whether there was a seasonality effect during the 2017 season [11]. 11 samples tested positive for APMV-1

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based on a 121 nt region in the matrix protein. Most of the PCR positive samples were from the Anseriformes order (n=8), however, Chardiiformes (n=2) and Pelecaniformes (n=1) also tested positive (**Table S3**). The mean infection rate (by PCR detection) of APMV-1 across species was relatively low, however, White Pelicans did have a 2.43% mean infection rate. Large outbreaks of virulent APMV has led to large die-offs in cormorants and gulls, therefore infection in white pelicans that also occur in marine or coastal habitats is not surprising [40,41]. Circulation of APMV in gulls and pelicans can facilitate transmission if they are near poultry or other domesticated fowl farms [41].

For APMV, RT-PCR based diagnostics are the most cost effective and efficient means of identifying the virus, however, hemagglutinin inhibition test has traditionally been used to determine a subtype. 11 isolates tested positive for avian avulavirus (APMV1 n =6; APMV-4 n=4; APMV-6 n=2; APMV-1/7 n=7) which is consistent with previous reports in Ukraine [11,18]. To resolve ambiguities in the HI assay, full genome sequencing of selected APMV-1 and APMV-6 positive samples was employed. Seven full-length genomes were pre-amplified using a tiling primer RT-PCR approach and sequenced on MinION. All 7 APMV genomes were isolated from the host order Anseriformes: five APMV-1 genomes were isolated from Mallard (n=2), Shelduck (n=2) and a Graylag Goose (n=1), and an additional two APMV-6 genomes were sequenced from a Mallard and from an unidentified, fecal (environmental) sample, respectively. For one of the 5 APMV-1 sequences (APMV-1/Mallard/Myt Kherson/1-4/4-09/17), the 3′ end of the genome was not assembled, most likely due to insufficient amplification of this region. APMV-1 samples (aside from APMV-1/Mallard/Myt Kherson/1-4/4-09/17) ranged in size from 15,085 - 15,168 nt. APMV-6 samples ranged in size from 16,234 - 16,235 nt and are consistent with APMV-6 genomes.

Upon further analysis of APMV-1 genomes, virulent strains of APMV-1 depend on the proteolytic cleavage of a multi-basic amino acid motif in the fusion protein that facilitates furin-like proteases to stimulate proteolytic cleavage and activation from the host cell [2,42]. Samples sequenced in the study display a mono-basic site suggesting all strains are avirulent [2]. While the viruses sequenced in this study are avirulent, a mild respiratory infection can still impact a flock and cause economic stress [43]. Previous evaluation of APMV-6 genomes suggests infections in chickens are asymptomatic or mild in other species [13,44]. Xiao et al. (2010) analyzed a GAGGGGGAAG motif located upstream of each protein in the untranslated region except for matrix protein which has GAGGGGAAC motif. This is consistent with the APMV-6 genomes sequenced in this study [13]. Motif's downstream of each protein are conserved among APMV-6 strains and are found in this study to be consistent [13].

Phylogenetic analysis revealed that all five Ukrainian APMV-1isolates were closely related to APMV-1 that were sampled from a large auk (*Uria aalge*) in Tyuleny Island in the Sea of Okhotsk, and an Eurasian teal (*Anas crecca*) from southwestern Siberia (Russian Federation). In addition, the Ukrainian APMV-1 isolates also geographically clustered with those sampled from mallards and a water rail (*Rallus aquaticus*) in South Korea and China. These bird species of migrate annually between breeding grounds in Asia and the Siberian tundra and natural habitats in southern Europe and Africa as part of the Black Sea-Mediterranean flyway route [45]. Therefore, it is likely that APMV viruses circulate annually in southern Ukraine, in particular in the Azov-Black Sea region, since many bird species use this region as a stopover on the flyway during migration periods. The reconstructed phylogeny was focused on evaluating of evolutionary history based on full length APMV genomes, thus the partial sequences previously obtained in Ukraine were not included in the analysis. The isolate APMV1/Mallard/Myt Kherson/1-4-4-09/2017 failed to full length assembled due to the low coverage depth at the beginning of the genome (3′-UTR region). Inadequate sequencing at the beginning and end of the genome in the noncoding region is consistent when sequencing other RNA viruses such as Zika using the tiling primer approach [27].

The reconstruction of evolutionary relationships for APMV-1 and APMV-6 were remarkably similar despite belonging to entirely different clades/groups. Evidence of circulation in wild water birds, primarily belonging to the Anseriformes originating from

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Eurasia was a consistent pattern between APMV groups. The Bayesian analysis indicated a TMRCA (the time of the most recent common ancestor) estimates that APMV-1 and APMV-6 emerged in the early 2000's, however, historical inconsistency of APMV sampling across countries makes it difficult to clearly represent the temporal origin of the viruses.

The connectivity of Ukraine to regions including Europe, Central Asia, and East Asia via the long-distance movement of that Ukraine plays an important role in the mixing of virus populations from Europe (west) and Asia (east), consistent with studies of AIV and APMV in wild bird reservoirs in the Azov-Black Sea wetland ecologies of southern Ukraine [11,17,18]. While both the maximum likelihood and Bayesian phylogenetic trees identified similar topologies, APMV sequences are likely under-sampled given their prevalence. Robust sampling efforts are required to classify an emerging viral event in detail. Since APMV identification and isolation have largely based on AIV surveillance efforts, there is a historical discrepancy between APMV sampling and sequencing, making it difficult to predict the exact location and timing of emergence of the viruses sequenced in our study.

5. Conclusions

Building from longitudinal surveillance of avian disease pathogens in Ukraine, this study was the first to isolate and sequence seven full length avian avulavirus (APMV) genomes from wild ducks and geese in the Azov-Black Sea region in Ukraine. Five novel APMV-1 and two APMV-6 genomes were sequenced and clustered with contemporary circulating strains in Russia, Europe, and East Asia, suggesting a broad but understudied reservoir and transmission pattern of these pathogens across the Eurasian continents.

Supplementary Materials: Supplementary Table S1: Avian sampling, Supplementary Table S2: infection of APMV-1 in wild birds by RT-PCR, Supplementary Table S3: isolation of APMV *in ovo*, Supplementary Table S4: samples tested by HI assay from wild birds, Supplementary Table S5: APMV Tiling primer sequences (spreadsheet .xlsx), Supplementary Table S6: APMV Reference sequences used to build APMV reference database (.fasta), Supplementary Table S7: APMV-1 and APMV-6 samples sequenced, Supplementary Table S8-S14: Amino acid substitution tables for novel APMV-1 and APMV-6 in comparison to reference genomes, Supplementary Figure S1. Genome assembly of APMV-1 sequences, Supplementary Figure S2. Genome assembly of APMV-6 sequences.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, E.B., D.M.; methodology, E.B., D.M., A.K., X.B., N.H., G.K.; software, A.K..; validation, A.K., G.K., N.H.; investigation, O.R., M.S., O.M., V.N., O.G., N.H., G.K., X.B., A.M., M.S., A.S., D.C..; data curation, D.M.D., M.B., A.K., N.H., A.L.D.; writing—original draft preparation, A.K.; writing—review and editing, A.K., E.B., D.M., D.M.D.; visualization, M.B.; supervision, B.S., A.M.; project administration, E.B., M.Sa., A.M., B.S., and D.M.; funding acquisition, E.B. and D.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Sequence data for AMPV-1 and APMV-6 genomes is available at NCBI GenBank under BioProject [*Registering*], Accession numbers [*pending*].

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