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臺灣禽類副黏液病毒分離、鑑定與親緣分析

Isolation, Identification, and Phylogenetic Analyses of Avian Paramyxoviruses in Taiwan

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國立臺灣大學博士學位論文
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臺灣禽類副黏液病毒分離、鑑定與親緣分析
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本論文係劉玉彬君（學號 D98629003）在國立臺灣大學獸醫學研究所完成之博士學位論文，於民國 109 年 12 月 30 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

在這些年博士班研究生涯中，最由衷感謝指導教授蔡向榮老師在學術研究及論文寫作的悉心指導與鼓勵，學生永銘於心。感謝周崇熙老師、連一洋老師、張伯俊老師、張紹光老師、鄭明珠老師與謝明昆老師在學生口試提供寶貴建議與細心審查斧正，使本篇論文能夠更臻完善，在此致上誠摯的謝忱。

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劉玉彬 謹誌

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中文摘要

禽類副黏液病毒為 *Paramyxoviridae* 科的 *Avulavirinae* 亞科下病毒群，目前已知共 22 型，且廣泛分布於世界眾多鳥種中。禽類副黏液病毒一型（亦稱為新城病病毒）為家禽新城病的致病原，已廣佈於全球，能夠感染兩百種以上的鳥類。目前已有許多即時反轉錄聚合酶反應針對新城病病毒不同標的基因偵測被研發出，但檢測的敏感性與效力仍待改進。在本研究中，於 2009 年至 2020 年期間由臺灣家禽、候鳥、輸入鳥類及本地留鳥檢體中分離禽類副黏液病毒，並進行病毒基因體或融合蛋白基因全長定序及分析。同時建立核鞘蛋白即時反轉錄聚合酶反應技術以檢測具高度變異性的新城病病毒核酸。本研究共分離 100 株禽類副黏液病毒株，分屬於一型（52 株）、二型（1 株）、四型（20 株）、六型（7 株）、十二型（2 株）、二十一型（2 株）以及二十二型（16 株）等 7 型。核酸序列分析顯示除了 10 株第一類禽類副黏液病毒一型及 2 株四型病毒屬於北美株外，其餘所有病毒均歸屬於歐亞株。禽類副黏液病毒一型的融合蛋白親緣分析顯示 52 株分離株中，50 株屬於第一類的第一基因型或第二類的第一、六及七基因型病毒、1 株為第一類新增的第二基因型及 1 株為無法分類之特異病毒；臺灣近十年的鴿子副黏液病毒流行株為第二類第六基因型的 2.1.1.2.1 與 2.1.1.2.2 亞型；造成臺灣家禽新城病疫情的病毒分離株均歸屬於第七基因型的 1.1 亞型。本研究在 2009 年的禽流感主動監測檢體中分離到一株特殊的副黏液病毒株（APMV/dove/Taiwan/AHRI33/2009），經基因體核酸序列比對，AHRI33 相近於第七型禽類副黏液病毒，但相似度僅 62.8%。依據 RNA 依賴性 RNA 聚合酶基因親緣性分析，AHRI33 病毒株應新成立為禽類副黏液病毒二十二型。本研究建立之核鞘蛋白即時反轉錄聚合酶反應檢測技術在不同型別新城病病毒株的測試下，全部 23 株均成功檢出，而經美國農業部認證之基質蛋白即時反轉錄聚合酶反應則未能檢出 8 株第一類及 2 株鴿子副黏液病毒株；新檢測方法對於 35 種非新城病病毒均無陽性反應，顯示高度之特異性；實際應用

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於臨床檢體時，與黃金標準的病毒分離檢測結果比對，其敏感性與特異性分別為100%與 96.61%。綜合上述，本篇研究證實臺灣禽鳥至少帶有 7 種不同型別之禽類副黏液病毒，而候鳥對於歐亞及北美間的跨洲際病毒傳播扮演重要之角色。AHRI33 病毒應於 *Avulavirinae* 亞科 *Metaavulavirus* 屬中設立為 *Avian metaavulavirus 22* 新病毒種；臺灣家禽與野鳥至少帶有 5 種不同基因型之新城病病毒；其中 AHRI67 病毒株應於第一類下新成立第二基因型；而新建立的核鞘蛋白即時反轉錄聚合酶反應技術具高敏感性、特異性，可檢出高度演化變異的新城病病毒。總結本篇研究結果可更深入瞭解臺灣禽類副黏液病毒之天然宿主、演化及遺傳多樣性。

關鍵詞：

禽類副黏液病毒、新城病、融合蛋白、跨洲際傳播、即時反轉錄聚合酶反應

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Abstract

Avian paramyxoviruses (APMVs) belonging to the subfamily *Avulavirinae* within the family *Paramyxoviridae* consist of twenty-two known species, and are constantly isolated from a wide variety of avian species around the world. Avian paramyxovirus 1, synonymous with Newcastle disease virus (NDV), is a worldwide viral agent that infects over 200 species of birds and responsible for outbreaks of Newcastle disease. Although a number of real-time reverse transcription polymerase chain reaction (rRT-PCR) assays have been developed for detecting different genes of NDV, diagnostic sensitivity and efficiency could be improved. In this study, the APMV isolates collected from poultry, migratory, imported and resident birds during 2009-2020 in Taiwan were genetically characterized by sequence analysis of the complete fusion protein gene or full-length genome. Furthermore, this study describes a nucleocapsid protein gene rRT-PCR screening assay based on TaqMan technology for the detection of divergent NDV strains and isolates. One hundred isolates belonging to seven species were identified as APMV-1 (n=52), APMV-2 (n=1), APMV-4 (n=20), APMV-6 (n=7), APMV-12 (n=2), APMV-21 (n=2) and APMV-22 (n=16). Genetic studies showed that the recovered APMVs isolates had highest homology with Eurasian isolates, except ten class I APMV-1 and two APMV-4 isolates related to North America strains. Our phylogenetic analysis of complete fusion protein gene of the APMV-1 isolates revealed that 50 of the 52 Taiwanese isolates were closely related to APMV-1 of class I genotype 1 or class II genotypes I, VI or VII, one isolate belonged to a group that can be classified as a novel genotype 2 within class I, and one isolate was grouped within class I viruses but formed a monophyletic lineage, with a genetic distance of 5.6% between them. Viruses placed in class II sub-genotype VI.2.1.1.2.1 and sub-genotype

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VI.2.1.1.2.2 were the dominant pigeon paramyxovirus 1 circulating in the last decade in Taiwan. All the Newcastle disease outbreak-associated isolates belonged to class II sub-genotype VII.1.1, which was mainly responsible for the present epizootic of Newcastle disease in Taiwan. In 2009, the isolate APMV/dove/Taiwan/AHRI33/2009 was isolated from swabs of red collared doves during active surveillance of avian influenza in resident birds in Taiwan. Nucleotide sequence comparisons 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. Using the NP-gene rRT-PCR assay, all 23 representative NDV strains of class I and II in the tested panel were detected, whereas eight class I and two class II NDV isolates cannot be detected by the USDA-validated matrix-gene assay. The new assay also has a high degree of specificity with no false-positive results of 35 non–NDV viruses. A total of 146 clinical specimens were also tested and the NP-gene assay gave high relative sensitivity (100%) and specificity (96.61%) when compared with virus isolation. We conclude that at least seven species of APMVs were obtained from multiple avian host species in Taiwan. Migratory birds may play an important role in intercontinental spread of APMVs between Eurasia and North America. The data suggest that the isolate APMV/dove/Taiwan/AHRI33/2009 should be considered as the prototype strain of the new species *Avian metaavulavirus 22* in the genus *Metaavulavirus* in the subfamily *Avulavirinae*. The current data confirm that at least five genotypes of APMV-1 circulate in both domestic and wild birds throughout Taiwan. One genetically divergent group of APMV-1 should be considered as a novel genotype within class I. Furthermore, the developed NP-gene rRT-PCR assay offers a sensitive, specific and rapid assay for

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detecting both class I and II NDV and could be used as part of a panel of diagnostic assays for this notifiable disease agent. Together, this study contributes to the knowledge of the distribution, evolution, and genetic diversity of APMVs in Taiwan.

Keywords:

avian paramyxovirus; Newcastle disease; fusion protein; intercontinental dispersal; real-time RT-PCR

doi:10.6342/NTU202100085

Contents

doi:10.6342/NTU202100085

doi:10.6342/NTU202100085

doi:10.6342/NTU202100085

doi:10.6342/NTU202100085

List of Figures

doi:10.6342/NTU202100085

List of Tables

doi:10.6342/NTU202100085

Chapter 1

Introduction

1.1 Background of avian paramyxoviruses

Avian paramyxoviruses (APMVs) are one of the most important viruses and have been reported from a wide variety of avian species around the world (Gogoi et al., 2017). To date, 21 different species of APMVs (APMV-1 to 21) have been reported and their complete genome sequences are available in GenBank. Avian paramyxoviruses belong to the subfamily *Avulavirinae* within the family *Paramyxoviridae*. APMV-1 (commonly termed Newcastle disease virus, NDV) is the most recognized species, but information on the distributions of other APMVs in domestic poultry and wild birds is limited. These findings suggest that wild birds and domestic poultry might harbor previously unrecognized genetic diversity of APMVs, and the full extent of the distribution, evolution, and host species of APMVs has remained unexplored.

Newcastle disease (ND) is one of the most important diseases of poultry and caused by virulent strains of avian paramyxovirus 1 (APMV-1), also known as Newcastle disease virus (NDV). Newcastle disease is an OIE (World Organization for Animal Health) notifiable disease that needs implementing control measures and trading restrictions to prevent the spread of the disease (OIE, 2012). Based on its genetic characteristics, the APMV-1 was re-assigned into the new genus *Orthoavulavirus* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* by the International Committee on Taxonomy of Viruses in 2019 (Amarasinghe et al., 2019). The virus has high genetic diversity and the infection of NDV has been reported in a wide variety of avian species around the world (Dimitrov et al., 2016).

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1.2 Research aims

In the present study, the APMVs isolates obtained from migratory birds and poultry in Taiwan were characterized by sequencing of complete fusion protein gene or full-length genome sequences and were compared to those available in GenBank. Based on the results of the phylogenetic analyses, we aim to i) illustrate the genetic diversity of APMVs in various avian hosts and present new epidemiological information on APMVs in Taiwan; ii) present new information on the genetic evolution and sub/genotype classification of NDV in Taiwan; iii) provide evidence for the potential intercontinental transmission of APMVs by migratory birds; iv) develop a rapid, sensitive, and reliable TaqMan rRT-PCR assay that could be used for detecting the NP gene from both class I and II NDV.

1.3 The layout and format of this dissertation

The dissertation comprises a series of studies that address different aspects of the evolution, current distribution and detection of different APMV species isolated from domestic poultry and wild birds in Taiwan. All the results chapters shown in (3 to 6) have already been published (3, 4 and 5) or have been prepared to submit to international journals. These result chapters contain the same text and structure as published or submitted to the respective journals. The formatting and referencing have been altered to form a standard style for all chapters. In the final chapter, a summary and discussion of all findings are presented.

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Chapter 2

Literature review

2.1 Avian paramyxovirus

2.1.1 Taxonomy of APMV

According to the taxonomy of the order *Mononegavirales*: updated in 2018, three genera, named *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus* has recently been created within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Amarasinghe et al., 2019). Avian paramyxoviruses (APMVs) belong to three genera, *Metaavulavirus* (APMV-2, -5, -6, -7, -8, -10, -11, -14, -15 and -20), *Orthoavulavirus* (APMV-1, -9, -12, -13, -16, APV-A, APV-B, APV-C and -21) and *Paraavulavirus* (APMV-3 and -4). The subfamily *Avulavirinae* comprises of all APMVs that have been isolated from avian species.

2.1.2 Virus characteristics

The APMVs contain a non-segmented, negative-sense, single-stranded RNA genome that ranges from 14,904 to 17,412 nucleotides (nt) in length (Aziz-ul-Rahman et al., 2018). For most of the APMVs, the genome encodes at least six proteins: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) except for APMV-6 that had an additional short hydrophobic (SH) region between F and HN genes (Chang et al., 2001). The order of the genes for these proteins in the genome is 3′ leader-N-P-M-F-HN-L-5′ trailer (Anderson andWang, 2011). The viral envelope is derived from the host cell membrane. The outer surface of the envelope consists of two

doi:10.6342/NTU202100085

glycoproteins, namely F and HN proteins. The HN protein mediates attachment by binding to the sialic acid receptor, has neuraminidase activity and plays a role in fusion promotion where as F protein directs the membrane fusion. The F and HN proteins play a defined role in the fusion, attachment and release from the cells. The cleavage site of the F protein acts as key determinant of virulence and existence of phenylalanine/leucine residue assists in classification of virulent and avirulent strains (Alexander, 2000). M protein lies below the envelope and is shown to play a major role in virus assembly and budding of NDV. The viral envelope encloses the ribonucleocapsid core that is formed by the nucleoprotein, the phosphoprotein and large polymerase protein bound with viral genomic RNA (Anderson andWang, 2011). Viral transcription begins at single promoter at the 3′ leader end, and the genes are copied into individual mRNAs by a start-stop-restart mechanism guided by conserved gene-start (GS) and gene-end (GE) transcription signals that flank the individual genes. For the APMVs and other members of the subfamily *Paramyxovirinae*, the genome nucleotide length is an even multiple of six, known as 'rule of six' which is required for efficient RNA replication and the precise packaging of the polynucleotide in the nucleocapsid (Kolakofsky et al., 1998).

2.1.3 Members of APMV

Until recently, the known APMV species were restricted to APMV-1 to -9, which were isolated and characterized based on haemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays in the 1970s (Alexander, 1987). The prototype virus, APMV-1 of genus *Orthoavulavirus*, also well known as Newcastle disease virus or NDV, is the most extensively studied virus in this group. NDV is a geographically widespread viral agent that infects wild and domestic avian hosts. Virulent strains of

doi:10.6342/NTU202100085

APMV-1 have significant global economic impacts on domestic poultry production and are notifiable to the World Organization for Animal Health (OIE). APMV-2 to -9 had been isolated from chicken, ducks, turkeys, and wild birds all over the world prior to the 1980s (Alexander, 2000). Following the expansion of viral surveillance initiatives and improvements in sequencing technology, 12 novel species have been designated since 2010. Many of these novel APMVs were isolated as a result of influenza virus surveillance programs. 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), 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). In 2018, one novel APMV species, APMV-21, was isolated from wild birds in South Korea (Jeong et al., 2018). These findings suggest that wild birds maintain a previously unrecognized genetic diversity of APMVs. Till date, 21 species of APMVs (APMV-1 to 21) are reported and their complete genome sequences are available in GenBank.

2.1.4 Natural host of APMV

doi:10.6342/NTU202100085

APMV-1 (commonly termed Newcastle disease virus, NDV) has a wide host range and is able to infect over 240 species of birds (Kaleta andBaldauf, 1988). In contrast, the information about the host ranges and the distribution of the other APMVs species in wild birds and domestic poultry is limited. The natural hosts for APMV-2 are turkeys, psittacines, and passerines, but chickens and rails act as incidental host (Alexander, 2000; Zhang et al., 2007). Apart from prototype strain Yucaipa, APMV-2 strains Bangor, England and Kenya are also characterized. APMV-2 strains Bangor, England and Kenya were isolated from a finch, chicken and gadwall duck, respectively. Apparently during a routine quarantine evaluation, APMV-2 strain Bangor was isolated and on its characterization, both biologically and serologically, it was observed that it might depict a different serotype or as a subgroup within serotype (Subbiah et al., 2010). APMV-3 and -7 have been isolated from captive caged birds and domestic poultry including chickens and turkeys; APMV-4 have mostly been isolated from waterfowl belonging to the order *Anseriformes*; APMV-5 have been isolated mostly from budgerigars; APMV-6 was first isolated from a healthy duck at a domestic duck farm in the year 1977 from Hong Kong; APMV-12 was first isolated from a cloacal swab of an infected Eurasian wigeon in Italy during the influenza surveillance programme in 2005 (Terregino et al., 2013) and was no other strains of APMV-12 have been reported till date; and the other APMVs appear to be more restricted to wild birds including ducks, geese, gulls and penguins, but very little is known regarding the disease potential and the epidemiology of these APMVs (Aziz-ul-Rahman et al., 2018).

2.1.5 Pathogenicity of APMV

APMV-1 is the best characterized virus among other APMVs due to the severity of disease caused by this virus in chickens. APMV-1 is also historically known as

doi:10.6342/NTU202100085

Newcastle disease virus (NDV) because of its origin from the seaport town of Newcastle upon Tyne in England. APMV-1 are categorized as lentogenic, mesogenic, and velogenic depending on clinical signs in chickens and the cleavage site amino acid sequence of fusion protein (Miller and Koch, 2013). Depending on its predilection site, velogenic NDV can be either viscerotropic or neurotropic (Alexander, 2000). Other APMVs are also associated with varying degrees of pathogenicity in chickens and other avian species. Most of these APMVs appear to be present in natural reservoirs of specific feral avian species, although other host species are usually susceptible. Only APMV-2 and APMV-3 viruses have made a significant disease and economic impact on poultry production. Both types of viruses cause respiratory disease and egg production losses which may be severe when exacerbated by other infections or environmental stresses. No report demonstrated natural infections of chickens with APMV-3 viruses (Alexander, 2000). APMV-2 infection can cause a decreased yield and decreased hatchability in turkeys (Bankowski et al., 1981). APMV-3 was isolated from turkeys which are showing signs of nasal discharge, coughing and swelling of the infra-orbital sinus (Kumar et al., 2010). APMV-6 seems to be apathogenic to chickens but can cause mild respiratory disease and a drop in egg production in turkeys (Alexander, 2000). The pathogenicity and the economic significance of other APMVs are not well understood.

2.1.6 Classification system of APMV

APMVs have been placed in different groups, or serotypes, based on their antigenic relation, and nine different groups were defined in this way (Alexander et al., 1983). However, there are multiple problems associated with the use of serology, including the inability to classify some APMVs by comparing them to the sera of the nine defined APMVs alone (Alexander et al., 1989). In addition, cross-reactivity

doi:10.6342/NTU202100085

between different serotypes have been documented for many years (Terregino et al., 2013). Because of obstacles imposed by the cross-reactivity as mentioned above and the limitation of available reference antiserum against each APMV serotype, analysis of complete genome sequences should be conducted to identify new APMV serotypes as suggested previously (Miller et al., 2010). The analysis of complete genome nucleotide identity is a simple method for identification of novel APMV, and up to 25% divergence may be suggestive of novel virus or strain (Terregino et al., 2013). This is evident in pairwise comparison of whole genome sequences of all APMVs where a substantial divergence was observed. The divergence in percentage nucleotide identity among inter-APMV can also be a marker of novel strain and may highlight the evolutionary distance of APMV. In this context, the complete genomes of all APMVs showed a high nucleotide divergence except in APMV-1, -9, -12, -16, -17, -18 and -19, which revealed a high nucleotide identity (70.6%-88.4%) compared to rest of APMVs (Aziz-ul-Rahman et al., 2018). Based on a sequence comparison of the RNA-dependent RNA polymerases (RdRps) of the viruses, APMVs are divided into twenty species according to the taxonomy of the order *Mononegavirales* updated in 2019 (ICTV, 2019). In the family *Paramyxoviridae*, the ICTV Study Group has decided that, since a number of other criteria are no longer applicable or have been applied inconsistently (Rima et al., 2018), the classification of the viruses should now be based on a sequence comparison of the RdRp gene. The primary criterion is the tree topology however using such trees for classification may require defining taxon-specific cut-off criteria.

2.2 Newcastle disease virus

2.2.1 Classification of APMV-1

Newcastle disease virus was assigned into the new genus *Orthoavulavirus* within

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the subfamily *Avulavirinae* of the family *Paramyxoviridae* by the International Committee on Taxonomy of Viruses in 2018 (Amarasinghe et al., 2018). The complete F gene sequence is considered as the main target for molecular epidemiological investigations and genotyping of APMV-1. Based on phylogenetic analyses of fusion protein gene, NDV has been divided into two clades, class I and class II (Czeglédi et al., 2006). An unified and objective classification system of APMV-1 was proposed in 2012 based on the coding sequences of the complete fusion protein gene (Diel et al., 2012), and this system and nomenclature criteria for APMV-1 were revised and updated by a global consortium in 2019 (Dimitrov et al., 2019). This system utilized the complete F gene coding sequences and incorporated a number of objective criteria for classification of NDV, including: i) New genotypes are created based on the phylogenetic tree topology (need to cluster into monophyletic branches) using the Maximum Likelihood method and the general time-reversible (GTR) model with gamma distribution; ii) Different genotypes have an average distance per site above 10%; iii) The bootstrap value at the genotype and sub-genotype defining node is 70% or above (≥70%); and iv) New genotypes are created only when four or more independent isolates, without a direct epidemiologic link (i.e. distinct outbreaks), are available. The use of Dimitrov et al. (2019) system and criteria led to the classification of class I isolates belong to a single ‘genotype’ (genotype 1) whereas class II isolates are divided into 21 genotypes. The classification system of APMV-1 then was applied to other species of APMVs to provide a more rational and scientific genotyping method for epidemiological studies (Yin et al., 2017; Chen et al., 2018; Tseren-Ochir et al., 2018).

2.2.2 Host, virulence, and genetic diversity of APMV-1

Almost all of the class I viruses are lentogenic strains and have been isolated

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primarily from waterfowl of the family *Anatidae* worldwide and occasionally from poultry in live bird markets (Kim et al., 2007). Class II viruses are genetically and phenotypically more diverse, frequently isolated from poultry with occasional spillovers into wild birds, and exhibit a wider range of virulence. Waterfowl, cormorants, and pigeons are natural reservoirs of all APMV-1 pathotypes, except viscerotropic velogenic viruses for which natural reservoirs have not been identified. APMV-1 isolates of class II, genotype I consist of lentogenic strains and have been widely recovered from a diversity of wild and domestic waterfowl. Genotypes I and II within class II include isolates of high and low virulence, the latter often being used as vaccines. Viruses of genotypes III and IX that emerged decades ago are now isolated rarely, but may be found in domestic and wild birds in China. Containing only virulent viruses and responsible for the majority of recent outbreaks in poultry and wild birds, viruses from genotypes V, VI, and VII, are highly mobile and have been isolated on different continents. A pigeon-adapted variant of genotype VI NDV, often termed pigeon paramyxovirus 1 (PPMV-1), is commonly isolated from columbids and can cause ND-like infectious disease in wild and domestic birds. Unlike any other NDV genotype, genotype VI viruses have been isolated in all continents, except Antarctica. Their potential to infect chickens has been demonstrated, but only rarely, and they appear to be highly adapted to some *Columbiform* birds (Aldous et al., 2014). Genotype VII is another large and genetically diverse group of viruses. Class II genotype VII viruses might emerge in the Far East in the1990s and subsequently spread to Europe, South Africa, South America, and Asia, including Taiwan (Dimitrov et al., 2016). All genotype VII viruses are predicted to be virulent based on deduced amino acid motifs and many have been shown to be velogenic via pathotyping. Genotype VII viruses have been associated with recurrent poultry outbreaks in Eastern Europe, the Middle East, and

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Asia and sporadic events in Africa and South America, making this large and diverse group of viruses of significant global economic importance (Miller et al., 2015). Conversely, virulent viruses of genotypes XI (Madagascar), XIII (mainly Southwest Asia), XVI (North America) and XIV-XXI appear to have a more limited geographic distribution and have been isolated predominantly from poultry (Dimitrov et al., 2016).

2.2.3 Molecular basis for pathogenicity

Although all NDV isolates belong to a single serotype, there is great genetic variability among different strains. The cleavability of protein F is the main determinant for viral virulence, but other genes such as HN and P are also believed to influence pathogenicity (deLeeuw et al., 2005). During replication, APMV-1 particles are produced with a precursor fusion glycoprotein, F0, which has to be cleaved to Fl and F2 for the virus particles to be infectious. This post translation cleavage is mediated by host cell proteases. Most APMV-1 viruses that are pathogenic for chickens have the sequence ^{112}R/K-R-Q/K/R-K/R-R ↓ F^{117} at their F protein cleavage site, whereas the lentogenic APMV-1 have sequences of ^{112}G/E-K/R-Q-G/E-R ↓ L^{117} (Choi et al., 2010). Therefore, predicted virulence based upon deduced amino acid motifs at the fusion cleavage site has been incorporated into the definition of notifiable APMV-1 strains by the OIE. The OIE definition for reporting an outbreak of ND is: Newcastle disease is defined as an infection of birds caused by a virus of APMV-1 that meets one of the following criteria for virulence: i) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks of 0.7 or higher (2.0 is maximum); ii) Multiple basic amino acids have been demonstrated in the virus at the C-terminus of the F2 protein and phenylalanine (F) at residue 117, which is the N-terminus of the F1 protein. The term ‘multiple basic amino

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acids' refers to at least three arginine (R) or lysine (K) residues between residues 113 and 116 (OIE, 2012).

2.2.4 Detection of APMV-1 by real-time RT-PCR assay

With its efficiency and high throughput, real-time reverse transcription polymerase chain reaction (rRT-PCR) has become one of the most widely applied assays for NDV diagnosis and surveillance (Hoffmann et al., 2009). At present, the U.S. Department of Agriculture (USDA) validated M-gene rRT-PCR assay described by Wise et al. (2004) is widely used in North America, Europe, and Taiwan for detecting most NDVs, mainly class II isolates. However, even within the more conserved genes of APMV-1 targeted in screening rRT-PCR assays, there is still considerably high genetic variation within APMV-1 to challenge primer/probe combinations to detect all contemporaneously circulating strains with sufficient diagnostic specificity/sensitivity. Recent data revealing significant heterogeneity in the genomes of APMV-1 suggests that some highly divergent viruses may not be detected (Khan et al., 2010). A large number of class I APMV-1 have been identified in samples recovered from waterfowl, shorebirds, and from poultry in live bird markets; however, the M-gene assay failed to detect the majority (73%) of these viruses (Kim et al., 2007). To minimize the false negatives produced by the USDA-validated M-gene assay, rRT-PCR assays targeting other NDV genomic regions were developed and evaluated. These assays include TaqMan rRT-PCR assays detecting the M gene (Hines et al., 2012), L gene (Fuller et al., 2010; Sutton et al., 2019), both L gene and M gene (Kim et al., 2008), along with many assays targeting the F gene, which also enable sequencing or pathotyping (Fuller et al., 2009; Yacoub et al., 2012). However, these assays cannot detect all NDV strains and a multiple testing approach may be needed for detecting the index case (OIE, 2012).

doi:10.6342/NTU202100085

2.3 Intercontinental dispersal of avian pathogens by migratory birds

There is a growing body of literature supporting a routine exchange of infectious agents between regions by migratory birds and the potential for identifying patterns of dispersal that may be useful to predict the spread of emerging avian pathogens. Based on the global phylodynamic analysis, a study provides evidence for East Asia representing a critically important node for the global dispersion of APMV-1 (Hicks et al., 2019). In a study of fusion protein gene sequences of two sub-genotypes of class II APMV-1 strains isolated from wild birds in Eurasia and North America, evidence of intercontinental dispersal by wild birds has been found (Ramey et al., 2013). The finding that APMV-1 sequences originating from isolates derived from waterfowl sampled in Alaska and a Delaware Bay shorebird were phylogenetically nested within mixed clades with samples from Eurasia is similar to findings of intercontinental gene flow in low-pathogenic avian influenza virus isolates (Koehler et al., 2008). The phylogentic study for global APMV-4 isolates presented limited evidence for historical viral movement between continents (Reeves et al., 2016). Phylogenetic network analysis also supported the introduction of Asia-origin clade 2.3.4.4 H5N8 avian influenza viruses into North America via intercontinental associations of waterfowl (Lee et al., 2015). Collectively, these findings suggest that migratory birds may play a potential role in the global spread of kinds of avian infectious agents.

2.4 APMV study in Taiwan

At least three major epidemics of ND have occurred in Taiwan, the first in 1969, the second in 1984, and the third in 1995 (Lu et al., 1986; Yang et al., 1997). Results obtained from a study of Taiwanese NDV isolates led Yang et al. (1997) to suggest that

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the 1969 outbreak of ND in Taiwan was caused by the genotype III virus, whereas the 1984 and 1995 outbreaks were caused by the genotype VII viruses. In Taiwan, despite that intensive vaccination programs against ND have been implemented for decades, NDV still has caused sporadic outbreaks among poultry flocks up to now. The antigenic and genetic characterization of velogenic NDV in Taiwan's poultry population has been studied previously (Tsai et al., 2004; Lien et al., 2007), and all of the 20 isolates (Lien et al., 2007) and 30 isolates (Ke et al., 2010) collected from ND outbreaks in Taiwan from 2003 to 2006 and from 2002 to 2008, respectively, were assigned to former class II genotype VIIe, with an exception of a VIIc isolate by phylogenetic analysis of partial fusion protein gene sequences. However, the full extent of the distribution, evolution, and host species of APMV-1 circulating in domestic and wild birds has remained unexplored. In Taiwan, an APMV-6 strain was isolated from domestic ducks which appeared to be healthy in Taiwan in the year 1998, as a result of influenza surveillance program. The complete genome of an APMV-6 strain isolated from ducks was reported in 2001 (Chang et al., 2001).

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Chapter 3

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

3.1 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 chapter), 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 (P. J.Miller et al.,

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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), 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.

3.2 Materials and methods

3.2.1 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℃ for 72 h. The allantoic fluid from each inoculated embryo was collected after two passages in eggs and individually

doi:10.6342/NTU202100085

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.

3.2.2 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℃. 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 (Cohen, 1992) and then examined using a JEOL JEM-1400 electron microscope (JEOL Ltd., Tokyo, Japan).

3.2.3 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

doi:10.6342/NTU202100085

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).

3.2.4 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).

3.2.5 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

doi:10.6342/NTU202100085

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.

3.2.6 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).

doi:10.6342/NTU202100085

3.2.7 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.

3.3 Results

3.3.1 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 collared 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 collared 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.

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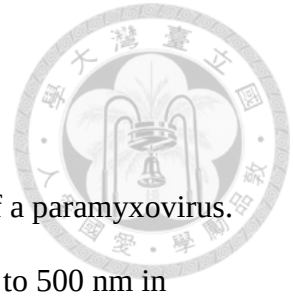

3.3.2 Electron microscopy

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. 3.1).

3.3.3 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.

3.3.4 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.

3.3.5 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

doi:10.6342/NTU202100085

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. 3.2), 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 (Anderson andWang, 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.

3.3.6 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 3.2). The highest inter-serotypic identity was 68.1% between APMV-1 and -16, and the lowest was 42.7% between

doi:10.6342/NTU202100085

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 3.3). 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 3.3).

Phylogenetic trees were constructed based on alignment of the full-length genome of AHRI33 (Fig. 3.3), the F gene (Fig. 3.4), and the HN gene (Fig. 3.5) with corresponding genes of representative APMV species. Phylogenetic analysis based on the full-length genome sequences (Fig. 3.3) 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 (Fig. 3.4) 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 (Fig. 3.4).

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. 3.6). 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.

3.4 Discussion

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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 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 3' end that is present in all APMVs) (Fig. 3.2). 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. 3.2). The length difference of 1,434 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. 3.3). 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

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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 3.2). 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.

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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-22 group, with the full name APMV-22/dove/Taiwan/AHRI33/2009.

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Table 3.1 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.

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Table 3.2 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	

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Table 3.3 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

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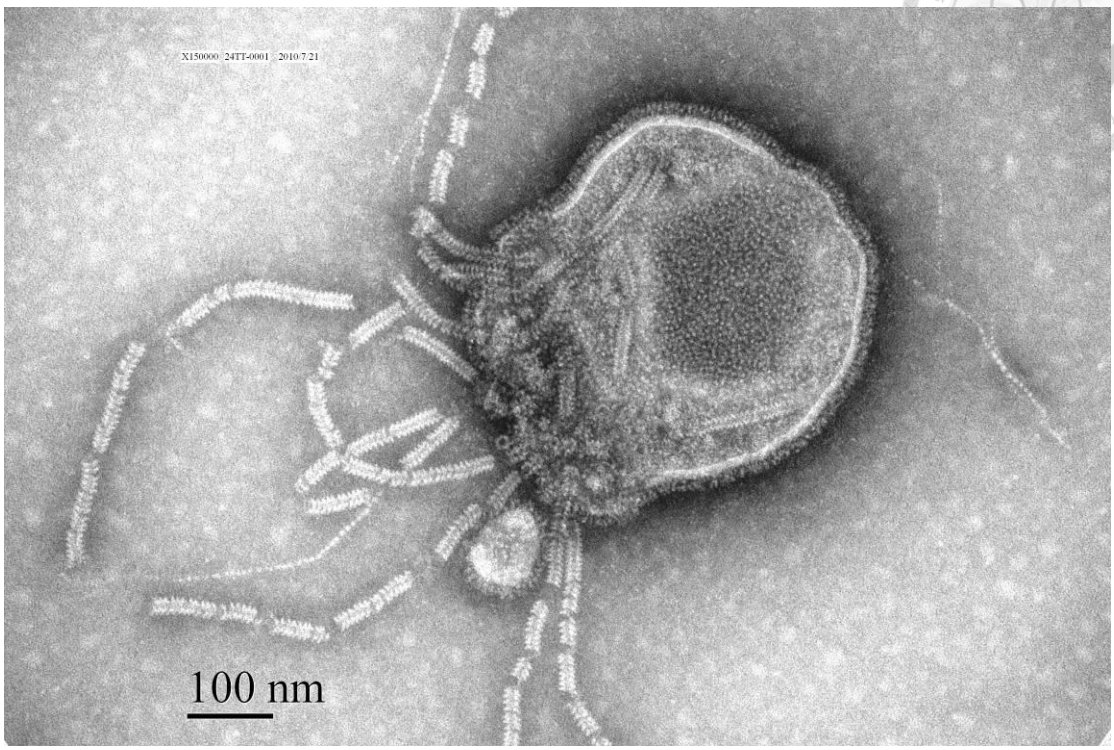

Figure 3.1 Paramyxovirus virion found in allantoic fluid of embryonated chicken egg inoculated with swab sample extract from a red collared dove in Taiwan. The virion consists of a fringed envelope, and the nucleoprotein helices protruded through the envelope. (×150,000.)

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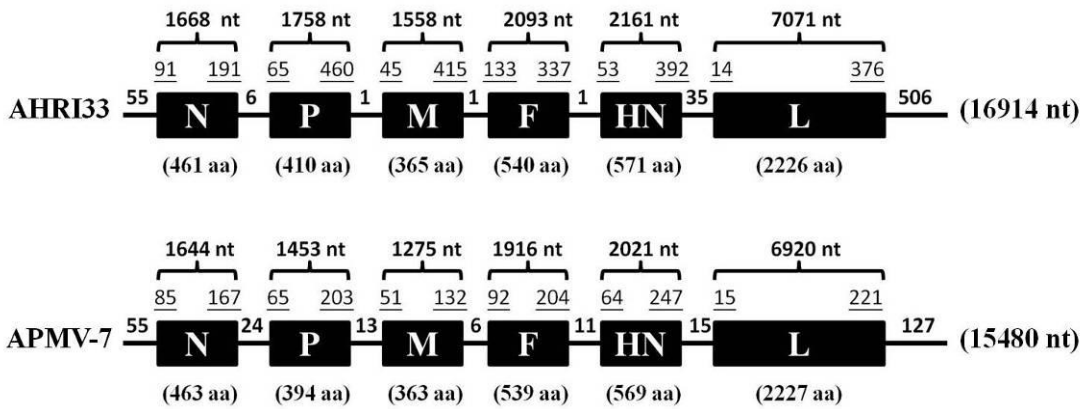

Figure 3.2 Schematic diagram of avian paramyxovirus (APMV) AHRI33 isolate and APMV-7 genome. Each rectangle indicates a gene and the letters within each rectangle represents the genes: N (nucleoprotein gene), P (phosphoprotein gene), M (matrix protein gene), F (fusion protein gene), HN (hemagglutinin-neuraminidase 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.

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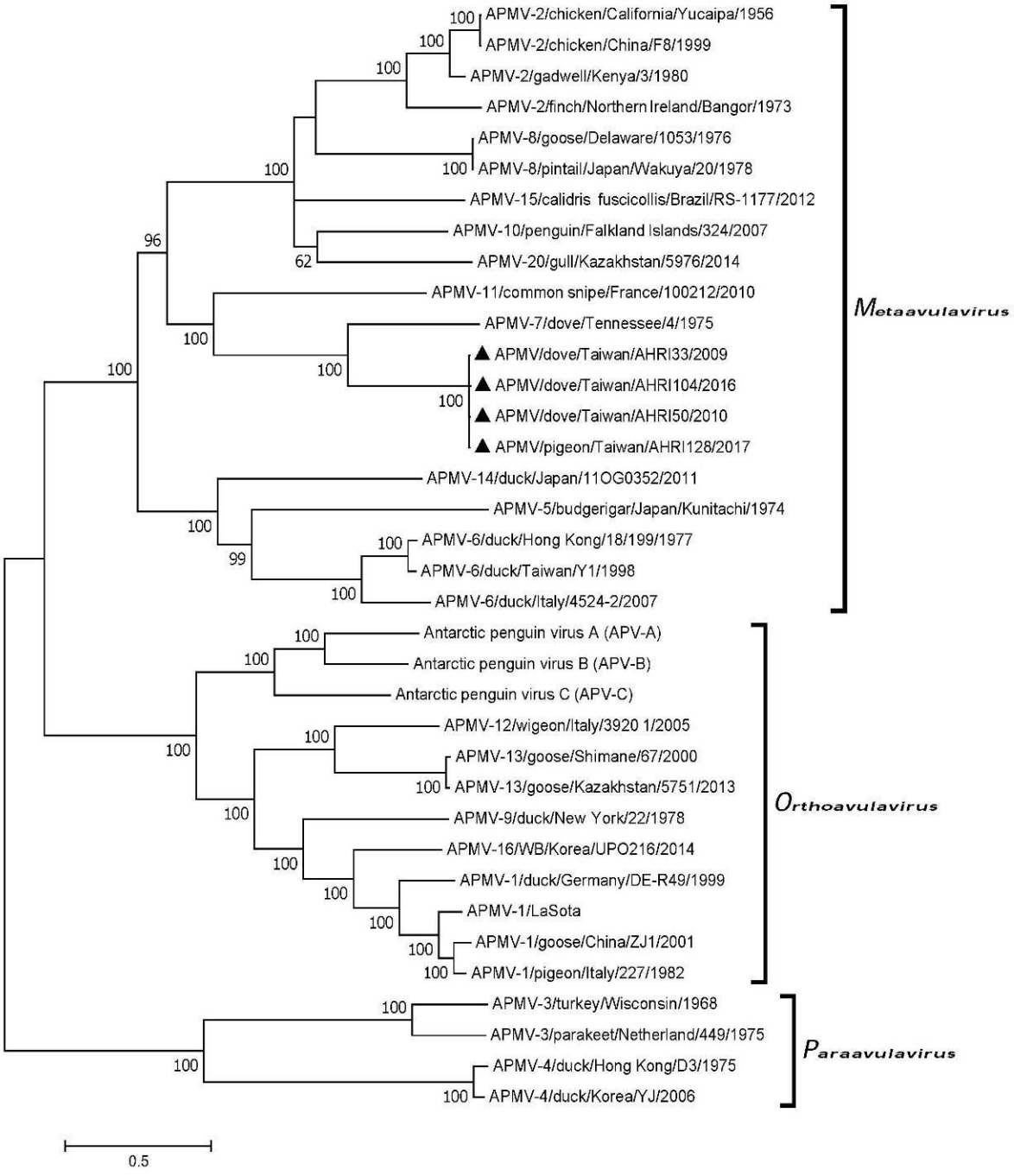

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Figure 3.3 Phylogenetic tree of the avian paramyxoviruses (APMVs), based on comparison of their full-length genomes. The solid triangles mark the isolates of APMV isolated from doves and pigeons in Taiwan in 2009-2017. The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites. 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.

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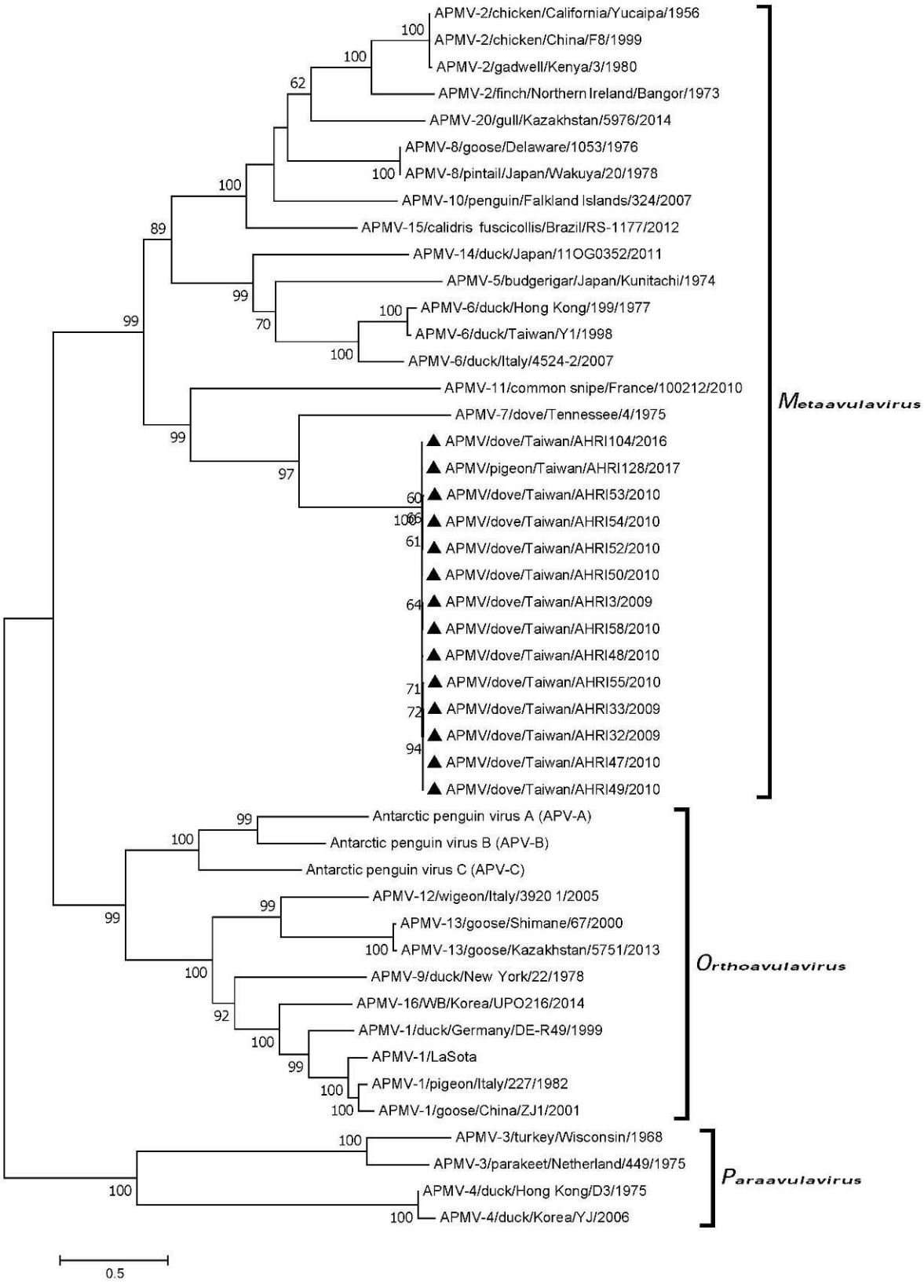

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Figure 3.4 Phylogenetic tree of the fusion gene. The solid triangles mark the isolates of APMV isolated from doves and pigeons in Taiwan in 2009-2017. The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites. There were a total of 1,455 positions in the final dataset.

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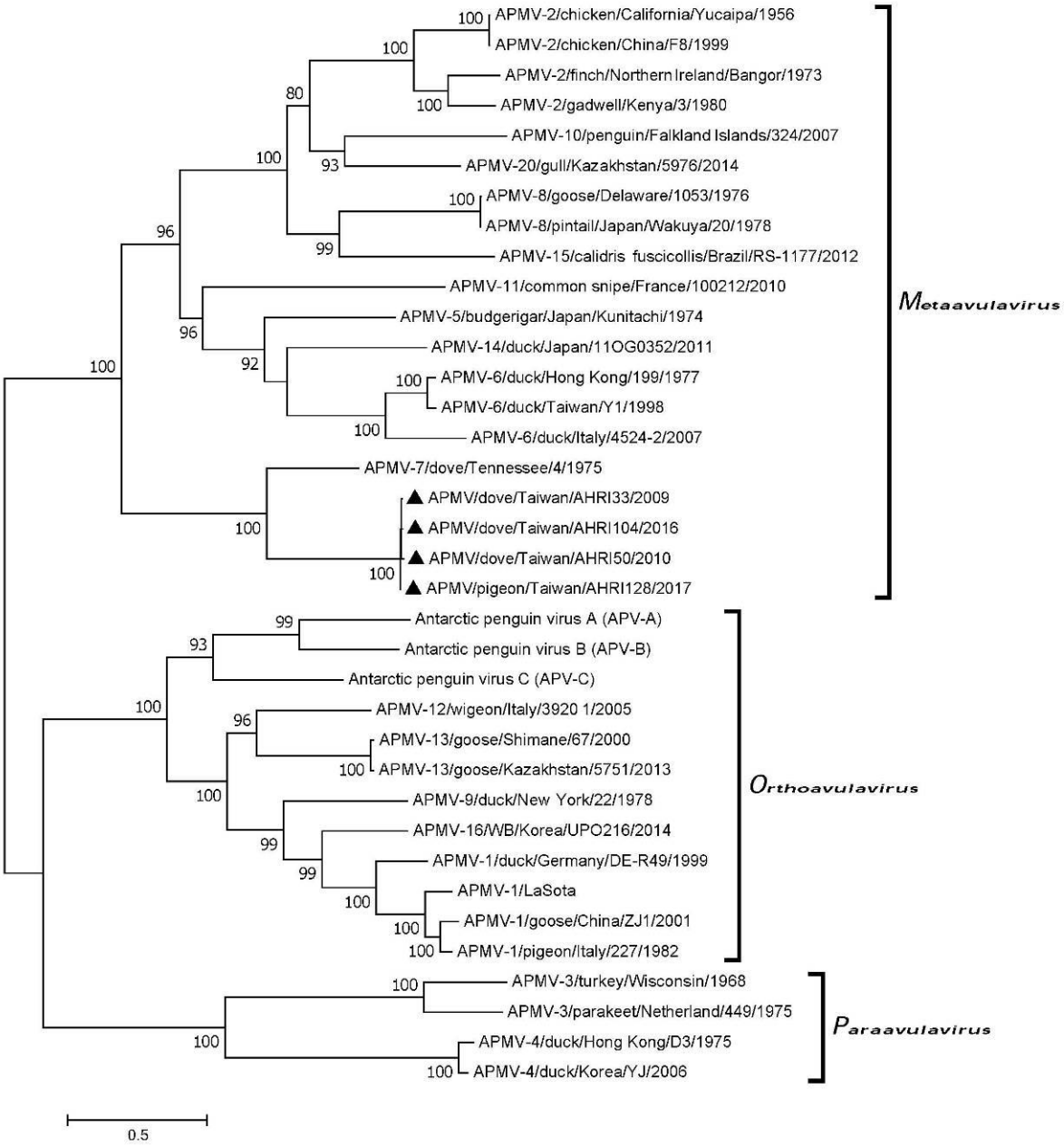

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Figure 3.5 Phylogenetic tree of the hemagglutinin-neuraminidase gene. The solid triangles mark four isolates of APMV isolated from doves and pigeons in Taiwan in 2009, 2010, 2016, and 2017. The evolutionary history was inferred by using the Maximum Likelihood method based on the general time reversible model with discrete gamma distribution and invariant sites (4 categories +G, parameter = 1.8716; [+I], 5.94% sites). The final dataset had a total of 1,514 positions.

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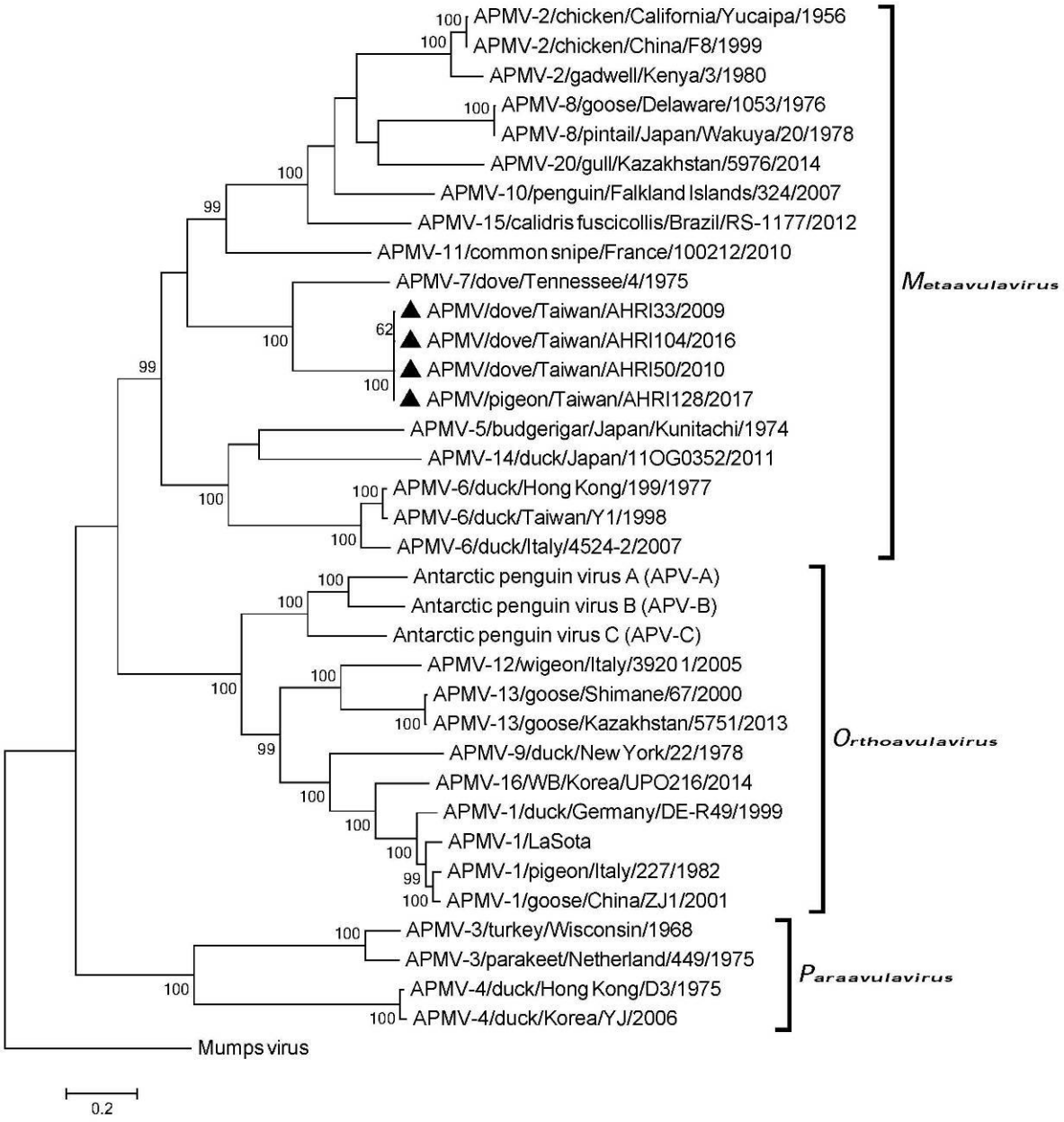

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Figure 3.6 Maximum Likelihood phylogenetic tree of the amino acid sequences of RdRp gene. The solid triangles mark the isolates of APMV isolated from doves and pigeons in Taiwan in 2009-2017. The evolutionary history was inferred by using the Maximum 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 2,344 positions.

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Chapter 4

Phylogenetic analysis of avian paramyxoviruses 1 isolated in Taiwan from 2010 to 2018 and evidence for their intercontinental dispersal by migratory birds

4.1 Introduction

Newcastle disease (ND) is one of the most important diseases of poultry and caused by virulent strains of avian paramyxovirus 1 (APMV-1), also known as Newcastle disease virus (NDV). Newcastle disease is classified as a notifiable disease by the World Organization for Animal Health (OIE) resulting in implementing control measures and trading restrictions to prevent the spread of the disease (OIE, 2012). Based on its genetic characteristics, the APMV-1 was re-assigned into the new genus *Orthoavulavirus* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* by the International Committee on Taxonomy of Viruses in 2019 (Amarasinghe et al., 2019). The virus has high genetic diversity and the infection of APMV-1 has been reported in a wide variety of avian species around the world (Dimitrov et al., 2016).

Based on phylogenetic analyses of nucleotide sequences of viral fusion protein gene, APMV-1 has been divided into two distinct clades, class I and class II (Czeglédi et al., 2006). An unified and objective classification system of APMV-1 was proposed in 2012 based on the coding sequences of the complete fusion protein gene (Diel et al., 2012), and this system (hereafter “former” for the purposes of this chapter) and nomenclature criteria for APMV-1 were revised and updated by a global consortium in 2019 (Dimitrov et al., 2019). The viruses within class I APMV-1 were assigned to a single genotype (genotype 1) and those within class II APMV-1 were further identified

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as 21 genotypes (genotype I-XXI) per criteria put forth by Dimitrov *et al*. APMV-1 are categorized as lentogenic, mesogenic, and velogenic depending on clinical signs in chickens and the cleavage site amino acid sequence of fusion protein (Miller and Koch, 2013). Almost all of the class I viruses are lentogenic strains and have been isolated primarily from waterfowl of the family *Anatidae* worldwide and occasionally from poultry in live bird markets (Kim et al., 2007). APMV-1 isolates of class II, genotype I consist of lentogenic strains and have been widely recovered from a diversity of wild and domestic waterfowl. A pigeon-adapted variant of genotype VI NDV, often termed pigeon paramyxovirus 1 (PPMV-1), is commonly isolated from columbids and can cause ND-like infectious disease in wild and domestic birds. Strains of genotype VII are regarded as the major pathogen responsible for the recent ND outbreaks in Europe, the Middle East, Africa, and Asia, including Taiwan (Dimitrov et al., 2016).

In Taiwan, despite that intensive vaccination programs against ND have been implemented for decades, NDV still has caused sporadic outbreaks among poultry flocks up to now. The antigenic and genetic characterization of velogenic NDV in Taiwan's poultry population has been studied previously (Tsai et al., 2004; Lien et al., 2007), and all of the 20 isolates (Lien et al., 2007) and 30 isolates (Ke et al., 2010) collected from ND outbreaks in Taiwan from 2003 to 2006 and from 2002 to 2008, respectively, were assigned to former class II genotype VIIe, with an exception of a VIIc isolate by phylogenetic analysis of partial fusion protein gene sequences. However, the full extent of the distribution, evolution, and host species of APMV-1 circulating in domestic and wild birds has remained unexplored. Moreover, the emerging virulent NDV isolates from sub-genotypes VIIi and VIIh were rapidly spreading throughout Asia in recent years and had potential to cause a new ND panzootic (Miller et al., 2015).

Based on the global phylodynamic analysis, a study provides evidence for East

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Asia representing a critically important node for the global dispersion of APMV-1 (Hicks et al., 2019). In a study of fusion protein gene sequences of two sub-genotypes of class II APMV-1 strains isolated from wild birds in Eurasia and North America, evidence of intercontinental dispersal by wild birds has been found (Ramey et al., 2013). The phylogentic study for global APMV-4 isolates presented limited evidence for historical viral movement between continents (Reeves et al., 2016). Phylogenetic network analysis also supported the introduction of Asia-origin clade 2.3.4.4 H5N8 avian influenza viruses into North America via intercontinental associations of waterfowl (Lee et al., 2015). Collectively, these findings suggest that migratory birds may play a potential role in the global spread of kinds of avian infectious agents.

In the present study, the APMV-1 isolates obtained from migratory birds and poultry in Taiwan were characterized by sequencing of complete fusion protein gene sequences and were compared to those available in GenBank. Based on the results of the phylogenetic analyses, we aim to illustrate the genetic diversity of APMV-1 in various avian hosts, present new epidemiological information on ND in Taiwan, and provide evidence for the potential intercontinental transmission of APMV-1 by migratory birds.

4.2 Materials and methods

4.2.1 Sample collection and virus isolation

The samples of this study were collected from migratory, resident, and domestic birds in Taiwan as part of an avian influenza surveillance program and clinical cases submitted to Animal Health Research Institute from 2010 to 2018. The cloacal swab samples, fecal samples from healthy birds and tissue samples of the brain, trachea, lung, liver, spleen, heart, and kidney from clinical cases were inoculated into the allantoic

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cavities of 9- to 11-day-old specific-pathogen-free embryonated chicken eggs (Animal Drugs Inspection Branch, Animal Health Research Institute, Miaoli, Taiwan) and then incubated at 37℃ for 72 hr. The allantoic fluid from each inoculated embryo was examined for hemagglutination (HA) activity. If no HA activity was detected, a second passage was then performed. When HA activity was positive, then the allantoic fluid was tested by a commercial rapid test strip, NDV Ag Test Kit (BioNote Inc., Hwaseong-si, South Korea). Samples that were tested positive by the kit were subjected to further analyses.

4.2.2 RNA extraction and reverse transcription-polymerase chain reaction (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. The detection of APMV-1 RNA was performed using specific fusion protein gene-targeting RT-PCRs with SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) to generate amplicons of either 328 bp (class I) or 292 bp (class II). The primers to amplify the specific region and the complete coding region of the fusion protein genes of class I and class II APMV-1 isolates are listed in Table 4.1. The full-length genome sequences of the representatives of each genotype and virulent class II genotype VII NDVs were determined with five different sets of primers according to the genotypes of isolates, and the sequences of theses primers are available upon request. The cycling parameters were reverse transcription at 50°C for 40 min, followed by heating at 95°C for 2 min, 35 cycles of denaturing at 95℃ for 40 sec, annealing at 50℃ for 50 sec, and extension at 72℃ for

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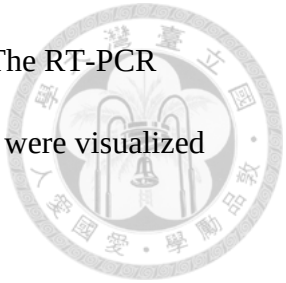
1 min, and completed with a final extension step at 72℃ for 7 min. The RT-PCR products were separated by electrophoresis using 2% agarose gel and were visualized with ethidium bromide stain and ultraviolet transillumination.



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

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 with the Lasergene 6.0 software package (DNASTAR Inc., Madison, WI, USA).

4.2.4 Phylogenetic analysis

The curated complete fusion protein gene of class I and class II datasets, provided by Dimitrov *et al.* (2019) and four referenced sequences of class I novel genotype isolates retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank) were analyzed with those obtained in this study. For the construction of the phylogenetic trees, the evolutionary history was inferred using the maximum-likelihood (ML) method based on the general time reversible model with discrete gamma distribution and invariant sites by using RaxML version 8.2.12 (Stamatakis, 2014) with 1,000 bootstrap replicates through the CIPRES Science Gateway (Miller et al., 2010). The parameters for building Maximum likelihood trees using the CIPRES Science Gateway were set according to the step-by-step guidelines (Dimitrov et al., 2019). Trees were visualized

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using Molecular Evolutionary Genetics Analysis version 7, or MEGA 7 (Kumar et al., 2016).

The estimates of average evolutionary distance between class I genotype 1 and genotype 2 were inferred using MEGA 7 (Kumar et al., 2016). Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter=1). The analysis involved 292 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

4.3 Results

4.3.1 Sample collection and virus isolation

Forty APMV-1 isolates from different avian species were collected in this study (Table 4.2). The isolates were confirmed to be APMV-1 by isolation in specific-pathogen-free embryonated eggs with hemagglutination activity, NDV rapid test strip, specific F gene RT-PCR and sequencing. Vaccine-like isolates of NDV obtained from samples of chickens, turkeys and pet parrots were excluded from the dataset in order to analyze only APMV-1 representing natural circulation and evolution in Taiwan during 2010-2018.

4.3.2 GenBank accession numbers

GenBank accession numbers of the APMV-1 strains described in this study are listed in Tables 4.2. The accession numbers for the full-length genome sequences of 18 isolates are MN632509-MN632526. The accession numbers for the complete coding region of the fusion protein genes of the other 22 isolates are MN632527-MN632548.

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4.3.3 Genetic analysis of class I APMV-1

All of the 10 class I isolates were obtained from the samples of waterfowl of the order *Anseriformes* and domestic ducks. The fusion protein gene sequences of the 10 class I isolates were most closely related to those previously identified as former sub-genotype 1c (n = 2), sub-genotype 1d (n = 7) or phylogenetically divergent (n = 1) from class I genotypes 1, illustrated in the ML phylogenetic tree (Fig. 4.1). The sequences of two former sub-genotype 1c isolates were grouped with those derived from wild bird samples collected in Japan, China, Russia, Kazakhstan, Germany, and Finland. The sequences of seven former sub-genotype 1d isolates were grouped with those originated from samples collected in Alaska, Connecticut, Delaware, Florida, Idaho, Louisiana, Massachusetts, Michigan, Minnesota, New Jersey, Ohio, Oregon, Pennsylvania, and Texas of the United States. The fusion protein gene sequence of the isolate, Anseriformes/Taiwan/AHRI67/2011, and other four sequences of APMV-1 collected from China, France, and Finland formed a strongly supported monophyletic clade. In estimated evolutionary mean distance analyses, this undesignated clade (genotype 2) had average distances of 0.246 (standard error 0.018) base pairs per site as compared to the sequences in genotype 1.

The deduced amino acid motif at the fusion protein cleavage site sequence for nine class I genotype 1 isolates were ^{112}E(R/Q)QER ↓ L^{117}, and that of one genotype 2 isolate was ^{112}ERQGR ↓ L^{117}.

4.3.4 Genetic analysis of class II APMV-1

The fusion protein gene sequences of class II isolates in this study were most

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closely related to those previously identified as genotype I (n = 9), genotype VI (n = 16), or genotype VII (n = 5). The nine genotype I isolates were obtained from waterfowl (*Anseriformes*), and shorebirds (*Charadriiformes*), except one isolate, AHRI137, from sparrow (*Passeriformes*). The fusion protein gene sequences of the nine isolates were assigned to genotype Ib with scattered distribution in the clade and were grouped with those derived from samples collected in China, Japan, Russia, and South Korea in ML phylogenetic analyses (Fig. 4.2). The deduced amino acid motif at the fusion protein cleavage site of the nine sub-genotype I.2 (former Ib) isolates was ^{112}GKQGR ↓ L^{117}, except that of one isolate from domestic ducks was ^{112}GEQGR ↓ L^{117}.

Of the 16 isolates of pigeon paramyxovirus 1 (PPMV-1), a genetic variant of NDV that belongs to genotype VI, 15 were obtained from birds in the family *Columbidae* (pigeon, red collared dove, spotted dove, and rufous turtle dove) and 1 from that of the family *Corvidae* (magpie). Two of the 16 PPMV-1 isolates were placed in sub-genotype VI.2.1.1.2.1 (former VIj) and the remaining 14 isolates in sub-genotype VI.2.1.1.2.2 (former VIk). Both sub-genotypes of the PPMV-1 isolates from Taiwan were related to the viruses previously circulating in pigeons and doves in China, and seem to originate from the ancestral pigeon/Belgium/248/1998 (JX901110) and pigeon/Belgium/3936-8/2005 (JX901120) strains, respectively (Fig. 4.3). The deduced amino acid motif at the fusion protein cleavage site sequence of all PPMV-1 isolates was ^{112}RRQKR ↓ F^{117}.

Five isolates of genotype VII APMV-1 were all obtained from chicken farms. Based on the phylogenetic analyses, the viruses responsible for the ND endemic in Taiwan were classified into sub-genotype VII.1.1 and grouped together within an exclusive and independent monophyletic branch (Fig. 4.4). The deduced amino acid

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motif at the fusion protein cleavage site sequence of the group of NDVs was ^{112}RRKKR ↓ F^{117}.

4.4 Discussion

For a long time, the distribution, circulation and evolution of APMV-1 in Taiwan have been largely unknown, and only two complete coding sequences of fusion protein gene of had been available in GenBank (U62620 and AF358786). To investigate genetics of APMV-1 in Taiwan, 40 APMV-1 isolates obtained from poultry, migratory birds, and resident birds were characterized genetically. Twenty-two sequences of complete fusion protein gene and eighteen sequences of full-length genomes were determined in this study. Our results revealed that the North America-origin APMV-1 strains have been introduced into Taiwan wild bird populations since 2014 and have kept circulating until now. Moreover, our genetic analyses supported the designation of the novel APMV-1 class I genotype 2 and illustrated the genetic diversity of APMV-1 in Taiwan.

The result provided the evidence to suggest the intercontinental dispersal of APMV-1, from North America to East Asia. In 2017, a previously undescribed class I former sub-genotype 1d APMV-1 had been characterized phylogenetically and isolated exclusively from the United States during 1998-2014 (Ramey et al., 2017). In our study, seven APMV-1 isolates of this sub-genotype were obtained from waterfowl in Taiwan and found to be closely related to those isolated in the United States. Since the first isolation of the former sub-genotype 1d APMV-1 (Anseriformes/Taiwan/AHRI85/2014) from wild birds in March 2014, APMV-1 of this sub-genotype had been constantly obtained every year in Taiwan. Related APMV-1 isolates were obtained from wild birds in East Asia and North America, supporting possible intercontinental lentogenic

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APMV-1 spread by migratory birds.

Viruses of the proposed novel genotype within APMV-1 class I were identified in Taiwan. The APMV-1 isolate Anseriformes/Taiwan/AHRI67/2011 and the other four closely related isolates, teal/Finland/13111/2008 (Lindh et al., 2012), teal/France/100011/2010 (Briand et al., 2013), egret/China/SD18/2013 (KY284861), and Sheldrake duck/China/SD19/2013 (MK516204), were clustered independently from all the other studied viruses with a bootstrap value of 95%, indicating a statistically well-supported grouping (Fig. 4.1). The average distance between these two groups within class I suggested a very distant genetic relationship between them (24.6%). The epidemiological and phylogenetic data supported the designation of a new genotype, genotype 2, in class I as per the nomenclature criteria for APMV-1 proposed in 2019 (Dimitrov et al., 2019), which included an average distance per site above 10%, a bootstrap value at the genotype defining node above 70%, and at least four independent isolates without a direct epidemiologic link. The isolate Anseriformes/Taiwan/AHRI67/2011, together with the four Euroasian isolates, met these criteria and were distinct from those viruses within the genotype 1 of class I.

Our findings indicated that viral transmission may occur between migratory birds (*Anseriformes* and *Charadriiformes*) and domestic ducks. The 18 isolates obtained from migratory birds and domestic ducks in this study belonged to genotypes 1 and 2 of class I, or genotype I of class II. These isolates possessed fusion protein cleavage site motifs consistent with the previously reported lentogenic strains (Kim et al., 2007; Ramey et al., 2013), except that the isolate mule duck/Taiwan/AHRI77/2013 contained the unique motif ^{112}GEQGR ↓ L^{117}. Phylogenetically, the viruses of class II genotype I consisted of genetically divergent viruses were clustered with the viruses isolated from domestic ducks and mallards in the Eurasian countries such as China, Japan, Russia, Finland, and

doi:10.6342/NTU202100085

Germany. These results were consistent with previous findings (Kim et al., 2007) and indicated that wild and domestic waterfowl share the same APMV-1 gene pool, implying a putative transmission across these species.

The PPMV-1 was continuing endemic in Taiwan among birds of the family *Columbidae*. In this study, the PPMV-1 isolates were nearly exclusively obtained from pigeons and doves (*Columbidae*) with the exception that was obtained from common magpie (*Corvidae*). These PPMV-1 isolates were obtained from dead birds sent to The Animal Health Research Institute (AHRI, New Taipei City, Taiwan), and nervous symptoms were recorded in some birds' case description. All of these PPMV-1 isolates were virulent by definition since they contain three basic amino acid residues at positions 113-116 and a phenylalanine residue at position 117 of the fusion protein cleavage site. Phylogenic analysis showed that these isolates belonged to sub-genotypes VI.2.1.1.2.1 (2010-2012) and VI.2.1.1.2.2 (2017-2018) and were closely related to those from China, as shown in Fig. 4.3. Moreover, this analysis also suggested that Taiwanese and Chinese PPMV-1 had common ancestors from Europe. The viruses of these two sub-genotypes were introduced into Taiwan and had become dominant in pigeon and dove populations. The closely phylogenetic relationship demonstrated an epidemiological link between Taiwan and neighboring countries and highlighted the importance of constant surveillance for pathogenic microorganisms carried by pigeons.

Sporadic outbreaks of ND in Taiwan from year to year were majorly due to the infection of a virulent strain of sub-genotypes VII.1.1 (former VIIe), which was only restrictively obtained from land-based poultry and had not been detected in wild birds and waterfowl. Phylogenetic analysis of the complete fusion protein gene sequences suggests that the five outbreak-associated NDV in this study were 95.8% to 96.9% similar to the ancestral chicken/Taiwan/2000 strain (AF358786). There was one lineage

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of genotype VII NDVs maintained in chicken farms and NDVs of this lineage were continuously evolving independently. No evidence suggested new introduction of emerging sub/genotypes NDV from abroad.

In summary, our findings supported the intercontinental transmission of lentogenic APMV-1 between Eurasia and North America by wild birds. This investigation provided the information of previously unrecognized genetic diversity and distribution of class I and class II APMV-1 isolates in nature and viral evolution of class II sub-genotype VII.1.1 outbreak-associated isolates in Taiwan poultry farms. In addition, according to the criteria of the updated classification system for APMV-1 isolates, a novel genotype within class I was identified.

doi:10.6342/NTU202100085

Table 4.1 List of RT-PCR primers.

Class	Designation	Primer sequence (5'-3')	Position[a)]	Fragment size
Class I	APMV-1 C I 272F	TCCTYRCCCCRCTYGG	4,827-4,842	328 bp
	APMV-1 C I 599R	ATRCAGTCRATYTCYTGKGCTGT	5,132-5,154	
	APMV-1 C I fusion-1F	CACGGGTAGAAGGTATGGG	4,509-4,527	1,004 bp
	APMV-1 C I fusion-1R	CACTAATGCGGATGCGAATCC	5,492-5,512	
	APMV-1 C I fusion-2F	TGGGAGTGGGTAATAATCAGC	5,313-5,333	1,039 bp
	APMV-1 C I fusion-2R	CTCCGACTGTTCTACCCGTA	6,332-6,351	
Class II	APMV-1 C II 208F	CCYARRGAYAARGARGCRTG	4,751-4,770	292 bp
	APMV-1 C II 499R	CRTGYACRGCYTCATTRGTYGC	5,021-5,042	
	APMV-1 C II fusion-1F	GCACACCATTGCYAAATACAATCC	4,348-4,371	1,052 bp
	APMV-1 C II fusion-1R	GTATRCCCAAGAGTTGAGTCTG	5,378-5,399	
	APMV-1 C II fusion-2F	GCTGGTGGCAAYATGGATTAC	5,267-5,287	1,076 bp
	APMV-1 C II fusion-2R	CTYCTCTGACCGTTCTACC	6,324-6,342	

[a)] Nucleotide positions of class I and class II APMV-1 were based on the complete genomes of duck/Germany/DE-R49/99 strain (GenBank accession number DQ097393) and chicken/U.S./LaSota/46 strain (AF077761), respectively.

doi:10.6342/NTU202100085

Table 4.2 Isolate details.

Strains	Type	Class	Sub/genotype (Diel et al., 2012)	Sub/genotype (Dimitrov et al., 2019)	Cleavage site of fusion protein	Accession no.
Anseriformes/Taiwan/AHRI76/2013	Migratory	I	1c	1.2	ERQER ↓ L	MN632509[a)]
Mule duck/Taiwan/AHRI79/2013	Domestic	I	1c	1.2	ERQER ↓ L	MN632510[a)]
Anseriformes/Taiwan/AHRI85/2014	Migratory	I	1d	1.2	ERQER ↓ L	MN632511[a)]
Mule duck/Taiwan/AHRI95/2015	Domestic	I	1d	1.2	ERQER ↓ L	MN632527[b)]
Anseriformes/Taiwan/AHRI98/2015	Migratory	I	1d	1.2	ERQER ↓ L	MN632528[b)]
Anseriformes/Taiwan/AHRI102/2015	Migratory	I	1d	1.2	EQQER ↓ L	MN632529[b)]
Anseriformes/Taiwan/AHRI106/2016	Migratory	I	1d	1.2	ERQER ↓ L	MN632512[a)]
Anseriformes/Taiwan/AHRI130/2017	Migratory	I	1d	1.2	ERQER ↓ L	MN632513[a)]
Anseriformes/Taiwan/AHRI132/2018	Migratory	I	1d	1.2	ERQER ↓ L	MN632514[a)]
Anseriformes/Taiwan/AHRI67/2011	Migratory	I	2	2	ERQGR ↓ L	MN632515[a)]
Charadriiformes/Taiwan/AHRI44/2010	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632516[a)]
Anseriformes/Taiwan/AHRI59/2010	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632530[b)]
Anseriformes/Taiwan/AHRI63/2011	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632531[b)]
Mule duck/Taiwan/AHRI77/2013	Domestic	II	Ib	I.2	GEQGR ↓ L	MN632517[a)]
Charadriiformes/Taiwan/AHRI84/2013	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632532[b)]
Anseriformes/Taiwan/AHRI108/2016	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632533[b)]
Anseriformes/Taiwan/AHRI136/2018	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632534[b)]
Sparrow/Taiwan/AHRI137/2018	Resident	II	Ib	I.2	GKQGR ↓ L	MN632535[b)]
Anseriformes/Taiwan/AHRI138/2018	Migratory	II	Ib	I.2	GKQGR ↓ L	MN632536[b)]
Pigeon/Taiwan/AHRI43/2010	Resident	II	VIj	VI.2.1.1.2.1	RRQKR ↓ F	MN632518[a)]
Pigeon/Taiwan/AHRI68/2012	Resident	II	VIj	VI.2.1.1.2.1	RRQKR ↓ F	MN632519[a)]

doi:10.6342/NTU202100085

Table 4.2 (continued)

Strains	Type	Class	Sub/genotype (Diel et al., 2012)	Sub/genotype (Dimitrov et al., 2019)	Cleavage site of fusion protein	Accession no.
Pigeon/Taiwan/AHRI107/2016	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632520[a)]
Pigeon/Taiwan/AHRI111/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632521[a)]
Dove/Taiwan/AHRI113/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632537[b)]
Pigeon/Taiwan/AHRI114/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632538[b)]
Dove/Taiwan/AHRI115/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632539[b)]
Dove/Taiwan/AHRI116/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632540[b)]
Dove/Taiwan/AHRI117/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632541[b)]
Dove/Taiwan/AHRI118/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632542[b)]
Dove/Taiwan/AHRI120/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632543[b)]
Pigeon/Taiwan/AHRI121/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632544[b)]
Dove/Taiwan/AHRI123/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632545[b)]
Magpies/Taiwan/AHRI125/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632546[b)]
Dove/Taiwan/AHRI126/2017	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632547[b)]
Pigeon/Taiwan/AHRI133/2018	Resident	II	VIk	VI.2.1.1.2.2	RRQKR ↓ F	MN632548[b)]
Chicken/Taiwan/AHRI70/2012	Domestic	II	VIIe	VII.1.1	RRKKR ↓ F	MN632522[a)]
Chicken/Taiwan/AHRI91/2015	Domestic	II	VIIe	VII.1.1	RRKKR ↓ F	MN632523[a)]
Chicken/Taiwan/AHRI103/2016	Domestic	II	VIIe	VII.1.1	RRKKR ↓ F	MN632524[a)]
Chicken/Taiwan/AHRI105/2016	Domestic	II	VIIe	VII.1.1	RRKKR ↓ F	MN632525[a)]
Chicken/Taiwan/AHRI131/2017	Domestic	II	VIIe	VII.1.1	RRKKR ↓ F	MN632526[a)]

[a)] GenBank accession numbers of full-length genome sequences.

[b)] GenBank accession numbers of complete fusion protein gene sequences.

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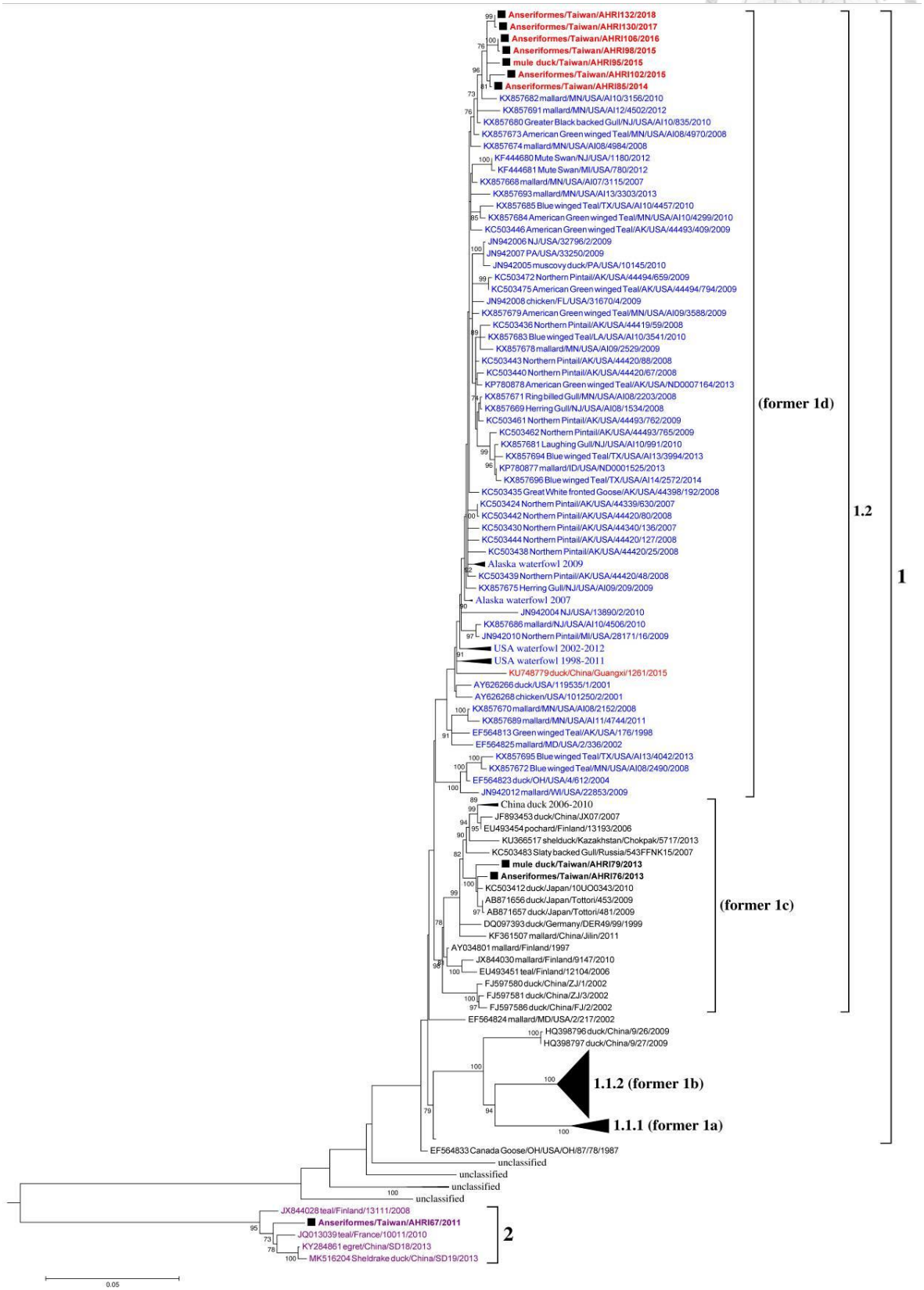

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Fig. 4.1 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class I (n = 298). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The sub-tree was rooted with four historical APMV-1 class II isolates, avian/Mukteswar/1940 (EF201805), fowl/UK/Herts/1933 (AY741404), chicken/Malaysia/AF2240/1960 (AF048763), and chicken/Mexico/Queretaro/452/1947/1947 (JX915243). The solid square marks the isolate of APMV-1 obtained from birds in Taiwan in this study. The former sub-genotype 1d isolates in North America and Asia are shown in blue and red, respectively. The novel genotype 2 isolates are shown in purple.

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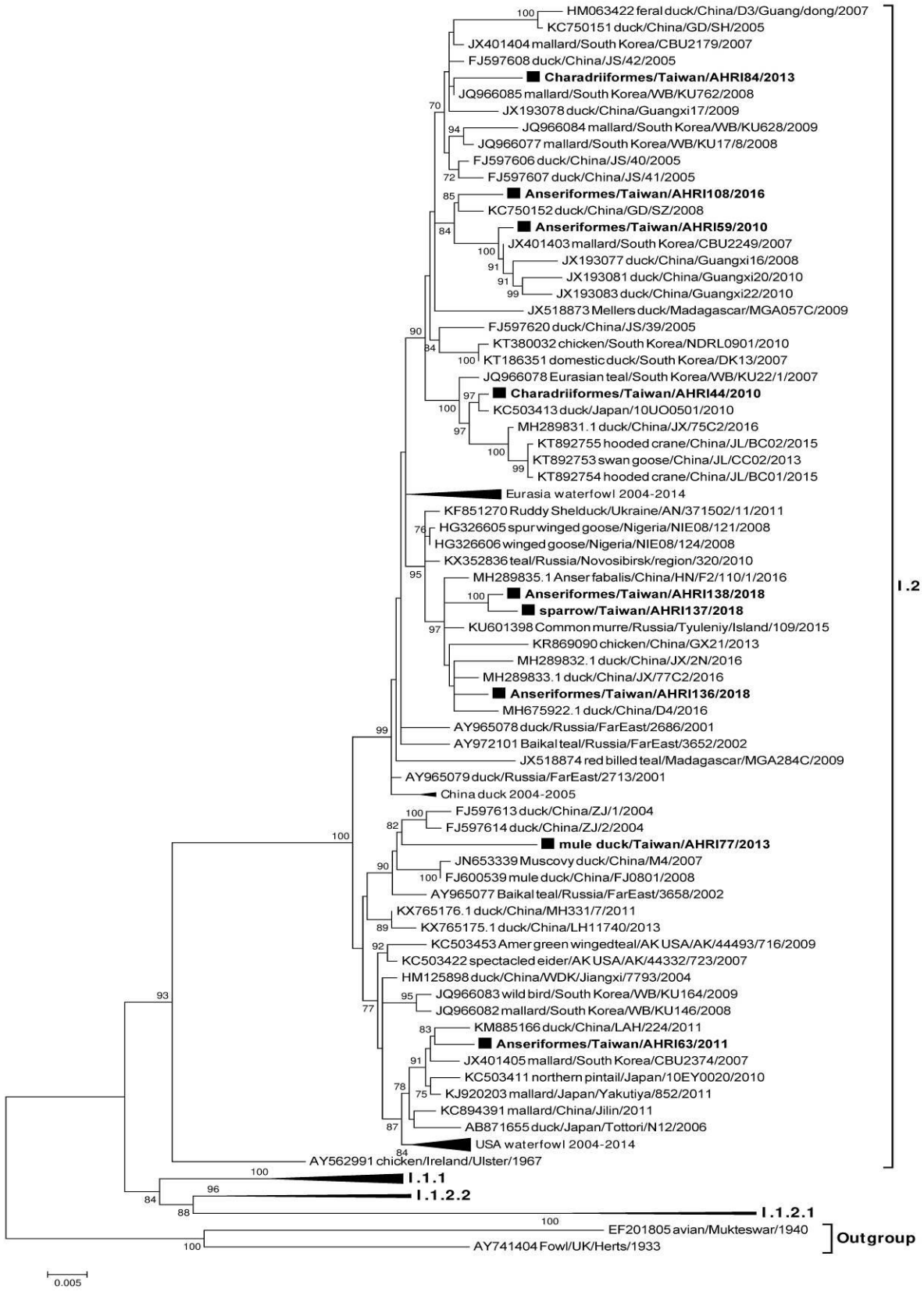

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Fig. 4.2 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype I (n = 129). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid square marks the isolate of APMV-1 obtained from birds in Taiwan in this study.

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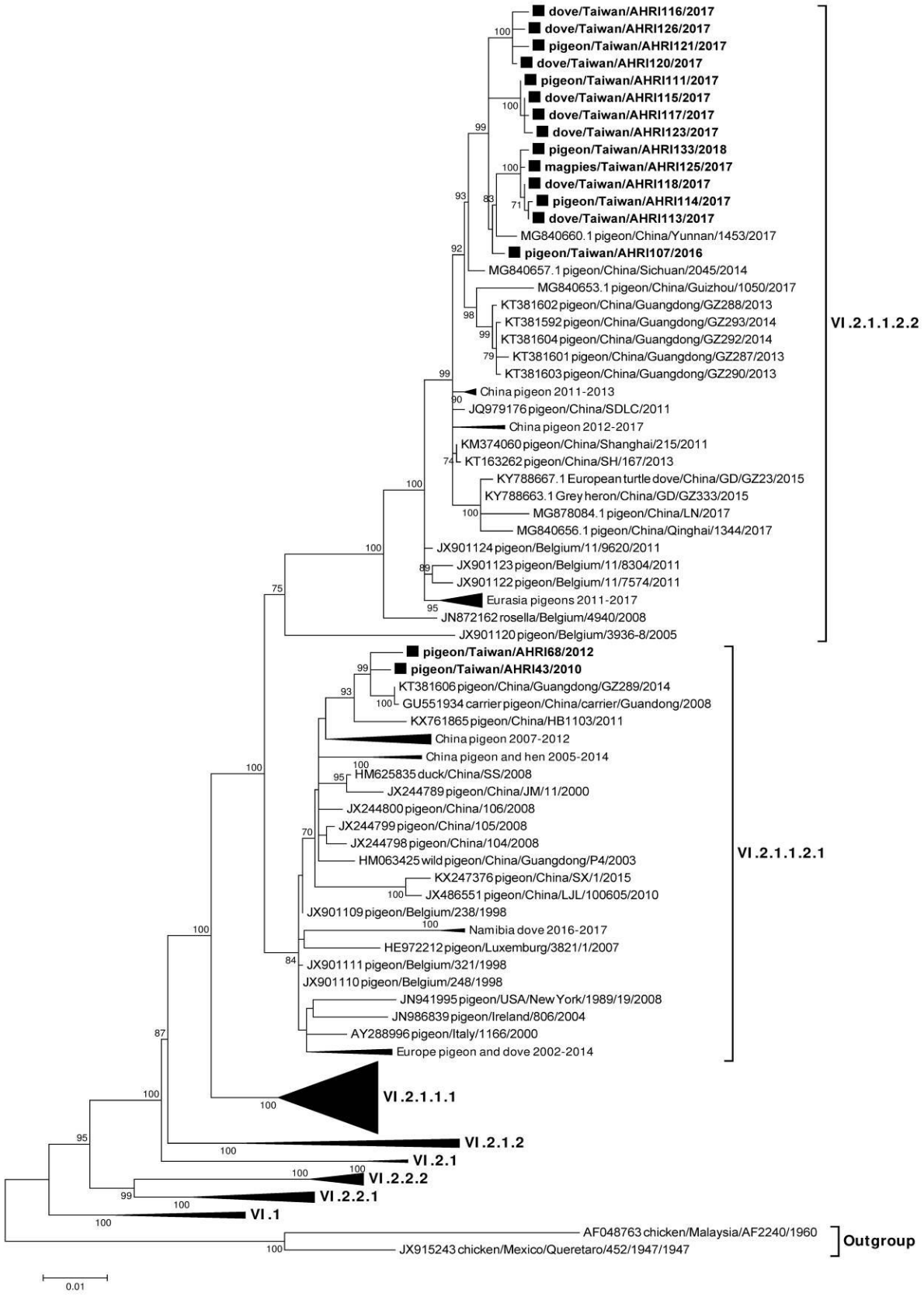

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Fig. 4.3 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype VI (n = 278). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid square marks the isolate of APMV-1 obtained from birds in Taiwan in this study.

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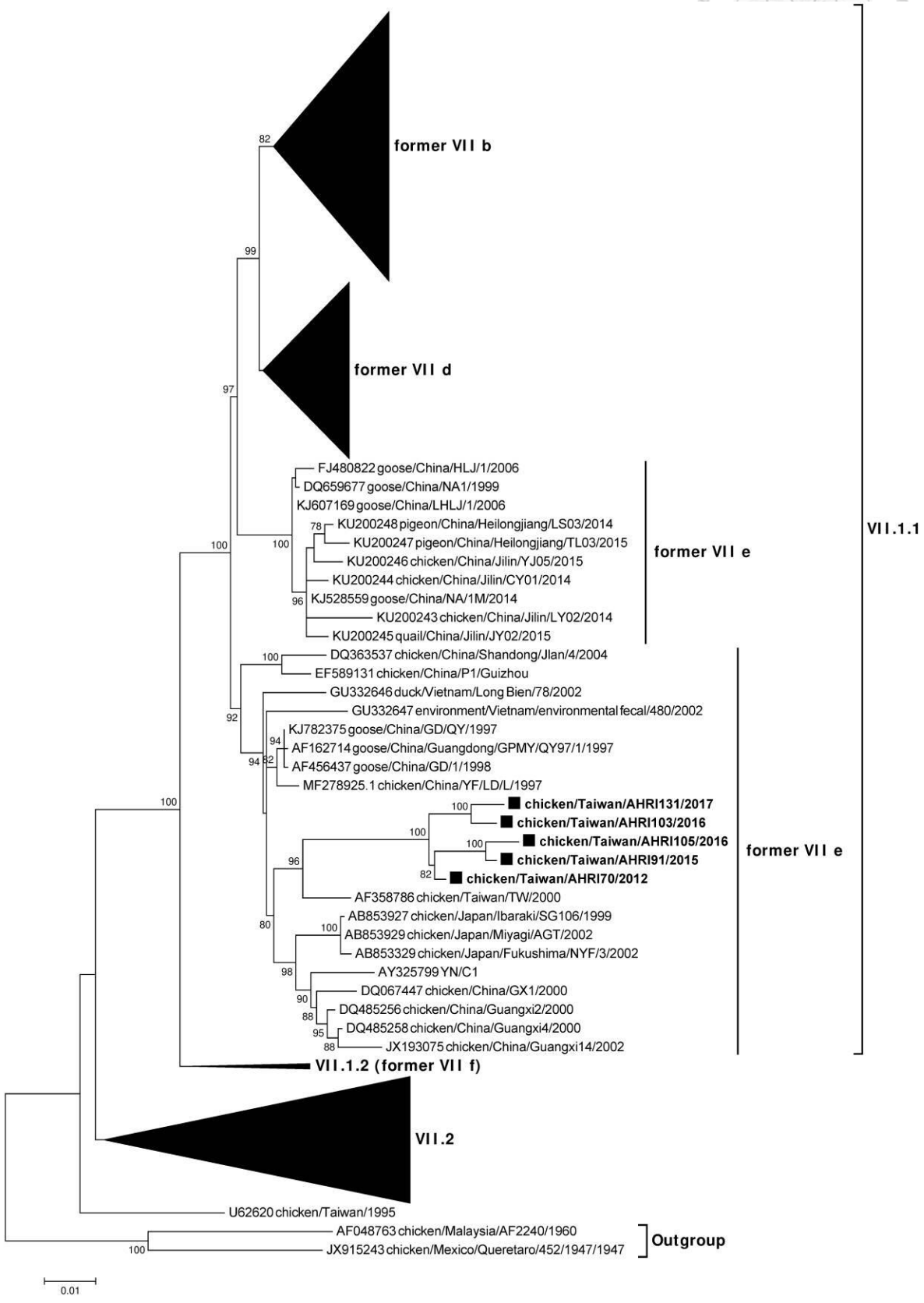

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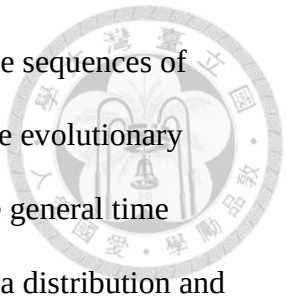

Fig. 4.4 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype VII (n = 777). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. The number of branch nodes in the tree presents the bootstrap value and the scale bar presents per-site substitution. The solid square marks the isolate of APMV-1 obtained from birds in Taiwan in this study.

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Chapter 5

A highly sensitive real-time reverse transcription polymerase chain reaction for detecting nucleocapsid protein gene of both classes I and II of Newcastle disease virus

5.1 Introduction

Newcastle disease (ND) is one of the most prevalent and economically important poultry diseases and caused by Newcastle disease virus (NDV), virulent strains of avian paramyxovirus 1 (APMV-1). Newcastle disease is an OIE (World Organization for Animal Health) notifiable disease resulting in implementing control measures and trading restrictions to prevent the spread of the disease (OIE, 2012). The virus has high genetic diversity and the infection of NDV has been reported in a wide variety of avian species around the world (Dimitrov et al., 2016).

Newcastle disease virus was assigned into the new genus *Orthoavulavirus* within the subfamily *Avulavirinae* of the family *Paramyxoviridae* by the International Committee on Taxonomy of Viruses in 2018 (Amarasinghe et al., 2018). Based on phylogenetic analyses of fusion protein gene, NDV has been divided into two clades, class I and class II (Czeglédi et al., 2006). The viruses within class I were identified as a single genotype (genotype 1) and those within class II were further assigned to 21 genotypes (genotype I-XXI) per criteria put forth by a global consortium in 2019 (Dimitrov et al., 2019). Newcastle disease virus contains one single-stranded negative sense RNA genome and the genome encodes at least six proteins: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) (Kattenbelt et al., 2006).

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With its efficiency and high throughput, real-time reverse transcription polymerase chain reaction (rRT-PCR) has become one of the most widely applied assays for NDV diagnosis and surveillance (Hoffmann et al., 2009). At present, the U.S. Department of Agriculture (USDA) validated M-gene rRT-PCR assay described by Wise et al. (2004) is widely used in North America, Europe, and Taiwan for detecting most NDVs, mainly class II isolates. However, even M gene is highly conserved among NDV strains, variability of the gene may result in failures of detecting some NDVs (Kim et al., 2007; Khan et al., 2010). To minimize the false negatives produced by the USDA-validated M-gene assay, rRT-PCR assays targeting other NDV genomic regions were developed and evaluated. These assays include TaqMan rRT-PCR assays detecting the M gene (Hines et al., 2012), L gene (Fuller et al., 2010; Sutton et al., 2019), both L gene and M gene (Kim et al., 2008), along with many assays targeting the F gene, which also enable sequencing or pathotyping (Fuller et al., 2009; Yacoub et al., 2012). However, these assays cannot detect all NDV strains and a multiple testing approach may be needed for detecting the index case (OIE, 2012).

To improve detection of NDV in clinical specimens of poultry and wild bird, a new auxiliary assay is needed for detecting a broader range of NDV isolates. The objective of the current study was to develop and evaluate a rapid, sensitive, and reliable TaqMan rRT-PCR assay that could be used for detecting the NP gene of both class I and II NDV. This assay could provide a first-line screening tool for the detection of NDV in clinical specimens.

5.2 Materials and methods

5.2.1 Probe and primers design

To design the TaqMan probe, forward primer, and reverse primer, all available

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full-length NP gene sequences of NDV isolated worldwide were obtained from GenBank database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), and the 546 sequences were aligned using the program Megalign (DNASTAR Inc., Madison, Wisconsin, USA). Design of the probe and primers targeting highly conserved regions within the NP gene were using a combination of Microsoft Excel and manual selection (Table 5.1). The oligonucleotides of the probe and primers were evaluated for their possible secondary structures including hairpin, self-dimer, and hetero-dimer using the software OligoAnalyzer (Integrated DNA Technologies, INC., Coralville, Iowa, USA). The specificity of these probe and primers was identified using BLAST search (Altschul et al., 1997). The probe used in the study was synthesized by Thermo Fisher Scientific (Waltham, Massachusetts, USA) and the primers were synthesized by Mission Biotech (Taipei, Taiwan).

5.2.2 Virus isolates and characterization

A panel composed of 23 NDV isolates (Table 5.2) selected from the sample repository held at the Animal Health Research Institute was used for an initial evaluation of the NP-gene rRT-PCR assay in this study. These lentogenic and velogenic NDV isolates consisting of the viruses isolated from poultry and wild birds between 2010 and 2019 were selected to represent the genetic diversity of contemporary NDV strains circulating in Taiwan. All of the isolates used in this study were propagated in 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 characterized as NDV using F-gene RT-PCR and sequencing. The primers to amplify the specific regions containing the F0 cleavage site of the F genes of class I or class II NDV are listed in Table 5.1.

doi:10.6342/NTU202100085

5.2.3 RNA extraction and rRT-PCR

Viral RNA was extracted from infective allantoic fluid using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. The rRT-PCR was performed in a 25 μl reaction volume with SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). For each reaction, the assay was optimized to 12.5 μl of 2X reaction mix, 4.8 μl of RNase-free water, 1 μl of primer mix (10 μM of each primer), 0.5 μl of probe (10 μM), 1 μl of SuperScript III RT/Platinum Taq mix, 0.2 μl of RNasin Ribonuclease Inhibitor (Promega, Madison, Wisconsin, USA), and 5 μl of template RNA. The reaction was carried out using a Roche LightCycler 480 system (Roche, Mannheim, Germany) with the following program parameters: reverse transcription at 50℃ for 30 min, followed by heating at 94℃ for 3 min and 45 cycles of denaturing at 94℃ for 10 sec, annealing at 56℃ for 30 sec, and extension at 72℃ for 10 sec. Fluorescence data were collected during the annealing step. Cycle threshold (Ct) values were analyzed using the second derivative maximum method in Light Cycler 480 Software 1.5.1.62. In this study, Ct values larger than 35 were considered suspect, and a Ct value of 0 indicated a negative result. The performance characteristics of the new assay were compared with the USDA-validated M-gene rRT-PCR described by Wise et al. (2004).

5.2.4 Limit of detection

Amplification efficiency and analytical sensitivity of the newly NP-gene rRT-PCR assay were evaluated through limit of detection (LOD) testing on a median embryo

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infectious dose (EID_{50}) titered virus (velogenic NDV strain, chicken/Taiwan/AHRI148/2019). Ten-fold serial dilutions were prepared from extracted viral RNA and tested in triplicate using the same extracted material. The concentration corresponding to the final dilution at which all three replicates of the 10-fold serial dilutions were considered positive was used to determine the LOD.

5.2.5 Specificity testing

For assessment of the assay's specificity, the following 35 non-NDV respiratory pathogens were included in this testing: 11 isolates of the other APMV types (one APMV-2, three APMV-4, two APMV-6, one APMV-7, one APMV-9, and three APMV-21), 20 isolates of avian influenza viruses (subtypes H1N1, H1N3, H3N8, H4N6, Mexican-like H5N2, clade 2.3.4.4 H5N2, clade 2.3.4.4 H5N6, clade 2.3.4.4 H5N8, H6N1, H7N7, H7N9, H8N4, H9N2, H10N3, H10N7, H11N2, H12N1, H12N5, H13N6, and H15N9), two isolates of infectious bronchitis viruses (IBV), and two isolates of infectious laryngotracheitis viruses (ILTV), as detailed.

5.2.6 Comparison with virus isolation in clinical specimens

The potential for the rRT-PCR assay to detect NDV RNA in clinical specimens was assessed by detecting clinical tissue samples (comprising brain, heart, lung and trachea, liver, kidney and spleen) submitted to AHRI for notifiable disease investigation. Parallelly, these samples were also tested by virus isolation with embryonated chicken eggs, as the gold standard method for comparison. Allantoic fluid from the incubated eggs was harvested either when the embryos were killed or after two 4-day blind passages. Harvested fluid was then tested for its hemagglutination activity according the OIE protocols (OIE, 2012); the presence of NDV was confirmed by the F-gene RT-PCR

doi:10.6342/NTU202100085

and sequencing as described (Table 5.1).

5.3 Results

5.3.1 Probe and primers design

After the alignment of all 546 nucleotide sequences of NDV NP gene available in GenBank, the probe and primer sites with less than three nucleotide mismatches were chosen to maximize binding potential. A 25-mer hydrolysis probe was labeled at 5’ end with 6-carboxyfluorescein (FAM) and 3’ end with fluorescence quencher (QSY), and a compatible primer set giving an amplicon size of 94 base pairs was chosen.

5.3.2 Virus isolates and characterization

A panel of 23 NDV isolates corresponding to different genotypes of the two classes (8 isolates of class I and 15 isolates of class II) was tested to evaluate and optimize our NP-gene rRT-PCR assay. The extracted RNA of each isolate grown in embryonated chicken eggs was diluted 100-1000 fold to give a concentration approximately equal to those present in clinical specimens. After detecting the panel of 23 NDV isolates, the Ct values obtained from the NP-gene rRT-PCR assay and those from the USDA-validated M-gene rRT-PCR assay were compared, as shown in Table 5.2. All of the eight NDV isolates of class I and two genotype VI isolates (pigeon paramyxovirus 1, PPMV-1) of class II could not be detected by the current USDA-validated M-gene assay. The M-gene assay only detected 56.52% (13/23) isolates of the tested panel, while the NP-gene assay successfully detected 100% of isolates (23/23).

5.3.3 Limit of detection

The analytical sensitivity of the NP-gene rRT-PCR assay was evaluated using the

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RNA extracted from infective allantoic fluid that had been quantified by EID_{50}. The genotype VII NDV isolate (chicken/Taiwan/AHRI148/2019) of class II was selected to represent the currently endemic strain in poultry farms in Taiwan and determined the initial titer of $10^{9.5}$ EID_{50}/mL. Ct values obtained from the NP-gene assay in relation to virus titers are shown in Figure 5.1. The LOD was $10^{3.5}$ EID_{50}/mL. The standard curve of Ct values against the logarithmic dilutions showed good linearity with an R^2 value of 0.99 in the range of $10^{3.5}$ – $10^{9.5}$ EID_{50}/mL, where the slope value of -3.24 corresponded to the amplification efficiency of 103.54%.

5.3.4 Specificity testing

The specificity of the NP-gene rRT-PCR assay was evaluated to confirm that this assay only reacted with the targeted sequence. All 35 non-NDV isolates derived from allantoic fluid were not detected using the NP-gene rRT-PCR assay.

5.3.5 Comparison with virus isolation in clinical specimens

The results of our NP-gene rRT-PCR assay are compared to those obtained using the current ‘gold standard’ diagnostic approach (virus isolation using embryonated chicken eggs, VI), as showed in Table 5.3. Among the 146 specimens from poultry or wild birds, 142 specimens gave concordant results by the rRT-PCR and VI (28 positive and 114 negative), while 4 were NP-gene rRT-PCR positive but VI negative. The relative diagnostic sensitivity and specificity for the NP-gene rRT-PCR assay compared to VI was 100% and 96.61% respectively.

5.4 Discussion

Newcastle disease is a notifiable disease which can have a devastating impact in

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poultry industries; therefore availability of rapid, sensitive, and specific diagnostic assays is critically important for surveillance and immediate response to outbreaks. In this study, an NP-gene rRT-PCR assay using TaqMan technology was developed for the detection of divergent NDV genotypes and the capability of the assay was compared with that of a previously published assay (Wise et al., 2004). This developed NP-gene rRT-PCR assay showed an improved diagnostic sensitivity, enabling the detection of NDV of class I and PPMV-1 of class II isolates which have been difficult to be detected using the USDA-validated M-gene assay.

Our developed NP-gene rRT-PCR assay was able to detect all NDV isolates of classes I and II testing in this study. The NP gene was targeted due to its highly genetic conservation within distinct genotypes of class I and II NDV. Previously, Tan et al. developed an real-time PCR targeting the same gene (Tan et al., 2009), but the real-time PCR uses a SYBR green-based detection with less specificity compared to TaqMan probes. To improve the NP-gene detection for NDV, we developed a new rRT-PCR assay based on TaqMan technology, and redesigned the probe and primers using all 546 available sequences in GenBank to optimize binding potential. To evaluate our NP-gene rRT-PCR assay as a diagnostic tool by direct comparison to previously established M-gene assays, the 23 NDVs used in the optimization stages of development were specifically chosen to reflect the currently known genetic diversity of the NDV population in Taiwan. The NP-gene rRT-PCR assay developed was successful in detecting this broad spectrum of NDVs (from all testing genotypes of both classes), indicating the potential of this assay as an initial screening test for the detection of any suspect NDV infection. Failure to detect all of 8 class I NDV isolates and 2 class II PPMV-1 isolates by USDA-validated rRT-PCR (Table 5.2) was due to considerable sequence variability causing 3 to 6 nucleotide mismatches in the forward primer and

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probe of the M-gene assay. These results support the findings by Kim et al., (2008) and illustrate the importance of our newly development NP-gene rRT-PCR assay in detection efficiency for highly divergent isolates of NDV.

Limit of detection of our NP-gene rRT-PCR rRT-PCR assay was comparable to those of similar assays. Limit of detection of the NP-gene assay was estimated utilizing serial dilutions of extracted RNA of a velogenic strain isolated from an ND outbreak farm in Taiwan. The capacity of the assay to detect a concentration of $10^{3.5}$ EID_{50}/mL appeared to be equivalent to that observed for the different target-gene assays reported in previous studies (Fuller et al., 2009, 2010; Hines et al., 2012; Sutton et al., 2019). The resulting correlation coefficient (R^2=0.99 based on three replicate virus dilution series) and rRT-PCR efficiency (103.54%) demonstrates near theoretical test properties over a linear dynamic range of seven 10-fold dilutions ($10^{3.5}$–$10^{9.5}$ EID_{50}/mL). An R^2 value of >0.98 and the slope corresponded to efficiency in the range 90–110% indicated an optimized rRT-PCR protocol (Broeders et al., 2014).

Our developed assay has a better diagnostic sensitivity than that of virus isolation. Virus isolation is the ‘gold standard’ diagnostic techniques for NDV but it can be time consuming to give results. On the contrary, results of NP-gene rRT-PCR can be obtained in less than three hours, and gave high relative sensitivity (100%) and specificity (96.61%) when compared with VI (Table 5.3). When employing our assay to detect the clinical specimens, our NP-gene assay represented a higher number of positive rRT-PCR results than VI, since four specimens were detected by rRT-PCR but not by VI. Based on amplicon sequencing to provide the evidence for presence of NDV RNA, it is likely that the NP-gene assay is detecting non-infectious virus genomes present in the samples. While VI remains the ‘gold standard’, the TaqMan rRT-PCR has been used extensively for the detection of NDV RNA extracted directly from clinical specimens,

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where a greater sensitivity in comparison with VI was previously observed (Fuller et al., 2010; Sutton et al., 2019). As well as for avian influenza, high-throughput, rapid, and accurate diagnostics is highly important to avoid unnecessary delay of implementing control measures in the event of an ND outbreak or ongoing surveillance.

In conclusion, the NP-gene rRT-PCR assay developed in this study demonstrated a test of rapid, sensitive, and specific screening capability and can be used as a parallel method that promotes the diagnostic efficacy for a broad range of NDV infection in clinical specimens from poultry and wild birds. Further continually evaluating the performance characteristics of the developed assay shall be guaranteed to assure the assay can detect newly emerging viruses.

doi:10.6342/NTU202100085

Table 5.1 Nucleotide sequences of the primers and probe used in the NP-gene rRT-PCR and fusion gene RT-PCR assay.

Class/Gene	Primer/probe Designation	Nucleotide sequence (5'-3')	Position[a]	Amplicon size
Class I and II / Nucleocapsid	NDV-NP gene-f1	CGTCTGTCTTCGATGAATACGAGC	126-149	91-94 bp
	NDV-NP gene-f2	GTCTTCCGTATTYGATGARTACGARC	124-149	
	NDV-NP gene-r1	TTTAGCGTGCTCCCTTTCTCCC	195-216	
	NDV-NP gene-r2	TTTKARRGTRCTICCYTTCTCTCC	194-217	
	NDV-NP gene-probe	[FAM] CTCCTYGCIGCTCAGACICGMCCYA [QSY]	152-176	
Class I / Fusion	CI-NDV-F gene-4663f	AGGAATAGTAGTCACAGGAGAC	4,663-4,684	480 bp
	CI-NDV-F gene-5142r	ATRCAGTCRATYTCYTGKGCTGT	5,120-5,142	
Class II / Fusion	CII-NDV-F gene-4698f	TATACACCTCATCYCAGACAGG	4,698-4,719	509 bp
	CII-NDV-F gene-5206r	GAATACTGTAGTCAATTCRGTTAGG	5,182-5,206	

[a]Nucleotide positions were based on the complete genome of chicken/U.S./LaSota/46 strain (GenBank accession number AF077761).

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Table 5.2 Panel of reference Newcastle disease viruses used in rRT-PCR optimization studies and their cycle threshold (Ct) values.

Isolate details	Host	Class	Sub/genotype	M-gene assay Ct	NP-gene assay Ct
Anseriformes/Taiwan/AHRI76/2013	wild bird	I	1.2	No Ct	18.52
Mule duck/Taiwan/AHRI79/2013	poultry	I	1.2	No Ct	19.94
Anseriformes/Taiwan/AHRI158/2019	wild bird	I	1.2	No Ct	19.55
Anseriformes/Taiwan/AHRI102/2015	wild bird	I	1.2	No Ct	20.41
Mule duck/Taiwan/AHRI139/2019	poultry	I	1.2	No Ct	21.56
Duck/Taiwan/AHRI149/2019	poultry	I	1.2	No Ct	20.95
Charadriiformes/Taiwan/AHRI145/2019	wild bird	I	unclassified	No Ct	22.70
Anseriformes/Taiwan/AHRI67/2011	wild bird	I	2	No Ct	17.67
Chicken/Taiwan/AHRI94/2015 (V4 strain)	poultry	II	I.1.1	20.87	20.88
Chicken/Taiwan/AHRI72/2012 (PHY-LMV42 strain)	poultry	II	I.1.2.1	18.57	19.22
Chicken/Ishii/AHRI89/2014 (Ishii strain)	poultry	II	I.1.2.2	20.52	21.04
Charadriiformes/Taiwan/AHRI84/2013	wild bird	II	I.2	16.48	19.15
Anseriformes/Taiwan/AHRI108/2016	wild bird	II	I.2	22.75	27.23
Charadriiformes/Taiwan/AHRI146/2019	wild bird	II	I.2	20.05	22.16
Chicken/Taiwan/AHRI71/2012 (LaSota strain)	poultry	II	II	18.17	23.73
Pigeon/Taiwan/AHRI43/2010	wild bird	II	VI.2.1.1.2.1	No Ct	26.81
Pigeon/Taiwan/AHRI68/2012	wild bird	II	VI.2.1.1.2.1	No Ct	19.64
Pigeon/Taiwan/AHRI114/2017	wild bird	II	VI.2.1.1.2.2	22.96	25.93
Dove/Taiwan/AHRI122/2017	wild bird	II	VI.2.1.1.2.2	20.99	24.45
Dove/Taiwan/AHRI144/2019	wild bird	II	VI.2.1.1.2.2	22.73	25.24
Chicken/Taiwan/AHRI91/2015	poultry	II	VII.1.1	18.36	17.78
Chicken/Taiwan/AHRI131/2017	poultry	II	VII.1.1	20.05	19.54
Chicken/Taiwan/AHRI148/2019	poultry	II	VII.1.1	19.82	19.97

doi:10.6342/NTU202100085

Table 5.3 Two-by-two tables comparing the relative sensitivity and specificity of NP-gene rRT-PCR assay with gold standard (virus isolation in embryonated chicken eggs).

	Virus isolation (gold standard)		
NP-gene rRT-PCR	+	−	Total
+	28	4	32
−	0	114	114
Total	28	118	146
Sensitivity (%)	100.00%		
Specificity (%)	96.61%		

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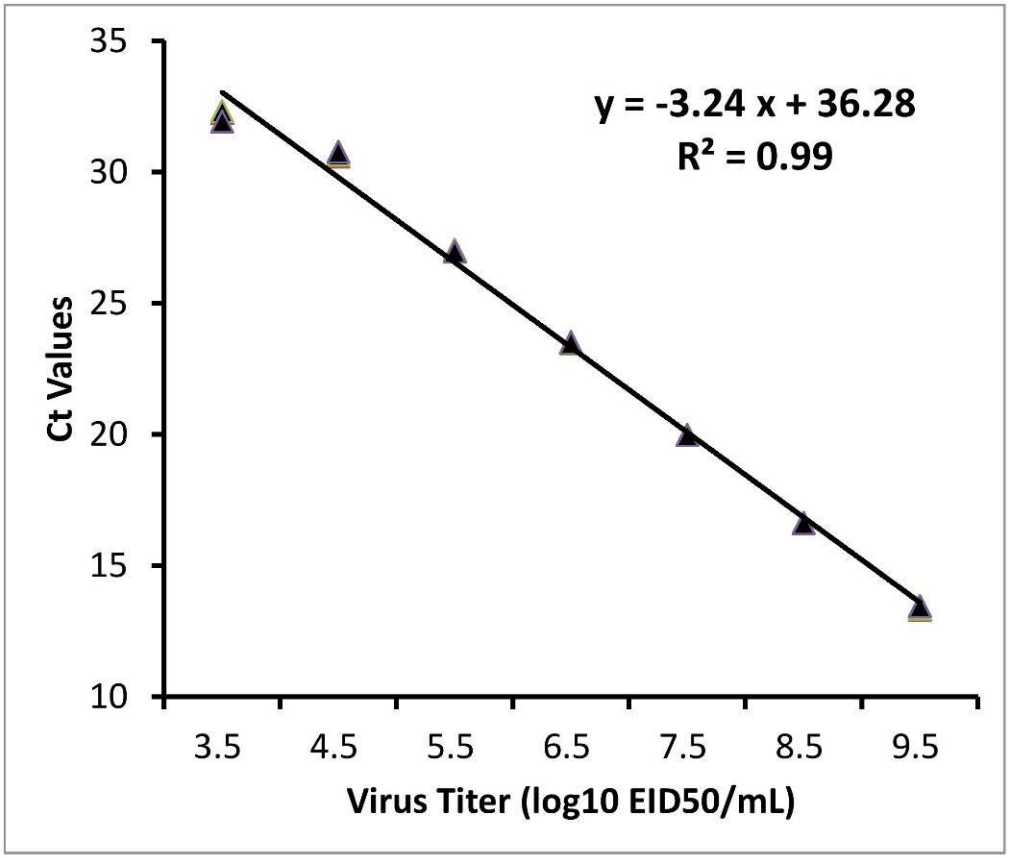

Figure 5.1 Limit of detection of the nucleocapsid protein (NP) gene rRT-PCR assays. Cycle threshold (Ct) values plotted against serial dilutions of isolates chicken/Taiwan/AHRI148/2019 (log_{10} EID_{50}/mL). The standard curve was generated using the Ct values of three replicates of 10-fold serial dilutions. The correlation coefficient (R^2) and slope of the standard curve are shown in the graph.

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Chaper 6

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

6.1 Introduction

According to the taxonomy of the order *Mononegavirales*: updated in 2018, three genera, named *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus* has recently been created within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Amarasinghe et al., 2019). Avian paramyxoviruses (APMVs), which belong to the newly assigned subfamily *Avulavirinae*, have been isolated from a wide variety of avian species across the globe. Till date, 21 species of APMVs (APMV-1 to 21) are reported and their complete genome sequences are available in GenBank. Recently, a new species of APMV-22 was proposed in previous study (Liu et al., 2019).

The APMVs contain a non-segmented, negative-sense, single-stranded RNA genome that ranges from 14,904 to 17,412 nucleotides (nt) in length (Aziz-ul-Rahman et al., 2018). For most of the APMVs, the genome encodes at least six proteins: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase (L). The order of the genes for these proteins in the genome is 3′ leader-N-P-M-F-HN-L-5′ trailer (Anderson andWang, 2011). The complete F gene sequence is considered as the main target 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-populational evolutionary distances of the complete F gene sequence, with cut-off values more than 10% to assign new genotypes (Diel et al., 2012), and this

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system and nomenclature criteria for APMV-1 were revised and updated by a global consortium in 2019 (Dimitrov et al., 2019). The classification system of APMV-1 then was applied among other species of APMVs to provide a more rational and scientific genotyping method for epidemiological studies (Yin et al., 2017; Chen et al., 2018; Tseren-Ochir et al., 2018).

APMV-1 (commonly termed Newcastle disease virus, NDV) has a wide host range and is able to infect over 240 species of birds (Kaleta andBaldauf, 1988). In contrast, the information about the host ranges and the distribution of the other APMVs species in wild birds and domestic 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 (Aziz-ul-Rahman et al., 2018).

In Taiwan, the previous studies provided the information of genetic diversity and distribution of class I and class II APMV-1 isolates in 2010-2018 (Liu et al., 2020) and identified novel APMV-22 isolates from the birds of family *Columbidae* in 2009-2017 (Liu et al., 2019). These findings suggest that wild birds and domestic poultry maintain previously unrecognized genetic diversity of APMVs, and the full extent of the distribution, evolution, and host species of APMVs has remained unexplored. In the present study, the APMVs isolates obtained from migratory birds and poultry in Taiwan were characterized by sequencing of complete fusion protein gene or full-length genome sequences and were compared to those available in GenBank. Based on the results of the phylogenetic analyses, we aim to illustrate the genetic diversity of APMVs in various avian hosts, present new epidemiological information on APMVs in Taiwan, and provide evidence for the potential intercontinental transmission of APMVs by

doi:10.6342/NTU202100085

migratory birds.

6.2 Materials and methods

6.2.1 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 the World Organisation for Animal Health procedures (OIE, 2012). The cloacal swab samples, fecal samples from 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-pathogen-free embryonated chicken eggs (Animal Drugs Inspection Branch, Animal Health Research Institute, Miaoli, Taiwan) and then incubated at 37℃ for 72 hr. The allantoic fluid from each inoculated embryo was examined for hemagglutination (HA) activity. If no HA activity was detected, a second passage was then performed. The HA-positive allantoic fluid was tested by avian influenza virus real-time reverse transcription polymerase chain reaction (Spackman et al., 2002). If tested negative, then the samples will be subjected to further analyses.

6.2.2 RNA extraction and seminested reverse transcription-polymerase chain reaction (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 seminested assay, we used the SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA)

doi:10.6342/NTU202100085

with the primers Pan-APMV-F and Pan-APMV-1R to amplify the conservative region of L gene (Table 6.1). 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℃ for 40 sec, annealing at 50℃ for 50 sec, and extension at 72℃ for 40 sec, and completed with a final extension step at 72℃ for 7 min. For the second PCR in the seminested 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 amplification and sequencing to identify viral species. The cyclic conditions for the second amplification were same as of first amplification. The PCR products were separated by electrophoresis using 2% agarose gel and were visualized with ethidium bromide stain and ultraviolet transillumination.

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

To amplify the complete coding region for the F protein gene of APMV-1, RT-PCR was conducted as previously described (Liu et al., 2020). The primers to amplify the complete coding region for the F protein gene of APMV-2, APMV-4, APMV-6, APMV-12, APMV-21, and APMV-22 isolates are listed in Table 2. The full-length genome sequences were determined with different sets of primers according to the species of APMVs, and the sequences of theses primers are available upon request. 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

doi:10.6342/NTU202100085

Technologies, Carlsbad, CA) by a commercial sequencing service (Mission Biotech, Taipei, Taiwan). Sequences were assembled and edited with the Lasergene 6.0 software package (DNASTAR, Madison, WI).

6.2.4 Phylogenetic analysis

The sequences of complete fusion protein gene of the isolated APMV were aligned with the sequences of APMV-1 to APMV-22 representative viruses retrieved from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using ClustalW in Molecular Evolutionary Genetics Analysis version 7, or MEGA 7 (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 estimated of evolutionary distance between each species of APMVs was measured by MEGA 7 using the maximum composite likelihood model (Tamura et al., 2004). 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.

6.3 Results

6.3.1 Sample collection and virus isolation

Forty-six APMVs isolates from different avian species were collected in this study (Table 2 and Table 3). The isolates were confirmed to be APMVs by isolation in specific-pathogen-free embryonated eggs with hemagglutination activity, specific L gene RT-PCR and sequencing. After sequencing of PCR products and subsequent

doi:10.6342/NTU202100085

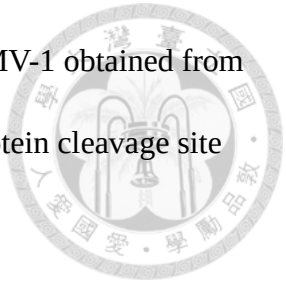
BLAST analysis it was determined that twelve isolates belonged to APMV-1, twenty isolates to APMV-4, seven isolates to APMV-6, two isolates to APMV-12, two isolates to APMV-21, and two isolates to APMV-22. Seven different species of APMVs were isolated from the samples.

6.3.2 Genetic analysis of APMV-1

In 2019, twelve viruses sequenced for this study were identified as members of APMV-1 (Table 2). Five isolates obtained from wild birds and domestic ducks were identified as genotype 1 (n = 4), or unclassified (n = 1) within class I (Fig. 1). The APMV-1 AHRI139, AHRI149, and AHRI155 isolates of genotype 1 viruses were grouped with those originated from samples collected in the United States. The APMV-1 AHRI158 isolates of genotype 1 virus was clustered with those derived from wild bird samples collected in Japan, China, Russia, Kazakhstan, Germany, and Finland. The APMV-1 AHRI145 isolates was grouped within class I viruses but formed a monophyletic lineage, with a genetic distance of 5.6% between them. The deduced amino acid motif at the fusion protein cleavage site sequence for five class I isolates were ^{112}ERQER ↓ L^{117}. Seven class II viruses of APMV-1 in this study were identified as sub-genotype I.2 (n = 4), sub-genotype VI.2.1.1.2.2 (n = 2), or sub-genotype VII.1.1 (n = 1). The four genotype I.2 isolates were obtained from waterfowl (*Anseriformes*), and shorebirds (*Charadriiformes*), and the deduced amino acid motif at the fusion protein cleavage site was ^{112}GKQGR ↓ L^{117}. 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, were obtained from birds in the family *Columbidae* (pigeon and spotted dove), and the deduced amino acid motif at the fusion protein cleavage site sequence

doi:10.6342/NTU202100085

was ^{112}(K/R)RQKR ↓ F^{117}. One isolates of sub-genotype VII.1.1 APMV-1 obtained from domestic chicken, and the deduced amino acid motif at the fusion protein cleavage site sequence was ^{112}RRKKR ↓ F^{117}.



6.3.3 Genetic analysis of APMV-2

One virus isolated from the birds of the family *Psittacidae* in this study were classified as members of APMV-2 (Fig. 2). The isolate, APMV-2/macaw/Singapore/AHRI35/2009 (APMV-2 AHRI35), obtained from samples of the blue-and-gold macaw (*Ara ararauna*) imported from Singapore by routine examination during quarantine in Taiwan in 2009. Phylogenetic analysis based on F gene sequences (Fig. 2) revealed that the APMV-2 AHRI35 isolate was clustered closely to APMV-2/Finch/N. Ireland/Bangor/73 (APMV-2 Bangor, GenBank accession number HM159995) within APMV-2 group, and demonstrated that the APMV-2 strains represent two genotypes, including the prototype strain Yucaipa and strains England and Kenya as genotype I, and strain Bangor and the APMV-2 AHRI35 isolate as genotype II. The complete genome sequences of the APMV-2 AHRI35 isolate, determined by an amplification strategy combined APMV-2-specific primer set and rapid amplification of cDNA ends method (RACE), was 15,024 nt, which was identical to that of the APMV-2 Bangor strain. The genome of APMV-2 AHRI35 isolate showed 91.5% identity with that of APMV-2 Bangor but there were six single-nucleotide insertion-deletion combination in the genome positions of 8487-9200, 9655-9892, 11573-11616, 12634-12710, 13519-13547, and 14011-14105 nt. The deduced amino acid motif at the fusion protein cleavage site of APMV-2 AHRI35 was ^{102}LPSSR ↓ F^{107}, similar with APMV-2 Bangor as ^{102}LPSAR ↓ F^{107}. The amino acid sequence identities of each

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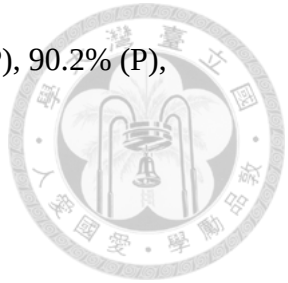
APMV-2 AHRI35 gene to those of APMV-2 Bangor were 98.2% (NP), 90.2% (P), 97.0% (M), 95.6% (F), 94.7% (HN), and 81.3% (L), respectively.



6.3.4 Genetic analysis of APMV-4

Twenty viruses obtained in this study were classified as members of APMV-4, the most prevalent species of APMVs besides APMV-1. All of these APMV-4 isolates were isolated from wild birds of the order *Anseriformes* in Taiwan between 2009 and 2020. Phylogenetic analysis demonstrated that there were at least three circulating groups of APMV-4 in Taiwan (Fig. 3). Nine of these viruses studies here grouped with the isolates from ducks sampled in the Asian countries of China, Japan, and South Korea and clustered within sub-genotype Ia. Nine viruses were grouped with the isolates from domestic and wild birds sampled in China, Russia, South Korea, Italy, Ukraine, and South Africa which located in three different continents, Asia, Europe, and Africa and clustered within sub-genotype Ib. The remaining two viruses were grouped with the isolates from waterfowls sampled in Alaska, Minnesota, and Texas of United States within genotype II. The deduced amino acid motif at the fusion protein cleavage site of the 20 APMV-4 isolates was ^{116}DIQPR ↓ F^{121}, except those of two isolate, APMV-4/Anseriformes/Taiwan/AHRI60/2011 and APMV-4/Anseriformes/Taiwan/AHRI66/2011, obtained in 2011 were ^{116}DIRPR ↓ F^{121} and ^{116}DVQPR ↓ F^{121}, respectively.

6.3.5 Genetic analysis of APMV-6

Seven viruses isolated from wild birds of the order *Anseriformes* in this study were identified as members of APMV-6. Phylogenetic analysis revealed that two groups of

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APMV-6 circulating in Taiwan (Fig. 4). Five isolates here were fell into genotype I of APMV-6 and closely related to viruses obtained in China, Kazakhstan, Russia, South Korea, Belgium, and Italy during 2003-2015. The remaining two isolates were fell into genotype II of APMV-6 and closely related to viruses obtained in Japan, Russia, South Korea, 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}.

6.3.6 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), sequenced in this study were most related with the prototype virus of 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 complete genome sequences of the two viruses, determined by an amplification strategy combined APMV-12-specific primer set, primer walking, and RACE, were both 15,228 nucleotides (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 sequence identity, and showed 64.5% and 64.4% identity with that of APMV-12 3920-1 strain. The deduced amino acid motif at the fusion protein cleavage site of APMV-12 AHRI101 isolate was ^{103}TAQPR ↓ L^{108}, and APMV-12 AHRI143 isolate was ^{103}VTQPK ↓ L^{108}.

doi:10.6342/NTU202100085

6.3.7 Genetic analysis of APMV-21

Two APMV isolates, APMV-21/Anseriformes/Taiwan/AHRI83/2013 (APMV21 AHRI83) and APMV-21/Anseriformes/Taiwan/AHRI141/2019 (APMV21 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 complete genome sequences of the two viruses, determined by an amplification strategy combined APMV-21-specific primer set and RACE, were both 15,408 nt in length equal to that of APMV-21 Cheonsu1510 strain. The genome of APMV-21 AHRI83 and AHRI141 shared 86.4% nt sequence identity, and showed 98.2% and 86.2% identity 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. The deduced amino acid motif at the fusion protein cleavage site of APMV-21 AHRI83 and AHRI141 isolates was ^{107}DREGR ↓ L^{112}, the same as APMV-21 Cheonsu1510.

6.3.8 Genetic analysis of putative APMV-22

Two viruses isolated from the birds of the family *Columbidae* in this study were classified as members of putative APMV-22 (Fig. 2). The isolate, APMV-22/dove/Taiwan/AHRI140/2019 (APMV-22 AHRI140), obtained from a resident dove grouped with the putative APMV-22 isolates described in our previous study conducted between 2009 and 2017 (Liu et al., 2019), and shared 98.8-99.5% nt sequence identity of F gene with them. Another isolate,

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APMV/pigeon/Netherlands/AHRI97/2015 (APMV-22 AHRI97), obtained from samples of pigeons imported from the Netherlands during routine quarantine in Taiwan. Phylogenetic analysis based on F gene sequences (Fig. 2) revealed that the APMV-22 AHRI140 isolate was clustered closely to reference strain APMV-22/dove/Taiwan/AHRI33/2009 (APMV-22 AHRI33), and the APMV-22 AHRI97 isolate imported from the Netherlands was again clustered close to but more distantly related to the reference strain AHRI33. The complete genome sequences of the APMV-22 AHRI97 isolate, determined by an amplification strategy combined APMV-22-specific primer set and RACE, was 16,908 nt, which was short than that of the APMV-22 AHRI33 strain due to a six-nucleotide deletion (position 16441–16446) in the trailer at the 5' end. The genome of APMV-22 AHRI97 isolate showed 80.2% identity with that of APMV-22 AHRI33. The deduced amino acid motif at the fusion protein cleavage site of APMV-22 AHRI97 and AHRI140 isolates was ^{103}TQQER↓L^{108}, the same as APMV-22 AHRI33.

6.4 Discussion

For a long time, the distribution, circulation and evolution of different APMVs with the exception of APMV-1 in Taiwan have been largely unknown. Here we present the results of the first molecular investigation of APMVs circulation in Taiwan. Forty-six APMV isolates obtained from wild birds and domestic poultry characterized genetically. Nineteen sequences of full-length genome and twenty-seven sequences of complete fusion protein gene were determined in this study. Viruses from 7 of the 21 known APMVs species were isolated over a ten year period. Our genetic analyses supported the designation of the novel variant APMVs and illustrated the genetic diversity of APMV in Taiwan. Phylogenetic analysis of the isolates demonstrated that

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several species of APMVs were represented by more than one genotype; some sub/genotypes were clearly distinct from previously characterized strains. Moreover, our results revealed that both APMV-1 and APMV-4 of North America-origin strains have been introduced into the wild bird populations of Taiwan.

Here, eleven APMV-1 isolates related to those of described in our previous study conducted in 2010-2018 (Liu et al., 2020) were constantly circulated and subsequently evolved in wild birds and domestic poultry in Taiwan in 2019, and one unique isolate obtained from the *Charadriiformes* birds should be a unclassified virus within class I. The previous study of the genetic diversity of APMV-1 in Taiwan indicated the presence of five different sub-genotypes of APMV-1 circulating in multiple avian host species. In this study, the class I sub-genotype 1.2 viruses closely related to those isolated from wild birds in North America, the class II sub-genotype I.2 viruses clustered with those isolated from domestic ducks and mallards in the Eurasian countries, the sub-genotype VI.2.1.1.2.2 viruses the predominant PPMV-1 strains, and the sub-genotype VII.1.1 viruses caused the epizootic of Newcastle disease in Taiwan were successively obtained and identified in 2019. Moreover, one unique isolate, APMV-1 AHRI145, was obtained from the samples of migratory birds. The genome length of APMV-1 AHRI145 isolate was 15,198 nt, a characteristic of class I viruses with a 12 nt insertion in the phosphoprotein gene (Czeglédi et al., 2006). Based on the phylogentic analysis, the APMV-1 AHRI145 isolate, lacked branch support (44%), and sufficient number of independent isolates, was a unclassified virus within class I.

In this study, the APMV-2 AHRI35 isolate closely related to the APMV-2 Bangor strain was genetic characterized, and these two viruses represented the genotype 2 of APMV-2. APMV-2 was first isolated from chickens in 1956, and then the viruses were reported worldwide from wild birds, captive caged birds, and domestic poultry. Till now,

doi:10.6342/NTU202100085

there was no report for the isolation of APMV-2 viruses from avian species in Taiwan. In 2009, one related virus, APMV-2 AHRI35, was obtained from the sample of psittacine birds imported from Singapore by routine examination during quarantine. Phylogenetic analysis of the complete fusion gene sequences suggests that the APMV-2 AHRI35 and APMV-2 Bangor strain cluster together on a branch that was distinct from those of viruses clustered with the APMV-2 prototype strain Yucaipa. Based on the mean inter-populational evolutionary distances of the full F protein sequences, the APMV-2 isolated were divided into two genotypes (I and II, with the distance of 24.4%). To compare the sequence of APMV-2 Bangor strain with those of all available APMV-2 sequences in GenBank and the AHRI35 in this study, four untranslated amino acid codons containing ambiguous nucleotides were presented in the positions of 8, 147, 1175, 1176 aa of L gene. Moreover, six single-nucleotide insertion-deletion combinations presented in the genome sequences of the APMV-2 Bangor strain caused a frame-shift mutation and made a unreasonable misalignment in the positions of 160-397, 549-628, 1189-1202, 1543-1567, 1837-1846, and 2002-2030 aa of L gene. Here, the full-length genome sequence of the APMV-2 AHRI35 was reported and could be the representative strain of APMV-2 genotype II for the genetic analysis of APMVs in further studies.

The results of phylogenetic analysis indicate the existence of a high APMV-4 genetic diversity among wild birds in Taiwan, and provide the evidence for the intercontinental dispersal of APMV-4 with sequences from Taiwan nested within North America genotype II (Fig. 3.), which identical to results for APMV-1. Besides APMV-1, APMV-4 was the most prevalent species of APMVs in Taiwan, and all of these APMV-4 isolates were isolated from waterfowl. APMV-4 recently was suggested to classify into three genotypes (I to III), that were almost exclusively monophyletic by continent of

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sample origin (Yin et al., 2017). The existing information and result demonstrate that at least one genotype and two sub-genotypes of APMV-4 isolates circulating in Taiwan: sub-genotype Ia comprised of sequences for viruses from East Asian countries, sub-genotype Ib comprised of sequences for viruses from Eurasian and African countries, and genotype II comprised of sequences for viruses from the North America. Related APMV-4 isolates were obtained from wild birds in East Asia and North America, supporting possible intercontinental APMVs spread by migratory birds. Wild birds shared the same breeding areas among the East Asian–Australian Flyway and the Pacific Americas Flyway providing a possible mechanism through which APMV-4 may be exchanged between these continents. Collectively, these findings suggest that migratory birds may play a potential role in the global spread of kinds of avian infectious agents.

Currently, two recognized genotypes of APMV-6, genotype I and II were classified based on the phylogenetic analysis of the all available complete F gene sequences in GenBank (Chen et al., 2018), and here isolates of both two genotypes were obtained from the samples of waterfowl in Taiwan during 2010-2019. In Taiwan, the APMV-6 genotype I viruses have been previously isolated from domestic ducks in 1998 and the complete genome sequence were determined (Chang et al., 2001). In this study, five related genotype I APMV-6 isolates were obtained, and additional two isolates of genotype II closely related to viruses circulating in Eurasian regions. All the isolates of APMV-6 had one or two basic residues at the cleavage site and a leucine residue at the first position of F1 subunit which were typically found in lentogenic APMV-1 strains (Miller and Koch, 2013).

Two unique isolate, APMV-12 AHRI101 and AHRI143, were more closely related to APMV-12 3920-1, the prototype virus of the species of *Avian Orthoavulavirus 12*,

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based on estimates of the nucleotide sequence identities of the genome between each prototype of APMVs, with a sequence identity of 64.4% and 64.5%, respectively, but showed limited evidence to be a new species in the genus *Orthoavulavirus*. Currently, no criteria are proposed for the classification of APMVs. 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 so obvious, only above the value of 37.5% between APMV-1 and APMV-16. However, 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 value of 43.3% between the subgroup within APMV-3. There is no unified criterion for differentiating APMVs based on evolutionary divergence or the nucleotide sequence identities of the full genome, and it is urgent to develop new guidelines of classification for APMVs, and need to integrate genomic analysis, serological assays, and evaluation of ecosystem the virus originates from.

In this study, we reported the second and third full-length genome sequences of APMV-21 isolates, APMV-21 AHRI83 and AHRI141. The first full-length genome of the prototype strain, APMV-21 Cheonsu1510, has been published in 2018 (Jeong et al., 2018), and no other APMV-21 strains have been reported till date. 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% identity with Cheonsu1510 strain and AHRI83 isolate, respectively. The deep divergence and the six-year gap between the two Taiwanese isolates demonstrated that the continuous

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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. HN C-terminal length variations have previously been seen among the APMV-9 viruses and been used to categorize them into different lineages (Dundon et al., 2010). Whether the C-terminal extensions could be a feature of different lineages of APMV-21 or be responsible for a change in tissue tropism and or virulence need further investigation to a better understanding.

The viruses of novel APMV-22 species described in our previous study (Liu et al., 2019) were constantly circulated and subsequently evolved in *Columbidae* birds in Taiwan, and a related European isolate obtained from imported pigeons demonstrated the extensive distribution of the species of viruses. Since the prototype strain of APMV-22 AHRI33 were first isolated in 2009, fourteen viruses in 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 a period of one decade indicated that the species of viruses had been endemic in Taiwan but not reported elsewhere in other regions of the world. In 2015, one related virus of APMV-22 AHRI97 was obtained from the sample of pigeons imported from the Netherlands by routine examination during quarantine. Phylogenetic analysis of the complete fusion gene sequences suggests that the prototype APMV-22 AHRI33 strain and AHRI97 isolate cluster together on a branch that was distinct from those of the

doi:10.6342/NTU202100085

other species of APMVs. Both APMV-22 AHRI33 and AHRI97 have identical genome organizations and 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 AHRI97 isolate was closest to APMV-22, with calculated distance value of 0.137, which is under value considered to differentiate other species (observed minimum was 0.375 between APMV-1 and APMV-16). However, the AHRI97 isolate was 11.7% distant from the AHRI33 strain, which meet the classification criteria for consideration as new genotypes. This indicated that APMV-22 strains represent two genotypes and we propose that the prototype strain AHRI33 represent genotype I while strain AHRI97 represents genotype II. The isolation of APMV-22 AHRI97 from imported birds in quarantine revealed that the viruses were not exclusive in Taiwan, and additional sequencing of historical and prospective APMV isolates will provide a more detailed characterization of the viral distribution and genetic diversity.

In summary, a broad range of circulating APMVs with a very high degree of genetic diversity was identified in Taiwan. Our findings provided more evident 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 F gene sequences, three novel sub/genotypes within APMV-2, APMV-12, and APMV-22 were identified.

doi:10.6342/NTU202100085

Table 6.1 List of RT-PCR primers.

Species	Designation	Primer sequence (5'-3')	Gene	Position	Fragment size	Accession no. of reference sequence
APMV Universal	Pan-APMV-F	TGACHACWGAYYTIIARAARTAYTG	Large polymerse	10,293-10,317	355 bp	AF077761
	Pan-APMV-1R	GCIATIRCYTGRTTRTCICCYTG	Large polymerse	10,625-10,647		
	Pan-APMV-2Ra	CCATAGTTTYTGTTGCAGRCCTTC	Large polymerse	10,532-10,555	263 bp	
	Pan-APMV-2Rb	CCACAKYTTYTGRCAYARICCYTC	Large polymerse	10,532-10,555		
APMV-2	APMV-2-1Fa	CTCCAGACTAAGTGGGTGG	Fusion	4,310-4,328	978 bp	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 bp	
	APMV-2-2Ra	TGCTGCCAGGTTCTCCC	Fusion	6,107-6,124		
	APMV-2-2Rb	WTYIGTGAGGTTCTCTCTKG	Fusion	6,104-6,124		

doi:10.6342/NTU202100085

Table 6.1 (continued)

Species	Designation	Primer sequence (5'-3')	Gene	Position	Fragment size	Accession no. of reference sequence
APMV-4	APMV-4-1F	GGARTTGATTGGGTGTCTAAAC	Fusion	4,331-4,352,	988 bp	FJ177514
	APMV-4-1R	CRACCCTCGTATTCTGGAC	Fusion	5,300-5,318		
	APMV-4-2F	GATCTGTCACAAGTCARTTGG	Fusion	5,172-5,192	1,000 bp	
	APMV-4-2R	CCAAYCGGCCTTGTGACAC	Fusion	6,153-6,171		
APMV-6	APMV-6-GI-1F	CTTCCTARCTRTTCCTYCCTTAG	Fusion	4,478-4,500	1,036 bp	EU622637
	APMV-6-GI-1R	CAAYTCTGTCAGTCGCAACC	Fusion	5,493-5,512		
	APMV-6-GI-2F	CTTAATCAATGGCAGAATCATTC	Fusion	5,404-5,426	1,042 bp	
	APMV-6-GI-2R	GTTGGGCTGTTAGATTATTCTGC	Fusion	6,423-6,445		
	APMV-6-GII-1F	GCCAYAGACCACAAAAGAGC	Fusion	4,548-4,567	1,005 bp	GQ406232
	APMV-6-GII-1R	CTTTACCCTCTCCAGCAG	Fusion	5,535-5,552		
	APMV-6-GII-2F	CAGATAATGGTCATTCAAGTCTC	Fusion	5,444-5,466	944 bp	
	APMV-6-GII-2R	GCAATTTACGGCTAATCAACTG	Fusion	6,366-6,387		

doi:10.6342/NTU202100085

Table 6.1 (continued)

Species	Designation	Primer sequence (5'-3')	Gene	Position	Fragment size	Accession no. of reference sequence
APMV-12	APMV-12-1Fa	GGTKGAWCYTGAACCAATACGG	Fusion	4,552-4,573	963 bp	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 bp	
	APMV-12-2R	GRGACYSYCYCSTTCTGCC	Fusion	6,415-6,433		
APMV-21	APMV-21-1F	TGAGAGYGATACGGGTAGG	Fusion	4,776-4,794	934 bp	MF594598
	APMV-21-1R	AGAACTCCCTTGAGATTCCC	Fusion	5,690-5,709		
	APMV-21-2F	TCTWGGAGCAGACAACAGC	Fusion	5,569-5,587	1,026 bp	
	APMV-21-2R	GCRCACCACCTTCCTACC	Fusion	6,577-6,594		
APMV-22	APMV-22-1Fa	GTACAAGAGTCAAAGTAGAAACAG	Fusion	5,127-5,150	914 bp	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 bp	
	APMV-22-2Ra	ATGAGTCAATGTGCAATGAGG	Fusion	6,839-6,859		
	APMV-22-2Rb	ATGATTCAGTGTGTGATAAGG	Fusion	6,839-6,859		

doi:10.6342/NTU202100085

Table 6.2 Details of APMV-1 isolates since 2019.

Isolate	Origin	Class	Sub/genotype	Cleavage site of fusion protein	Sequence Target
APMV-1/mule duck/Taiwan/AHRI139/2019	Domestic	I	1.2	^{112}ERQER ↓ L^{117}	Fusion
APMV-1/mule duck/Taiwan/AHRI149/2019	Domestic	I	1.2	^{112}ERQER ↓ L^{117}	Fusion
APMV-1/Anseriformes/Taiwan/AHRI155/2019	Migratory	I	1.2	^{112}ERQER ↓ L^{117}	Genome
APMV-1/Anseriformes/Taiwan/AHRI158/2019	Migratory	I	1.2	^{112}ERQER ↓ L^{117}	Fusion
APMV-1/Charadriiformes/Taiwan/AHRI145/2019	Migratory	I	Unclassfied	^{112}ERQER ↓ L^{117}	Genome
APMV-1/Anseriformes/Taiwan/AHRI142/2019	Migratory	II	I.2	^{112}GKQGR ↓ L^{117}	Fusion
APMV-1/Charadriiformes/Taiwan/AHRI146/2019	Migratory	II	I.2	^{112}GKQGR ↓ L^{117}	Fusion
APMV-1/Anseriformes/Taiwan/AHRI151/2019	Migratory	II	I.2	^{112}GKQGR ↓ L^{117}	Fusion
APMV-1/Anseriformes/Taiwan/AHRI156/2019	Migratory	II	I.2	^{112}GKQGR ↓ L^{117}	Fusion
APMV-1/dove/Taiwan/AHRI144/2019	Resident	II	VI.2.1.1.2.2	^{112}RRQKR ↓ F^{117}	Fusion
APMV-1/pigeon/Taiwan/AHRI147/2019	Resident	II	VI.2.1.1.2.2	^{112}KRQKR ↓ F^{117}	Fusion
APMV-1/chicken/Taiwan/AHRI148/2019	Domestic	II	VII.1.1	^{112}RRKKR ↓ F^{117}	Genome

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Table 6.3 Details of non-APMV-1 isolates during 2009-2020.

Isolate	Origin	Cleavage site of fusion protein	Sequence Target
APMV-2			
APMV-2/macaw/Singapore/AHRI35/2009	Quarantine	^{102}LPSSR ↓ F^{107}	Genome
APMV-4/			
APMV-4/Anseriformes/Taiwan/AHRI36/2009	Migratory	^{116}DIQPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI37/2009	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI38/2009	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI40/2009	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI41/2009	Migratory	^{116}DIQPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI57/2010	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI60/2011	Migratory	^{116}DI**R**PR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI62/2011	Migratory	^{116}DIQPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI66/2011	Migratory	^{116}D**V**QPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI78/2013	Migratory	^{116}DIQPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI88/2014	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI93/2015	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI100/2015	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI110/2016	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI135/2018	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI150/2019	Migratory	^{116}DIQPR ↓ F^{121}	Genome
APMV-4/Anseriformes/Taiwan/AHRI152/2019	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI153/2019	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI157/2019	Migratory	^{116}DIQPR ↓ F^{121}	Fusion
APMV-4/Anseriformes/Taiwan/AHRI159/2020	Migratory	^{116}DIQPR ↓ F^{121}	Genome

doi:10.6342/NTU202100085

Table 6.3 (continued)

Isolate	Origin	Cleavage site of fusion protein	Sequence Target
APMV-6			
APMV-6/Anseriformes/Taiwan/AHRI45/2011	Migratory	^{104}IREPR ↓ L^{109}	Fusion
APMV-6/Anseriformes/Taiwan/AHRI56/2014	Migratory	^{114}APEPR ↓ L^{119}	Fusion
APMV-6/Anseriformes/Taiwan/AHRI65/2011	Migratory	^{114}APEPR ↓ L^{119}	Genome
APMV-6/Anseriformes/Taiwan/AHRI90/2014	Migratory	^{114}APEPR ↓ L^{119}	Genome
APMV-6/Anseriformes/Taiwan/AHRI99/2015	Migratory	^{114}APEPR ↓ L^{119}	Fusion
APMV-6/Anseriformes/Taiwan/AHRI109/2016	Migratory	^{104}IREPR ↓ L^{109}	Genome
APMV-6/Anseriformes/Taiwan/AHRI154/2019	Migratory	^{114}APEPR ↓ L^{119}	Fusion
APMV-12			
APMV/Anseriformes/Taiwan/AHRI101/2015	Migratory	^{103}TAQPR ↓ L^{108}	Genome
APMV/Anseriformes/Taiwan/AHRI143/2019	Migratory	^{103}VTQPK ↓ L^{108}	Genome
APMV-21			
APMV-21/Anseriformes/Taiwan/AHRI83/2013	Migratory	^{107}DREGR ↓ L^{112}	Genome
APMV-21/Anseriformes/Taiwan/AHRI141/2019	Migratory	^{107}DREGR ↓ L^{112}	Genome
APMV-22 (putative)			
APMV-22/pigeon/Netherlands/AHRI97/2015	Quarantine	^{103}TQQER ↓ L^{108}	Genome
APMV-22/dove/Taiwan/AHRI140/2019	Resident	^{103}TQQER ↓ L^{108}	Fusion

doi:10.6342/NTU202100085

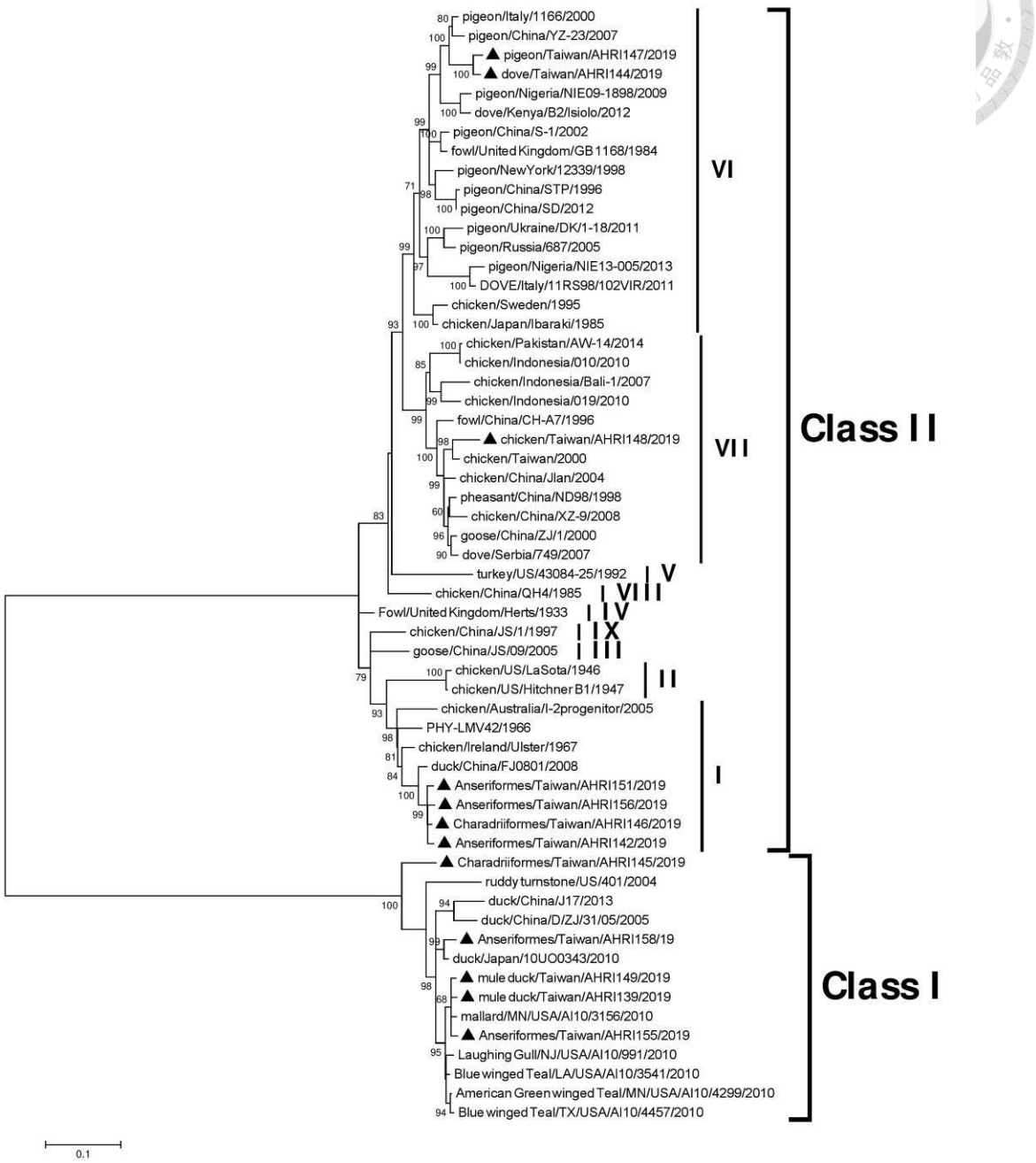

Fig. 6.1 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of the avian paramyxovirus 1 (APMV-1). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. A discrete Gamma distribution was used to model evolutionary rate differences among

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sites (4 categories (+*G*, parameter = 0.6710)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 23.84% sites). The analysis involved 58 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,657 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 isolate of APMVs obtained from birds in Taiwan in this study.

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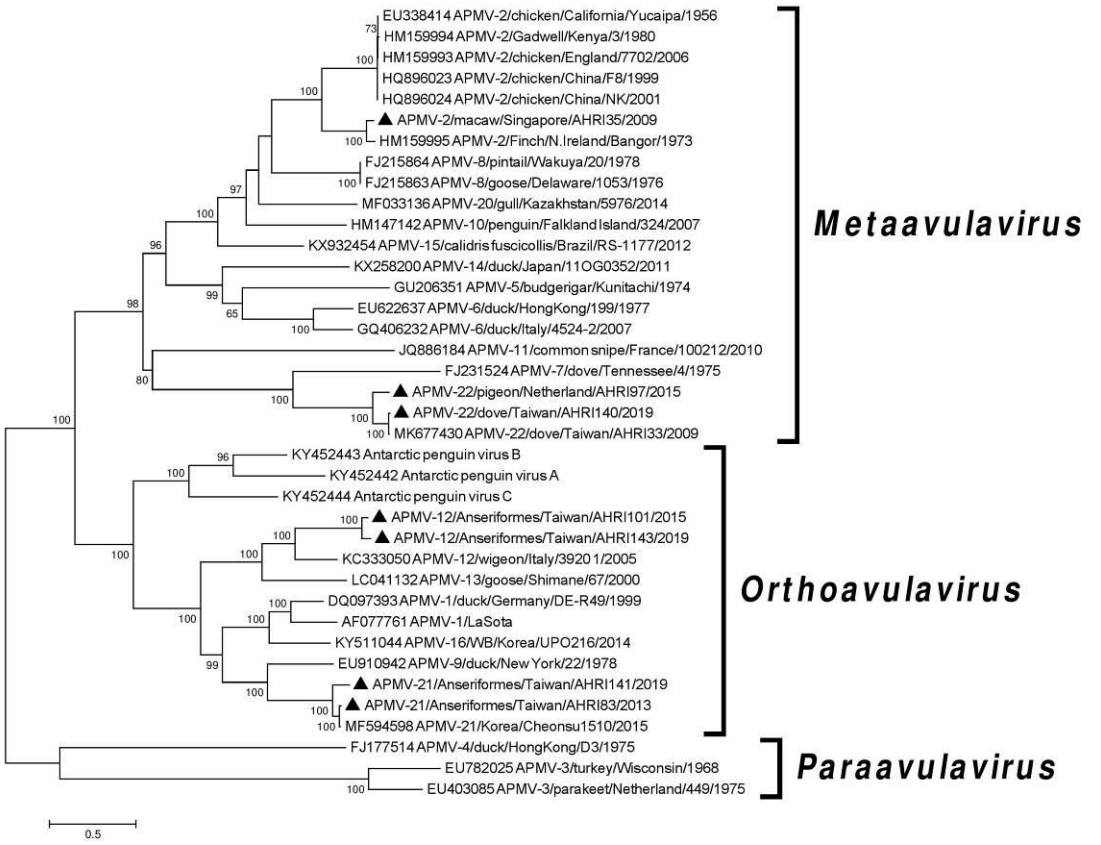

Fig. 6.2 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of the avian paramyxoviruses (APMVs). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 1.8009)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 5.61% sites). The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,516 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 isolate of APMVs obtained from birds in Taiwan in this study.

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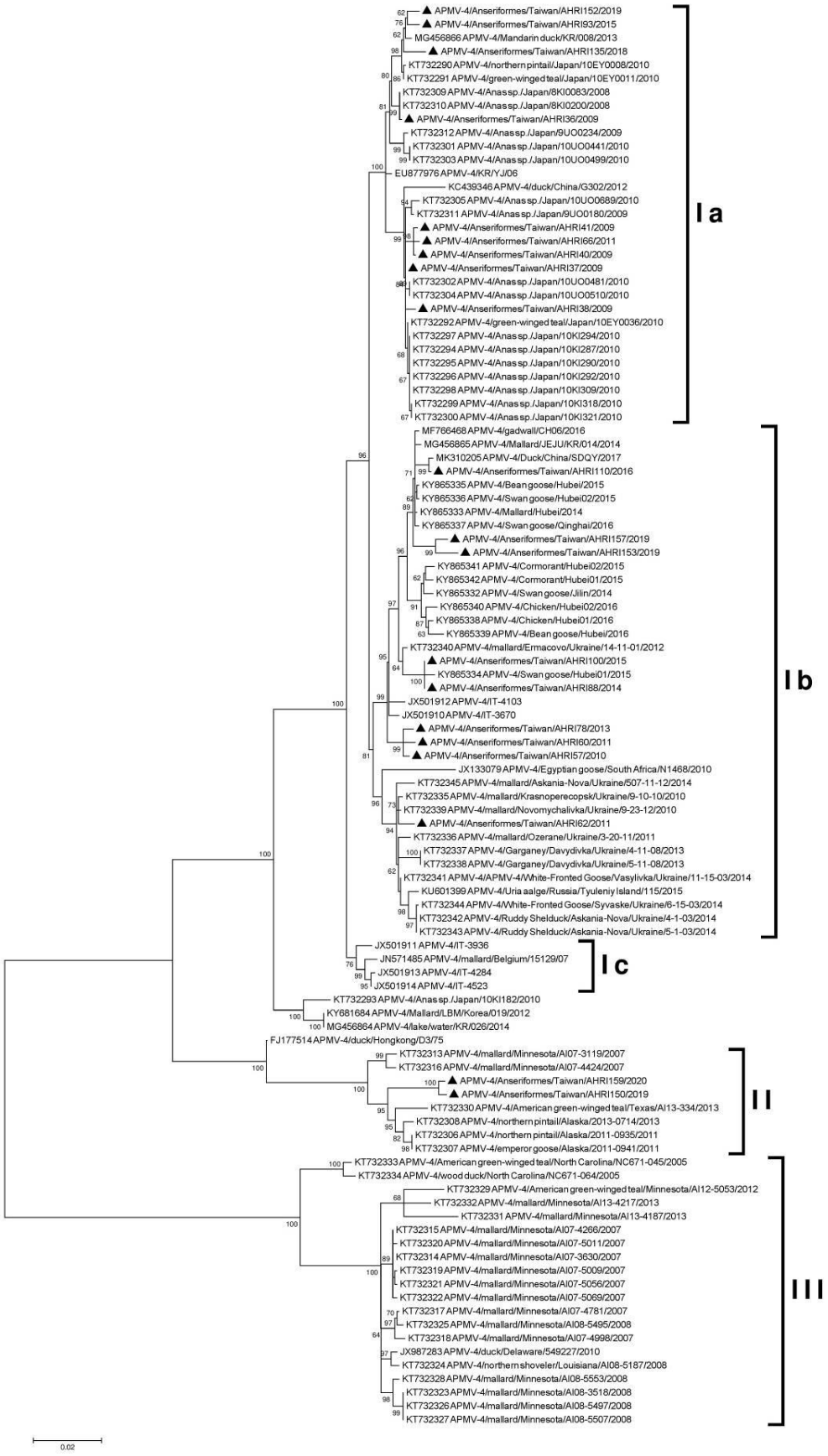

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doi:10.6342/NTU202100085

Fig. 6.3 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 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. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.4385)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 33.27% sites). The analysis involved 105 nucleotide sequences. 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 isolate of APMV-4 obtained from birds in Taiwan in this study.

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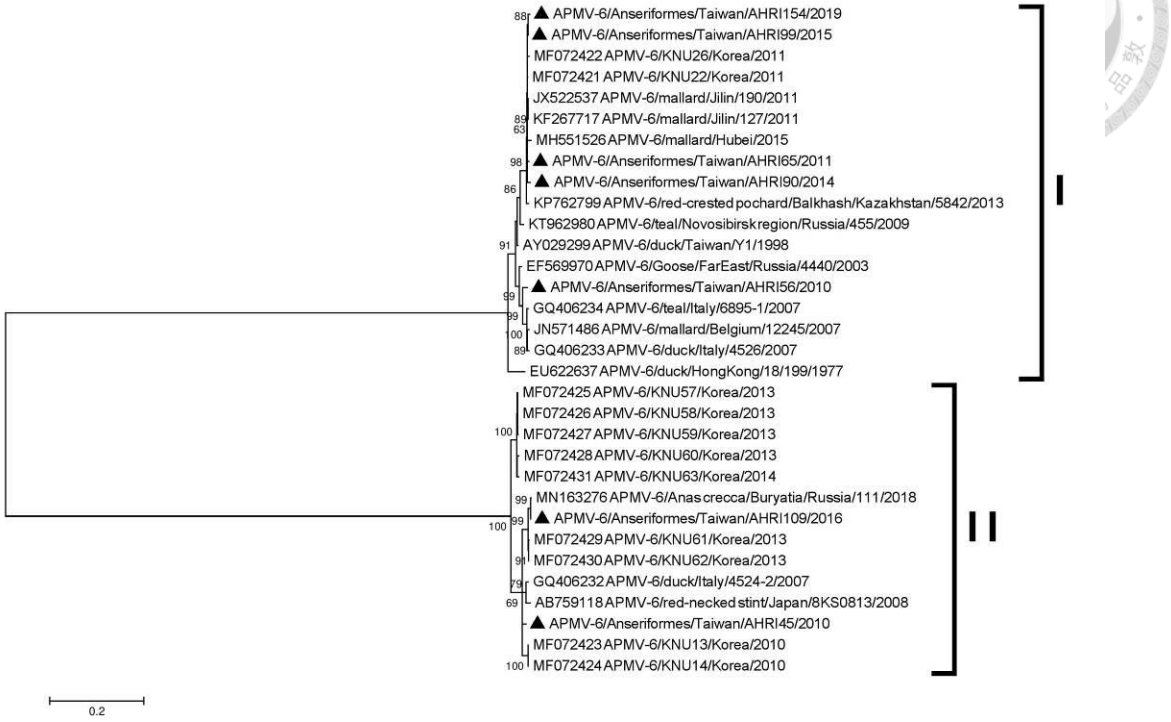

Fig. 6.4 Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 6. The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution and invariant sites. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.5624)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 32.39% sites). The analysis involved 32 nucleotide sequences. 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 isolate of APMV-6 obtained from birds in Taiwan in this study.

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Chapter 7

Conclusion

For a long time, the distribution, circulation and evolution of APMVs with the exception of APMV-1 in Taiwan have been largely unknown. Here we present the results of the first molecular investigation of APMVs circulation in Taiwan. One hundred APMV isolates obtained from migratory, resident, imported, and domestic birds were characterized genetically. Forty-one sequences of full-length genome and fifty-nine sequences of complete fusion protein gene were determined in this study. Viruses from 7 of the 22 known APMVs species were isolated over a ten year period. Phylogenetic analysis of the isolates demonstrated the presence of several APMV species that were represented by more than one genotype, and some sub/genotypes were clearly distinct from previously characterized strains. Moreover, our results revealed that both APMV-1 and APMV-4 of North America-origin strains have been introduced into the wild bird populations of Taiwan. In addition, an NP-gene rRT-PCR assay using TaqMan technology was developed for the detection of divergent NDV genotypes.

The reported large scale surveillance resulted in isolation of the APMVs belonging to seven species APMV-1, 2, 4, 6, 12, 21, and 22 will enhance the understanding of the APMVs circulation in Taiwan. APMV-1 was the most prevalent species of APMVs in Taiwan and constantly obtained from samples of wild birds and domestic poultry. In 2009, the APMV-2 AHRI35 isolate was obtained from the sample of psittacine birds imported from Singapore by routine examination during quarantine. Till now, there were no complete and accurate genome sequence of APMV-2 genotype II in GenBank and the full-length genome sequence of APMV-2 AHRI35 could be the representative

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strain for the genetic analysis of APMVs in further studies. This study confirmed the primary role of wild birds as a reservoir of APMV-4, and 6 in nature and demonstrated that the related viruses circulated in the wild birds of Asia and Europe. Two unique isolates were closest to APMV-12, with genome sequence identity of 38.8% and 38.9%, respectively. There is no unified criterion for differentiating APMVs based on evolutionary divergence or the nucleotide sequence identities of the full genome, and it is urgent to develop new guidelines of classification for APMVs, and to integrate genomic analysis, serological assays, and evaluation of ecosystem the virus originates from. In this study, we reported the second and third full-length genome sequences of APMV-21 isolates, and 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. Based on sequence comparison of the RdRps of APMVs, phylogenetic tree topology and the branch length, the AHRI33-like isolates met the criteria for designation as distinct species, APMV-22. In addition, the isolation of APMV-22 AHRI97 from imported birds in quarantine revealed that the viruses were not exclusive in Taiwan, and additional sequencing of historical and prospective APMV isolates will provide a more detailed characterization of the viral distribution and genetic diversity.

The large diversity of genetic, temporal, and host distribution of genotypes containing virulent and non-virulent strains of APMV-1 in Taiwan suggests that many factors may contribute to the maintenance, evolution, and dispersal of this viral agent. The results demonstrated the presence of at least five different genotypes of APMV-1 circulating in multiple avian host species. The epidemiological and phylogenetic data of this study supported the designation of a new genotype, genotype 2, in class I as per the nomenclature criteria for APMV-1 proposed in 2019. Moreover, the presence of

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identical genotypes/ sub-genotypes in wild birds and poultry supports transmission across the wild bird-poultry interface (e.g., class I genotype 1 and class II genotypes I). The closely phylogenetic relationship demonstrated an epidemiological link of PPMV-1 between Taiwan and neighboring countries and highlighted the importance of constant surveillance for pathogenic microorganisms carried by pigeons. Sporadic outbreaks of ND in Taiwan from year to year were majorly due to the infection of a virulent strain of sub-genotypes VII.1.1 (former VIIe), which was only restrictively obtained from land-based poultry and had not been detected in wild birds and waterfowl. No evidence suggested new introduction of emerging sub/genotypes NDV from abroad.

The result provided the evidence to suggest the intercontinental dispersal of APMV-1 and APMV-4 from North America to East Asia. Since the first isolation of North American strains of APMVs in 2014, this group of viruses have been constantly obtained from wild birds every year in Taiwan. Related APMVs isolates were obtained from wild birds in East Asia and North America, supporting possible intercontinental APMVs spread by migratory birds. Wild birds shared the same breeding areas among the East Asian–Australian Flyway and the Pacific Americas Flyway providing a possible mechanism through which APMVs may be exchanged between these continents. Collectively, these findings suggest that migratory birds may play a potential role in the global spread of kinds of avian infectious agents.

We developed NP-gene rRT-PCR assay which was able to detect all testing NDV isolates of classes I and II and has a better diagnostic sensitivity than that of virus isolation. In this study, the USDA M-gene rRT-PCR failed to detect the class I NDV isolates and some PPMV-1 isolates and the results illustrated the importance of our newly development NP-gene rRT-PCR assay in detection efficiency for highly divergent isolates of NDV. While virus isolation remains the ‘gold standard’ diagnostic techniques

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for NDV, results of our NP-gene rRT-PCR assay can be obtained in less than three hours, and gave high relative sensitivity and specificity. As well as for avian influenza, high-throughput, rapid, and accurate diagnostics is highly important to avoid unnecessary delay of implementing control measures in the event of an ND outbreak or ongoing surveillance.

In summary, this investigation provided the information of previously unrecognized genetic diversity and distribution of APMVs isolates in nature and viral evolution of ND outbreak-associated isolates in Taiwanese poultry farms. Our findings provided more evidence for the intercontinental transmission of lentogenic APMV-1 and APMV-4 between Eurasia and North America by wild birds. In addition, according to the criteria of the classification system based on complete F gene or RdRp gene sequences, one novel species of APMV-22 and three novel genotypes within APMV-1 class I, APMV-12, and APMV-22 were identified. Finally, the NP-gene rRT-PCR assay developed in this study is a test with rapid, sensitive, and specific screening capability and can be used as a parallel method that promotes the diagnostic efficacy for a broad range of NDV infection in clinical specimens from poultry and wild birds.

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1. Liu, Y.P., Kuo, S.T., Chiou, C.J., Terregino, C., Tsai, H.J., 2019. Novel avian metaavulavirus isolated from birds of the family *Columbidae* in Taiwan. Vet. Microbiol. 236, 108377.
2. Liu, Y.P., Chang, C.Y., Lee, F., Chiou, C.J., Tsai, H.J., 2020. Phylogenetic analysis of avian paramyxoviruses 1 isolated in Taiwan from 2010 to 2018 and evidence for their intercontinental dispersal by migratory birds. J. Vet. Med. Sci. 82, 1366–1375.
3. Liu, Y.P., Chang, C.Y., Lee, F., Chiou, C.J., Tsai, H.J., 2020. A highly sensitive real-time reverse transcription polymerase chain reaction for detecting nucleocapsid protein gene of both classes I and II of Newcastle disease virus. Taiwan Vet. J. 46, 49-55.
4. Liu, Y.P., Lee, F., Chang, C.Y., Chiou, C.J., Tsai, H.J. Genetic diversity of avian paramyxoviruses isolated from wild birds and domestic poultry in Taiwan between 2009 and 2020. (In progress)

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