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ORIGINAL PAPER

RNA sequencing of avian paramyxovirus (Paramyxoviridae, *Avulavirinae*) isolates from wild mallards in Belgium, 2021: complete genomes and coinfections

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Abstract

We used untargeted RNA sequencing to characterize three *Avulavirinae* isolates from pooled samples obtained from wild mallards in Belgium in 2021. The complete genome sequences of two avian Orthoavulavirus-1 (AOAV-1) strains and one avian Paraavulavirus-4 (APMV-4) strain were determined confirming hemagglutination inhibition testing of the virus isolates. In addition, the applied sequencing strategy identified an avian influenza virus (AIV) coinfection in all three virus isolates, confirming weak-positive AIV realtime RT-PCR results from the original sample material. In one AOAV-1 isolate, partial sequences covering all genome segments of an AIV of subtype H11N9 could be de novo assembled from the sequencing data. Besides an AIV coinfection, RNA metagenomic data from the APMV-4 isolate also showed evidence of *Alpharetrovirus* and *Megrivirus* coinfection. In total, two AOAV-1 of Class II, genotype I.2 and one APMV-4 complete genome sequences were assembled and compared to publicly available sequences, highlighting the importance of surveillance for poultry pathogens in wild birds. Beyond the insights from full genome characterization of virus isolates, untargeted RNA sequencing strategies provide additional insights in the RNA virome of clinical samples as well as their derived virus isolates that are particularly useful when targeting wild avifauna reservoirs of poultry pathogens.

Keywords Avulavirinae · Complete genome · Next generation sequencing · Avian paramyxovirus · Avian influenza · Mallard

Introduction

Avian paramyxoviruses (*Paramyxoviridae*, *Avulavirinae*) infect a variety of wild and domestic bird species and present 21 different serotypes (reviewed in Ref. [1–3]). They demonstrate a variable pathogenicity in chickens and other domestic birds [1]. The subfamily *Avulavirinae* was recently divided in three genera based on phylogenetic relationships of complete large (L) protein amino acid sequences ([4], http://www.ictv.global/report/paramyxoviridae). The genus *Metaavulavirus* regroups a variety of previously identified avian paramyxoviruses (APMV-2, 5–8 10–11, 14–15, 20, 22) isolated from wild and domestic birds with variable pathogenicity in chickens, while *Paraavulavirus* consists of the antigenically closely related APMV-3 and APMV-4 viruses [4]. The genus *Orthoavulavirus* includes, in addition to related Orthoavulavirus species (APMV-9, 12, 16, 17, and 21), the species avian Orthoavulavirus-1 (AOAV-1). AOAV-1 is known to circulate in a diversity of wild bird species and is the etiological agent of Newcastle disease (ND) in poultry [5], responsible for some of the most economically devastating poultry panzootic events. As a result, global molecular characterization efforts of avian paramyxoviruses focus mostly on avian Orthoavulavirus-1. Knowledge about the distribution, biological characteristics, pathogenicity, and diversity (both genetic and antigenic) of other avian

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paramyxoviruses remains fragmentary [1]. However, other members of the subfamily *Avulavirinae* are also associated with variable degree of pathogenicity in chickens and other avian species but their prevalence remains relatively poorly studied [1]. Over the last decade, high throughput sequencing technologies have significantly contributed to increasing the number of complete genome sequences for poorly represented avian paramyxovirus species (e.g., Refs. [6–11]) and to the identification of novel deviating species (e.g., Refs. [12–18]), facilitating new insights and leading to an improved taxonomic classification [4] of *Avulavirinae*. An international working group recently updated the taxonomy of AOAV-1, confirming two major classes [19]. Class I AOAV-1 circulation is mostly restricted to wild avifauna, while the class II can be found in both domestic and wild birds. AOAV-1 Class II is subdivided in several genotypes and subgenotypes, some of which are velogenic and cause Newcastle disease in poultry [19]. Clear (sub)genotype demarcation criteria have been identified and reference datasets have been made available ([19], https://github.com/NDVconsortium/NDV_Sequence_Datasets/) to standardize and facilitate both the subgenotyping and the detailed molecular characterization of AOAV-1 based on complete F gene sequences [19].

Wild waterbirds are known as an essential reservoir in the ecology of the causative viruses of important poultry diseases including avian influenza [20] and Newcastle disease [21]. Belgium's national avian influenza virus (AIV) and Newcastle disease virus (NDV) surveillance in wild birds is currently limited to passive surveillance (based on unexpected mortality). Regional programs supplement this with active surveillance including swabbing of both live captured and hunted wild birds (including *Anas plathyrhynchos*). In the context of this active surveillance, three avian paramyxovirus virus isolates were obtained from wild mallards in 2021. The objectives of this study were the use of untargeted RNA sequencing to characterize the RNA viral metagenome of these isolates and the full genome characterization of the isolated viruses.

Methods

Samples, targeted testing and virus isolation

Three pooled cloacal swab samples in viral transport medium (BHI 37 g/L, supplemented with 107 U/L penicillin, 2 g/L streptomycin, 1 g/L gentamycin, and 650 mg/L kanamycin) from wild mallards (*Anas plathyrhynchos*) were obtained in the context of wild bird surveillance for avian influenza virus (AIV). Two samples (1388_0006 and 1728_0002) consisted of pooled cloacal swabs from 5 individual hunted mallards (pooled per sampling location; likely a mix of wild mallards and mallards bred in captivity and released for hunting), while one sample consisted of 4 pooled cloacal swabs from healthy mallards collected for active surveillance by bird ringers (sample 116665_0002). All three sample pools tested weak-positive (Cq values between 26.72 and 29.93, Table 1) in matrix gene targeted AIV real time RT-PCR [22], and were submitted for virus isolation in embryonated chicken eggs consisting of one (samples 1388_0006 and 1728_0002) or two (sample 116665_0002) 5-day amplification rounds in the allantoic cavity of 9–11-day-old-specific pathogen-free embryonated chicken eggs [3]. Hemagglutination inhibition testing using reference sera following standard procedures [3] excluded AIV but identified two samples as AOAV-1 and one sample as APMV-4 (Table 1). The three obtained virus isolates were included this RNA metagenomic study.

Sequencing

The chorioallantoic fluids were centrifuged for 5 min at 10,000 × *g* in a precooled (4 °C) centrifuge prior to collection of the supernatant. RNA was extracted from the supernatant using the Nucleospin RNA virus kit (Macherey Nagel) using 4 µL of synthetic carrier (GenElute™-LPA, Sigma) instead of the kit-supplied polyA carrier RNA. DNA was removed using 3 U of Baseline-ZERO™ DNase (Lucigen) for a starting volume of 50 µL of extracted nucleic acids following manufacturer's instructions and the treated RNA was purified using the RNA Clean & Concentrator-5 kit (Zymo Research). cDNA was synthesized using SuperScript IV reverse transcriptase (Thermo Fisher Scientific) and random hexamer primers, followed by double-strand cDNA synthesis using the NEBNext mRNA second-strand synthesis module (New England BioLabs). Sequencing libraries were prepared using the Nextera XT kit (Illumina) and standard Nextera XT index adapters (Illumina) and sequenced using a MiSeq reagent kit version 3 (Illumina) with 2 × 300-bp paired-end sequencing aiming for a minimum of 1-million read pairs per sample. Metagenomic NGS data were generated for 3 samples. The resulting fastq raw metagenomics datasets are publicly available in the Sequence Read Archive (SRA) under BioProject ID PRJNA949131.

Bioinformatic analysis

In preparation of metagenomic read classification, raw NGS reads were trimmed using Trimmomatic v0.38 [23] to remove adapter sequences and low quality bases (setting the 'ILLUMINACLIP 2:30:10', 'SLIDINGWINDOW:4:20', and 'MINLEN:50' options). Only paired reads were retained for further analysis. Classification of trimmed reads was performed with Kraken2 (Galaxy version 2.0.7) as previously described [24]. A customized Kraken database was built using all available RefSeq "Complete Genome" sequences

Table 1 Avian influenza virus and *Avulavirus* identification in original samples and virus isolates

Sample identification	1388_0006	1728_0002	11665_0002
Realtime RT-PCR and hemagglutination inhibition (HI) results			
Original sample	Pooled cloacal swabs, 5 mallards	Pooled cloacal swabs, 5 mallards	Pool of cloacal swabs, 4 mallards
AIV Cq original sample	27.47	26.72	29.93
NDV Cq original sample	33.54	nd	nd
Sequenced isolate	ECE 1	ECE 1	ECE 2
AIV Cq isolate	neg	19.21	neg
NDV Cq isolate	13.26	13.73	Neg
HI identification isolate	AOAV-1	AOAV-1	APMV-4
RNA sequencing results isolate			
Trimmed reads	1,616,838	1,207,759	585,749
% viral reads	36	44	25
AOAV-1 RPM (% of viral reads)	356626.33 (98.8%)	424626.11 (95.77%)	–
APMV4 RPM (% of viral reads)	–	–	252195.05 (99.84%)
AIV RPM (% of viral reads)	52.57 (0.01%)	12936.36 (2.92%)	134.87 (0.05%)
Avian Orthoavulavirus-16* RPM	(3866.19)*	(4167.22)*	–
Avian Orthoavulavirus-18* RPM	(217.71)*	(1596.34)*	–
Avian leucosis virus (Alpharetrovirus) RPM	–	–	105.85 (0.04%)
Megrivirus (Picornaviridae) RPM	–	–	102.43 (0.04%)

The realtime RT-PCR identification, hemagglutination inhibition characterization and metagenomics characterization are reported. From the metagenomic viral read classification, normalized read counts are reported (reads per million reads of sequencing effort, RPM). Only taxa reporting a minimum of 50 RPM were reported (arbitrary cutoff). In addition, the size of the total trimmed dataset of each sample as well as its % viral reads is reported. ECE 1 and ECE 2: virus isolated using 1, resp. 2 passages in embryonated chicken eggs

nd not tested

*Avian Orthoavulavirus-16 and -18 reads were discarded by further bioinformatic investigations as mapping only to conserved Avulavirus M gene regions

of six targeted taxonomic groups (archaea, bacteria, fungi, protozoa, and viral, as well as selected avian genomes (*Gallus gallus* GCF-000002315; *Columba livia* GCF-00037935; *Meleagris gallopavo* GCF-000146605; *Anas Platyrhynchos* GCF-000355855; *Numida meleagris* GCF-002078875) downloaded from RefSeq Genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/) on 22/08/2022. An arbitrary cutoff of minimum 50 reads per million of trimmed reads was used for reporting the presence of taxa, as the metagenomics experiment was purposed to characterized virus isolates. For exploration of the isolate's virome, the % of viral reads for each reported taxon were calculated.

In preparation of the de novo assembly and characterization of complete viral genomes, raw sequence data was trimmed using Trim Galore! V0.5.0 ($q=25$, $l=50$, paired; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). For each sample, viral contigs were de novo assembled from a random paired subset ($2\times50{,}000$ reads; optimum subsample to maximize contig length) of the reads using SPAdes v3.9.0 [25] and IVA v1.0.0 [26]. Relevant contigs (as determined using a Blastn search of all contigs longer than 500 nucleotides against the NCBI Nucleotide database) were joined using CAP3 [27]. The full trimmed dataset was mapped to the finished full genome length contig using Minimap2 [28]. The annotation of assembled genomes and partial genomes was done using GATU [29] relative to references KU601398 and KX352836 for AOAV-1 and MW880773 for APMV-4.

Phylogenetic analysis

The avian Orthoavulavirus-1 (AOAV-1) pilot references dataset maintained by the NDV consortium ([19]; https://github.com/NDVconsortium/NDV_Sequence_Datasets/; 128 full Fusion gene sequences of Class II, version 9 may 2022) was used for a preliminary subtyping of the AOAV-1 genomes. For further detailed characterization all genotype I references from the complete F gene dataset (https://github.com/NDVconsortium/NDV_Sequence_Datasets/, version 9 may) were supplemented with all Class II genotype I full F gene data submitted in Genbank between 9 may and 18 august 2022 and root sequences AY741404 and EF 201805 as suggested by Ref. [19]. The final dataset included 134 sequences of 1662 nucleotides long and sequences were aligned using MAFFT v7.310 [30, 31]. After selection of the most suitable evolutionary model (lowest Bayesian

Information Criterion score, BIC), a maximum likelihood phylogenetic tree was inferred by using the Maximum Likelihood method in Mega X [32] (T92 + G [33], partial deletion of missing data and gaps; 500 bootstrap replicates). For visual clarification, a subtree representing the identified subgenotype I.2, itself supported by a bootstrap value of 99%, was extracted from the full-dataset phylogenetic tree.

All avian Paraavulavirus-4 (APMV-4, NCBI:txid2560328) Fusion gene coding sequences with minimum length of 1500 nt were downloaded from the NCBI Nt database (on 18 august 2022) and supplemented with the F gene coding sequence from the complete genome determined in the present study (APMV-4/Anas_platyrhynchos/Belgium/11665_002/2021). The final dataset included 125 sequences of 1523 nucleotides long and sequences were aligned using MAFFT v7.310 [30, 31]. After selection of the most suitable evolutionary model (lowest BIC), an unrooted phylogenetic tree was inferred by using the Maximum Likelihood method in Mega X [32] (T92 + I [33], partial deletion of missing data and gaps; 500 bootstrap replicates).

Results

RNA sequencing resulted in 1,084,126, 1,207,759 and 1,616,838 trimmed reads for isolates 11665_0002, 1728_0002, and 1388_0006, respectively (Table 1, complete datasets submitted to Sequence Read Archive (SRA) under BioProject ID PRJNA949131). The majority of reads originated from the virus isolation host (*Gallus gallus*), while between 25 and 44% of the sequencing effort represented viral reads (Table 1). Eliminating taxa with a normalized read count below 50 as well as bacteriophages, the isolates' virome was dominated by *Avulavirinae* (Table 1). 98.8% of viral reads originating from isolate 1338_0006 classified as avian Orthoavulavirus-1, while only 0.01% of viral reads represented influenza A virus (IAV). 95.77% of viral reads originating from isolate 1728_0002 classified as avian Orhoavulavirus-1, while a coinfection with influenza A virus (2.92%) was evident (Table1). The RNA virome of isolate 11665_0002 was dominated by avian Paraavulavirus-4 (99.84%), with traces of Alpharetrovirus (avian leucosis virus) as well as Megrivirus (Table 1). Close examination of read alignments to reference sequences in the metagenomic classification database revealed that all reads classified by Kraken2 as avian Orthoavulavirus -16 and -18 mapped to conserved *Avulavirinae* genome regions and thus most likely represent avian Orthoavulavirus-1.

Our denovo assembly strategy allowed the reconstruction of two avian Orthoavulavirus-1 complete genomes (one from each isolate 1388_0006 and 1728_0002, having a nucleotide sequence similarity of 97.4%) and one Paraavulavirus-4 (from isolate 11665_002) complete coding sequence (near complete genome) from randomly subsampled datasets of 50,000 paired reads. These avulavirinae genomes were submitted to genbank under accession numbers OQ714710 (AOAV-1/Anas_platyrhynchos/Belgium/1388_0006/2021), OQ714709 (AOAV-1/Anas_platyrhynchos/Belgium/1728_0002/2021) and OQ714711 (APMV-4/Anas_platyrhynchos/Belgium/11665_002/2021). Genome annotation confirmed the presence of the complete coding sequence of the 6 open reading frames (5'-N-P-M-F-HN-L-3') of Paramyxoviridae in all three genomes.

In addition, the de novo assembly of 50,000 random subsampled paired reads from isolate 1728_0002 resulted in 8 contigs representing partial sequences (segment coverage width ranging from 58 to 97%) for all 8 genome segments of an influenza A virus of subtype H11N9 (submitted under GISAID EpiFlu isolate number EPI_ISL_17298410).

The complete fusion gene phylogenetic analysis confirms that both AOAV-1 isolates are classified as Class II, genotype I.2 according to the recently proposed unified nomenclature [19]. Both 2021 Belgian AOAV-1 isolates cluster in a well-supported clade (bootstrap support 84) of genotype I.2 viruses detected since 2008 in birds of the orders Anseriformes and Galliformes in a wide geographical area (Asia, Europe and Africa, Fig. 1).

The complete F gene coding sequence of APMV-4/Anas_platyrhynchos/Belgium/11665_002/2021 clusters in a well-supported (bootstrap 96) clade of APMV-4 recently (2017–2020) isolated from birds of the order Anseriformes in Asia. The single other Belgian APMV-4 genome from 2007 previously sequenced by our group [6] is unrelated to the APMV-4/Anas_platyrhynchos/Belgium/11665_002/2021 but clustered in a clade with predominant European circulation (Fig. 2).

Discussion

This study determined two AOAV-1 of Class II, genotype I.2 complete genome sequences from belgian wild mallards. Our phylogenetic analyses confirm the continued circulation of class II genotype I.2 AOAV-1 in healthy wild waterbirds in Europe. Viruses from this subgenotype are known to sporadically infect domestic birds like chickens (cf. genbank accession number KR869090.1, China, 2013), highlighting a continued risk for the global poultry industry. Genotype I NDV vaccines (Ulster like) are expected to have acceptable antigenic match with more recent wild type genotype I.2 viruses [34].

Although most species from the subfamily Avulavirinae are globally distributed, their genetic characterization remains patchy. Recent advances in sequencing technologies have resulted in more complete genomes for several Avulavirinae (e.g., Refs. [6–11]) but also in the identification of

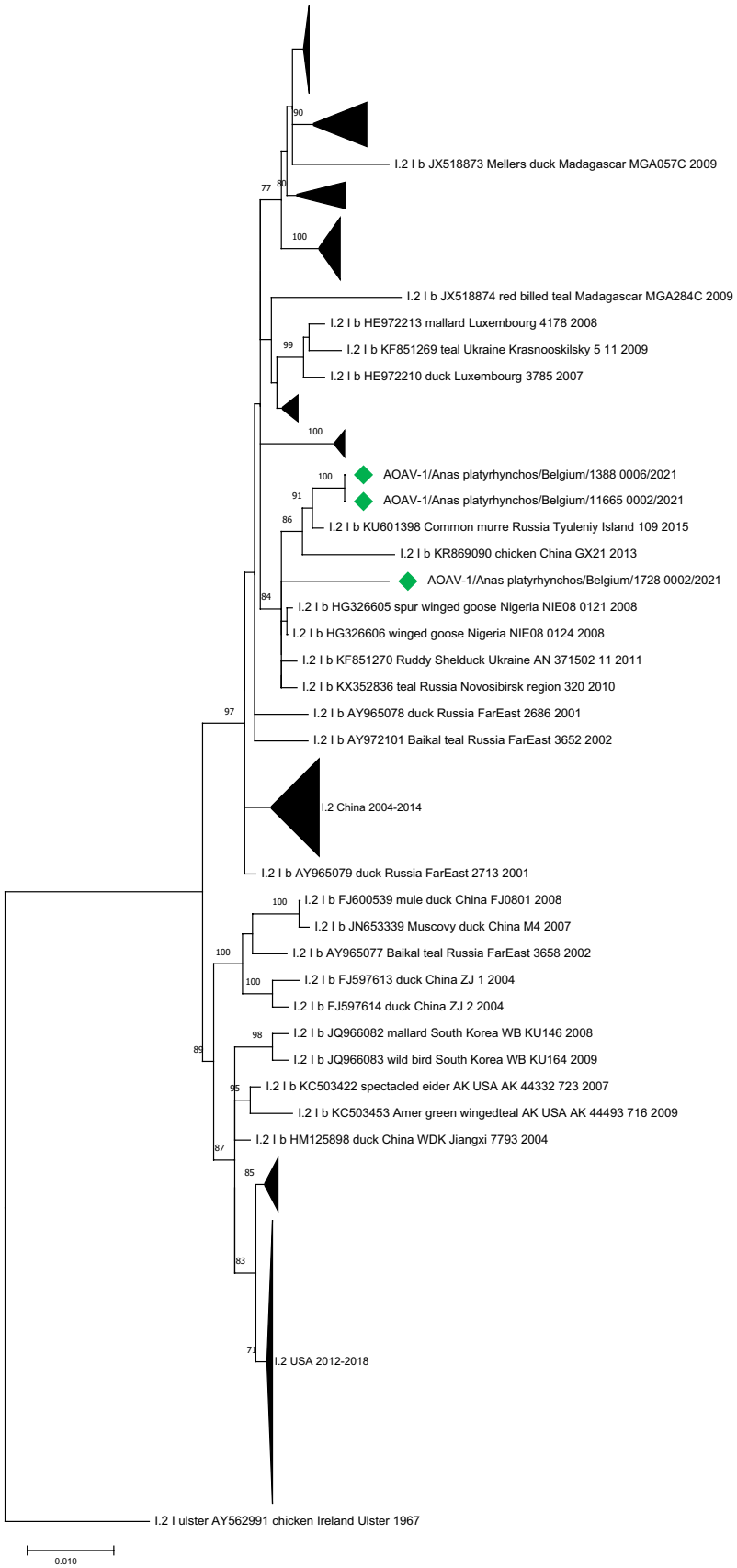
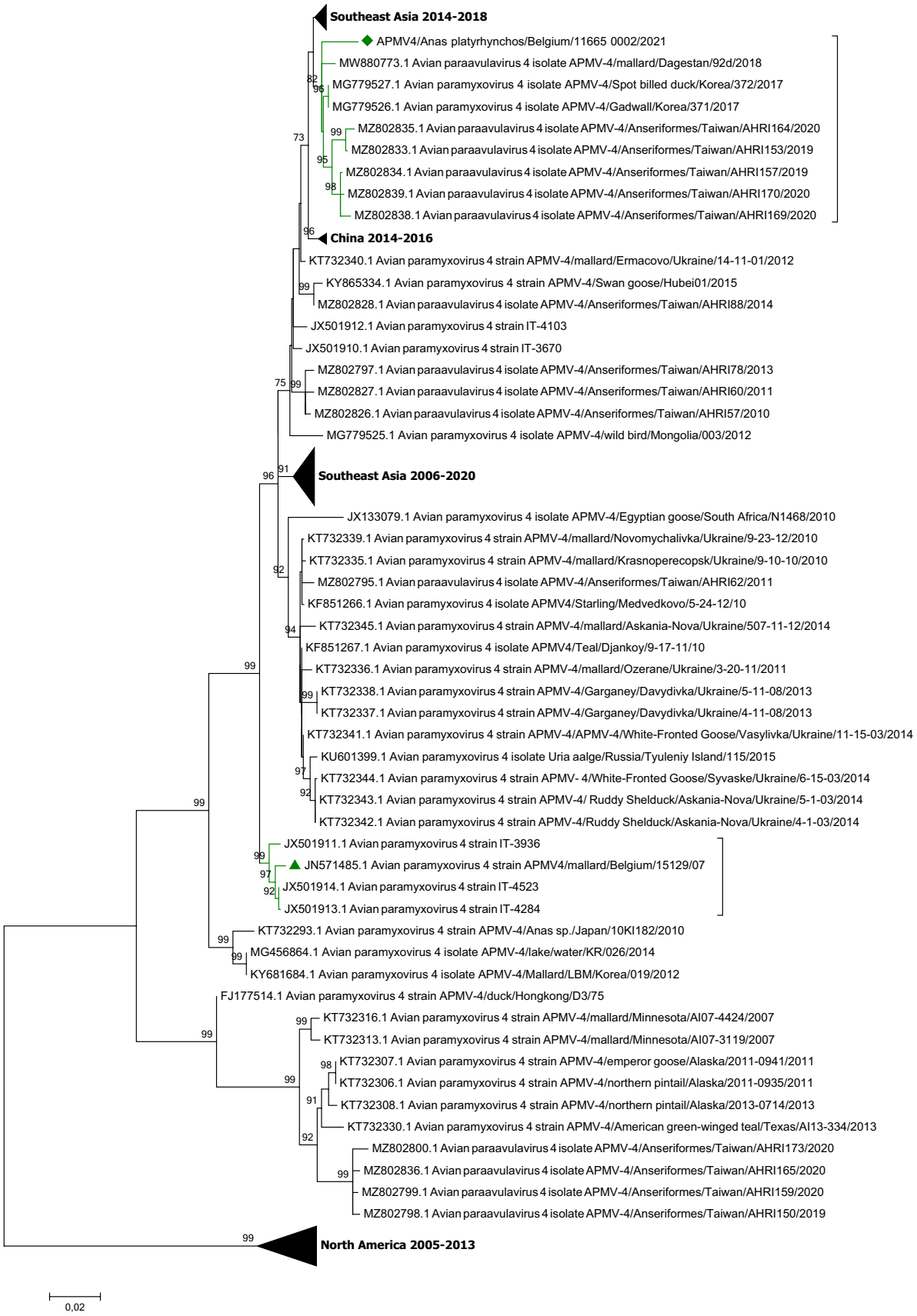


Fig. 1 Phylogenetic characterization of avian Orthoavulavirus-1. As suggested by Ref. [19], a ML tree containing all publicly available sequences from genotype I of class II was inferred and rooted in a single genotype III + a single genotype IV strain. For visual clarification, only the subtree representing subgenotype I.2 is shown here from the tree based on all 134 sequences. Taxa with viral strains only circulating in a limited geographical region were collapsed for visual simplification. ◆: sequenced in this study


Southeast Asia 2014-2018
APMV4/Anas platyrhynchos/Belgium/11665 0002/2021
MW880773.1 Avian paraavulavirus 4 isolate APMV-4/mallard/Dagestan/92d/2018
MG779527.1 Avian paramyxovirus 4 isolate APMV-4/Spot billed duck/Korea/372/2017
MG779526.1 Avian paramyxovirus 4 isolate APMV-4/Gadwall/Korea/371/2017
MZ802835.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI164/2020
MZ802833.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI153/2019
MZ802834.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI157/2019
MZ802839.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI170/2020
MZ802838.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI169/2020
China 2014-2016
KT732340.1 Avian paramyxovirus 4 strain APMV-4/mallard/Ermacovo/Ukraine/14-11-01/2012
KY865334.1 Avian paramyxovirus 4 strain APMV-4/Swan goose/Hubei01/2015
MZ802828.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI88/2014
JX501912.1 Avian paramyxovirus 4 strain IT-4103
JX501910.1 Avian paramyxovirus 4 strain IT-3670
MZ802797.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI78/2013
MZ802827.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI60/2011
MZ802826.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI57/2010
MG779525.1 Avian paramyxovirus 4 isolate APMV-4/wild bird/Mongolia/003/2012
Southeast Asia 2006-2020
JX133079.1 Avian paramyxovirus 4 isolate APMV-4/Egyptian goose/South Africa/N1468/2010
KT732339.1 Avian paramyxovirus 4 strain APMV-4/mallard/Novomychalivka/Ukraine/9-23-12/2010
KT732335.1 Avian paramyxovirus 4 strain APMV-4/mallard/Krasnoperecopsk/Ukraine/9-10-10/2010
MZ802795.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI62/2011
KF851266.1 Avian paramyxovirus 4 isolate APMV4/Starling/Medvedkovo/5-24-12/10
KT732345.1 Avian paramyxovirus 4 strain APMV-4/mallard/Askania-Nova/Ukraine/507-11-12/2014
KF851267.1 Avian paramyxovirus 4 isolate APMV4/Teal/Djankoy/9-17-11/10
KT732336.1 Avian paramyxovirus 4 strain APMV-4/mallard/Ozerane/Ukraine/3-20-11/2011
KT732338.1 Avian paramyxovirus 4 strain APMV-4/Garganey/Davydivka/Ukraine/5-11-08/2013
KT732337.1 Avian paramyxovirus 4 strain APMV-4/Garganey/Davydivka/Ukraine/4-11-08/2013
KT732341.1 Avian paramyxovirus 4 strain APMV-4/APMV-4/White-Fronted Goose/Vasylivka/Ukraine/11-15-03/2014
KU601399.1 Avian paramyxovirus 4 isolate Uria aalge/Russia/Tyuleniy Island/115/2015
KT732344.1 Avian paramyxovirus 4 strain APMV- 4/White-Fronted Goose/Syvaske/Ukraine/6-15-03/2014
KT732343.1 Avian paramyxovirus 4 strain APMV-4/ Ruddy Shelduck/Askania-Nova/Ukraine/5-1-03/2014
KT732342.1 Avian paramyxovirus 4 strain APMV-4/Ruddy Shelduck/Askania-Nova/Ukraine/4-1-03/2014
JX501911.1 Avian paramyxovirus 4 strain IT-3936
JN571485.1 Avian paramyxovirus 4 strain APMV4/mallard/Belgium/15129/07
JX501914.1 Avian paramyxovirus 4 strain IT-4523
JX501913.1 Avian paramyxovirus 4 strain IT-4284
KT732293.1 Avian paramyxovirus 4 strain APMV-4/Anas sp./Japan/10KI182/2010
MG456864.1 Avian paramyxovirus 4 isolate APMV-4/lake/water/KR/026/2014
KY681684.1 Avian paramyxovirus 4 isolate APMV-4/Mallard/LBM/Korea/019/2012
FJ177514.1 Avian paramyxovirus 4 strain APMV-4/duck/Hongkong/D3/75
KT732316.1 Avian paramyxovirus 4 strain APMV-4/mallard/Minnesota/AI07-4424/2007
KT732313.1 Avian paramyxovirus 4 strain APMV-4/mallard/Minnesota/AI07-3119/2007
KT732307.1 Avian paramyxovirus 4 strain APMV-4/emperor goose/Alaska/2011-0941/2011
KT732306.1 Avian paramyxovirus 4 strain APMV-4/northern pintail/Alaska/2011-0935/2011
KT732308.1 Avian paramyxovirus 4 strain APMV-4/northern pintail/Alaska/2013-0714/2013
KT732330.1 Avian paramyxovirus 4 strain APMV-4/American green-winged teal/Texas/AI13-334/2013
MZ802800.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI173/2020
MZ802836.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI165/2020
MZ802799.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI159/2020
MZ802798.1 Avian paraavulavirus 4 isolate APMV-4/Anseriformes/Taiwan/AHRI150/2019
North America 2005-2013
0,02

◂**Fig. 2** Phylogenetic analysis of avian Paraavulavirus-4. Maximum likelihood phylogenetic tree based on complete F gene coding sequences. Taxa with viral strains only circulating in a limited geographical region were collapsed for visual simplification. ◆: sequenced in this study, ▲: previously sequenced Belgian APMV-4 isolate

novel species (e.g., Refs. [12–18]) and several re-classifications of Avulavirinae [4]. APMV-4 has been predominantly isolated from wild birds (reviewed in Ref. [1]), although mild disease has been documented in experimentally infected chickens [35]. To date, only 23 complete *Paraavulavirus-4* genomes were published, albeit with a heavy geographical sampling bias to Southeast Asia. The addition of further complete APMV-4 genomes from other geographical areas is needed. Our phylogenetic analysis confirms the global distribution and exchange of this species. In particular, APMV-4/Anas_platyrhynchos/Belgium/11665_002/2021 sequenced in the present study clustered with viruses from Asian sampling locations while the only previous Belgian APMV-4 sequence clustered with viruses with predominant European circulation.

The hemagglutination inhibition and targeted NDV realtime RT-PCR testing of these three virus isolates suggested successful isolation of paramyxoviruses instead of avian influenza. Not surprisingly, the majority of viral reads from our RNA sequencing experiments were classified as Avulavirinae. However, these paramyxovirus isolates obtained through inoculation of embryonated chicken eggs proved to contain additional viruses. The detection of AIV reads in the isolated paramyxoviruses confirms their realtime RT-PCR detection in the original pooled swab samples. However, only in isolate 1728_0002, AIV realtime RT-PCR results and AIV read numbers seem to suggest successful co-isolation of the AIV together with the isolated paramyxovirus. In both other isolates, AIV dropped below realtime RT-PCR detection limits and resulted in only marginally significant normalised AIV read numbers, suggesting differences in the co-isolation of paramyxovirus and orthomyxoviruses using routine diagnostic virus isolation assays. The virus isolate from sample 1728_002 yielded more than 12,000 avian influenza virus reads which could be de novo assembled into a partial genome covering between 58 and 95% of all segments of an H11N9 subtype avian influenza virus.

In addition, the APMV-4 isolate's RNA sequencing data showed significant evidence for the presence of *Alpharetrovirus* (avian leucosis virus) and *Megrivirus* reads, both taxa previously suspected as etiological agents of poultry disease [36, 37]. We have previously documented co-isolation of different avian paramyxovirus species using metagenomics methods [6] as well as the coinfection of avian paramyxovirus isolates with circoviruses [38]. These coinfections in lab-isolated virus preparations highlight the importance of thorough validation of reference material used in diagnostic or research efforts, where possible including verification of presence of non-AOAV-1 species. Random RNA sequencing of virus isolates is an untargeted approach with a great added diagnostic value such applications of quality control and thorough characterization of virus isolates.

The sequenced RNA viromes in the present study resulted from isolates obtained from pooled cloacal swab specimens from healthy wild mallards. Unfortunately, nor the pooled samples, nor the individual cloacal swab samples were available at the time of the genetic investigations. Consequently, we could not find out which of the animals in the pool were infected and whether we were dealing with a mixed infection in a single bird. Individual wild mallards have been previously documented to frequently being coinfected with avian paramyxoviruses, influenza viruses and coronaviruses [39]. Moreover, a careful and non-quantitative interpretation of the metagenomic read classification is needed, as virus isolation may bias the composition of the detected virome.

Although a lot of countries carry out targeted AIV and AOAV-1 surveillance as these concern notifiable diseases under international animal health law [3], this active surveillance effort is often targeted to poultry. More surveillance is needed to characterize the circulation of AOAV-1 and other Avulavirinae in wild bird reservoirs. The exploitation of part of these sample and isolate collections by untargeted sequencing methods may provide a first window on the circulation of other viruses of yet unknown epizootic potential and provides a powerful tool to identify coinfections.

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Author contributions SVB, performed experiments and data analysis; SVB, MS, VR and BL designed the experiment and wrote the manuscript; AL, DV, and VD provided resources, samples and data and reviewed the manuscript.

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Availability of data and material Raw NGS RNA sequencing data are publicly available in the Sequence Read Archive (SRA) under BioProject ID PRJNA949131, Viral genome sequences and partial genome sequences are available under Genbank accession numbers OQ714709-OQ714711, and GISAID Epiflu isolate number EPI_ISL_17298410.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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