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
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.
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 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 | TGACHACWAGYTIARAAARTAYTG | Large polymerase | 10,293–10,317 | 355 | AF077761 |
| Pan-APMV-1R | GCAIARRYTGRTTRTCICCTYG | Large polymerase | 10,625–10,647 | | |
| Pan-APMV-2Ra | CCATAGGTTYTGTGACRCCCTTC | Large polymerase | 10,532–10,555 | 263 | |
| Pan-APMV-2Rb | CCAKYTTYTTGCAYARICCCTYC | Large polymerase | 10,532–10,555 | | |
| APMV-2 | | | | | |
| APMV-2-1Fa | CTCCAGACTAATGGGCTGG | Fusion | 4,310–4,328 | 978 | EU338414 |
| APMV-2-1Fb | CCAAAAAACRYCSCCGAG | Fusion | 4,310–4,328 | | |
| APMV-2-1Fc | CCATAGCAAACCTGGCC | Fusion | 4,310–4,326 | | |
| APMV-2-1R | GTCAVCACYCTRCTYGWGC | Fusion | 5,268–5,287 | | |
| APMV-2-2F | GTRTCWTCAMMAGTGTGTSCT | Fusion | 5,157–5,176 | 968 | |
| APMV-2-2Ra | TCTGTGCAAGTGTCTCC | Fusion | 6,107–6,124 | | |
| APMV-2-2Rb | WTYGGTGAAGTCTCTCT | Fusion | 6,104–6,124 | | |
| APMV-4 | | | | | |
| APMV-4-1F | GAGTCTGACAAATCGTGG | Fusion | 4,331–4,352 | 988 | FJ177514 |
| APMV-4-1R | CRACCCTCGTATCTCGAG | Fusion | 5,300–5,318 | | |
| APMV-4-2F | GATCTGCAAACTGCGAC | Fusion | 5,172–5,192 | 1,000 | |
| APMV-4-2R | CCAAYCGGCTTGACAC | Fusion | 6,153–6,171 | | |
| APMV-6 | | | | | |
| APMV-6-1F | GAGCCTGATCTCTTCTTGGTAG | Fusion | 4,478–4,500 | 1,036 | EU622637 |
| APMV-6-1Fb | CCAAAAAACRYCSCCGAG | Fusion | 5,493–5,512 | 1,042 | |
| APMV-6-1Fb | CCAAAAAACRYCSCCGAG | Fusion | 5,493–5,512 | 1,042 | |
| APMV-6-1R | GCTGCAAGTGTCTCC | Fusion | 6,107–6,124 | | |
| APMV-6-2F | GAGTCTGACAAATCGTGG | Fusion | 4,331–4,352 | 988 | FJ177514 |
| APMV-6-2R | CRACCCTCGTATCTCGAG | Fusion | 5,300–5,318 | | |
| APMV-6-2F | GATCTGCAAACTGCGAC | Fusion | 5,172–5,192 | 1,000 | |
| APMV-6-2R | CCAAYCGGCTTGACAC | Fusion | 6,153–6,171 | | |
| APMV-12 | | | | | |
| APMV-12-1Fa | GTGCGAGWCTGAAACCATACCG | Fusion | 4,552–4,573 | 963 | NC_025363 |
| APMV-12-1Fb | GAGAAAAACTGACGACGAGAC | Fusion | 4,552–4,573 | | |
| APMV-12-2F | TRGGYATTGAMGRGACRCAGC | Fusion | 5,361–5,381 | 1,073 | |
| APMV-12-2R | GRCGACYSYCYCSTTCTGCC | Fusion | 6,415–6,433 | | |
| APMV-21 | | | | | |
| APMV-21-1F | TGAGAGYGTAGCGGTTAGG | Fusion | 4,776–4,794 | 934 | MF594598 |
| APMV-21-1R | AGAACTCCCTGGAAGACCTC | Fusion | 5,690–5,709 | | |
| APMV-21-2F | GTCAGGACGAGACGAGACG | Fusion | 5,569–5,587 | 1,026 | |
| APMV-21-2R | GCRCACACCTTCTACC | Fusion | 6,577–6,594 | | |
| APMV-22 | | | | | |
| APMV-22-1Fa | GTGCGAGWCTGAAACCATACCG | Fusion | 5,127–5,150 | 914 | MK677430 |
| APMV-22-1Fb | GTGCGAGWCTGAAACCATACCG | Fusion | 5,127–5,150 | | |
| APMV-22-2F | GAGAAAAACTGACGAGACG | Fusion | 6,018–6,047 | | |
| APMV-22-2R | ATGAGTCAGGATGCAATAG | Fusion | 6,839–6,859 | | |
| APMV-22-2Rb | ATGAGTCAGGATGCAATAG | Fusion | 6,839–6,859 | | |
dove), and the deduced amino acid motif at the F cleavage site was 112
\[ \text{RRKKR}↓\text{F} \]

variant of NDV that belongs to sub-genotype VI.2.1.1.2.2, was obtained from birds in the family (pigeon and spotted Columbidae), and shorebirds (Charadriiformes Anseriformes waterfowl (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

A 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

AHRI145/2019 (APMV-1 AHRI145) isolate was grouped within class I viruses but formed a monophyletic lineage with a genetic distance of 17.8% from the others within class I. The APMV-1/Anseriformes/Taiwan/AHRI142/2019 isolate was characterized by a deletion in the fusion protein and a single-nucleotide insertion in the M gene, and the deduced amino acid motif at the F cleavage site was 112
\[ \text{RRKKR}↓\text{F} \]

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 I (n=7), or unclassified (n=1) within class I (Fig. 1). Five isolates of genotype I viruses were grouped with those originated from the USA. The APMV-1/Anseriformes/Taiwan/AHRI1158/2019 and APMV-1/Anseriformes/Taiwan/AHRI177/2020 isolates of genotype I viruses were clustered with those derived from wild birds in Japan, China, Russia, Kazakhstan, Germany, and Finland. The APMV-1/Charadriiformes/Taiwan/AHRI1145/2019 (APMV-1 AHRI1145) 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
\[ \text{RRKKR}↓\text{F} \]

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-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

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.
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

| Isolate | Origin | Cleavage site of fusion protein | Sequence coverage | GenBank accession no. |
|---------|--------|---------------------------------|-------------------|-----------------------|
| APMV-6/Anseriformes/Taiwan/AHRI145/2010 | Migratory | $^{104}$IREPR↓L$^{109}$ | Fusion | MZ802840 |
| APMV-6/Anseriformes/Taiwan/AHRI156/2010 | Migratory | $^{114}$APEPR↓L$^{119}$ | Fusion | MZ802841 |
| APMV-6/Anseriformes/Taiwan/AHRI165/2011 | Migratory | $^{114}$APEPR↓L$^{119}$ | Genome | MZ802801 |
| APMV-6/Anseriformes/Taiwan/AHRI190/2014 | Migratory | $^{114}$APEPR↓L$^{119}$ | Genome | MZ802802 |
| APMV-6/Anseriformes/Taiwan/AHRI199/2015 | Migratory | $^{114}$APEPR↓L$^{119}$ | Fusion | MZ802842 |
| APMV-6/Anseriformes/Taiwan/AHRI1109/2016 | Migratory | $^{104}$IREPR↓L$^{109}$ | Genome | MZ802803 |
| APMV-6/Anseriformes/Taiwan/AHRI1154/2019 | Migratory | $^{114}$APEPR↓L$^{119}$ | Fusion | MZ802843 |
| APMV-6/Anseriformes/Taiwan/AHRI168/2020 | Migratory | $^{114}$APEPR↓L$^{119}$ | Fusion | MZ802844 |
| 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/Anseriformes/Taiwan/AHRI183/2013 | Migratory | $^{107}$DREGR↓L$^{112}$ | Genome | MZ802806 |
| APMV-21/Anseriformes/Taiwan/AHRI141/2019 | Migratory | $^{107}$DREGR↓L$^{112}$ | Genome | MZ802807 |
| 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 |

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

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=.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.
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 \(\text{APEPR}_{\downarrow}L\) from 114. 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 \(\text{IREPR}_{\downarrow}L\) from 104.

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 shorter 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 1.2 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 1a comprised East Asian viruses, sub-genotype 1b 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
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 AHRRI33 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-22 in the wild bird reservoir. Of note, the F gene sequence of APMV-21 AHRRI141 isolate was almost 10% (9.9%) distant from the Cheonsu1510 strain and AHRRI33 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 AHRRI140 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 AHRRI33 isolate and the Q97-NL isolate clustered together on a branch that was distinct from those of the other APMV species. Both APMV-22 AHRRI33 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 AHRRI33 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 AHRRI33 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.

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