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FULL PAPER

Avian Pathology

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

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ABSTRACT. Avian paramyxovirus 1 (APMV-1), synonymous with Newcastle disease virus (NDV), is a worldwide viral agent that infects various avian species and responsible for outbreaks of Newcastle disease. In this study, 40 APMV-1 isolates collected from poultry, migratory birds, and resident birds during 2010–2018 in Taiwan were characterized genetically. Our phylogenetic analysis of complete fusion protein gene of the APMV-1 isolates revealed that 39 of the 40 Taiwanese isolates were closely related to APMV-1 of class I genotype 1 or class II genotype**s** I, VI or VII, and one isolate belonged to a group that can be classified as a novel genotype 2 within class I. The fusion protein gene sequences of a branch (former 1d) nested within class I sub-genotype 1.2 were closely related to those isolated from wild birds in North America. Viruses placed in class II sub-genotype VI.2.1.1.2.1 and sub-genotype VI.2.1.1.2.2 were the dominant pigeon paramyxovirus 1 (PPMV-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. We conclude that at least five sub/genotypes of APMV-1 circulate in multiple avian host species in Taiwan. One genetically divergent group of APMV-1 should be considered as a novel genotype within class I. Migratory birds may play an important role in intercontinental spread of lentogenic APMV-1 between Eurasia and North America.

KEY WORDS: avian paramyxovirus 1, fusion protein, intercontinental dispersal, Newcastle disease, novel genotype

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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 [17]. 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 [1]. 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 [6].

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 [3]. An unified and objective classification system of APMV-1 was proposed in 2012 based on the coding sequences of the complete fusion protein gene [4], and this system (hereafter "former") and nomenclature criteria for APMV-1 were revised and updated by a global consortium in 2019 [5]. 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 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 [16]. 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 [9]. 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

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are regarded as the major pathogen responsible for the recent ND outbreaks in Europe, the Middle East, Africa, and Asia, including Taiwan [6].

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 [12, 23], and all of the 20 isolates [12] and 30 isolates [8] 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 [15].

Based on the global phylodynamic analysis, a study provides evidence for East Asia represent a critically important node for the global dispersion of APMV-1 [7]. 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 [19]. The phylogentic study for global APMV-4 isolates presented limited evidence for historical viral movement between continents [20]. 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 [11]. 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.

MATERIALS AND METHODS

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 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°C 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.

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 1. The full-length genome sequences of the representatives of each genotype and virulent class II genotype VII NDVs were determined with five different

Table 1. List of reverse transcription-polymerase chain reaction (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.

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°C for 40 sec, annealing at 50°C for 50 sec, and extension at 72°C for 1 min, and completed with a final extension step at 72°C 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.

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).

Phylogenetic analysis

The curated complete fusion protein gene of class I and class II datasets, provided by Dimitrov *et al.* [5] 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 [21] with 1,000 bootstrap replicates through the CIPRES Science Gateway [14]. The parameters for building Maximum likelihood trees using the CIPRES Science Gateway were set according to the step-by-step guidelines [5]. Trees were visualized using Molecular Evolutionary Genetics Analysis version 7, or MEGA 7 [10].

The estimates of average evolutionary distance between class I genotype 1 and genotype 2 were inferred using MEGA 7 [10]. Analyses were conducted using the Maximum Composite Likelihood model [22]. 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.

RESULTS

Sample collection and virus isolation

Forty APMV-1 isolates from different avian species were collected in this study (Table 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.

GenBank accession numbers

GenBank accession numbers of the APMV-1 strains described in this study are listed in Table 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.

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. 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}.

Genetic analysis of class II APMV-1

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

Table 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)]
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.

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. 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 turtle 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. 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). The deduced amino acid motif at the fusion protein cleavage site sequence of the group of NDVs was ^{112}RRKKR↓F^{117}.

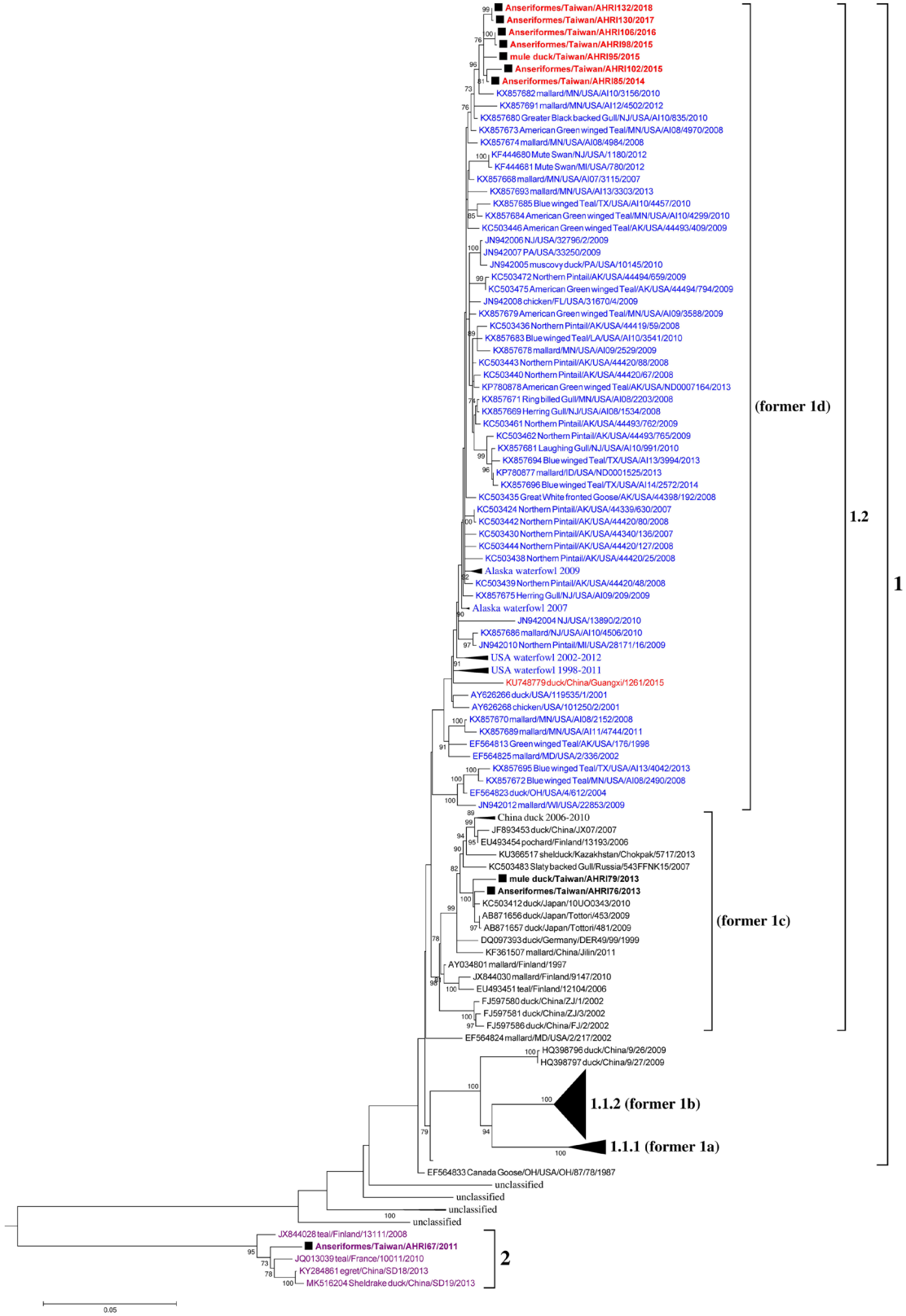

Fig. 1. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class I (n=298). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete gamma distribution 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 avian paramyxovirus 1 (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.

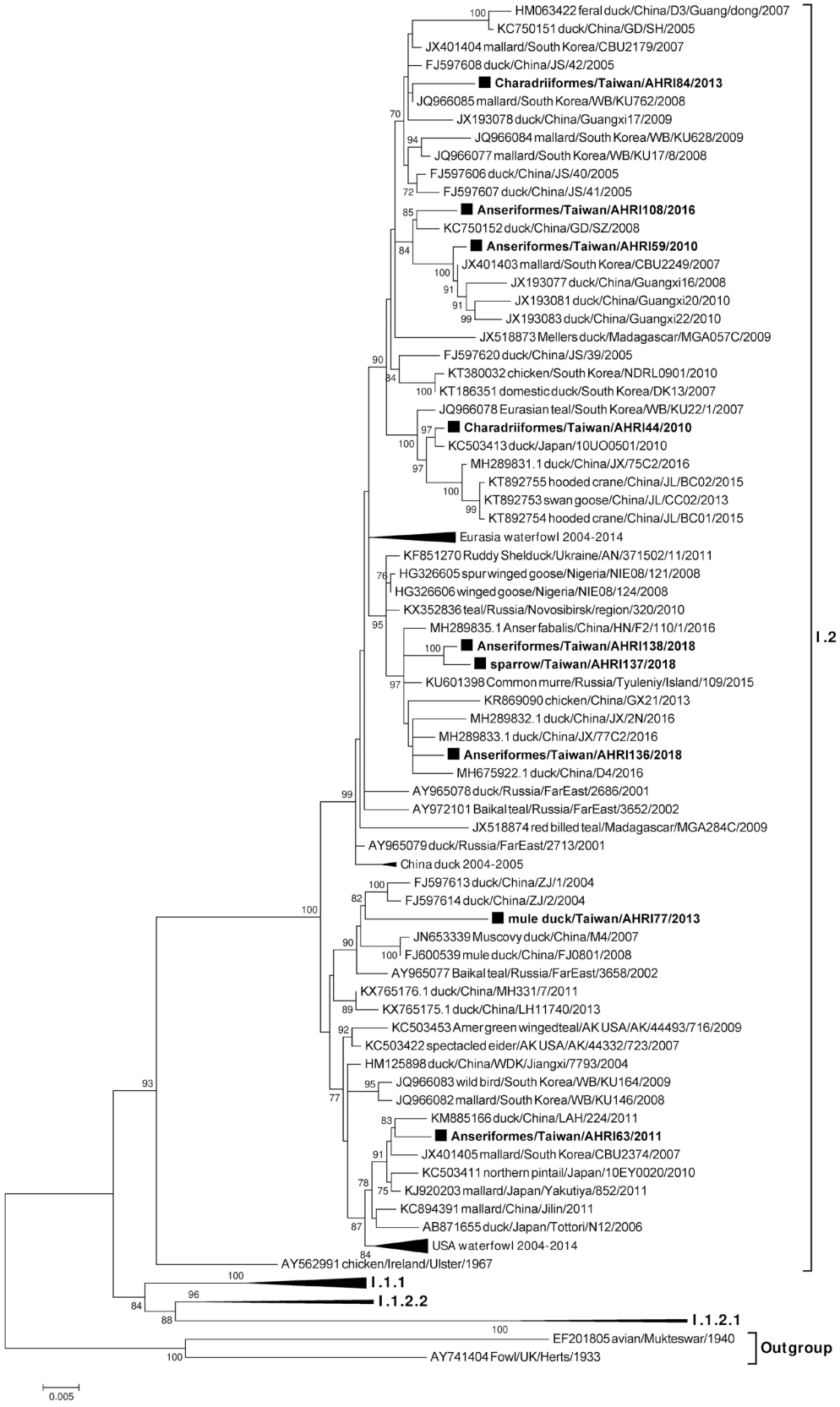

Fig. 2. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype I (n=129). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete 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 avian paramyxovirus 1 (APMV-1) obtained from birds in Taiwan in this study.

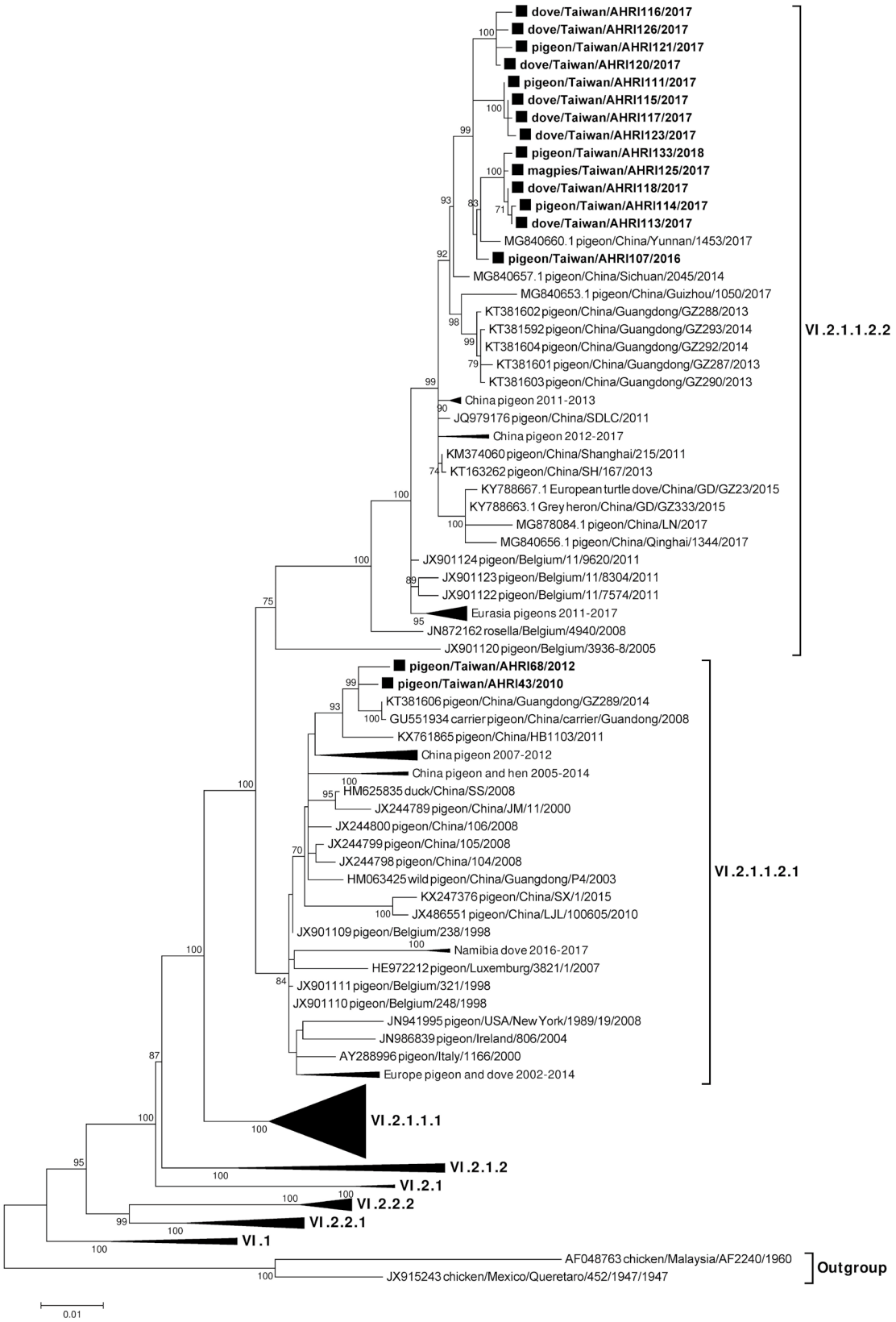

Fig. 3. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype VI (n=278). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete 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 avian paramyxovirus 1 (APMV-1) obtained from birds in Taiwan in this study.

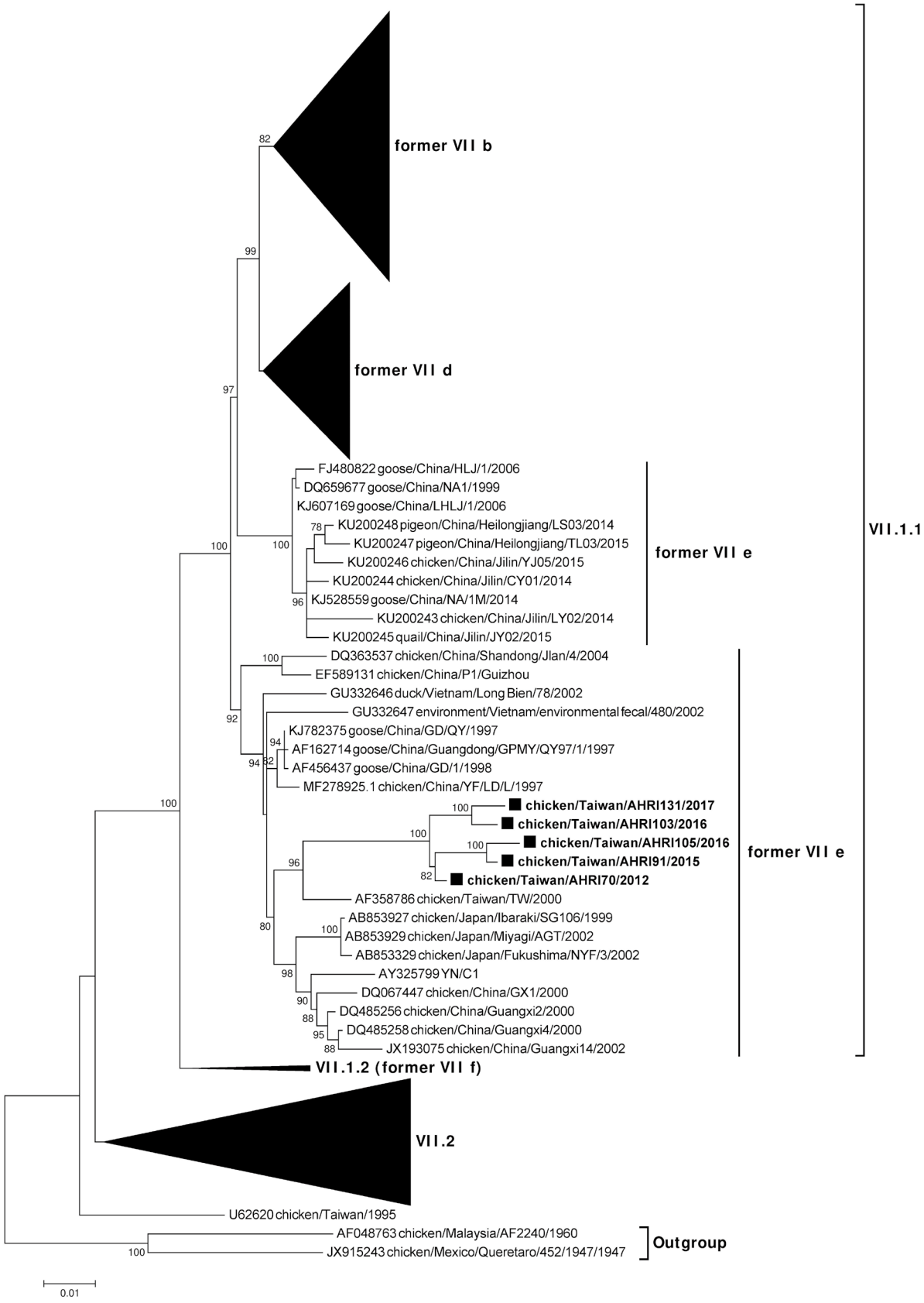

Fig. 4. Phylogenetic tree based on the complete fusion protein gene sequences of isolates of avian paramyxovirus 1 class II genotype VII (n=777). The evolutionary history was inferred by the maximum likelihood method based on the general time reversible model using 1,000 bootstrap replicates with discrete 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 avian paramyxovirus 1 (APMV-1) obtained from birds in Taiwan in this study.

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 [18]. 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 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 [13], teal/France/100011/2010 [2], 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. 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 [5], 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 [9, 19], 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 Germany. These results were consistent with previous findings [9] 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 obtaibed 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. 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 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.

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