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Novel avian metaavulavirus isolated from birds of the family *Columbidae* in Taiwan

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ABSTRACT

Avian paramyxoviruses (APMVs) consist of twenty known species and have been isolated from domestic and wild birds around the world. In 2009, the isolate APMV/dove/Taiwan/AHRI33/2009 was isolated from swabs of red turtle doves (*Streptopelia tranquebarica*) during active surveillance of avian influenza in resident birds in Taiwan, and it was initially identified as paramyxovirus based on electron microscopy. Hemagglutination inhibition assays indicated antigenic heterogeneity of AHRI33 with the known APMV-1, -2, -3, -4, -6, -8, and -9 species, only showing weak but measurable cross-reactivity with APMV-7. Pathogenicity ICPI test revealed that the virus was avirulent for chickens. The AHRI33 virus genome revealed a typical APMV structure consisting of six genes 3′-NP-P-M-F-HN-L-5′, and the length of the genome was 16,914 nucleotides, the third longest among the members of the subfamily *Avulavirinae*. Estimates of the nucleotide sequence identities of the genome between each prototype of APMVs had shown AHRI33 to be more closely related to APMV-7 than to the others, with a sequence identity of 62.8%. Based on topology of the phylogenetic tree of RdRp genes and the branch length between the nearest node and the tip of the branch, AHRI33 met the criteria for designation as distinct species. Together, the data suggest that the isolate APMV/dove/Taiwan/AHRI33/2009 should be considered as the prototype strain of the new species *Avian metaavulavirus 21* in the genus *Metaavulavirus* in the subfamily *Avulavirinae*.

1. Introduction

The International Committee on Taxonomy of Viruses has recently created and renamed three new genera, *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus*, within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Amarasinghe et al., 2019). Viruses in the subfamily *Avulavirinae*, commonly known as avian paramyxoviruses (APMVs, used hereafter for the purposes of this paper), have been reported from a wide variety of avian species around the world (Gogoi et al., 2017). APMVs contain a non-segmented negative sense single-stranded RNA genome ranging from 15 to 17 kb in length (Aziz-Ul-Rahman et al., 2018). According to the taxonomy of the order *Mononegavirales* updated in 2019, APMVs are divided into twenty species based on a sequence comparison of the RNA-dependent RNA polymerases (RdRps) of the viruses (ICTV, 2019). APMV-1 (commonly termed Newcastle disease virus, NDV) is the most recognized species, but information on the distributions of all other APMVs in domestic poultry and wild birds is limited.

Until recently, the known APMV species were restricted to APMV-1 to -9, which were isolated and characterized in the 1970s (Alexander, 1987). Following the expansion of viral surveillance initiatives and improvements in sequencing technology, 11 novel species have been designated since 2010. A virus isolated from rockhopper penguins was antigenically and genetically distinct from all known APMV-1 to -9 and considered to represent the prototype of a new species, APMV-10 (Miller et al., 2010). A further novel APMV-11 isolate was obtained in France from a common snipe in 2010 (Briand et al., 2012). APMV-12 was isolated in Northern Italy in 2005 from an Eurasian widgeon (Terregino et al., 2013). In 2015 and 2016, three publications described a novel APMV-13 species found in three separate regions of Eurasia: Japan, Kazakhstan and Ukraine (Yamamoto et al., 2015; Karamendin et al., 2016; Goraichuk et al., 2016). In 2017, seven novel APMV species were announced: APMV-14 from ducks in Japan (Thampaisarn et al., 2017), APMV-15 from shorebirds in Brazil (Thomazelli et al., 2017),

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and APMV-16 from ducks in Korea (Lee et al., 2017). Three novel species, Antarctic penguin virus A, B, and C (APV-A, -B, and -C), were simultaneously isolated from Antarctic penguins (Neira et al., 2017), and APMV-20 from gulls in Kazakhstan (Karamendin et al., 2017). These findings suggest that wild birds maintain a previously unrecognized genetic diversity of APMVs. In Taiwan, the complete genome of an APMV-6 strain isolated from ducks was reported in 2001 (Chang et al., 2001). In the present article, we present the antigenic and genetic characterization of a novel APMV isolated from doves in Taiwan, which represents a previously unknown species.

2. Materials and methods

2.1. Sample collection and virus isolation

The samples of this study were collected from domestic poultry and wild birds in Taiwan as part of an avian influenza (AI) surveillance program. The tracheal/cloacal swabs and tissue samples of the trachea, lung, liver, spleen, heart, and kidney were inoculated into the allantoic cavities of 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Animal Drugs Inspection Branch, Animal Health Research Institute, Miaoli, Taiwan) and then incubated at 37°C for 72 h. The allantoic fluid from each inoculated embryo was collected after two passages in eggs and individually examined for hemagglutination assay (HA). The HA-positive allantoic fluid was tested by AIV (Spackman et al., 2002) and NDV (Wise et al., 2004) real-time reverse transcription polymerase chain reaction (rRT-PCR). Samples that tested negative were subjected to further analyses.

2.2. Electron microscopy

HA-positive allantoic fluid which tested negative for AIV and NDV by rRT-PCR was centrifuged at 3000 × g for 20 min at 4°C. The supernatant was separated and then subjected to ultracentrifugation at 100,000 × g for 5 min. The pellet was resuspended in 25 μL of distilled water and stained with 2% phosphotungstic acid in a copper grid (Bozzola and Russell, 1992) and then examined using a JEOL JEM-1400 electron microscope (JEOL Ltd., Tokyo, Japan).

2.3. Determination of nucleotide sequence of full-length APMV genome

Viral RNA from HA-positive allantoic fluid which tested negative for AIV and NDV by rRT-PCR was isolated using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Amplification of the APMV RNA was performed using *Rubulavirus-Avulavirus* genus subgroup-specific RT-PCR (Tong et al., 2008) with SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, California, USA). To determine the nucleotide sequence of the full-length genome, a combination of random primers and primer walking was used to generate PCR amplicons covering the genome (except for the 3′ and 5′ ends). The sequences of both ends of the genome were amplified using the rapid amplification of cDNA ends method (RACE) (Qiu et al., 2009). According to the assembling contig of primer-walking and RACE amplicons, we redesigned 17 primer sets to amplify 17 overlapping segments covering the entire genome. All amplified products were separated on 2% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). These products were cloned with the TOPO TA Cloning kit (Invitrogen) using the standard protocol, and inserted cDNA segments were amplified using M13 forward and reverse primers provided with the kit. The PCR products with expected lengths were sequenced with the 3700XL DNA analyzer (Applied Biosystems, Life Technologies, Carlsbad, California, USA) by a commercial sequencing service (Mission Biotech, Taipei, Taiwan).

2.4. Intracerebral pathogenicity index (ICPI)

To determine the virulence of an APMV isolate, the ICPI test for NDV (OIE, 2012) was employed in day-old SPF chicks (Animal Drugs Inspection Branch, Animal Health Research Institute).

2.5. Serological characterization

In view of the difficulties of obtaining a whole set of APMV-1 to APMV-9 reference antisera and antigen, the new APMV isolate was submitted to the OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico, Italy. Hemagglutination inhibition (HI) tests (OIE, 2012) were performed using antigens and chicken polyclonal antisera of representative APMV-1 to APMV-9 (except APMV-5). The antiserum against the new APMV isolate used in reverse HI assay was produced in adult SPF leghorn chickens by subcutaneously inoculating 0.2 mL of inactivated, adjuvanted virus emulsion twice with a 2-week interval, and the antiserum was collected two weeks after the second inoculation. The reverse HI assays were conducted for characterizing new the APMV isolate with APMV-7 reference antigen and antiserum, which were kindly shared by the Italian OIE reference laboratory. The antigenic relationship between the two viruses was analyzed with the following formula (Archetti and Horsfall, 1950): $R = (r1 \times r2)^{1/2}$ where r1 is the ratio of the heterologous HI titer divided by the homologous HI titer for virus 1, and r2 is the ratio of the heterologous HI titer divided by the homologous HI titer for virus 2.

2.6. Phylogenetic analysis

The sequences of the full-length genome, fusion (F), and hemagglutinin-neuraminidase gene (HN) of the isolated APMV were aligned with the sequences of APMV-1 to APMV-20 representative viruses retrieved from the GenBank database using ClustalW in Molecular Evolutionary Genetics Analysis version 7, or MEGA7 (Kumar et al., 2016). For the construction of the phylogenetic trees, the evolutionary history was inferred using the Maximum Likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites (Nei and Kumar, 2000) by 1000 bootstrap replicates.

The phylogenetic analyses based on complete amino acid sequences of RNA-dependent RNA polymerase (RdRp or L gene) of the viruses were conducted as previously described (ICTV, 2019). The alignment prepared with MEGA7 software with a gap-opening penalty of 5 and a gap extension penalty of 1. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

2.7. Nucleotide sequence accession number

The complete genome sequences of APMV/dove/Taiwan/AHRI33/2009 and three AHRI33-like isolates are available from GenBank under the accession numbers MK677430-MK677433. GenBank accession numbers for the complete coding region of the fusion genes for the other ten AHRI33-like isolates sequenced as part of this study are as follows: MK677434–MK677443.

3. Results

3.1. Sample collection and virus isolation

As a result of the avian influenza surveillance program on resident birds in Taiwan, a novel virus, AHRI33, showing hemagglutination activity was isolated from a clinically healthy red turtle dove (*Streptopelia tranquebarica*) in 2009. The AHRI33 virus propagated well in embryonated chicken eggs, and the harvested infective allantoic fluid had an HA titer of 32. Moreover, we also obtained additional 13 AHRI33-like virus isolates from resident doves and pigeons in two different time periods. In 2009–2010, a research project for surveillance of AIV in resident birds was conducted. Swab samples were mainly collected from sparrows, doves, pigeons, and Chinese bulbuls, the dominant species of resident birds in Taiwan, and 11 AHRI33-like strains were isolated only from red turtle doves. Since the 2015 outbreak of the clade 2.3.4.4 H5Nx highly pathogenic avian influenza (HPAI) viruses in Taiwan, dead birds found in public places have been sent to AHRI for detection of AIV. Two AHRI33-like strains were isolated. Among them, AHRI104 was obtained from a dead oriental turtle dove (*Streptopelia orientalis*) in 2016 and AHRI128 from a dead pigeon (*Columba livia*) in 2017.

3.2. Electron microscopy

The fine structure of the virion showed typical characteristics of a paramyxovirus. The virion was pleomorphic, roughly spherical, and varied from 150 to 500 nm in diameter. Herringbone-like nucleocapsids were encased in a fragile envelope coated with approximately 10 nm long spikes (Fig. 1).

3.3. Intracerebral pathogenicity index

After inoculation of the AHRI33 virus into the cerebrums of one-day-old chickens, the obtained ICPI value was zero, indicating no clinical signs. This result suggested that the AHRI33 isolate was avirulent to chickens.

3.4. Serological characterization

As shown in Table 1, the titers of antisera against each representative APMV species were higher with the homologous virus. The AHRI33 isolate only showed titers of 1:4 to 1:16 with the reference antisera against APMV-1, -2, -3, -4, -6, -8, and -9, but it was antigenically similar to APMV-7 on the basis of HI typization (4-fold difference in HI titers). The R-value between AHRI33 and APMV-7 was then calculated based on the HI titers obtained from the cross-HI assay, and the R-value of 0.125 indicated antigenic similarity between these viruses, although the R-value was less than would be expected between viruses of the same serogroup.

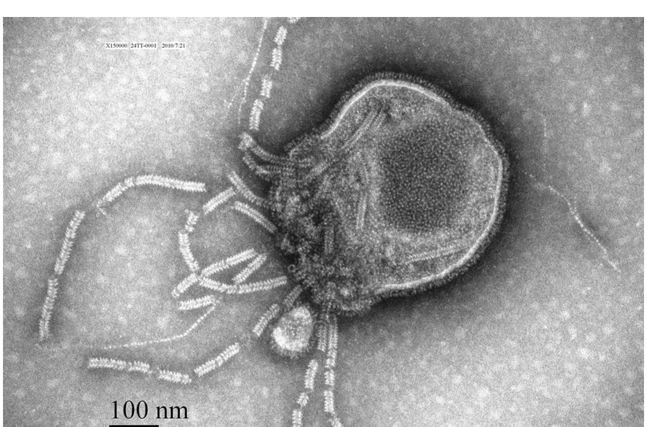

Fig. 1. Paramyxovirus virion found in allantoic fluid of embryonated chicken egg inoculated with swab sample extract from a red turtle dove in Taiwan. The virion consists of a fringed envelope, and the nucleoprotein helices protruded through the envelope. (×150,000).

3.5. Determination of nucleotide sequences of full-length APMV genome

The complete genome sequence of the AHRI33 isolate was characterized using a Sanger sequencing approach. Assembly of the sequences produced a contig of 16,914 nucleotides (nt), making it close in size to APMV-5 (GU206351, 17,262 nt, 348 nt longer than that of AHRI33), APMV-11 (NC_025407, 17,412 nt, 498 nt longer), and APMV-3/Netherlands (EU403085, 16,272 nt, 642 nt shorter). Other APMV genome sequences range in length from 14,880 nt for APMV-2/F8 (HQ896023) to 17,412 nt for APMV-11.

Six genes, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L), were identified in the genome of the AHRI33 virus, showing a typical gene arrangement of APMVs 3′-leader-NP-P-M-F-HN-L-trailer-5′ (Fig. 2), which predicted six viral proteins: NP, 461 amino acids (aa); P, 410 aa; M, 365 aa; F, 540 aa; HN, 571 aa, and L, 2226 aa. The 3′ leader sequence of the AHRI33 isolate was 55 nt in length, as conserved among most APMV species (Samal, 2011). The length of the trailer at the 5′ end was 506 nt. The first 13 nt of the leader sequence and the last 13 nt of the 5′ trailer sequence showed complete complement. The conserved sequences for the gene start (GS) and gene end (GE) of the AHRI33 virus were CCCCCNNUN and $AAUNNU_{6-7}$, respectively. The lengths of the intergenic region sequences ranged from 1 to 35 nt. The deduced amino acid sequence of the F gene cleavage site was STQQER/LFG, which lacked multiple basic residues at the C-terminus of the F2 protein and a phenylalanine residue at the N-terminus of the F1 protein.

3.6. Sequence comparison and phylogenetic analysis

Comparison of the AHRI33 full-length genome with those of known APMV species revealed that AHRI33 was more closely related to APMV-7 (62.4% nucleotide identity) than to the other APMVs (43.5–53.7%) (Table 2). The highest inter-serotypic identity was 68.1% between APMV-1 and -16, and the lowest was 42.7% between APMV-4 and -6.

The nucleotide sequence identities of each AHRI33 gene to the corresponding genes of other APMVs ranged from 39.3% (P) to 68.0% (N) (Table 3). All six genes of AHRI33 were most similar to those of APMV-7, with identities of 55.7% to 68.0%. The amino acid identities were consistent with the corresponding nucleotide identities (Table 3).

Phylogenetic trees were constructed based on alignment of the full-length genome of AHRI33 (Fig. 3), the F gene (Supplementary Fig. 1), and the HN gene (Supplementary Fig. 2) with corresponding genes of representative APMV species. Phylogenetic analysis based on the full-length genome sequences (Fig. 3) revealed that the four AHRI33-like isolates were grouped along with APMV-7 and -11 but were distinguishable from these two species. Within this group, AHRI33-like isolates appeared to be more closely related to APMV-7 than to APMV-11. Phylogenetic analysis based on F gene sequences (Supplementary Fig. 1) gave similar results. The other 13 AHRI33-like viruses isolated in Taiwan were highly similar to AHRI33, with 98.9% to 99.9% nucleotide identities of the fusion gene. All of them were assigned to the same cluster in the phylogenetic analyses (Supplementary Fig. 1).

Phylogenetic analysis of amino acid sequences of the RdRp revealed that all APMVs clustered together in a group designated as subfamily *Avulavirinae*, when compared with the closely related Mumps virus as an outgroup (Fig. 4). Three main clades designated as genera *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus* were observed within the subfamily *Avulavirinae*. The AHRI33-like isolates characterized in this study nested within genus *Metaavulavirus*, and the branch length between the nearest node and the tip of the branch is 0.28.

4. Discussion

This study demonstrated that a unique APMV strain has been long-term circulating in resident doves and pigeons in Taiwan. In addition to the novel AHRI33 virus, 13 AHRI33-like APMVs were isolated from

Table 1
Antigenicity between newly isolated AHRI33 virus and representative avian paramyxoviruses (APMVs), measured by cross-hemagglutination inhibition tests. The following representative viruses were used as antigens and to prepare homologous chicken antisera: APMV-1/Ulster/2C/70, APMV-2/chicken/California/Yucaipa/56, APMV-3/parakeet/Netherlands/449/75, APMV-4/duck/Hong Kong/D3/75, APMV-6/duck/Hong Kong/199/77, APMV-7/dove/Tennessee/4/75, APMV-8/goose/Delaware/1053/75, and APMV-9/duck/New York/22/78.

Virus	APMV-1 antiserum	APMV-2 antiserum	APMV-3 antiserum	APMV-4 antiserum	APMV-6 antiserum	APMV-7 antiserum	APMV-8 antiserum	APMV-9 antiserum	AHRI33 antiserum
APMV-1	512[a]								
APMV-2		256							
APMV-3			1024						
APMV-4				1024					
APMV-6					512				
APMV-7						4096			8
APMV-8							1024		
APMV-9								256	
AHRI33	16	4	16	16	4	1024	16	4	128

[a] HI titres are expressed as the reciprocal of the highest dilution causing inhibition of 4 HA units of virus.

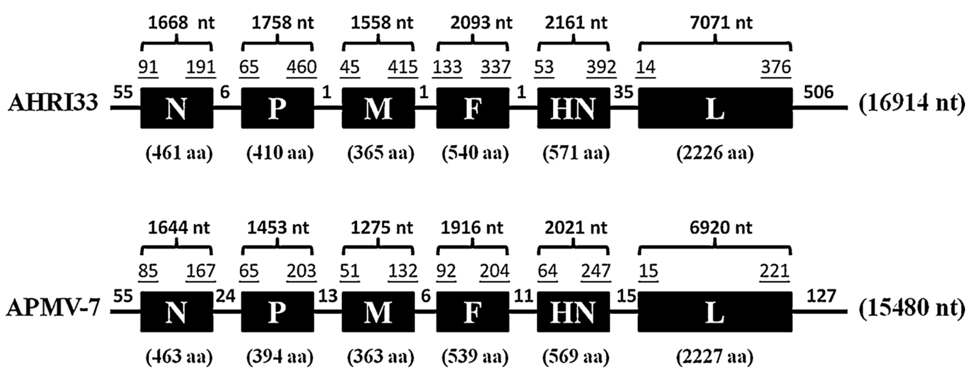

Fig. 2. Schematic diagram of avian paramyxovirus (APMV) AHRI33 isolate and APMV-7 genome. Each rectangle indicates a gene and the letters within each rectangle represents the genes: N (nucleoprotein gene), P (phosphoprotein gene), M (matrix protein gene), F (fusion protein gene), HN (hemagglutinin-neuraminidase gene), and L (large polymerase gene). The length of the genes and predicted proteins are shown above and under the rectangle, respectively. The lengths of the non-translated upstream and downstream regions are underlined. Intergenic regions are located between each box.

Table 2
Percentage identity of nucleotide sequences of genome (lower left) and amino acid sequences of RdRp gene (upper right). Shaded cells represent the inter-species heterogeneous levels that are lower than those between APMV-7 and AHRI33.

APMV	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11	-12	-13	-14	-15	-16	-17	-18	-19	-20	AHRI33
-1		39.1	33.8	33.4	38.7	39.1	39.2	38.9	61.9	38.7	39.8	56.3	55.1	37.2	38.0	75.8	52.0	52.0	52.2	38.6	39.1
-2	46.9		35.2	35.6	44.5	45.1	45.2	60.7	39.5	62.4	49.7	39.1	38.2	42.8	59.4	39.5	40.6	40.7	40.4	61.3	45.3
-3	44.7	44.4		41.7	36.0	37.0	37.0	35.6	33.9	35.5	37.7	34.5	33.5	34.9	35.5	33.8	34.2	35.0	34.2	36.2	37.2
-4	44.0	44.9	47.7		34.5	34.7	35.7	34.8	32.8	34.9	36.0	33.5	32.9	32.2	35.1	34.1	33.5	34.3	34.1	34.9	34.9
-5	46.7	50.4	44.3	43.6		51.7	46.3	44.7	39.3	44.1	47.1	38.8	38.0	50.1	44.7	38.7	39.5	40.1	40.4	45.1	44.7
-6	44.9	48.2	43.3	42.7	56.4		45.6	44.6	40.4	45.6	47.3	39.6	39.0	50.4	44.5	40.1	40.4	40.9	40.6	45.2	45.2
-7	45.8	50.3	44.8	43.9	50.5	48.3		46.4	39.5	46.5	48.6	39.9	40.7	43.9	46.0	39.5	39.6	39.7	40.1	45.6	**62.2**
-8	46.7	60.8	44.6	43.9	50.9	48.0	50.8		39.7	60.7	49.9	39.8	39.2	45.0	57.9	39.9	39.7	39.5	39.9	60.8	46.0
-9	61.0	46.0	44.5	43.6	46.4	44.4	46.0	46.8		40.1	39.9	54.2	54.5	39.0	38.8	62.9	52.3	51.5	51.6	40.2	39.2
-10	46.1	60.6	44.1	43.6	51.4	49.0	50.8	60.1	46.6		49.8	39.3	38.8	44.3	61.3	39.6	39.8	39.4	38.9	61.9	47.1
-11	45.8	51.1	44.8	44.2	51.9	48.6	53.7	51.2	46.4	51.7		41.2	40.7	46.2	49.8	40.4	41.1	40.5	40.9	49.1	47.4
-12	57.9	46.4	44.5	44.2	46.6	44.4	46.1	46.9	56.7	45.9	46.5		66.5	38.4	39.9	57.9	53.3	53.1	52.6	39.7	39.5
-13	57.4	45.9	45.2	44.4	46.4	45.3	46.6	46.8	56.5	46.0	46.4	64.5		38.7	39.8	55.5	52.4	52.1	52.0	39.3	38.6
-14	46.2	50.5	44.4	43.9	54.4	51.6	49.7	51.8	46.0	51.3	49.9	46.5	46.5		43.5	39.1	38.3	38.9	38.9	42.9	44.0
-15	46.0	59.0	43.6	43.9	50.6	48.8	50.1	59.5	46.1	60.1	51.7	46.3	46.0	51.5		39.7	40.0	40.9	39.9	58.1	46.0
-16	68.1	46.5	44.0	43.8	46.0	45.0	46.0	47.0	60.8	46.2	46.4	57.6	58.0	46.7	46.6		53.0	53.5	53.9	39.3	39.5
-17	53.8	46.9	44.1	44.5	46.1	44.8	46.4	46.8	53.4	46.0	46.1	54.4	54.0	46.1	47.3	53.9		75.8	67.1	40.0	40.0
-18	53.9	45.9	44.3	44.3	46.0	44.4	45.1	45.8	53.4	45.9	45.9	54.1	53.5	46.2	45.7	54.1	67.4		67.0	40.4	39.9
-19	54.1	45.7	44.3	44.8	45.9	44.7	45.1	46.0	53.3	45.5	46.0	53.5	54.4	45.8	46.3	54.0	62.2	62.7		40.3	40.4
-20	45.8	59.3	45.1	44.5	51.5	48.7	50.7	59.8	47.0	60.4	51.6	47.1	46.6	51.6	58.6	47.0	46.7	46.1	45.6		46.0
AHRI33	46.1	49.7	44.6	43.5	50.2	47.8	**62.4**	50.1	46.5	50.1	53.7	46.4	46.2	49.4	50.5	46.7	46.0	45.9	45.7	50.4	

Table 3
Percentage nucleotide (nt) and deduced amino acid (aa) sequence identities between the AHRI33 isolate and avian paramyxoviruses (APMVs) representing the species in the subfamily *Avulavirinae*.

Virus	N		P		M		F		HN		L	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
APMV-1	49.3	43.0	43.6	25.1	43.7	33.8	47.1	39.3	43.5	36.7	47.7	39.1
APMV-2	56.2	56.5	41.1	28.7	49.2	41.4	49.3	41.7	48.9	44.0	50.6	45.3
APMV-3	44.7	35.5	39.3	23.1	43.3	33.2	40.4	29.7	45.2	35.9	45.2	37.2
APMV-4	44.4	35.7	40.0	26.4	41.6	29.0	43.2	32.8	45.4	35.7	44.5	34.9
APMV-5	57.1	52.4	47.0	30.0	49.9	46.8	49.1	39.9	49.7	43.2	50.9	44.7
APMV-6	56.3	53.8	45.2	28.7	53.0	46.2	49.1	41.8	51.2	44.5	50.5	45.2
APMV-7	68.0	75.7	55.7	41.7	65.5	65.4	60.4	56.8	63.1	66.5	62.5	62.2
APMV-8	56.8	54.7	44.2	29.0	51.8	40.8	49.5	41.7	48.0	40.6	50.9	46.0
APMV-9	49.1	41.4	42.6	28.7	44.5	32.7	46.2	39.1	44.7	35.2	48.2	39.2
APMV-10	56.0	54.9	45.2	31.6	47.3	37.5	49.9	44.8	49.0	40.2	52.3	47.1
APMV-11	58.9	57.9	44.9	31.9	49.5	39.4	50.3	38.5	50.5	42.3	54.4	47.4
APMV-12	48.1	41.4	43.5	25.1	44.9	32.1	46.9	37.7	44.8	34.4	48.0	39.5
APMV-13	49.5	41.4	41.2	25.1	43.5	29.6	47.1	38.3	44.7	36.8	47.5	38.6
APMV-14	54.4	50.1	43.3	26.1	50.8	41.7	49.9	42.0	50.7	43.6	50.1	44.0
APMV-15	59.2	57.0	43.5	27.4	49.0	39.7	49.9	44.0	47.1	38.3	51.5	46.0
APMV-16	50.9	44.2	43.9	28.7	40.7	31.5	47.3	39.9	45.4	36.8	48.5	39.5
APV-A	48.8	42.8	41.5	28.0	43.5	33.0	44.9	37.1	46.3	38.3	47.5	40.0
APV-B	50.7	43.2	41.8	30.6	44.4	35.8	45.8	37.3	44.4	36.3	46.8	39.9
APV-C	48.6	43.2	44.3	30.3	43.8	34.9	46.2	38.7	45.9	38.7	46.4	40.4
APMV-20	57.5	57.0	43.2	30.0	48.9	39.7	50.0	41.7	48.8	41.2	51.7	46.0

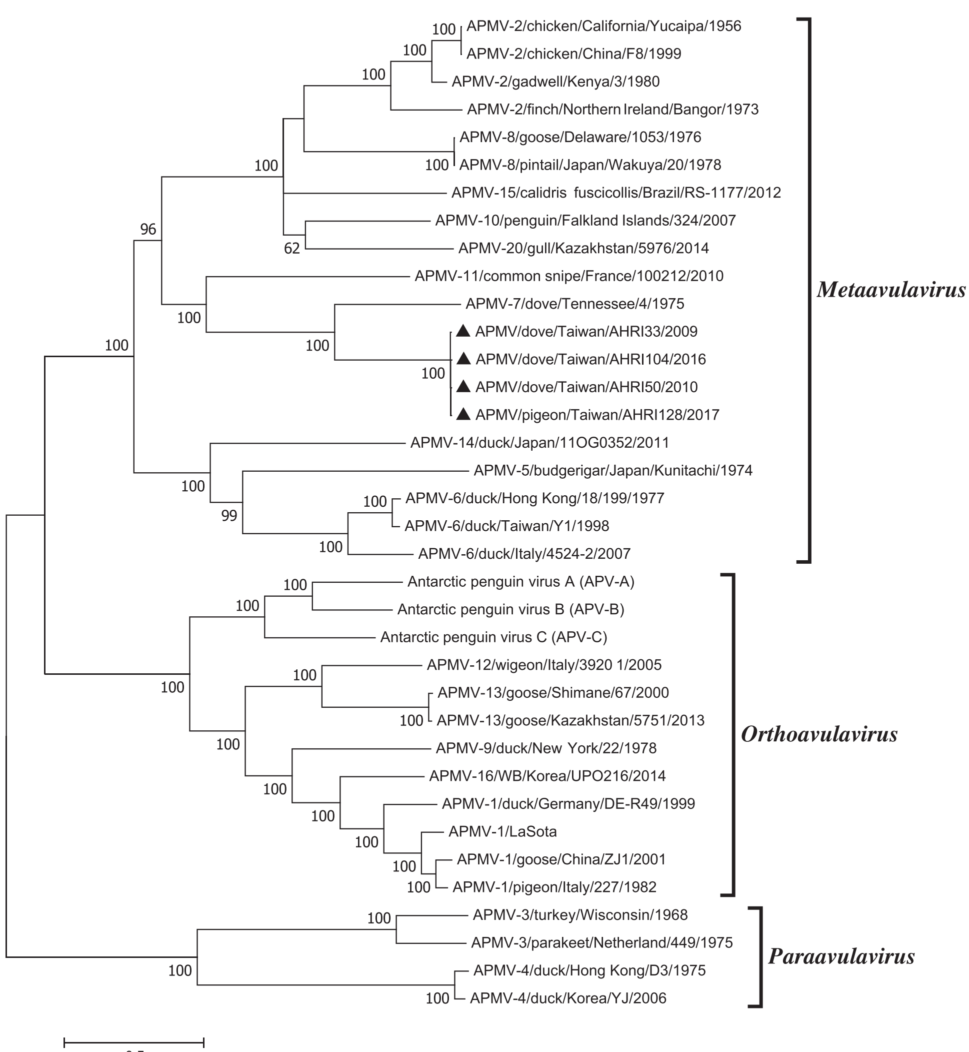

Fig. 3. Phylogenetic tree of the avian paramyxoviruses (APMVs), based on comparison of their full-length genomes. The solid triangles mark the isolates of APMV isolated from doves and pigeons in Taiwan in 2009-2017. The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites (4 categories + G, parameter = 1.5932; [+I], 5.54% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The final dataset had a total of 12,555 positions. Numbers at the nodes indicate bootstrap confidence value (1000 replicates) for the group composed of the viruses right to the node.

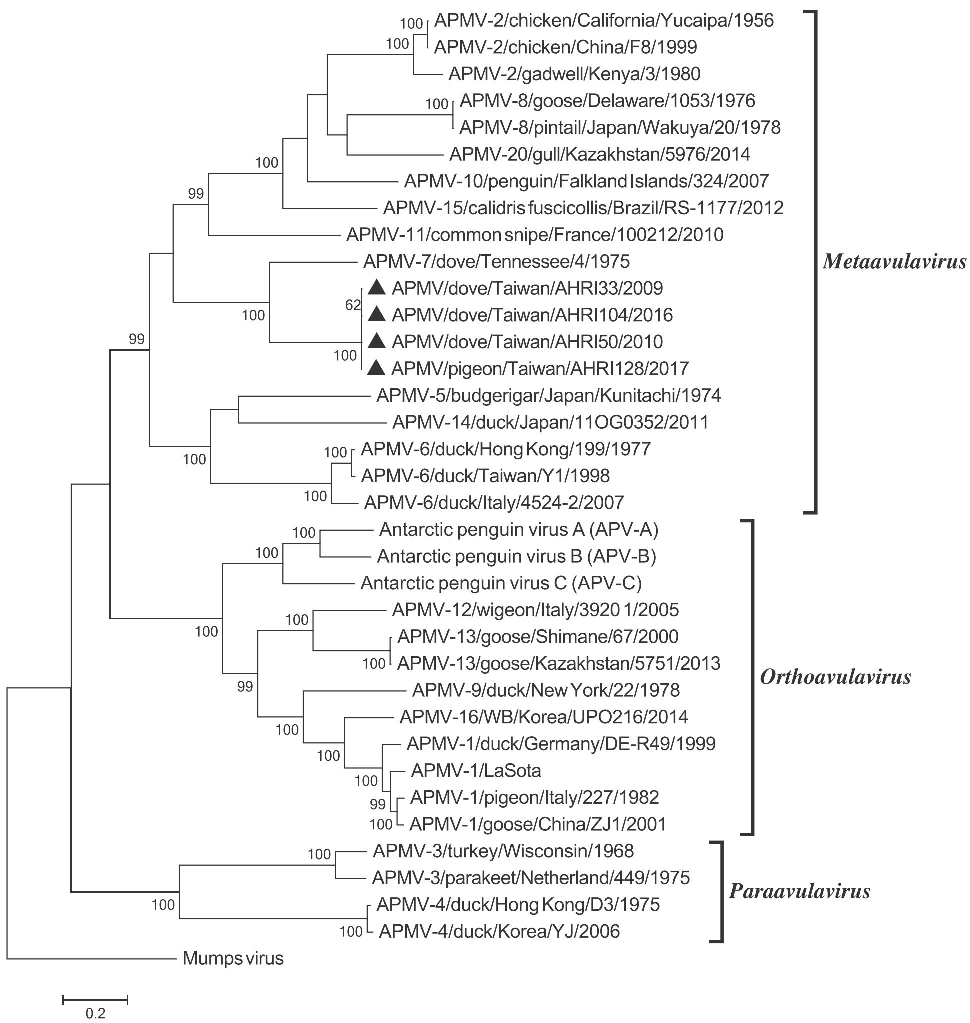

Fig. 4. Maximum Likelihood phylogenetic tree of the amino acid sequences of RdRp gene. The solid triangles mark the isolates of APMV isolated from doves and pigeons in Taiwan in 2009-2017. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-92897.20) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The final dataset had a total of 2344.

three different species of the family *Columbidae* in 2009-2017. Although the samples taken in the avian influenza surveillance program covered many species of domestic poultry, migratory birds, resident birds, and imported birds, the novel APMV strain was isolated from members of the family *Columbidae*, implying that pigeons and doves may be one of the natural hosts of AHRI33-like viruses. Future surveillance of wild birds in Taiwan may help to better elucidate the host distribution of AHRI33-like viruses.

Sequencing of the viral genome of the AHRI33 isolate revealed characteristic APMV coding regions and the non-coding terminal sequences (e.g., the 55 nucleotide non-coding leader sequence at the 5′ end that is present in all APMVs) (Fig. 2). The genome length of 16,914 nt is compatible with the “rule of six”, which plays an important role in the replication of APMVs (Kolakofsky et al., 1998), and it is the third longest among the twenty species of APMVs reported to date, shorter than only those of APMV-11 and APMV-5. The schematic diagram of AHRI33 and the protovirus APMV-7 genome made clear the significant disparities in length of the complete genome, all six of genes, the intergenic regions, and the 5′-trailer region (Fig. 2). The length difference of 1434 nt between AHRI33 and APMV-7 (15,480 bp) is much greater than the largest difference, 120 nt, between intra-species of APMV-2/Yucaipa and APMV-2/Bangor. The phylogenetic relationship with APMV-7 is consistent throughout the genome, forming a monophyletic group, suggesting that these viruses share a more recent common ancestor than do other lineages (Fig. 3). The deduced amino acid sequence of the F-gene cleavage site was STQQER/LFG, which was significantly different from that of APMV-7/Tennessee (LPSSR/FAG) and all other APMVs. This motif lacks multiple basic residues and phenylalanine at the N-terminus of the F1 protein, a characteristic that typically corresponds with non-pathogenic variants, which is concordant with the results of the ICPI test.

Traditionally, APMVs were classified based on their antigenic differences, and nine serotypes were defined by HI assay in the 1970s (Alexander, 1987). In the present study, HI assay revealed weak cross-reactivity between APMV-7 and AHRI33 (R = 0.125), and there were extremely low relationships between AHRI33 and representatives of the other species. HI cross-reactivity is not rare between different species of APMV (e.g., between APMV-1 and APMV-12 (Terregino et al., 2013), and APMV-9 and APMV-16 (Lee et al., 2017)), and lack of HA activity observed in APMV-5 (Samuel et al., 2010) and one novel APMV-6 (Chen et al., 2018), and all this makes classification into serotypes problematic.

In contrast, Terregino et al. (2013) proposed a classification based on nucleotide sequence identities of the whole genome as one simple method. According to this classification method based on genome identities, AHRI33 is closest to APMV-7, at 62.4%, which less than those between APMV-1 and -16 (68.1%), APMV-A and -B (67.4%), APMV-12 and -13 (64.5%), and APMV-B and -C (62.7%) (Table 2).

These results of genetic analyses indicate that the AHRI33 isolate evolved from a common ancestor of APMV-7 and -11 and is now a distinct branch of the APMV groups.

In the last proposal for taxonomy changes of the family *Paramyxoviridae*, the ICTV Study Group has decided that the classification should be based on a sequence comparison of the RdRps of the viruses (ICTV, 2019). Based on the phylogenetic tree topology (clustered into monophyletic branch within the clade of the genus *Metaavulavirus*) and the branch length measured in the number of substitutions per site above 0.03, the AHRI33-like isolates met the criteria for designation as distinct species.

To sum up, we identified new APMVs from the birds of family *Columbidae* in Taiwan from 2009 to 2017. The new APMV isolates are more closely related to APMV-7 based on estimates of nucleotide identities of the full-length genome; however, these heterogeneous levels are comparable to, or even greater than, those of several inter-species distances separating other accepted species. This, together with the analysis according to new RdRp phylogeny-based classification system, suggests that the newly-isolated APMV should be considered as a novel species and the prototype strain of a new APMV-21 group, with the full name APMV-21/dove/Taiwan/AHRI33/2009.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.029.

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