The Journal of Veterinary Medical Science

FULL PAPER

Avian Pathology

Genetic diversity of avian paramyxoviruses isolated from wild birds and domestic poultry in Taiwan between 2009 and 2020

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J. Vet. Med. Sci.
84(3): 378–389, 2022
doi: 10.1292/jvms.21-0608

Received: 12 November 2021
Accepted: 10 January 2022
Advanced Epub:
27 January 2022

ABSTRACT. Avian paramyxoviruses (APMVs) belonging to the subfamily *Avulavirinae* within the family *Paramyxoviridae*. APMVs consist of twenty-two known species and are constantly isolated from a wide variety of avian species around the world. In this study, the APMV isolates obtained from wild birds and domestic poultry during 2009–2020 in Taiwan were genetically characterized by phylogenetic analysis of their complete fusion protein gene or full-length genome. As a result, 57 APMV isolates belonging to seven different species were obtained during this period and subsequently identified as APMV-1 (n=17), APMV-2 (n=1), APMV-4 (n=25), APMV-6 (n=8), APMV-12 (n=2), APMV-21 (n=2) and APMV-22 (n=2). Sanger sequencing was performed to provide 22 full-length genome sequences and 35 complete fusion protein gene sequences for the APMV isolates. Phylogenetic analysis showed that the recovered viruses were closely related to Eurasian strains, except five class I APMV-1 and four APMV-4 isolates were related to North America strains. Our findings provided more evidence for the intercontinental transmission of APMVs between Eurasia and North America by wild birds. In addition, according to the criteria of the classification system based on complete fusion protein gene sequences, three novel genotypes within APMV-2, APMV-12, and APMV-22 were identified. Together, this investigation provided a broader perspective on the genetic diversity, evolution, and distribution of APMVs in multiple avian host species sampled in Taiwan.

KEY WORDS: avian paramyxovirus, fusion protein, genetic diversity, intercontinental dispersal

Three genera, named *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus* have recently been created within a new subfamily *Avulavirinae* of the family *Paramyxoviridae*, according to the taxonomy of the order *Mononegavirales*: updated in 2018 [1]. Avian paramyxoviruses (APMVs), which belong to the newly assigned subfamily *Avulavirinae*, have been isolated from a wide variety of avian species across the globe. Twenty-one species of APMVs (APMV-1 to 21) are reported and their full-length genome sequences are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank). Recently, a new species of APMV-22 was proposed [15].

The APMV contains a non-segmented, negative-sense, single-stranded RNA genome that ranges from 14,904 to 17,412 nucleotides (nt) in length [3]. Most of the APMVs' genomes encode at least six proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase (L). The order of these protein genes in the genome is 3′ leader-N-P-M-F-HN-L-5′ trailer [2]. Among these genes, the F gene has been used frequently for molecular epidemiological investigations and genotyping of APMVs. Meanwhile, an unified and objective classification system of APMV-1 was proposed in 2012 based on the mean inter-population evolutionary distances of the complete F gene sequence, with cut-off values more than 10% to assign new genotypes [8], and this system and nomenclature criteria were revised and updated by a global consortium in 2019 [9]. This classification system of APMV-1 was then applied among other species of APMVs to provide a more rational and scientific genotyping method for epidemiological studies [6, 19, 20].

APMV-1, commonly termed Newcastle disease virus or NDV, has a wide host range and is able to infect over 240 species of birds [12]. In contrast, the information about the host ranges and distribution of the other APMV species in wild birds and domestic

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poultry is limited. APMV-2, -3 and -7 have been isolated from captive caged birds and domestic poultry including chickens and turkeys; APMV-5 has been isolated mostly from budgerigars; and the other APMVs appear to be more restricted to wild birds including ducks, geese, gulls and penguins [3].

With regard to APMVs in Taiwan, our previous studies outlines genetic diversity and distribution of class I and class II APMV-1 isolates in 2010–2018 [14] and identified novel APMV-22 isolates from the birds of family *Columbidae* in 2009–2017 [15]. These findings suggest that wild birds and domestic poultry maintain previously unrecognized genetic diversity of APMVs. Meanwhile, what we know about host spectrum, distribution, and evolution of APMVs has remained limited. In the present study, the APMV isolates obtained from wild birds and domestic poultry in Taiwan were characterized by sequencing complete F protein gene or full-length genome sequences and were compared to those available in GenBank. Through these efforts, we aim to illustrate the genetic diversity of APMVs in various avian hosts sampled in Taiwan and to provide more evidence for the intercontinental transmission of APMVs by migratory birds.

MATERIALS AND METHODS

Sample collection and virus isolation

The samples of this study were collected from migratory, resident, imported, and domestic birds in Taiwan as part of an avian influenza surveillance program and clinical cases submitted to Animal Health Research Institute from 2009 to 2020. Virus isolation was conducted in accordance with Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [16]. The oropharyngeal and cloacal/fecal swabs from apparently healthy birds and tissue samples of the brain, trachea, lung, liver, spleen, heart, and kidney from clinical cases were inoculated into the allantoic cavities of 9- to 11-day-old specific-antibody-negative embryonated chicken eggs (Animal Drugs Inspection Branch, Animal Health Research Institute, Miaoli, Taiwan) and then incubated at 37°C for 72 hr. The allantoic fluid from each inoculated embryo was collected and examined for its hemagglutination (HA) activity. If HA activity was not detected, a second passage was then performed. The HA-positive allantoic fluid was subjected to further analyses.

RNA extraction and semi-nested reverse transcription-polymerase chain reaction (semi-nested RT-PCR)

Viral RNA was extracted from infective allantoid fluid using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

For the first RT-PCR in the semi-nested assay, we used the SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with the universal primers, Pan-APMV-F and Pan-APMV-1R (Table 1), to amplify the conservative region of L gene of APMV-1 to 22. The cycling parameters were reverse transcription at 50°C for 40 min, followed by heating at 94°C for 2 min, 35 cycles of denaturing at 94°C for 40 sec, annealing at 50°C for 50 sec, and extension at 72°C for 40 sec, and completed with a final extension step at 72°C for 7 min. For the second PCR in the semi-nested assay, we used the Quick Taq™ HS DyeMix kit (TOYOBO, Osaka, Japan) with the primers Pan-APMV-F, Pan-APMV-2Ra, and Pan-APMV-2Rb for amplifying and sequencing to identify viral species. The cyclic conditions for the second PCR were the same as those of the first RT-PCR. The PCR products were separated by electrophoresis using 2% agarose gel and were visualized with ethidium bromide stain and ultraviolet transillumination.

Nucleotide sequencing of complete fusion protein gene and full-length genome

To determine the nucleotide sequence of complete coding region for the F gene of APMV-1, amplification of the coding region by RT-PCR was conducted as previously described [14]. To amplify the same region of APMV-2, APMV-4, APMV-6, APMV-12, APMV-21, and APMV-22 isolates, the corresponding RT-PCR primers are listed in Table 1. The full-length genome sequences were determined with various sets of primers according to the APMV species, and the sequences of these primers are available upon request. The sequences of both ends of the genome were amplified using the rapid amplification of cDNA ends method (RACE) [17]. The RT-PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). These products were then cloned with TOPO TA Cloning kit (Invitrogen) using the standard protocol, and the inserted cDNA segments were amplified using M13 forward and reverse primers provided by the kit. Amplified products with expected size were sequenced using the 3700XL DNA analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) by a commercial sequencing service (Mission Biotech, Taipei, Taiwan). Sequences were assembled and edited using the Lasergene 6.0 software package (DNASTAR, Madison, WI, USA).

Phylogenetic analysis

The sequences of complete F gene or full-length genome of the isolated APMVs were aligned with the sequences of APMV-1 to APMV-22 viruses retrieved from GenBank using ClustalW in Molecular Evolutionary Genetics Analysis version 7, or MEGA 7 [13]. 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 by 1,000 bootstrap replicates.

The estimated evolutionary distance between each APMV species was measured by MEGA 7 using the maximum composite likelihood model [18]. The rate variation among sites was modeled with a gamma distribution (shape parameter=1). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

Table 1. List of RT-PCR primers

Assay/Designation	Primer sequence (5′-3′)	Gene	Position in reference sequence	Fragment size (bp)	Accession no. of reference sequence
Avian paramyxoviruses (APMV) universal					
Pan-APMV-F	TGACHACWGAYYTIIARAARTAYTG	Large polymerse	10,293–10,317	355	AF077761
Pan-APMV-1R	GCIATIRCYTGRTTRTCICCYTG	Large polymerse	10,625–10,647		
Pan-APMV-2Ra	CCATAGTTTYTGTTGCAGRCCTTC	Large polymerse	10,532–10,555	263	
Pan-APMV-2Rb	CCACAKYTTYTGRCAYARICCYTC	Large polymerse	10,532–10,555		
APMV-2					
APMV-2-1Fa	CTCCAGACTAAGTGGGTGG	Fusion	4,310–4,328	978	EU338414
APMV-2-1Fb	CCCAAAAAACRYYCCGAG	Fusion	4,310–4,328		
APMV-2-1Fc	CCCATAGCAACCTGGCC	Fusion	4,310–4,326		
APMV-2-1R	GTCAVCACYCTRCTYTGWGC	Fusion	5,268–5,287		
APMV-2-2F	GTRTCWTACCCMAGTGTSTC	Fusion	5,157–5,176	968	
APMV-2-2Ra	TGCTGCCAGGTTCTCCC	Fusion	6,107–6,124		
APMV-2-2Rb	WTYIGTGAGGTTCTCTCTKG	Fusion	6,104–6,124		
APMV-4					
APMV-4-1F	GGARTTGATTGGGTGTCTAAAC	Fusion	4,331–4,352	988	FJ177514
APMV-4-1R	CRACCCTCGTATTCTGGAC	Fusion	5,300–5,318		
APMV-4-2F	GATCTGTCACAAGTCARTTGG	Fusion	5,172–5,192	1,000	
APMV-4-2R	CCAAYCGGCCTTGTGACAC	Fusion	6,153–6,171		
APMV-6					
APMV-6-GI-1F	CTTCCTARCTRTTCCTYCCTTAG	Fusion	4,478–4,500	1,036	EU622637
APMV-6-GI-1R	CAAYTCTGTCAGTCGCAACC	Fusion	5,493–5,512		
APMV-6-GI-2F	CTTAATCAATGGCAGAATCATTC	Fusion	5,404–5,426	1,042	
APMV-6-GI-2R	GTTGGGCTGTTAGATTATTCTGC	Fusion	6,423–6,445		
APMV-6-GII-1F	GCCAYAGACCACAAAAGAGC	Fusion	4,548–4,567	1,005	GQ406232
APMV-6-GII-1R	CTTTACCCTCTCCAGCAG	Fusion	5,535–5,552		
APMV-6-GII-2F	CAGATAATGGTCATTCAAGTCTC	Fusion	5,444–5,466	944	
APMV-6-GII-2R	GCAATTTACGGCTAATCAACTG	Fusion	6,366–6,387		
APMV-12					
APMV-12-1Fa	GGTKGAWCYTGAACCAATACGG	Fusion	4,552–4,573	963	NC_025363
APMV-12-1Fb	GAAAAAACTGATACTGCCACGG	Fusion	4,552–4,573		
APMV-12-1R	TCRAGKAGAGTYGCWCGTGC	Fusion	5,495–5,514		
APMV-12-2F	TRGGYATTGAMGRGACRCAGC	Fusion	5,361–5,381	1,073	
APMV-12-2R	GRGACYSYCYCSTTCTGCC	Fusion	6,415–6,433		
APMV-21					
APMV-21-1F	TGAGAGYGATACGGGTAGG	Fusion	4,776–4,794	934	MF594598
APMV-21-1R	AGAACTCCCTTGAGATTCCC	Fusion	5,690–5,709		
APMV-21-2F	TCTWGGAGCAGACAACAGC	Fusion	5,569–5,587	1,026	
APMV-21-2R	GCRCACCACCTTCCTACC	Fusion	6,577–6,594		
APMV-22					
APMV-22-1Fa	GTACAAGAGTCAAAGTAGAAACAG	Fusion	5,127–5,150	914	MK677430
APMV-22-1Fb	GTGTAAATATTACCACCAAGTTAG	Fusion	5,127–5,150		
APMV-22-1R	TAGTGTTGCTATGCTAGGAAG	Fusion	6,018–6,040		
APMV-22-2F	GAGAAATAYGGTTATAARCAAGC	Fusion	5,904–5,926	956	
APMV-22-2Ra	ATGAGTCAATGTGCAATGAGG	Fusion	6,839–6,859		
APMV-22-2Rb	ATGATTCAGTGTGTGATAAGG	Fusion	6,839–6,859		

RESULTS

Sample collection and virus isolation

Fifty-seven APMV isolates were obtained from various avian species in this study (Tables 2 and 3). The isolates were confirmed to be APMVs by the HA test, semi-nested RT-PCR targeting L gene, and Sanger sequencing. With subsequent BLAST analysis, 17 isolates belonged to APMV-1, 1 isolate to APMV-2, 25 isolates to APMV-4, 8 isolates to APMV-6, 2 isolates to APMV-12, 2 isolates to APMV-21, and 2 isolates to APMV-22.

Table 2. Genotype and pathotype of avian paramyxovirus 1 (APMV-1) isolates in 2019 and 2020

Isolate	Origin	Class	Sub/genotype	Cleavage site of fusion protein	Sequence coverage	GenBank accession no.
APMV-1/mule_duck/Taiwan/AHRI139/2019	Domestic	I	1.2	^{112}ERQER↓L^{117}	Fusion	MZ802810
APMV-1/mule_duck/Taiwan/AHRI149/2019	Domestic	I	1.2	^{112}ERQER↓L^{117}	Fusion	MZ802811
APMV-1/Anseriformes/Taiwan/AHRI155/2019	Migratory	I	1.2	^{112}ERQER↓L^{117}	Genome	MZ802788
APMV-1/Anseriformes/Taiwan/AHRI158/2019	Migratory	I	1.2	^{112}ERQER↓L^{117}	Fusion	MZ802812
APMV-1/Anseriformes/Taiwan/AHRI171/2020	Migratory	I	1.2	^{112}ERQER↓L^{117}	Genome	MZ802789
APMV-1/Ardea_alba/Taiwan/AHRI172/2020	Migratory	I	1.2	^{112}ERQER↓L^{117}	Fusion	MZ802813
APMV-1/Anseriformes/Taiwan/AHRI177/2020	Migratory	I	1.2	^{112}ERQER↓L^{117}	Fusion	MZ802814
APMV-1/Charadriiformes/Taiwan/AHRI145/2019	Migratory	I	Unclassified	^{112}ERQER↓L^{117}	Genome	MZ802790
APMV-1/Anseriformes/Taiwan/AHRI142/2019	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802815
APMV-1/Charadriiformes/Taiwan/AHRI146/2019	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802816
APMV-1/Anseriformes/Taiwan/AHRI151/2019	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802817
APMV-1/Anseriformes/Taiwan/AHRI156/2019	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802818
APMV-1/Anseriformes/Taiwan/AHRI174/2020	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802819
APMV-1/Anseriformes/Taiwan/AHRI176/2020	Migratory	II	I.2	^{112}GKQGR↓L^{117}	Fusion	MZ802820
APMV-1/dove/Taiwan/AHRI144/2019	Resident	II	VI.2.1.1.2.2	^{112}RRQKR↓F^{117}	Fusion	MZ802821
APMV-1/pigeon/Taiwan/AHRI147/2019	Resident	II	VI.2.1.1.2.2	^{112}KRQKR↓F^{117}	Fusion	MZ802822
APMV-1/chicken/Taiwan/AHRI148/2019	Domestic	II	VII.1.1	^{112}RRKKR↓F^{117}	Genome	MZ802791

GenBank accession numbers

GenBank accession numbers of the APMV isolates described in this study are listed in Tables 2 and 3.

Genetic analysis of APMV-1

From 2019 to 2020, 17 isolates sequenced for this study were identified as members of APMV-1 (Table 2). Eight of the 17 isolates obtained from wild birds and domestic ducks were identified as either genotype 1 (n=7), or unclassified (n=1) within class I (Fig. 1). Five isolates of genotype 1 viruses were grouped with those originated from the USA. The APMV-1/Anseriformes/Taiwan/AHRI158/2019 and APMV-1/Anseriformes/Taiwan/AHRI177/2020 isolates of genotype 1 viruses were clustered with those derived from wild birds in Japan, China, Russia, Kazakhstan, Germany, and Finland. The APMV-1/Charadriiformes/Taiwan/AHRI145/2019 (APMV-1 AHRI145) isolate was grouped within class I viruses but formed a monophyletic lineage with a genetic distance of 5.6% between the others within class I. Nine class II viruses of APMV-1 in this study were identified as sub-genotype I.2 (n=6), sub-genotype VI.2.1.1.2.2 (n=2), or sub-genotype VII.1.1 (n=1). The six genotype I.2 isolates were obtained from waterfowl (*Anseriformes*), and shorebirds (*Charadriiformes*). Of the two isolates of pigeon paramyxovirus 1 (PPMV-1), a genetic variant of NDV that belongs to sub-genotype VI.2.1.1.2.2, was obtained from birds in the family *Columbidae* (pigeon and spotted dove), and the deduced amino acid motif at the F cleavage site was ^{112}(K/R) RQKR↓F^{117}. One isolate of sub-genotype VII.1.1 APMV-1 was obtained from domestic chicken, and the deduced amino acid motif at the F cleavage site was ^{112}RRKKR↓F^{117}.

Genetic analysis of APMV-2

One virus isolated in this study was classified as APMV-2, which was from a bird of the family *Psittacidae* (Fig. 2). The isolate, APMV-2/macaw/Taiwan/Q35-SG/2009 (APMV-2 Q35-SG), was obtained from a blue-and-gold macaw (*Ara ararauna*) imported from Singapore during quarantine in Taiwan in 2009. Phylogenetic analysis based on full-length genome sequences (Fig. 2) revealed that the APMV-2 Q35-SG isolate was clustered closely to APMV-2/Finch/N.Ireland/Bangor/1973 (APMV-2 Bangor, GenBank accession number HM159995) and other APMV-2. These APMV-2 isolates represented two genotypes based on the mean inter-population evolutionary distance of 24.4%: the genotype I composed of the prototype northern American (Yucaipa) isolate and the isolates of China, England, and Kenya; the genotype II composed of Bangor isolate and the Q35-SG isolate. The genome sequence of the APMV-2 Q35-SG isolate, determined by an amplification strategy combined APMV-2-specific primer set and rapid amplification of cDNA ends method (RACE), was 15,024 nt in length which was identical to that of the Bangor isolate. The genome of APMV-2 Q35-SG isolate showed 91.5% identity with that of Bangor isolate and six single-nucleotide insertion-deletion combinations at the genome positions of 8,487–9,200, 9,655–9,892, 11,573–11,616, 12,634–12,710, 13,519–13,547, and 14,011–14,105. The amino acid sequence identities of each APMV-2 Q35-SG gene to those of Bangor isolate were 98.2% (NP), 90.2% (P), 97.0% (M), 95.6% (F), 94.7% (HN), and 81.3% (L).

Genetic analysis of APMV-4

Twenty-five viruses obtained in this study were classified as members of APMV-4, the most prevalent species of APMVs besides APMV-1. All of these Taiwanese APMV-4 isolates were isolated from wild birds of the order *Anseriformes* in 2009–2020. Phylogenetic analysis demonstrated that there were three groups of APMV-4 circulating in wild birds from the wetlands of Taiwan (Fig. 3). Among these viruses, ten viruses were grouped with the isolates from ducks sampled in China, Japan, and South Korea

Table 3. Detailed information on non-avian paramyxovirus 1 (non-APMV-1) isolates between 2009 and 2020

Isolate	Origin	Cleavage site of fusion protein	Sequence coverage	GenBank accession no.
APMV-2				
APMV-2/macaw/Taiwan/Q35-SG/2009	Quarantine	^{102}LPSSR↓F^{107}	Genome	MZ802792
APMV-4				
APMV-4/Anseriformes/Taiwan/AHRI36/2009	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802793
APMV-4/Anseriformes/Taiwan/AHRI37/2009	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802823
APMV-4/Anseriformes/Taiwan/AHRI38/2009	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802824
APMV-4/Anseriformes/Taiwan/AHRI40/2009	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802825
APMV-4/Anseriformes/Taiwan/AHRI41/2009	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802794
APMV-4/Anseriformes/Taiwan/AHRI57/2010	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802826
APMV-4/Anseriformes/Taiwan/AHRI60/2011	Migratory	^{116}DIRPR↓F^{121}	Fusion	MZ802827
APMV-4/Anseriformes/Taiwan/AHRI62/2011	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802795
APMV-4/Anseriformes/Taiwan/AHRI66/2011	Migratory	^{116}DVQPR↓F^{121}	Genome	MZ802796
APMV-4/Anseriformes/Taiwan/AHRI78/2013	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802797
APMV-4/Anseriformes/Taiwan/AHRI88/2014	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802828
APMV-4/Anseriformes/Taiwan/AHRI93/2015	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802829
APMV-4/Anseriformes/Taiwan/AHRI110/2016	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802830
APMV-4/Anseriformes/Taiwan/AHRI135/2018	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802831
APMV-4/Anseriformes/Taiwan/AHRI150/2019	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802798
APMV-4/Anseriformes/Taiwan/AHRI152/2019	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802832
APMV-4/Anseriformes/Taiwan/AHRI153/2019	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802833
APMV-4/Anseriformes/Taiwan/AHRI157/2019	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802834
APMV-4/Anseriformes/Taiwan/AHRI159/2020	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802799
APMV-4/Anseriformes/Taiwan/AHRI164/2020	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802835
APMV-4/Anseriformes/Taiwan/AHRI165/2020	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802836
APMV-4/Anseriformes/Taiwan/AHRI166/2020	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802837
APMV-4/Anseriformes/Taiwan/AHRI169/2020	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802838
APMV-4/Anseriformes/Taiwan/AHRI170/2020	Migratory	^{116}DIQPR↓F^{121}	Fusion	MZ802839
APMV-4/Anseriformes/Taiwan/AHRI173/2020	Migratory	^{116}DIQPR↓F^{121}	Genome	MZ802800
APMV-6				
APMV-6/Anseriformes/Taiwan/AHRI45/2010	Migratory	^{104}IREPR↓L^{109}	Fusion	MZ802840
APMV-6/Anseriformes/Taiwan/AHRI56/2010	Migratory	^{114}APEPR↓L^{119}	Fusion	MZ802841
APMV-6/Anseriformes/Taiwan/AHRI65/2011	Migratory	^{114}APEPR↓L^{119}	Genome	MZ802801
APMV-6/Anseriformes/Taiwan/AHRI90/2014	Migratory	^{114}APEPR↓L^{119}	Genome	MZ802802
APMV-6/Anseriformes/Taiwan/AHRI99/2015	Migratory	^{114}APEPR↓L^{119}	Fusion	MZ802842
APMV-6/Anseriformes/Taiwan/AHRI109/2016	Migratory	^{104}IREPR↓L^{109}	Genome	MZ802803
APMV-6/Anseriformes/Taiwan/AHRI154/2019	Migratory	^{114}APEPR↓L^{119}	Fusion	MZ802843
APMV-6/Anseriformes/Taiwan/AHRI168/2020	Migratory	^{114}APEPR↓L^{119}	Fusion	MZ802844
APMV-12				
APMV-12/Anseriformes/Taiwan/AHRI101/2015	Migratory	^{103}TAQPR↓L^{108}	Genome	MZ802804
APMV-12/Anseriformes/Taiwan/AHRI143/2019	Migratory	^{103}VTQPK↓L^{108}	Genome	MZ802805
APMV-21				
APMV-21/Anseriformes/Taiwan/AHRI83/2013	Migratory	^{107}DREGR↓L^{112}	Genome	MZ802806
APMV-21/Anseriformes/Taiwan/AHRI141/2019	Migratory	^{107}DREGR↓L^{112}	Genome	MZ802807
APMV-22				
APMV-22/pigeon/Taiwan/Q97-NL/2015	Quarantine	^{103}TQQER↓L^{108}	Genome	MZ802808
APMV-22/dove/Taiwan/AHRI140/2019	Resident	^{103}TQQER↓L^{108}	Genome	MZ802809

and clustered within sub-genotype Ia. Eleven viruses, clustered in sub-genotype Ib, were grouped with the isolates sampled from domestic and wild birds in China, Russia, South Korea, Italy, Ukraine, and South Africa. The remaining four viruses were grouped with the isolates sampled from waterfowls in Alaska, Minnesota, and Texas of the United States (during 2007–2013), classified as genotype II. The deduced amino acid motif at the F cleavage site of the 25 APMV-4 isolates was ^{116}DIQPR↓F^{121}, except that two isolates, APMV-4/Anseriformes/Taiwan/AHRI60/2011 and APMV-4/Anseriformes/Taiwan/AHRI66/2011, possessed ^{116}DIRPR↓F^{121} and ^{116}DVQPR↓F^{121}, respectively.

Genetic analysis of APMV-6

Eight viruses isolated from wild birds of the order *Anseriformes* in this study were identified as APMV-6. Phylogenetic analysis

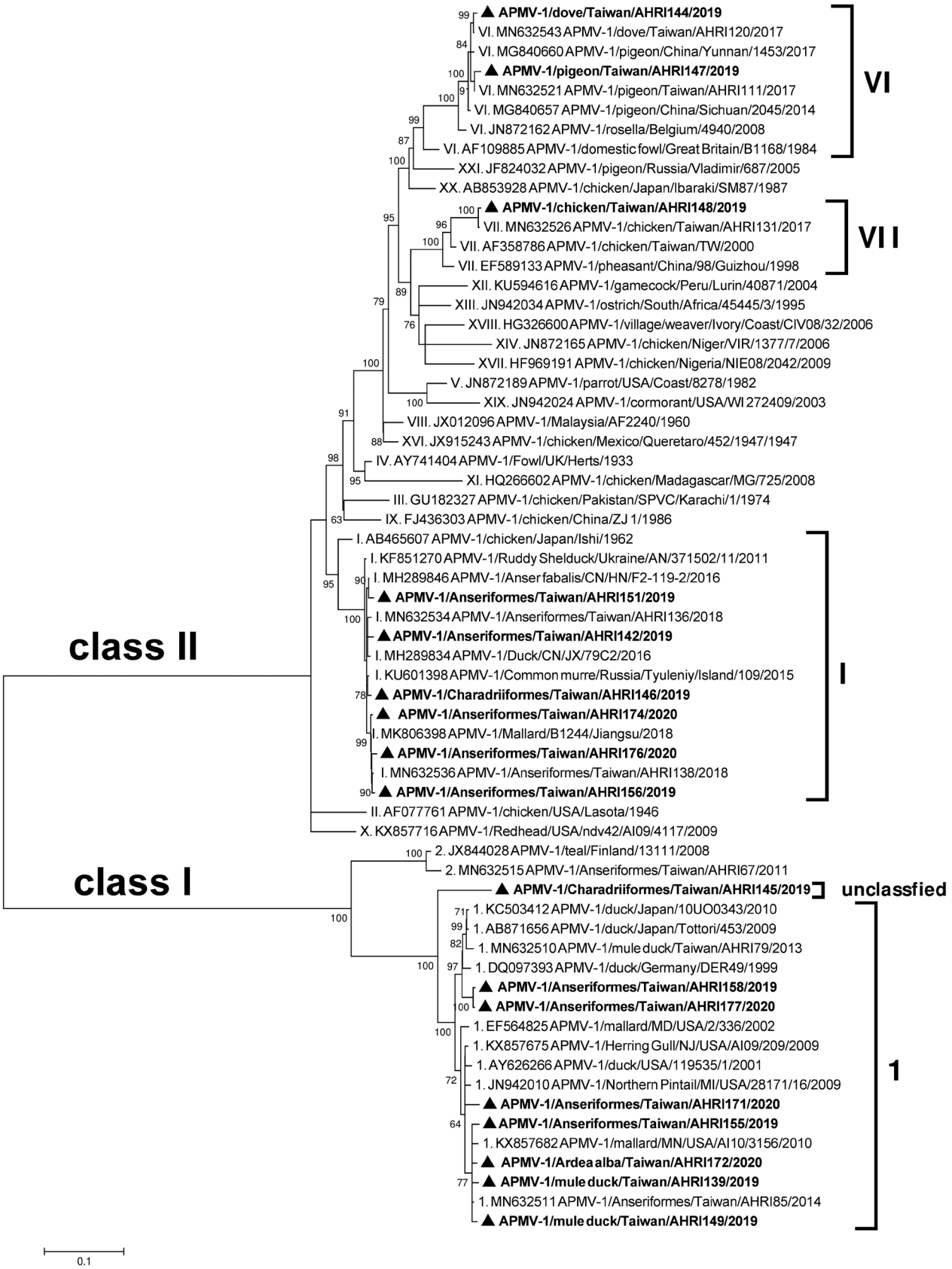

Fig. 1. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of the avian paramyxovirus 1 (APMV-1). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites (4 categories + G, parameter=0.7044; [+I], 23.99% sites). All positions containing gaps and missing data were eliminated. There were a total of 1,659 positions in the final dataset. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid triangle marks the isolates of APMV-1 obtained from birds sampled in Taiwan in this study.

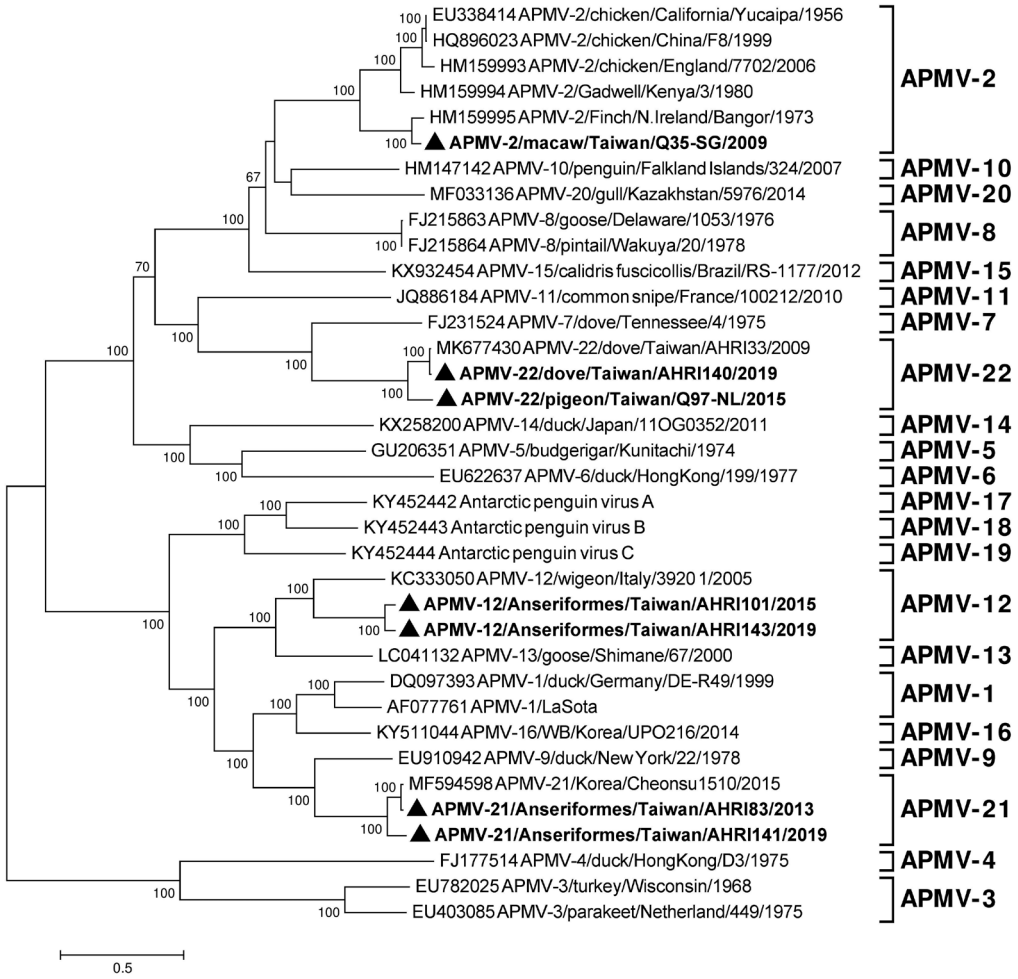

Fig. 2. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of the avian paramyxoviruses (APMVs). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites (4 categories + G, parameter=1.6630; [+I], 5.61% sites). All positions containing gaps and missing data were eliminated. There were a total of 12,494 positions in the final dataset. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid triangle marks the isolates of APMVs obtained from birds in sampled Taiwan in this study.

revealed that two genotypes of APMV-6 were circulating in wild birds sampled on the wetlands of Taiwan (Fig. 4). Six isolates were fell into genotype I and closely related to viruses obtained in China, South Korea, Russia, Kazakhstan, Belgium, and Italy during 2003–2015. The remaining two isolates fell into genotype II and closely related to the viruses obtained in Japan, South Korea, Russia, and Italy during 2007–2018. The isolates of genotype I contained an open reading frame (ORF) of the F gene, consisting of a 1,668 nt fragment and the deduced amino acid motif of cleavage site was ^{114}APEPR↓L^{119}. The isolates of genotype II had an N-truncated ORF (1,638 nt) of the F gene, and the deduced amino acid motif of cleavage site was ^{104}IREPR↓L^{109}.

Genetic analysis of APMV-12

Two unique APMV isolates, APMV-12/Anseriformes/Taiwan/AHRI101/2015 (APMV-12 AHRI101) and APMV-12/Anseriformes/Taiwan/AHRI143/2019 (APMV-12 AHRI143), were most related with the prototype virus, APMV-12/wigeon/Italy/3920-1/2005 (APMV-12 3920-1, GenBank accession number NC_025363) isolated from wigeon in Italy in 2005 (Fig. 2). The full-length genome sequences of the two viruses were both 15,228 nt in length, 84 nt shorter than that of APMV-12 3920-1. The genome of APMV-12 AHRI101 and AHRI143 shared 91.6% nt identity, and showed 64.5% and 64.4% identities with that of APMV-12 3920-1 strain, respectively.

Genetic analysis of APMV-21

Two APMV isolates, APMV-21/Anseriformes/Taiwan/AHRI83/2013 (APMV-21 AHRI83) and APMV-21/Anseriformes/Taiwan/AHRI141/2019 (APMV-21 AHRI141) sequenced in this study, were most related with the prototype virus of APMV-21/wild birds/Korea/Cheonsu1510/2015 (APMV-21 Cheonsu1510, GenBank accession number MF594598) isolated from wild birds in South Korea in 2015 (Fig. 2). The full-length genome sequences of the two viruses were both 15,408 nt in length equal to that of APMV-

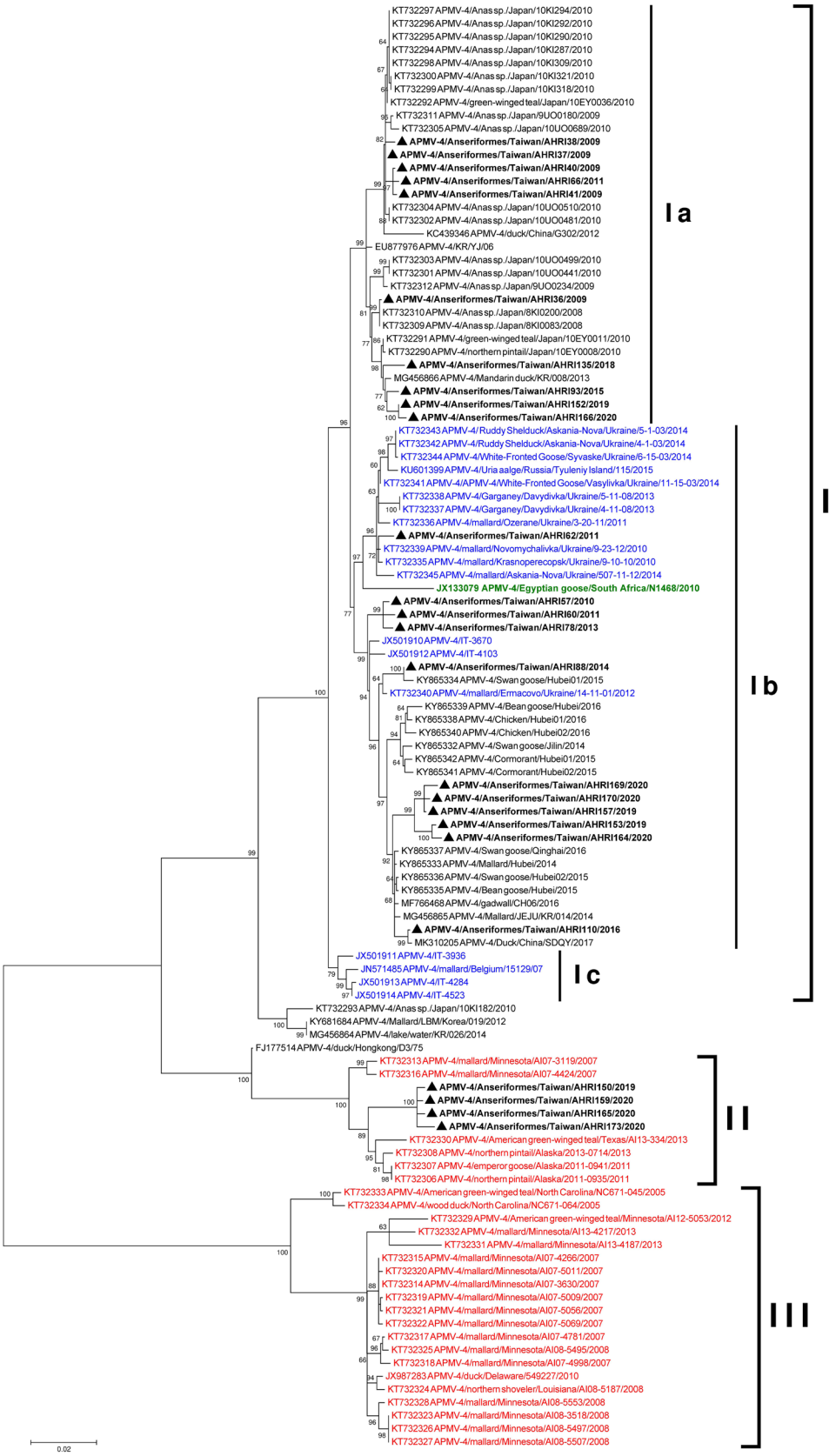

Fig. 3. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 4 (APMV-4). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites (4 categories + G, parameter=0.4394; [+I], 33.04% sites). All positions containing gaps and missing data were eliminated. There were a total of 1,695 positions in the final dataset. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid triangle marks the isolates of APMV-4 obtained from birds sampled in Taiwan in this study. The APMV-4 isolated from samples collected in Asia, Europe, Africa, and North America are shown in black, blue, green, and red, respectively.

21 Cheonsu1510 strain. The genome of APMV-21 AHRI83 and AHRI141 shared 86.4% nt sequence identity, and showed 98.2% and 86.2% identities, respectively, with that of APMV-21 Cheonsu1510. The viral genome contained six transcriptional units (3′-NP-P-M-F-HN-L-5′) of 1,482 nt, 1,194 nt, 1,101 nt, 1,638 nt, 1,740 nt, and 6,600 nt in length, respectively. The lengths of the six transcriptional units of APMV-21 AHRI83 and AHRI141 were all equal to APMV-21 Cheonsu1510 except that of HN protein gene was 159-nt longer than the prototype virus.

Genetic analysis of APMV-22

Two viruses isolated from the birds of the family *Columbidae* in this study were classified as members of APMV-22. One of the isolates, APMV-22/dove/Taiwan/AHRI140/2019 (APMV-22 AHRI140) obtained from a resident dove, was grouped with the APMV-22 isolates described in our previous study conducted between 2009 and 2017 [15], and shared 98.8–99.5% nt identity of F gene with them. The other isolate, APMV-22/pigeon/Taiwan/Q97-NL/2015 (APMV-22 Q97-NL), was obtained from samples of pigeons imported from the Netherlands during the routine quarantine in Taiwan in 2015. Phylogenetic analysis based on the genome sequences (Fig. 2) revealed that the APMV-22 AHRI140 isolate clustered with the reference strain APMV-22/dove/Taiwan/AHRI33/2009 (APMV-22 AHRI33), and the APMV-22 Q97-NL isolate was also clustered to the reference strain APMV-22 AHRI33, but more distantly. The full-length genome of the APMV-22 Q97-NL isolate was 16,908 nt in length, which was short than that of the APMV-22 AHRI33 strain due to a six-nucleotide deletion (positions 16441–16446) in the trailer at the 5′ end. The genome of the APMV-22 Q97-NL isolate showed 80.2% identity with that of APMV-22 AHRI33.

DISCUSSION

For a long time, the distribution, circulation, and evolution of APMVs isolated in Taiwan have been largely unknown. Here we present the results of the first molecular investigation of APMVs circulating in Taiwan. Twenty-two sequences of full-length genome and thirty-five sequences of complete F gene of fifty-seven APMV isolates obtained from wild birds and domestic poultry were determined in this study. The large-scale investigation over a ten-year period discovered that the isolated APMVs belonged to seven species: APMV-1, -2, -4, -6, -12, -21, and -22. Phylogenetic analysis of the isolates demonstrated that several species of APMVs were represented by more than one genotypes; some sub/genotypes were clearly distinct from previously characterized strains. Moreover, our results revealed that both APMV-1 and APMV-4 of North American strains have been introduced into the wild bird populations of Taiwan.

APMV-1 were constantly circulating and evolving in wild birds and domestic poultry in Taiwan. Sixteen APMV-1 isolates were identified in this study and one unique isolate, APMV-1 AHRI145, would be an unclassified virus within class I (Fig. 1). The previous study of the genetic diversity of APMV-1 in Taiwan indicates the presence of five different sub/genotypes of APMV-1 circulating in multiple avian species [14]. In this study, the class I sub-genotype 1.2 viruses were closely related to those isolated from wild birds in North America and Eurasia, and the class II sub-genotype I.2 viruses were clustered with those isolated from domestic ducks and mallards in the Eurasian countries. In addition, the sub-genotype VI.2.1.1.2.2 viruses were the predominant PPMV-1 strains and the sub-genotype VII.1.1 viruses that caused the Newcastle disease epidemic in Taiwan were successively obtained and identified for the past two years. Moreover, one unclassified APMV-1 was obtained from the samples of migratory birds. The genome length of APMV-1 AHRI145 isolate was 15,198 nt with a 12-nt insertion in the P gene, a characteristic of class I viruses [7]. Based on the phylogenetic analysis, the APMV-1 AHRI145 isolate, lacked branch support (44%), and sufficient number of independent isolates, was an unclassified virus within class I.

In this study, the APMV-2 Q35-SG isolate closely related to the APMV-2 Bangor isolate was genetically characterized, and the two viruses could be assigned to the genotype II of APMV-2. APMV-2 was first isolated from chickens in 1956 [4] and then the viruses were reported worldwide from wild birds, captive caged birds, and domestic poultry. There had been no report for the isolation of APMV-2 from avian species sampled in Taiwan. In 2009, one related virus, APMV-2 Q35-SG, was obtained from the sample of the psittacine birds imported from Singapore by routine examination during quarantine. Phylogenetic analysis of the genome sequences suggested that the APMV-2 Q35-SG and APMV-2 Bangor isolate were clustered together on a branch that was distinct from those of viruses clustered with the APMV-2 prototype Yucaipa strain. Through aligning the sequences of all available APMV-2 in GenBank and the sequence of Q35-SG isolate in this study, six single-nucleotide insertion-deletion combinations were identified in the genome sequences of the APMV-2 Bangor isolate. These combinations caused a frame-shift mutation and made an unreasonable misalignment of L gene. Moreover, four untranslated aa codons containing ambiguous nucleotides were presented at the positions of 8, 147, 1175, and 1176 aa of L gene of the APMV-2 Bangor isolate. Here, the flawless genome sequence was reported and could be the representative strain of APMV-2 genotype II for facilitating future evolutionary studies of APMVs.

The intercontinental dispersal of APMV-4 was evidenced by their high genetic diversity in waterfowl. Apart from APMV-1, APMV-4 was the most prevalent species of APMVs found and all of these APMV-4 isolates were obtained from waterfowl. APMV-4 recently was suggested to classify into three genotypes (I to III), that were almost exclusively monophyletic by continent of sample origin [20]. The existing information and results demonstrated that at least three sub/genotypes of APMV-4 circulating in wild birds sampled on the wetlands of Taiwan: sub-genotype Ia comprised East Asian viruses, sub-genotype Ib comprised Eurasian and African viruses, and genotype II comprised North American viruses. Our phylogenetic tree (Fig. 3) showed that Taiwan's APMV-4 isolates nested within North American viruses, supporting the possible intercontinental APMV spread by migratory birds. Wild birds shared the same breeding areas among the East Asian–Australian Flyway and the Pacific Americas Flyway and this sharing may provide a mechanism through which APMV-4 could be exchanged between these continents. Collectively, these

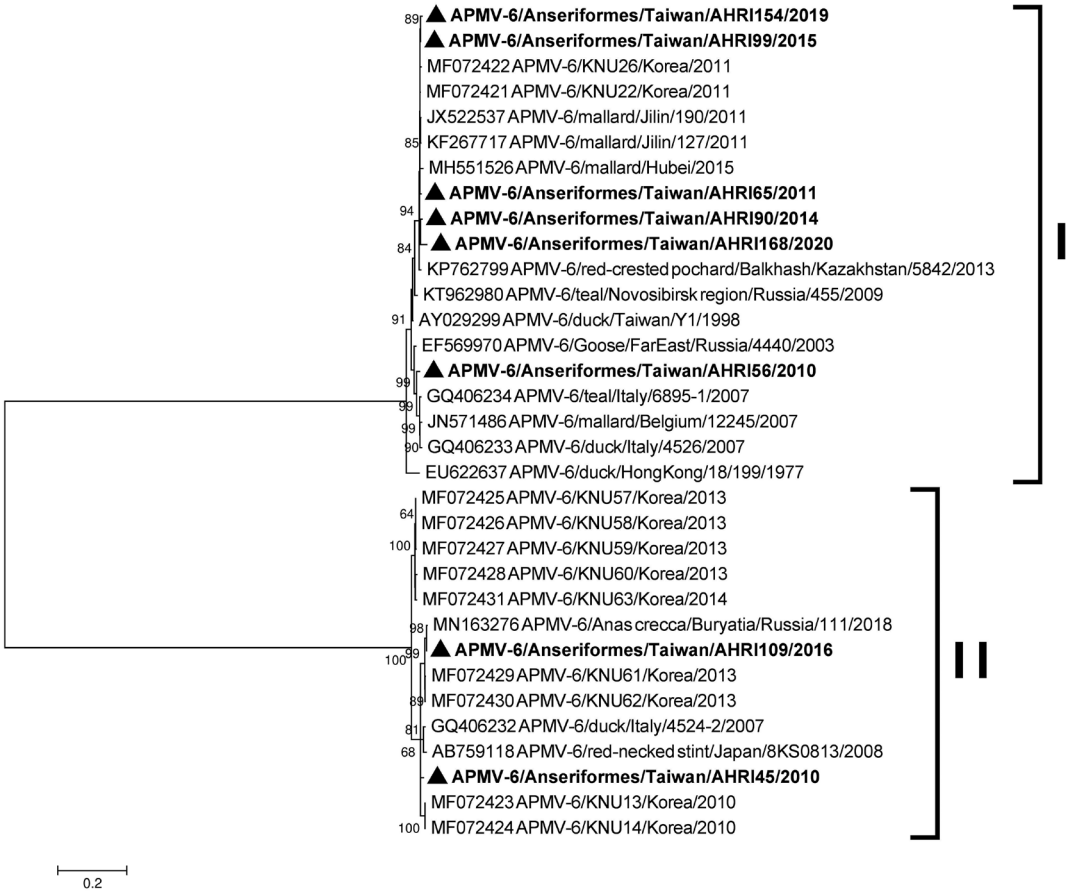

Fig. 4. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 6 (APMV-6). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites (4 categories + G, parameter=0.5571; [+I], 32.26% sites). All positions containing gaps and missing data were eliminated. There were a total of 1,638 positions in the final dataset. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid triangle marks the isolates of APMV-6 obtained from birds sampled in Taiwan in this study.

findings suggest that migratory birds may play a potential role in the global spread of APMVs.

Currently, two genotypes of APMV-6, genotypes I and II, were recognized based on the phylogenetic analysis of the all available complete F gene sequences in GenBank [6], and here the isolates of both genotypes were obtained from waterfowl sampled in Taiwan. The APMV-6 genotype I viruses have been isolated from domestic ducks in Taiwan in 1998 and the full-length viral genome sequences were determined [5]. In this study, six related APMV-6 isolates of genotype I were obtained, and so were additional two isolates of genotype II closely related to viruses circulating in Eurasian regions. All the APMV-6 isolates had one or two basic residues at the F cleavage site and a leucine residue at the first position of F1 subunit which were typically found in lentogenic APMV-1 strains. This study confirmed the primary role of wild birds as reservoirs of APMV-6 in nature and demonstrated that the related viruses circulated in the wild birds of Asia and Europe.

Two unique isolates, APMV-12 AHRI101 and AHRI143, were more closely related to APMV-12 3920-1, the prototype of *Avian Orthoavulavirus 12*, based on estimates of the evolutionary distance and limited evidence was identified to proposed as a new species in the genus *Orthoavulavirus*. The evolutionary distance of the nucleotide sequences of the genomes of APMVs revealed that APMV-12 AHRI101 and AHRI143 were closest to APMV-12 3920-1, with values of 38.8% and 38.9%, respectively, and these divergences were not distinct absolutely, only above the inter-species value of 37.5% between APMV-1 and APMV-16. On the other hand, estimates of the evolutionary distance of the nucleotide sequences of the fusion gene of APMVs revealed that APMV-12 AHRI101 and AHRI143 were closest to APMV-12 3920-1, with values of 39.3% and 41.1%, respectively, and the divergences were less than the intra-species value of 43.3% between the subgroups within APMV-3. Currently, no criteria are proposed for the classification of APMVs. More numbers of complete sequences of each species of APMVs isolated in different parts of the world deposited into GenBank will be the foundation for developing an unified phylogenetic classification system for APMVs.

The first full-length genome of the prototype strain, APMV-21 Cheonsu1510, has been published in 2018 [11], and we reported the second and third full-length genome sequences of APMV-21 isolates, AHRI83 and AHRI141 in this study. The APMV-21 AHRI83 isolates obtained from the samples of wild birds in 2013 showed a high level of 98.2% genome sequence identity with Cheonsu1510 strain. However, the APMV-21 AHRI141 isolate obtained in 2019 only showed 86.2% and 86.4% identities with

Cheonsu1510 strain and AHRI83 isolate, respectively. The deep divergence and the six-year gap between the two Taiwanese isolates demonstrated that the continuous evolution and previously unrecognized genetic diversity of APMV-21 in the wild bird reservoir. Of note, the F gene sequence of APMV-21 AHRI141 isolate was almost 10% (9.9%) distant from the Cheonsu1510 strain and AHRI83 isolate, and with the continuing evolution, will eventually meet the classification criteria for consideration as new genotypes. Of particular interest was the 53 amino acid carboxy-terminal extension of the HN protein that was identified among the two Taiwanese isolates resulting in a full-length protein of 620 aa compared to 567 aa for APMV-21 Cheonsu1510. The C-terminal length variations of HN have previously been seen among the APMV-9 viruses and has been used to categorize APMV-9 into different lineages [10]. Whether the C-terminal extensions could be a feature of different lineages of APMV-21 or cause a change in tissue tropism and or virulence need further investigation.

The newly proposed APMV-22 species described in our previous study [15] were constantly evolving in *Columbidae* birds in Taiwan, and one related European isolate obtained from imported pigeons demonstrated the extensive distribution of APMV-22. Since the prototype of APMV-22 was first isolated in 2009, fourteen viruses in our previous study and the AHRI140 isolates here had been successively detected from their natural reservoir host, resident doves and pigeons in Taiwan. The constant detection of APMV-22 over one decade indicated that the species had been endemic in Taiwan but not been reported elsewhere of the world. In 2015, the APMV-22 Q97-NL isolate was obtained from the sample of pigeons imported from the Netherlands during quarantine. Phylogenetic analysis of the full-length genome sequences suggested that the prototype APMV-22 AHRI33 isolate and the Q97-NL isolate clustered together on a branch that was distinct from those of the other APMV species. Both APMV-22 AHRI33 and Q97-NL have identical genome organizations, highly conserved genome terminal sequences, and gene start and stop sequences. Estimates of the evolutionary distances of the nt sequences of the genomes of APMVs have shown that the Q97-NL isolate was closest to APMV-22 with a calculated distance value of 0.137, which is lower than the value considered to differentiate other species (observed minimum was 0.375 between APMV-1 and APMV-16). However, the Q97-NL isolate was distant from the AHRI33 isolate, which meet the classification criteria for consideration as a new genotype. This indicated that APMV-22 may consist of two genotypes and we thus proposed that the prototype AHRI33 represents genotype I while the Q97-NL represents genotype II. The isolation of APMV-22 Q97-NL from import-quarantined birds revealed that the viruses were not exclusive in Taiwan, and additional sequencing of historical and prospective APMV isolates around the world will provide a more detailed characterization of the viral distribution and genetic diversity.

In summary, a broad range of circulating APMVs with a remarkable degree of genetic diversity was identified in this study. The previously unrecognized diversity of genetic, temporal, and host distribution of APMVs isolated in Taiwan suggests that multiple factors, including geographic regions, host species, wild bird migration, and domestication status, may contribute to the maintenance, evolution, and spread of these viruses. Our findings provided more evidence for the intercontinental transmission of APMV-1 and APMV-4 between East Asia and North America by wild birds. In addition, according to the criteria of the classification system based on complete F gene sequences, three novel sub/genotypes within APMV-2, APMV-12, and APMV-22 were identified. Our genetic analyses supported the designation of the novel variant APMVs and illustrated the genetic diversity of APMVs isolated in Taiwan.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

ACKNOWLEDGMENTS. This study was supported by grants from the Council of Agriculture, Taiwan: “Improvement of diagnosis and surveillance capacities of national veterinary laboratory system” [grant number 109AS-8.8.4-HI-H1].

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