Molecular and Epidemiological Studies on Avian Viruses Originated from Wild and Free-Ranging Birds in Western Japan

（西日本における野生および半野生鳥類由来
ウイルスの分子疫学的研究）

Eiji Yamamoto

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CONTENTS

GENERAL INTRODUCTION

It is well established that wild birds, including migratory species, have the potential to disperse certain avian pathogenic viruses [Nuttall, 1997; Wobeser, 1997]. Especially, wild birds, particularly aquatic birds, are the primary reservoir for transmission of avian influenza virus to domestic poultry.

However, relatively little is known about the ecology of the other avian viruses in wild birds, and the depths of data are inconsistent worldwide. It is possible that understanding the dissemination and maintenance of some avian pathogenic viruses in wild birds is important for understanding the factors that contribute to transmission of avian viruses from wild birds to poultry.

Therefore, in the present study, to understand the ecology of some avian pathogenic viruses in wild and free-ranging bird, surveillance studies were conducted in western Japan and the field isolates, for example avian paramyxoviruses, pigeon circoviruses and avian reoviruses, were biologically, serologically and genetically analyzed.

Overview of Avian Paramyxoviruses

Avian paramyxovirus (APMV) is a member of the genus Avulavirus in the subfamily *Paramyxovirinae* of the family *Paramyxoviridae*. It is generally believed that the first outbreak of a disease caused by APMV occurred in 1926, in Java, Indonasia and in Newcastle-upon-Tyne, England [Alexander, 1991]. Thereafter, the APMV was to be called APMV serotype 1 or Newcastle disease virus (NDV). In 1956, another type of APMV was isolated from chickens in Yucaipa, California

[Bankowski, 1960]. After that, many APMVs were isolated from the birds showing some symptoms or apparently healthy birds [Alexander, 2003]. Up to the present, APMV comprises nine known serotypes (APMV-1–APMV-9), based on hemagglutination inhibition and neuraminidase inhibition assay [Alexander, 2003]. Recently, new types of APMV have been isolated from rockhopper penguins (APVM-10), common snipes (APVM-11), and wigeon (APVM-12) [Briand *et al*., 2012; Miller *et al*., 2010; Terregino *et al*., 2013].

Negative contrast electron microscopy of NDV reveals very pleomorphic virus particles. Normally, they are rounded and 100-500 nm in diameter, though filamentous forms of about 100 nm across and of variable length are often seen. The virus is covered with projections about 8 nm in length [Alexander, 2003].

APMVs have a negative-sense, single-stranded RNA genome. The genome size of APMV ranged from approximately 14,900 to 17,260 nucleotides (nt) long [Briand *et al*., 2012; Miller *et al*., 2010; Samal, 2011; Terregino *et al*., 2013].

Overview of Circovirus

Circoviruses are among the smallest pathogenic DNA viruses known; this class exhibits the potential to infect various animal species. The family *Circoviridae* consists of two genuses: Circovirus and Gyrovirus. In genus Circovirus, there are 11 species: Beak and feather disease virus (BFDV), Canary circovirus, Duck circovirus, Finch circovirus, Goose circovirus, Gull circovirus, Pigeon circovirus (PiCV), Porcine circovirus-1, Porcine circovirus-2, Starling circovirus, and Swan circovirus. In contrast, genus Gyrovirus is composed of one species: Chicken infectious anemia virus

[international committee of taxonomy of virus 2014: http://ictvonline.org/ virusTaxonomy.asp].

Circoviruses are nonenveloped with a round to icosahedral morphology. Circovirus particles have a mean diameter of 14-17 nm, with the exception of chicken infectious anemia virus [Woods and Latimer, 2008]. The viral density of the BFDV was 1.378 g/cc on a cesium chloride equilibrium density gradient [Woods and Latimer, 2008].

There is considerable histologic and clinical evidence that immunosuppression is associated with circoviral infection in avian species [Woods and Latimer, 2008]. But experimental study to evaluate humoral and cell-mediated immune function in BFDV or PiCV infection in avian species has not reported yet.

Overview of Avian orthoreovirus

Avian orthoreoviruses (ARVs) are members of the Orthoreovirus genus in the *Reoviridae* family. In 1954, the first isolation of ARV from the respiratory tract of chickens with chronic respiratory disease was reported[Fahey et al., 1954; Petek *et al*., 1967]. Thereafter, many ARVs were isolated and recognized as one of the important pathogens of chickens and turkeys. Additionally, cases of various species of birds other than chickens and turkeys infected with ARVs were reported to date [Chen *et al*., 2012; Huhtamo *et al*., 2007; Palya *et al*., 2003; Perpiñán *et al*., 2010; Sakai *et al*., 2009; Senne *et al*., 1983; Yun *et al*., 2012].

ARVs are nonenveloped virus with an icosahedral symmetry and double-shelled capsid. Particles of ARVs have a diameter of 75 nm, and the viral density is 1.36 – 1.37 g/cc [Jones, 2008]. The viral genome of ARVs consists of 10 double stranded RNA

segments and the segments are grouped into three size classes: L (large), M (medium), and S (small) [Jones, 2008].

In this thesis, the author described the virological, serological and genetical analyses of avian pathogenic virus isolates, i.e. avian paramyxoviruses, circoviruses and orthoreoviruses, originated from wild or free-ranging birds in western Japan.

CHAPTER 1

Characterization of Novel Avian Paramyxovirus Strain APMV/Shimane67 Isolated from Migratory Wild Geese in Japan

Introduction

Avian paramyxovirus (APMV) belongs to the genus *Avulavirus* in the *Paramyxovirinae* subfamily of the family *Paramyxoviridae*. Members of family *Paramyxoviridae* are characterized by pleomorphic enveloped particles that contain a single-stranded, negative sense RNA genome [Lamb and Parks, 2007]. APMV is classified into nine distinct serotypes based on serological tests, such as hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests [Alexander, 2003]. APMV-1, also known as Newcastle disease virus (NDV), causes severe disease in chickens and huge economic losses for poultry industries. Thus, APMV-1 is the most extensively characterized APMV serotype. In addition to APMV-1, APMV-2, 3, 6 and 7 are reportedly associated with disease in poultry. APMV-2 causes respiratory disease in chickens and turkeys, while APMV-3, 6 and 7 cause respiratory disease or egg production disorder in turkeys. On the other hand, APMV-4, 5, 8 and 9 have not been reported to infect poultry. APMV-4, 8 and 9 were mainly isolated from waterfowl, such as ducks and geese, while APMV-5 was isolated from budgerigar and is associated with diarrhea and high mortality [Alexander, 2003]. Three APMVs isolated from the rockhopper penguin in the Falkland Islands, the common snipe in France and the Eurasian wigeon in Italy have recently been proposed to be new serotypes of APMV, APMV-10, 11 and 12, respectively [Briand *et al*., 2012; Miller *et al*., 2010; Terregino *et al*., 2013]. The intracerebral pathogenicity index test using one-day-old chicks suggested that APMV-10 and 12 showed little or no virulence in chickens, resembling the lentogenic NDV.

Since 1979, we have surveyed for influenza A viruses and APMVs in the San-in district of western Japan. During this surveillance, numerous influenza A viruses and

APMVs have been isolated from the feces of migratory waterfowl flying from Siberia or northern China [Fujimoto *et al*., 2010; Shengqing *et al*., 2002; Tsubokura *et al*., 1981]. APMV/Shimane/67/2000 (APMV/Shimane67) examined in this study was isolated from the feces of geese collected in 2000 in Shimane prefecture. Here, we report the biological, serological and genetic analyses of APMV/Shimane67. The results suggested that APMV/Shimane67 belongs to a new serotype APMV-13.

Materials and Methods

Viruses and cells

APMV/Shimane67 was isolated from goose fecal samples collected in Shimane prefecture, the wintering location for wild waterfowl, such as ducks, geese and swans. Virus isolation was performed as described previously [Shengqing *et al*., 2002].

Electron microscopy: Purification of APMV/Shimane67 was conducted by differential centrifugation and two successive sedimentations through a 10 to 50% sucrose gradient as described previously [Kida and Yanagawa, 1981]. The purified virus particles stained with 2% phosphotungstic acid (pH 6.8) were observed under the JEM-100CX electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Hemagglutination (HA) and hemagglutination inhibition (HI) test

HA and HI tests were performed using established procedures as described previously [Fujimoto *et al*., 2010]. For HI test, chicken hyperimmune sera against APMV/Shimane67, NDV/goose/Alaska/415/91 (NDV/AK/415), APMV-2/chicken/California/Yucaipa/56 (APMV-2/Yucaipa), APMV-3/turkey/Wisconsin/68 (APMV-3/WI), APMV-4/duck/Mississippi/320/75 (APMV-4/MS), APMV-6/duck/Hong Kong/

D199/77 (APMV-6/HK/D199) and APMV-7/dove/Tennessee/4/75 (APMV-7/TN) were prepared as described previously [Tsubokura, 1981].

Propagation of APMV/Shimane67 in cultured cells

Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), African green monkey kidney (Vero) and baby hamster kidney (BHK21) cells were grown in Eagle's minimal essential medium (E-MEM) containing 10% fetal bovine serum (FBS). Human embryonic kidney (293) and chicken embryo fibroblast (DF-1) cells were grown in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FBS. Cell monolayers prepared in 6-well plates were infected with 10^4 50% egg infectious dose (EID_{50}) of APMV/Shimane67. After adsorption for 1 hr at 37°C, cells were washed three times with phosphate buffered saline (PBS, pH7.2) and were incubated at 37°C in E-MEM or D-MEM containing 0.3% bovine serum albumin and 0.5 μg of TPCK-treated trypsin (Sigma-Aldrich, St. Louis, USA). The virus titer of each cell culture supernatant was determined as follows. Ten-fold diluted supernatant (100 μl) was inoculated into the allantoic cavities of five 10-day-old embryonated chicken eggs and were incubated at 37°C for 3 days unless death of the embryo was detected. After the inoculated eggs were chilled at 4°C, their allantoic fluids were tested for hemagglutination activity. The virus titer was calculated using the method of Reed and Muench [1938].

Pathogenicity test

The mean death time (MDT) at the minimum lethal dose for chicken embryos and the intracerebral pathogenicity index (ICPI) in 1-day-old chickens were measured in order to assess the virulence of this virus, as described previously [Allan *et al*., 1978].

Nucleotide sequencing and phylogenetic analysis

Viral RNAs were extracted from purified APMV/Shimane67 using the QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo, Japan), and were transcribed to cDNA using F10 primer and RAV-2 reverse transcriptase (TaKaRa Bio, Otsu, Japan) in accordance with the manufacturer's instructions. F10 primer was designed to bind with the gene-stop signal region of the matrix gene and gene-start signal of the F gene of NDV/AK/415 [Tsunekuni *et al*., 2010]. PCR was carried out using Pwo DNA polymerase (Roche, Mannheim, Germany) and a cycling sequence of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, which was then followed by a final extension at 72°C for 5 min. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN) and were sequenced using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, USA) and a Gene Rapid DNA sequencer (Amersham Pharmacia Biotech). Nucleotide sequences of PCR and sequencing primers are shown in Table 7. The nucleotide and deduced amino acid sequences of APMV/Shimane67 F gene were compared to those of published APMVs by Clustal X [Larkin *et al*., 2007]. Phylogenetic trees were generated using the Neighbor-Joining (NJ) method with 1,000 bootstraps.

Results

Electron microscopy

Electron microscopy of APMV/Shimane67 showed typical characteristics of a paramyxovirus. Virions were spherical or pleomorphic, enveloped particles (250-300

nm in diameter), and virion surfaces were covered with spiked projections of about 8-10 nm in length (Fig. 1).

HI test

In order to investigate the antigenic relationships between APMV/Shimane67 and other serotypes of APMV, HI tests were carried out (Table 8). As APMV-5 has no HA activity, and APMV-8 to 12 were not available in our laboratory, HI tests against these six APMVs were not performed. Antiserum against APMV/Shimane67 showed the highest HI titer (1: 1,280) with the homologous antigen, but little or no HI activity against other APMV serotypes. Reactivity of antisera against NDV/AK/415, APMV-2/Yucaipa, APMV-3/Wisconsin, APMV-4/Mississippi, APMV-6/Hong Kong and APMV-7/Tennessee on HI test was highest with each homologous antigen, while these antisera showed lower activity against APMV/Shimane67.

Growth in cell culture and pathogenicity of APMV/Shimane67

Growth of APMV/Shimane67 in avian and mammalian origin cell lines (DF-1, MDCK, MDBK, BHK, Vero and 293 cells) was measured. In the presence of trypsin, regardless of cell lines, APMV/Shimane67 efficiently grew and reached approximately $10^{5.0}$ EID_{50}/0.1 m*l* at 72 hr post-infection. Syncytium formation, a typical characteristic of paramyxovirus cytopathic effects, was clearly observed, particularly in MDBK cells (Fig. 2). In DF-1 cells without trypsin, titers of APMV/Shimane67 reached about $10^{3.5}$ EID_{50}/0.1 m*l* at 72 hr post-infection. These results indicate that external proteases are necessary for efficient cleavage of the F protein.

The MDT and ICPI of APMV/Shimane67 were 120 hr< and 0.0, respectively.

Nucleotide sequencing and phylogenetic analysis

A total of 1,878 nucleotides of the genome sequence of APMV/Shimane67 were determined. The putative initiation codon, which better matched the Kozak consensus sequence ((G/A)NNATGG) [Kozak *et al*., 1987], was the third ATG from 5'-end of the F10 primer binding site (data not shown). It contained a long open reading frame (ORF) composed of 1,638 nucleotides and encoded 545 amino acids. The nucleotide sequence of this ORF showed similarities with the F genes of NDVs and APMV-12/wigeon/Italy/3920_1/2005 (APMV-12/ITA) by discontiguous MegaBLAST search [Zhang *et al*., 2000], indicating that this ORF was the F gene of APMV/Shimane67. The nucleotide and deduced amino acid identities between the F gene of APMV/Shimane67 and other APMV serotypes ranged from 42.9% with APMV-3/NLD to 62.7% with APMV-12/ITA, and from 28.9% with APMV-3/NLD to 67.3% with APMV-12/ITA, respectively (Table 9). The deduced amino acid sequence of the putative cleavage site of the APMV/Shimane F gene was QVRENR/LVG (Fig. 3). This resembled the motif, a pair of single basic residues, of the lentogenic NDV. The phylogenetic tree of the F gene constructed with APMVs and members of subfamily *Paramyxovirinae* revealed that APMV/Shimane67 is a member of genus *Avulavirus* and is grouped with NDV, APMV-9 and APMV-12, while distinct from these APMV serotypes (Fig. 4).

Discussion

The typing of APMVs has mainly been conducted by HI test [Alexander *et al*., 2003]. In addition, because the nucleotide sequence data from various APMVs have been collected in DNA databases, genetic analysis has also been attempted to classify

new APMV isolates, and the results obtained have demonstrated the effectiveness of this approach [Briand *et al*., 2012; Miller *et al*., 2010; Terregino *et al*., 2013]. On HI test, the anti-APMV/Shimane67 serum reacted strongly (HI titer of 1: 1,280) with homologous virus and did not react with the other APMVs (HI titer of 1: <40). In addition, sera against other APMVs (NDV, APMV-2, 3, 4, 6 and 7) showed weaker reactivity with APMV/Shimane67 than with homologous viruses (Table 8). These results indicate that APMV/Shimane67 is serologically distinct from NDV, APMV-2, 3, 4, 6 and 7. Anti-NDV, APMV-4 and 7 sera showed some cross reactivity (HI titer of 1: 320 - 1: 1,280) with APMV/Shimane67. It is possible that this cross reactivity is caused by high titers of hyperimmune sera (HI titer of more than 1:2,560 against homologous viruses). The sera used in this study were prepared by multiple immunization of APMV antigen. Miller *et al.* [2010] also reported cross reactivity on HI test of APMVs using hyperimmune serum.

Analysis of the F gene of APMV/Shimane67 also revealed the relationship of this virus with other APMVs. The ORF of the APMV/Shimane67 F gene was 1,638 nucleotides long and encoded 545 amino acids. Although these nucleotide and amino acid lengths for the F gene were the same as those of APMV-6/duck/Italy/4524-2/07 [Xiao *et al*., 2010], the identities of nucleotide and amino acid sequences between APMV/Shimane67 and APMV-6/duck/Italy/4524-2/07 were low (45.3% and 37.3%, respectively) (Table 9). The amino acid sequence at the F protein cleavage site of APMV/Shimane67 was deduced as being QVRENR/LVG, which resembles the motif of lentogenic NDV (Fig. 3). This motif was observed in the F proteins of APMV/Shimane and NDV, APMV-9 and 12, and interestingly, these four APMVs containing conserved amino acid sequence motifs at the F protein cleavage site also formed one phylogenetic cluster (Fig. 4). The phylogenetic tree showed that

APMV/Shimane67 is a member of genus *Avulavirus*, is distinct from other APMVs and showed close relationship with APMV/wigeon/Italy/3920-1/2005 (APMV-12/ITA/3920-1). APMV-2, -3 and -6 viruses have antigenic differences constituting the antigenic subgroup within each serotype [Anderson *et al*., 1987; Kumar *et al*., 2010; Subbiah *et al*., 2010]. Similar situations in phylogenetic analysis were also observed in the nucleotide and amino acid identities of the F gene (Table 9). APMV/Shimane67 exhibited the highest nucleotide and amino acid identities (62.7% and 67.3%, respectively) with APMV-12. These identities were lower than those observed between the subgroups, APMV-2/Yucaipa and APMV-2/Bangor (69.8% in nucleotide and 79.1% in amino acid sequence), and APMV-3/NLD and APMV-3/WI (66.8% in nucleotide and 70.1% in amino acid sequence) (Table 9). These observations confirm a distant relationship corresponding to APMV serotype between APMV/Shimane67 and APMV-12/ITA/3920-1.

The pathogenicity of APMV/Shimane67 to chickens was evaluated by MDT and ICPI tests. MDT (120hr<) and ICPI (0.0) of APMV/Shimane67 were applicable to the lentogenic NDV and revealed that the virus had no virulence in chickens. The amino acid sequence at the F protein cleavage site is the major determinant of NDV pathogenicity to chickens. As described above, the putative cleavage site of the APMV/Shimane F protein is QVRENR/LVG, which resembles lentogenic NDV. Non-virulence of APMV/Shimane67 may be affected. The introduction of an amino acid sequence of velogenic NDV at the F protein cleavage site by reverse genetics did not increase the virulence of APMV-2, 4 and 7 in chickens [Kim *et al*., 2013; Subbiah *et al*., 2011; Xiao *et al*., 2012]. However, it has been reported that lentogenic NDV isolated from wild geese changed to velogenic virus by amino acid substitutions in the F protein cleavage site during serial passage in chickens [Shengqing *et al*., 2002].

Whether the virulent variant of APMV/Shimane67 will emerge with multiple passaging of APMV/Shimane67 in chickens is of interest.

Recently, whole genome sequences from various APMV serotypes have been reported, and the classification of APMVs using those whole genome sequence data has been proposed [Briand *et al*., 2012; Miller *et al*., 2010; Samuel *et al*., 2010; Terregino *et al*., 2013]. In this study, only the F gene of APMV/Shimane67 was identified; however, data obtained have suggested that APMV/Shimane67 is a novel APMV serotype, APMV-13. To emphasize this idea, the full-length genome sequence of APMV/Shimane67 needs to be determined.

Table 1 Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5' to 3')
For PCR	
F10	ATTAGAAAAAACACGGGTAGAA
FR1215	CAACGACAGATGACCTGTTG
F270	GAAACATTATCCCGCATTCTAAC
HNR39	GAATGAGCAATTTGATAACCCCAG
For Sequencing	
F10	ATTAGAAAAAACACGGGTAGAA
FR258	GCAATGATTGTTTGGCACAATC
F270	GAAACATTATCCCGCATTCTAAC
F588	TAACCCTTGAATCCAGGCTAGG
F905	GTTCAACCTTACTAGAAACATTAGC
F1090	CATGTCTCAATGGAAATCTAAGTGA
FR1215	CAACGACAGATGACCTGTTG
FR1545	GTGATTGCAATTATTAAGCATACCACTG
F1689	ACTCCCCCATCCAGCCACAT
FR1746	ACCTACATGTTGGAGTGCCA
HNR39	GAATGAGCAATTTGATAACCCCAG

Table 2 Antigenic analysis of APMV/Shimane67 by HI tests with antisera against representative APMV strains

Antigen	Antiserum to						
	APMV/Shimane67	NDV/AK/415	APMV-2/Yucaipa	APMV-3/WI	APMV-4/MI	APMV-6/HK	APMV-7/TN
APMV/Shimane67	1280[a)]	1,280	<40	<40	640	<40	320
NDV/AK/415	<40	5,120					
APMV-2/Yucaipa	<40		160				
APMV-3/WI	<40			160			
APMV-4/MI	<40				5,120		
APMV-6/HK	<40					640	
APMV-7/TN	<40						2,560

a) HI titre represents the reciprocal of the serum dilution inhibiting the activity of 4 hemagglutinating units of virus antigen. Blank: not tested.

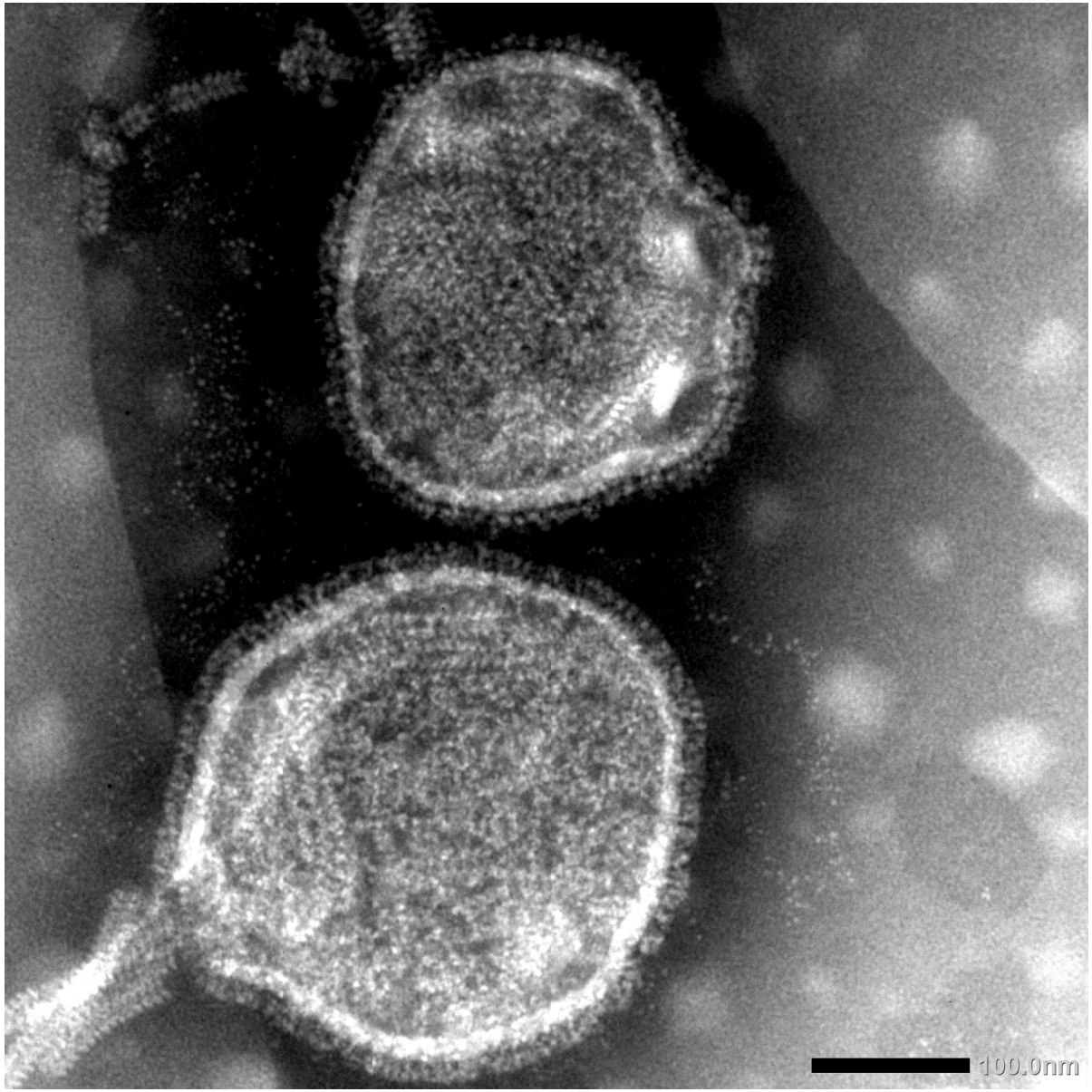
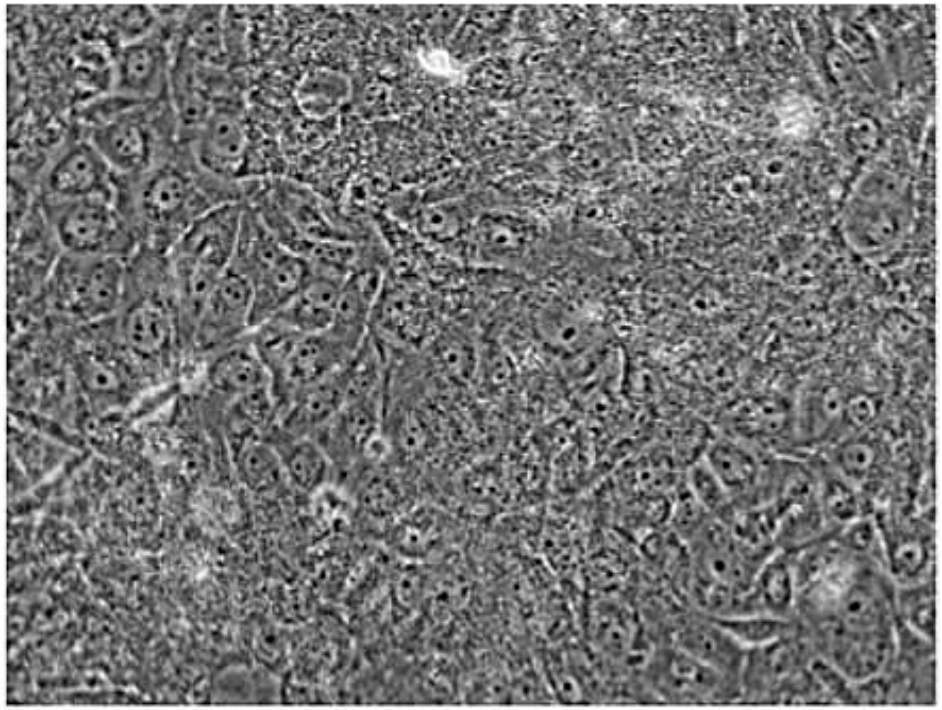
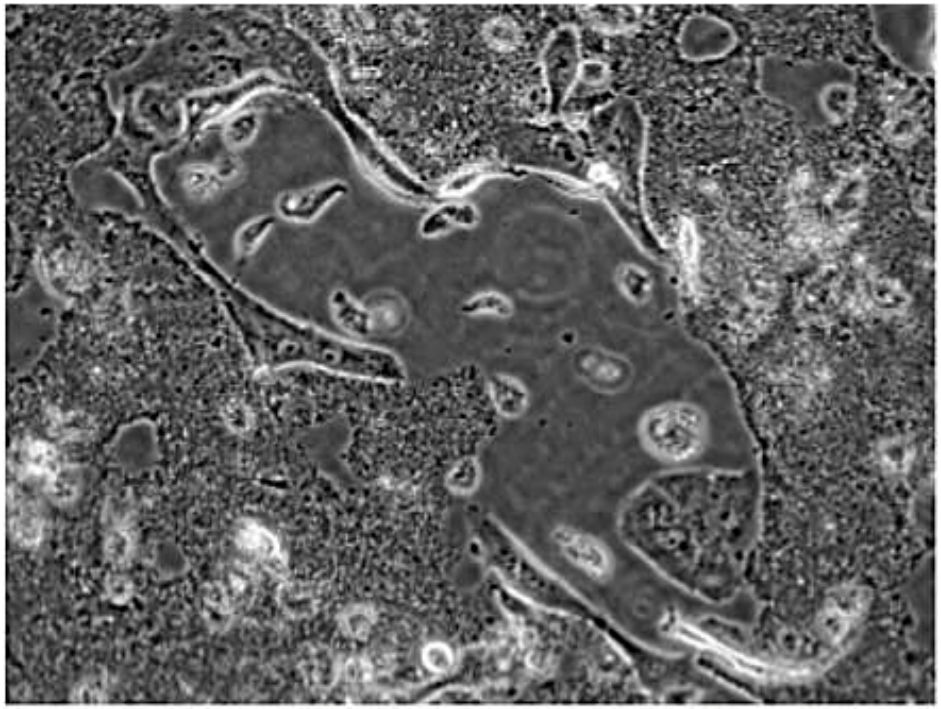


Fig. 1



Mock infected



APMV/Shimane67 (48 hr post infection)

Fig. 2

	F2←					→F1		
APMV/Shimane67	Q	V	R	E	N	R	L	V	G
NDV(Ientogenic)	G	G	R	Q	G	R	L	I	G
NDV(velogenic)	G	R	R	Q	R	R	F	I	G
APMV-2/Yucaipa	D	K	P	A	S	R	F	V	G
APMV-3/WI	P	R	P	S	G	R	L	F	G
APMV-4/HK/D3	V	D	I	Q	P	R	F	I	G
APMV-5/Kunitachi	G	K	R	K	K	R	F	V	G
APMV-6/TWN/Y1	P	A	P	E	P	R	L	I	G
APMV-7/TN	T	L	P	S	S	R	L	I	G
APMV-8/DE	T	Y	P	Q	T	R	L	I	G
APMV-9/NY	R	I	R	E	G	R	I	F	G
APMV-10/FLK/324	D	K	P	S	Q	R	I	I	G
APMV-11/FRA/100212	D	S	G	T	K	R	F	V	G
APMV-12/ITA/3920_1	R	G	R	E	P	R	L	V	G

Fig. 3

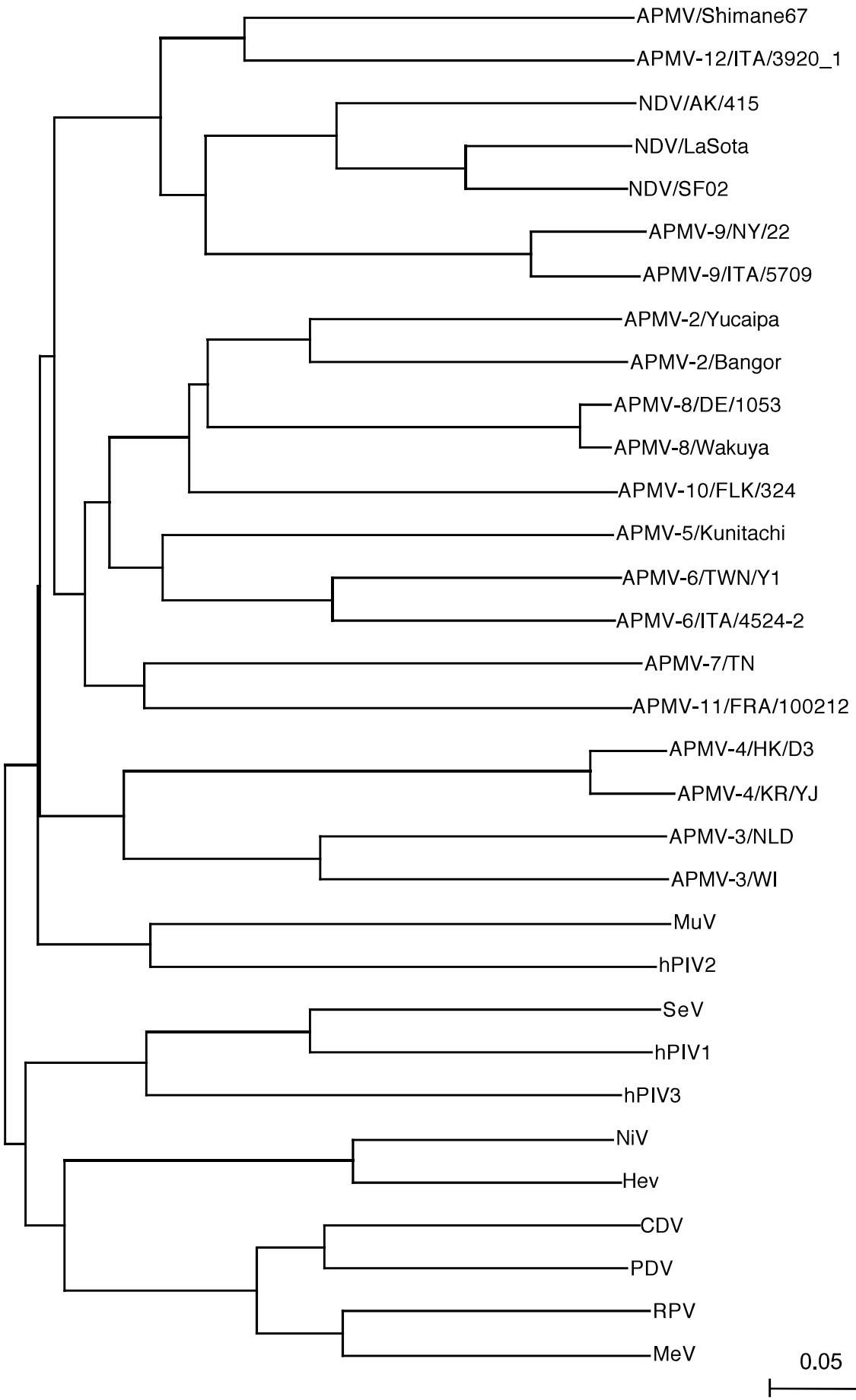

APMV/Shimane67
APMV-12/ITA/3920_1
NDV/AK/415
NDV/LaSota
NDV/SF02
APMV-9/NY/22
APMV-9/ITA/5709
APMV-2/Yucaipa
APMV-2/Bangor
APMV-8/DE/1053
APMV-8/Wakuya
APMV-10/FLK/324
APMV-5/Kunitachi
APMV-6/TWN/Y1
APMV-6/ITA/4524-2
APMV-7/TN
APMV-11/FRA/100212
APMV-4/HK/D3
APMV-4/KR/YJ
APMV-3/NLD
APMV-3/WI
MuV
hPIV2
SeV
hPIV1
hPIV3
NiV
Hev
CDV
PDV
RPV
MeV
0.05

Fig.4

Figure legends

Fig. 1. Negative contrast electron micrograph of APMV/Shimane67 virus showing a partially disrupted particle with nucleocapsid emerging. x200,000.

Fig. 2. MDBK cells infected with APMV/Shimane67.

Fig. 3. Amino acid sequences of F protein cleavage site from APMVs. NDV (lentogenic); strain LaSota, NDV (velogenic); strain Herts 33, APMV-4/HK/D3; APMV-4/duck/Hong Kong/D3/1975, APMV-5/Kunitachi; APMV-5/budgerigar/ Japan/Kunitachi/1974, APMV-6/TWN/Y1; APMV-6/duck/Taiwan/Y1/1998, APMV -8/DE; APMV-8/goose/Delaware/1053/1976, APMV-9/NY; duck/New York/22/1978, APMV-10/FLK/324; APMV10/penguin/Falkland Islands/324/2007, APMV-11/FRA/ 100212; APMV-11/common_snipe/France/100212/2010. Abbreviations of other APMV are given in the text.

Fig. 4. Phylogenetic tree of F gene sequences from subfamily *Paramyxovirinae*. The phylogenetic tree was generated using the neighbor-joining algorithm with 1,000 bootstrap replicates in Clustal X.

CHAPTER 2

Completion of Full Length Genome Sequence of Novel Avian Paramyxovirus Strain APMV/Shimane67 Isolated from Migratory Wild Geese in Japan

Introduction

Avian paramyxovirus (APMV) is a member of the genus *Avulavirus* in the subfamily *Paramyxovirinae* of the family *Paramyxoviridae*. APMV comprises nine known serotypes (APMV-1–APMV-9), based on hemagglutination inhibition and neuraminidase inhibition assay [Alexander, 2003]. APMV-1, also called Newcastle disease virus (NDV), is one of the most important pathogens for poultry because the infection of the virulent type of APMV-1 (velogenic) is highly lethal. Thus, APMV-1 is the most characterized virus among all other APMV serotypes. In addition to APMV-1, the association of APMV-2, -3, -6, and -7 with poultry disease were reported [Alexander, 2003]. APMV-2 causes respiratory disease in chickens and turkeys, whereas APMV-3, -6, and -7 cause respiratory disease or disorder in egg production of turkeys. Alternatively, APMV-4, -5, -8, and -9 have not been reported to infect poultry; APMV-4, -8, and -9 were mainly isolated from waterfowl, such as ducks and geese, whereas APMV-5 was isolated from budgerigar and was associated with diarrhea and high mortality. Recently, new types of APMV have been isolated from rockhopper penguins (APVM-10), common snipes (APVM-11), and wigeon (APVM-12) [Briand *et al*., 2012; Miller *et al*., 2010; Terregino *et al*., 2013]. Intracerebral pathogenicity index test using one-day-old chicks suggested that APMV-10 and -12 revealed little or no virulence in chickens, resembling the low or non-virulent (lentogenic) NDV.

APMVs are pleomorphic, enveloped viruses containing a negative-sense, single-stranded RNA genome. The genome size of APMV ranged from approximately 14,900 to 17,260 nucleotides (nt) long [Briand *et al*., 2012; Miller et al., 2010; Samal, 2011; Terregino *et al*., 2013]. The exact value for genome length is divisible by six

(rule of six), which is the basic feature for efficient replication of viral genome among members of the subfamily *Paramyxovirinae*. APMV genome contains nucleocapsid protein (N); phosphoprotein (P); matrix protein (M); hemagglutinin-neuraminidase (HN); and large polymerase protein (L) genes, similar to other members of the family Paramyxoviridae [Samal, 2011]. APMV-6 contains an additional gene that encodes the small hydrophobic protein (SH) [Chang *et al*., 2001]. Each gene encodes a single viral structural protein with the exception of P gene. mRNA transcribed from P gene has a potential to translate two additional nonstructural proteins, termed V and W proteins [Steward *et al*., 1993]. V or W proteins are translated from mRNA containing one or two guanine residue insertions, respectively, at the RNA editing site. The 3′- and 5′-ends of each gene possess the non-coding sequences, known as gene-start (GS) and gene-end (GE), which are conserved among similar types of APMVs and function as transcriptional promoters and terminators. The non-coding region boundaries between GE and GS are termed as intergenic sequence (IGS) that comprised various nucleotides. At the 3′- and 5′-ends of the APMV genome, non-coding leader (Le) and trailer (Tr) sequences exist and act as promoters for replication of genomic and antigenomic RNAs.

In the past, most of the available complete genome sequence of APMVs had been from APMV-1. However, recently, complete genome sequences from prototype of other APMV serotypes have been published [Kumar *et al*., 2008; Nayak *et al*., 2008; Paldurai *et al*., 2009; Samuel *et al*., 2009; 2010; Subbiah *et al*., 2008; Xiao *et al*., 2009; 2010]. Moreover, the number of reports on complete genome sequences from APMVs other than APMV-1 has been increasing in recent years [Briand *et al*., 2012; Chang *et al*., 2001; Jahanshiri *et al*., 2005; Jeon *et al*., 2008; Kho *et al*., 2004; Kumar *et al*., 2010; Miller *et al*., 2010; Rosseel *et al*., 2011; Subbiah *et al*., 2010; Terregino *et al*., 2013;

Tian *et al*., 2012]. In these studies, the phylogenetic trees that were constructed using whole genome or individual genes revealed the correlation between genetic classification and serotyping.

During our continuous surveillance for the presence of avian influenza A viruses and APMVs in wild birds, we isolated APMV/Shimane67 from the feces of geese collected in 2000 (Chapter 1). Serological and partial genome (F gene) analysis demonstrated the possibility that APMV/Shimane67 was distinct from the already existing APMVs. In this study, we completed the determination of whole genome sequences of APMV/Shimane67 to further understand its molecular characteristics.

Materials and Methods

Extraction of viral RNA, RT-PCR, and nucleotide sequencing

The isolation and characterization of APMV/Shimane67 have been previously described (Chapter 1). Viral genomic RNA was extracted from infected allantoic fluid using QIAamp Viral RNA Mini Kit (QIAGEN), according to the manufacturer's instructions manual. The cDNA of the APMV/Shimane67 genome was synthesized using Primescript Reverse Transcriptase (TaKaRa Bio) and amplified using SapphireAmp Fast PCR Master Mix (TaKaRa Bio) and pairs of oligonucleotide primers. The sequences of oligonucleotide primers used in this study are available upon request. After purification from agarose gel using QIAquick Gel Extraction Kit (QIAGEN), PCR products were sequenced using BigDye Terminater v3.1 Sequencing Kit (Applied Biosystems) and analyzed using the 3130xl Genetic Analyzer (Applied Biosystems).

Determination of 3′- and 5′-ends of the viral genome sequence

The 3′-end of the viral genome (Le region) sequence of APMV/Shimane67 was determined by a method previously described [Tsunekuni *et al*., 2010]. To determine the 5′-end of the viral genome (Tr region), cDNA from the 5′-end of the genome was amplified using SMART PCR cDNA Synthesis Kit (Clontech) and virus-specific primers, according to the manufacturer's instructions manual. The amplified cDNA was used for determining the nucleotide sequence as described above.

Analysis of nucleotide and deduced amino acid sequences

The molecular weight (MW) and isoelectric point (pI) of protein were calculated by Compute pI/Mw tool (http://web.expasy.org/compute_pi/) [Gasteiger *et al*., 2005]. The transmembrane region of HN protein was predicted by the SOSUI system (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) [Hirokawa *et al*., 1998]. The alignment of nt and deduced amino acid (aa) sequences and calculation of evolutionary distance in nt substitutions per site were conducted using the Clustal X program [Larkin *et al*., 2007]. Phylogenetic trees were generated by Neighbor-Joining method [Saitou and Nei, 1987] with 1,000 bootstraps using Clustal X program and then visualized with NJPlot [Perrière and Gouy, 1996]. The nucleotide sequence data reported in this study have been deposited in the DDBJ database under the accession numbers LC041132. Whole genome sequences of other APMVs for comparison with APMV/Shimane67 were from the following sources (abbreviation and accession number): APMV-1/goose/Alaska/415/91 (APMV-1/415, AB524405); APMV-1 strain LaSota (APMV-1/LaSota, AF077761); APMV-1 strain SF02 (APMV-1/SF02, AF473851); APMV-2/chicken/California/Yucaipa/56 (APMV-2/Yukaipa, EU338414); APMV-2/finch/Northern Ireland/Bangor/73 (APMV-2/Bangor, HM159995); APMV-3/parakeet/Netherland/449/75 (APMV-3/NLD, EU403085); APMV-3/turkey/Wisconsin/

68 (APMV-3/WI, EU782025); APMV-4/duck/Hong Kong/D3/75 (APMV-4/HK/D3, FJ177514); APMV-4/KR/YJ/06 (APMV-4/KR/YJ, EU877976); APMV-5/budgerigar/Kunitachi/74 (APMV-5/Kunitachi, GU206351); APMV-6/duck/Hong Kong/18/199/77 (APMV-6/HK/D199, EU622637); APMV-6/duck/Italy/4524-2/07 (APMV-6/ITA/4524-2, GQ406232); APMV-7/dove/Tennessee/4/75 (APMV-7/TN, FJ231524); APMV-8/goose/Delaware/1053/76 (APMV-8/DE, FJ215863); APMV-8/pintail/Wakuya/20/78 (APMV-8/Wakuya, FJ215864); APMV-9/duck/New York/22/78 (APMV-9/NY, EU910942); APMV-10/penguin/Falkland islands/324/2007 (APMV-10/FLK, HM147142); APMV-11/common snipe/France/100212/2010 (APMV-11/FRA, JQ886184); and APMV-12/wigeon/Italy/3920-1/2005 (APMV-12/ITA/3920-1, KC333050).

Results and Discussion

Genomic features of APMV/Shimane67

The genome characteristics of APMV/Shimane67 and some other APMVs are summarized in Table 4. The genome of APMV/Shimane67 comprised 16,146 nt that were slightly AU-rich (A 25.9%, C 22.1%, G 20.5%, and U 31.5%). The genome length was in agreement with the "rule of six" and could contribute to efficient replication of APMV/Shimane67 [Calain and Roux, 1993]. The genome of APMV/Shimane67 was the fifth longest among APMVs. With the exception of APMV/Shimane67, there are only four APMVs, which have genomes longer than 16,000 nt: APMV-3 with 16,182–16,272 nt; APMV-5 with 17,262 nt; APMV-6 with 16,174–16,236 nt; and APMV-11 with 17,412 nt [Briand *et al*., 2012; Chang *et al*., 2001; Kumar *et al*., 2008; 2010; Samuel *et al*., 2010; Tian *et al*., 2012; Xiao *et al*.,

2010]. The genome of APMV/Shimane67 contained six viral protein genes in the order 3′-N-P-M-F-HN-L-5′, which was identical to the other APMVs, except for APMV-6. The SH protein gene existing in the APMV-6 genome was not present in the APMV/Shimane67 genome. The full genome sequence of APMV/Shimane67 had the highest nt identity with APMV-12/ITA/3920-1 (62.2%), intermediate nt identities with APMV-1 and -9 (53.7%–55.0%), and lower nt identities with APMV-2, -3, -4, -5, -6, -7, -8, -10, and -11 (41.9%–44.8%) (Table 5). Among APMVs, except APMV/Shimane67, APMV-1 and APMV-9 had the highest inter-serotype genome identity of approximately 59%, whereas APMV-3/NLD and APMV-3/WI had the lowest intra-serotype genome identity of 67.1% (data not shown). Furthermore, three different levels of nt and aa identities between APMV/Shimane67 and APMV-12 (high), APMV-1 and -9 (intermediate), and the remaining other APMVs (low) were observed in the five genes (N, P, M, HN, and L) and F gene [Chapter 1].

The Le sequence of APMV/Shimane67 was 55 nt in length, which was the same as that of other APMVs. The 776 nt in length Tr sequence of APMV/Shimane67, which was the longest in the Avulavirus genus, contributed to the relatively large genome size of APMV/Shimane67. Marcos *et al*. [2007] reported that the first 18 nt and thrice-repeated motif (3′-NNNNGC-5′) at 73–90 nt of the APMV-1 genomic and antigenomic RNA functioned as promoters of viral genome replication. Moreover, similar sequence motifs were reported in APMV-2 genome [Subbiah *et al*., 2008]. Fourteen nt of the Le and Tr sequences, with the exception of the 9th nt from the 3′- and 5′-terminal of the genome, were complementary in the APMV/Shimane67 genome (Fig. 5a). Twelve nt of the 3′ Le and 11 nt of the 5′ Tr of the APMV/Shimane67 genome were relatively conserved with those of other APMVs and were completely matched with APMV-1 (Fig. 5b and 5c). The thrice-repeated motifs (3′-NNNNGC-5′) [Marcos

et al., 2007], 3′-GGUGGC-5′, 3′-ACAAGC-5′, and 3′-TCAGGC-5′ with 73–90 nt from the 3′-terminus of the genome and 3′-AUUUCC-5′, 3′-UCCAGC-5′, 3′-UUCAGC-5′ with 73–90 nt from the 3′ terminus of the antigenome were found in the APMV/Shimane67 genome. These sequences presumably act as the promoter for replication of APMV/Shimane67 genome as well as APMV-1. The GS signal of APMV/Shimane67 was well preserved and its consensus sequence was "ACGGGCAGAA" (Fig. 6a). The second nucleotide of the GS sequence of NP, P, M, F, and HN gene was C, whereas that of the L gene was replaced with G instead of C. In contrast, the preservation of GE signal sequences of APMV/Shimane67 was relatively low. The consensus sequence of the GE signal was $TTAAGA_{5\text{-}6}$, whereas that of the M gene diverged at positions 1 (A), 2 (A), 3 (T), and 5 (T). The HN gene had one nt difference at the fifth position (T); whereas the L gene also contained two nt differences at the second (A) and third (G) positions. Moreover, the less conservation of GE signal was reported in APMV-9 by Samuel *et al.* [2009]. Naylor *et al.* [2007] reported that nt substitution at the GE signal of avian metapneumovirus SH gene led to an increase in the level of readthrough dicistronic mRNA and reduced the level of expression of the downstream G gene product. The significance of sequence variation found in GE signal is unclear; however, alternation of GE signal could assist in optimal mRNA transcription and expression of APMV/Shimane67 viral proteins. APMV/Shimane67 GS and GE sequences had similarities with those of APMV-1, -9, and -12 (Fig. 6b). The IGS of APMV/Shimane67 varied in nucleotide sequence and length (Fig. 6a). The IGS length of NP-P, P-M, M-F, F-HN, and HN-L junctions were 14 nt, one nt, two nt, 14 nt, and 25 nt, respectively. There was no sequence similarity between APMV/Shimane67 and other APMVs, with the exception of IGS at P-M junction (T) and M-F junction (GT) that were also found in some other APMVs. The

last nt at IGS of APMV/Shimane67 was T at all times, and this could act with the GS sequences to initiate mRNA transcription.

N gene and N protein

The N gene of APMV/Shimane67 was 1,721 nt in length and contained an open reading frame (ORF) that encoded 493 aa, with MW of 54,045 Da and pI of 5.29. The nt sequence of APMV/Shimane67 N gene ORF was 67.6% identical with APMV-12/ITA/3920-1, 58.4%–59.0% identical with APMV-1 and -9, and 47.0%–49.1% identical with the rest of the APMVs. The predicted aa sequence of APMV/Shimane67 N gene demonstrated 74.4%, 56.0%–58.3%, and 36.9%–40.6% identities with APMV-12/ITA/3920-1, APMV-1 and -9, and the rest of the APMVs, respectively (Table 5). There were three highly conserved regions [region 1, QXW(I,V)XXXK(A,C)XT; region 2, FXXT(I,L)(R,K)Φ(G,A)(L,I,V)XT; and region 3, FXXXXYPXXΦSΦAMG] (where X represents any amino acid and Φ represents an aromatic amino acid and either of the residues in parentheses can be present at that position) in the central domain of the N protein of the subfamily *Paramyxovirinae* [Morgan, 1991]. Among these regions, region 3 was particularly important, because this region was thought to be involved in the N–N protein self-assembly. The replacement of ^{324}F, which is the first F residue of region 3, of the Sendai virus N protein to V or I demolished the self-assembly of the N protein [Paldurai *et al*., 2009]. The N protein of APMV/Shimane67 contained aa sequences similar to these motifs: ^{171}QIWVTLAKAMT^{181}, ^{266}FFLTLKYGINT^{277}, and ^{322}FAPAEYSLMYSFSMG^{336} (Fig. 7a). The 7th (P) and 13th (A) aa of region 3 motif were replaced with ^{328}S and ^{334}S, respectively. The aa at the position corresponding to ^{334}S was A in all other APMVs that agree with the conserved motif. The aa sequence of region 2 was completely

identical among APMV/Shimane67, APMV-1, and APMV-12. In the N protein of Sendai virus, ^{260}Y was important for N protein function [Myers *et al*., 1997]. Substitution of aa from ^{260}Y to D abolished N protein-RNA binding. The N protein of APMV/Shimane67 contained ^{258}Y, corresponding to ^{260}Y of the Sendai virus N protein that was similar with other APMVs. These results suggest that these conserved aa motifs presumably play important roles in APMV/Shimane67 N protein function as well as in that of other paramyxoviruses. By in vitro protein binding assay, the NP-P protein interactive domain was mainly located at the amino (N) terminal 25 aa of APMV-1 N protein [Kho *et al*., 2004].

Although only 10 aa were fully identical between APMV/Shimane67 and APMV-1/LaSota in this 25 aa region, six additional aa (2nd, 4th, 9th, 10th, 14th, and 17th aa) and three aa (21st, 23rd and 25th) were conserved as the "Clustal strong group (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW)" and "Clustal weak group (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY)" (refer to the on-line help; http://www.clustal.org/download/clustalx_help.html) (Fig. 7b), suggesting that the N-terminal 25 aa of APMV/Shimane67 likely functions as the NP-P binding domain.

P gene and P/V/W proteins

The P gene of APMV/Shimane67 genome was 1,512 nt long. The P gene of avulaviruses encodes three proteins P, V, and W [Samal, 2011]. The P protein is expressed from mRNA that is directly transcribed from the genomic RNA. The nt identities of APMV/Shimane67 P protein ORF were 61.4% with APMV-12/ITA/3920-1, 51.2%–53.8% with APMV-1 and -9, and 38.7%–42.9% with the other remaining APMVs (Table 5). The two residual proteins, V and W, are produced through mRNA

that contains insertion of one or two non-template G residues at the RNA editing site. Recently, Briand *et al.* [2012] found that the P gene of APMV-11/FRA genome primarily encoded V protein and by insertion of one or two Gs at the RNA editing site, W or P protein was expressed as observed with mumps virus and simian virus 5. The coding patterns of P, V, and W proteins of APMV/Shimane67 were identical with those of other APMVs, except for APMV-11. The nt at positions 436–444 of the P gene ORF, 5′-AAAAAAGGG-3′ (mRNA sense), was predicted as the RNA editing site of APMV/Shimane67 (Fig. 8a). This nt sequence was completely identical with that of APMV-5/Kunitachi, APMV-6/HK/D199, APMV-7/TN, APMV-9/NY, and APMV-12/ITA/3920-1. The deduced aa lengths of P, V, and W proteins were 397 aa (MW 41,992 Da, pI 6.22), 241 aa (MW 25,929 Da, pI 4.80), and 150 aa (MW 15,621 Da, pI 4.52), respectively. These three proteins shared the N-terminal 148 aa. The aa identities in P and V proteins between APMV/Shimane67 and other APMVs were comparatively low, ranging from 20.0% (APMV-7/TN) to 51.3% (APMV-12/ITA/3920-1) and 19.3% (APMV-3/WI) to 45.2% (APMV-12/ITA/3920-1), respectively (Table 5). Seven cysteine residues (^{198}C, ^{202}C, ^{214}C, ^{216}C, ^{219}C, ^{223}C, and ^{226}C) and the ^{179}HRRE^{182} and ^{197}WCNP^{200} motifs were conserved at the C-terminus of APMV/Shimane67 V protein as well as in other APMVs (Fig. 8b). These conserved aa would be important for V protein function. Jahanshiri *et al.* [2005] demonstrated that the 45 aa at positions 247–291 of the APMV-1 P protein involved in both P-P and P-NP protein interactions. The alignment of P protein aa sequences revealed a similarity between APMV/Shimane67 and APMV-1/LaSota (29 identical, eight “Clustal strong group,” and four “Clustal weak group” aa) (Fig. 8c), suggesting that this 45 aa region of APMV/Shimane67 may involve P-P and NP-P protein interactions.

M gene and M protein

The M gene of APMV/Shimane67 contained 1,335 nt and encoded a protein with 366 aa with MW of 39,902 Da and pI of 9.63. The ORF and predicted aa sequence of APMV/Shimane67 M gene had identities in 65.1% and 73.6% with APMV-12/ITA/3920-1, 55.3%–56.7% and 50.8%–53.9% with APMV-1 and -9, and 38.9%–44.2% and 24.4%–30.4% with the remaining APMVs, respectively (Table 5). There are two functional aa sequences, one is nuclear localization signal (NLS) and the other is the late domain in the M protein of APMV-1 [Coleman and Peeples, 1993; Duan *et al*., 2014]. NLS comprised a bipartite clustering of basic amino acids (*e.g.*, ^{247}RKGKKVTFDKLEKKIRS^{263} of APMV-1/LaSota M protein). In the APMV/Shimane67 M protein, the putative bipartite NLS motif (^{248}KGNKISVDKLELKIRR^{263}) was found at positions 248–263 aa (Fig. 9a). This aa sequence was located at almost the same position as APMV-1; however, the number of basic aa of APMV/Shimane67 was fewer by two or three than that of APMV-1/LaSota, 415 and SF02. Recently, Duan *et al.* [2013] demonstrated that although ^{262}R and ^{263}R were critical for nuclear localization of APMV-1 M protein, either ^{262}R or ^{263}R was sufficient. The additional putative bipartite NLS, which was found at the C-terminal region of NDV and APMV-3/NLD M proteins [Coleman and Peeples, 1993; Shihmanter *et al*., 2005], also existed at positions 349–364 aa (^{349}KIEKSGRNGKFNPFKK^{364}) in the M protein of APMV/Shimane67 (Fig 9a). However, Coleman and Peeples [1993] demonstrated that this C-terminal NLS motif did not act as NLS in the APMV-1 M protein. Although both regions in the M protein of all types of APMVs are rich in basic amino acids, the aa sequences are not strictly conserved. The FPIV late domain that contributes to efficient viral release and replication was at positions 23–26 aa of the APMV-1 M protein [Duan *et al*., 2013]. A

similar aa sequence motif (FPVV) was found at positions 23–26 aa in the M protein of APMV/Shimane67 (Fig. 9b). Furthermore, a comparable sequence motif in other APMV M proteins was reported [Samuel *et al*., 2010; Xiao *et al*., 2009; Xiao *et al*., 2010] and found by alignment of aa sequences from APMVs (Fig. 9b). These preserving properties in the APMV/Shimane67 M protein suggested that these regions could be functional homologues of NLS and late domain motif.

HN gene and HN protein

The HN gene for APMV/Shimane67 comprised 2,070 nt with one ORF that encoded a protein with 610 aa with MW of 67,492 Da and pI of 5.42. The nt and deduced aa of APMV/Shimane67 HN gene had the highest identities with APMV-12/ITA/3920-1 at 61.3% and 60.5%, followed by that with APMV-1 and -9 at 54.5%–56.9% and 54.7%–55.3%, and other APMVs at 42.9%–45.5% and 30.7%–37.9%, respectively (Table 5). The HN protein of paramyxoviruses is a type II transmembrane protein. The transmembrane region of APMV/Shimane67 HN protein was predicted at position 25–47 by the SOSUI system [Hirokawa *et al*., 1998]. Five N-glycosylation motifs (N-X-S/T) were found at positions 119, 341, 392, 481, and 604 aa of APMV/Shimane67 (Fig. 10a). Two of these, at positions 119 and 392 aa, were relatively conserved among avulaviruses because the HN proteins of APMV-1, -2, -5, -6, -7, -8, -9, -10, and -12, and APMV-2, -4, -5, -6, -7, -8, and -11 also contained the N-glycosylation motif at positions corresponding to the 119 and 392 aa, respectively. Furthermore, the N-glycosylation motif at positions 341 and 481 aa was found in the HN protein of APMV-1. Four N-glycosylation motifs at positions 119, 341, 433, and 481 of APMV-1 HN protein were actually glycosylated and influenced biological activities and viral pathogenicity [McGinnes and Morrison, 1995; Samuel *et al*., 2010].

These conserved potential N-glycosylation sites in the HN protein of APMV/Shimane67 may possibly be glycosylated and relate to HN protein function. The sialic acid-binding motif NRKSCS preserved among HN proteins of paramyxoviruses [Mirza *et al*., 1994] was identified at positions 234–239 of the HN protein of APMV/Shimane67. Twelve aa (^{174}R, ^{175}I, ^{198}D, ^{236}K, ^{258}E, ^{299}Y, ^{317}Y, ^{401}E, ^{416}R, ^{498}R, ^{526}Y, and ^{547}E) engaged in sialic acid-binding and neuraminidase (NA) activity of APMV-1 [Connaris *et al*., 2002; Crennell *et al*., 2000; Iorio *et al*., 2001] were also completely conserved in the HN protein of APMV/Shimane67 (data not shown). Moreover, the HN protein of other APMVs perfectly possessed these aa. The aa sequence alignment of HN protein from APMVs demonstrated that the HN protein of APMV/Shimane67, APMV-9/mallard/Italy/5709/2007, and APMV-12/ITA/3920-1 contained aa sequences corresponding to the 45 aa extension, which was found in the C-terminal end of HN protein of lentogenic APMV-1, such as strains D26, Ulster, and 415 (Fig. 10b). The removal of C-terminal 42 aa extension by proteolytic cleavage converts into the biologically active form of lentogenic APMV-1 HN protein [Nagai and Klenk, 1977; Nagai *et al*., 1976]. Recently, Yuan *et al.* [2012] demonstrated that the C-terminal 45 aa extension of lentogenic APMV-1 HN protein autoinhibited receptor binding and catalytic activities by blocking the NA-active and second sialic binding sites. In addition, the intermolecular disulfide bond formed by the cysteine residue at 596 aa in the C-terminal extension was critical for the expression of autoinhibition of HN activities. Although there was no cysteine residue and aa identity in the C-terminal extension region of APMV/Shimane67, the three extreme C-terminal residues (SWP), which masked the NA active site in the APMV-1 HN protein, were identical to APMV-1. Further studies should clear whether the HN protein of APMV/Shimane67 requires proteolytic cleavage to activate receptor binding

and NA activity.

L gene and L protein

The APMV/Shimane67 L gene was 6,763 nt long and encoded a single ORF, giving a deduced protein of 2,199 aa (MW 248,209 Da and pI 6.86). The ORF and aa sequences of APMV/Shimane67 L gene were 63.1% and 65.5% identical with APMV-12/ITA/3920-1, and 56.0%–57.0% and 53.4%–55.6% identical with APMV-1 and -9, respectively. In contrast, fewer identities (43.1%–47.6% in nt and 32.0%–40.3% in aa sequences) were observed between APMV/Shimane67 and APMV-2 to -8, APMV-10, and APMV-11 (Table 5). The L protein of non-segmented negative-strand RNA viruses contains six conserved aa domains [Poch *et al*., 1990]. Among these, domain III at positions 637–828 aa of APMV-1 was relatively highly conserved among avulaviruses, including APMV/Shimane67 (Fig. 11a). The QGDNQ sequence, identified in the motif C of domain III as the putative active site for nucleotide polymerization [Malur *et al*., 2002], was found on the APMV/Shimane67 L protein at positions 747–751 aa and was conserved among all APMVs. The putative ATP-binding site comprising K-$X_{18\text{-}21}$-G-X-G-X-G was found at positions 1756–1782 aa of domain IV of APMV/Shimane67 L protein (Fig. 11b) [Poch *et al*., 1990; Harcourt *et al*., 2001]. Amino acid differences were found in APMV-1 (K-X_{21}-A-X-G-X-G), APMV-3 (R-X_{21}-G-X-G-X-G), APMV-4 (R-X_{21}-G-X-G-X-G), APMV-5 (K-X_{21}-A-X-G-X-G), APMV-6 (K-X_{21}-A-X-S-X-G), APMV-7 (K-X_{21}-C-X-G-X-G), and APMV-9 (K-X_{21}-A-X-G-X-G).

Phylogenetic analysis

To understand genetic relationships, phylogenetic trees were constructed based

on the nt sequences of full length genome and the N, P, M, HN, and L genes of APMV/Shimane67 and other viruses from the five members of the family *Paramyxovirinae* (*Avulavirus*, *Henipavirus*, *Morbillivirus*, *Respirovirus*, and *Rubulavirus*) (Fig. 12). Essentially, all trees demonstrated similar grouping patterns except for APMV-7 and -11 and clearly divided according to the five classifications of paramyxoviruses. Furthermore, a similar phylogenetic tree was constructed by the F gene sequences [Chapter 1]. APMV/Shimane67 was classified as a member of the genus Avulavirus, but distinct from other APMVs. The APMV/Shimane67 fell into the group that comprised APMV-1, -9, and -12 and had the closest relationship with APMV-12/ITA/3920-1. This branching pattern was supported by high bootstrap values. When comparing full genome sequences, the evolutionary distance between APMV/Shimane67 and APMV-12/ITA/3920-1 was 0.357 nt substitutions per site. This distance was longer than those observed within serotypes, such as the distance between APMV-2/Yucaipa and APMV-2/Bangor at 0.291, APMV-3/NLD and APMV-3/WI at 0.292, and APMV-6/T WN/Y1 and APMV-6/ITA/4524-2 at 0.265. These strains were antigenically and genetically divided into subgroups in each serotype [Anderson *et al*., 1987; Kumar *et al*., 2010; Subbiah *et al*., 2010; Xiao *et al*., 2010]. Thus, the APMV/Shimane67 and APMV-12/ITA/3920-1 had greater degrees of genetic diversity than that found within the subgroups of APMV-2, -3, or -6.

In conclusion, in the present study, we determined the nt sequences of N, P, M, HN and L genes, Le, Tr, and IGS of APMV/Shimane67. Together with our previous study of the F gene sequencing [Chapter 1], whole genome sequencing of APMV/Shimane67 was completed. The genome organization and presence of putative functional aa sequences or motifs found in other paramyxovirus were basically conserved in APMV/Shimane67. However, the genome of APMV/Shimane67 had

differences with these APMVs in terms of some details, such as the nt length of whole genome, six genes (N, P, M, F, HN, and L) and IGS, and predicted aa length of six genes, which were almost unique in APMV/Shimane67. Although APMV/Shimane67 was phylogenetically grouped with APMV-1, APMV-9, and APMV-12, the Tr region of APMV/Shimane67 was obviously longer than that of APMV-1, APMV-9, and APMV-12. The homology analysis and phylogenetic trees indicated that APMV/Shimane67 had the closest relationship with APMV-12/ITA/3920-1; however, the distance of the relationship between APMV/Shimane67 and APMV-12/ITA/3920-1 was longer than that observed within the subgroups of APMV-2, -3, or -6. Although there are no defined genetic criteria to differentiate the typing of APMVs, recent studies attempted and demonstrated that classification based on genetic analysis was correlated with conventional serotyping [Briand *et al*., 2012; Miller *et al*., 2010;Samal, 2011; Terregino *et al*., 2013]. Our previous serological analysis demonstrated that APMV/Shimane67 was distinct from APMV-1, -2, -3, -4, -6, and -7 [Chapter 1]. Moreover, sequence analysis of F gene of APMV/Shimane67 indicated that APMV/Shimane67 was genetically diverse from other APMV serotypes. Thus, the results obtained in this study emphasized the possibility that APMV/Shimane67 would be a novel APMV type.

Table 4. Comparison of nucleotide and amino acid length between APMV/Shimane67, APMV-1, -9, and-12

Gene		APMV/ Shimane67	APMV-1/ LaSota	APMV-1/ 415	APMV-1/ SF02	APMV-9/ NY	APMV-12/ ITA/3920-1
Full genome (nt)		16,146	15,186	15,198	15,192	15,438	15,132
Leader (nt)		55	55	55	55	55	55
Trailer (nt)		776	114	114	114	47	60
N	3'-noncoding (nt)	60	66	66	66	66	66
	ORF (nt)	1,482	1,470	1,470	1,470	1,470	1,482
	5'-noncoding (nt)	179	210	210	217	192	162
	Total (nt)	1,721	1,746	1,746	1,753	1,728	1,710
	Amino acid	493	489	489	489	489	493
N-P intergenic (nt)		14	2	2	1	19	7
P	3'-noncoding (nt)	95	83	83	83	113	95
	ORF (nt)	1,194	1,188	1,200	1,188	1,260	1,218
	5'-noncoding (nt)	223	180	180	180	248	190
	Total (nt)	1,512	1,451	1,463	1,451	1,621	1,503
	Amino acid	397	395	399	395	419	405
P-M intergenic (nt)		1	1	1	1	6	3
M	3'-noncoding (nt)	34	34	34	34	34	34
	ORF (nt)	1,101	1,095	1,095	1,095	1,095	1,095
	5'-noncoding (nt)	200	112	112	112	161	151
	Total (nt)	1,335	1,241	1,241	1,241	1,290	1,280
	Amino acid	366	364	364	364	364	364
M-F intergenic (nt)		2	1	1	1	30	11
F	3'-noncoding (nt)	45	46	46	46	55	52
	ORF (nt)	1,638	1,662	1,662	1,662	1,656	1,641
	5'-noncoding (nt)	175	84	84	84	67	88
	Total (nt)	1,858	1,792	1,792	1,792	1,778	1,781
	Amino acid	545	553	553	553	551	546
F-HN intergenic (nt)		14	31	31	31	22	61
HN	3'-noncoding (nt)	92	91	91	91	97	91
	ORF (nt)	1,833	1,734	1,851	1,716	1,740	1,845
	5'-noncoding (nt)	145	177	59	195	293	136
	Total (nt)	2,070	2,002	2,001	2,002	2,130	2,072
	Amino acid	610	577	616	571	579	614
HN-L intergenic (nt)		25	47	48	47	0	42
L	3'-noncoding (nt)	13	11	11	11	11	11
	ORF (nt)	6,600	6,615	6,615	6,615	6,633	6,609
	5'-noncoding (nt)	150	77	77	77	69	106
	Total (nt)	6,763	6,703	6,703	6,703	6,713	6,727
	Amino acid	2,201	2,204	2,204	2,204	2,210	2,202

Table 5　Nucleotide (nt) and deduced amino acid (aa) sequence identities between APMV/Shimane67 and other APMVs (%)

Virus	Full genome	N		P		V	M		HN		L	
		nt	aa	nt	aa	aa	nt	aa	nt	aa	nt	aa
APMV-1/LaSota	55.0	58.6	57.9	53.8	42.3	38.5	56.7	53.3	56.9	55.1	56.6	54.4
APMV-1/415	55.0	59.0	58.3	53.6	40.5	34.9	56.5	53.9	56.1	55.3	56.7	55.6
APMV-1/SF02	54.6	58.4	56.4	52.3	42.8	37.6	56.3	52.8	56.1	55.1	57.0	55.5
APMV-2/Yucaipa	44.8	47.9	39.8	39.6	24.2	25.2	43.1	27.8	44.4	34.1	46.3	37.5
APMV-2/Bangor	44.6	47.0	39.3	40.1	22.9	24.4	43.8	27.8	43.3	34.5	46.4	32.0
APMV-3/NLD	41.9	48.5	38.7	41.0	23.7	20.5	40.2	25.2	43.8	37.2	43.6	33.2
APMV-3/WI	41.9	47.7	38.6	40.0	24.2	19.3	41.1	25.5	45.4	36.9	43.8	33.4
APMV-4/HK/D3	42.3	48.8	37.0	40.5	21.7	24.1	39.4	24.5	44.2	36.5	43.4	32.5
APMV-4/KR/YJ	42.3	48.4	37.0	39.7	22.5	25.1	40.8	24.4	44.0	36.3	43.1	32.6
APMV-5/Kunitachi	43.5	48.7	36.9	40.9	22.9	25.2	43.4	29.9	43.4	33.8	46.4	37.5
APMV-6/HK/D199	42.9	49.1	40.6	41.3	22.4	26.7	43.6	29.6	44.3	31.9	47.5	38.4
APMV-6/ITA/4524-2	43.4	49.0	40.2	42.9	22.7	26.1	43.6	30.4	42.9	30.7	47.6	38.5
APMV-7/TN	44.8	49.1	39.1	38.7	20.0	26.7	44.2	30.3	45.2	37.9	47.5	40.3
APMV-8/DE	44.8	47.8	39.2	40.6	24.9	27.2	42.4	29.5	43.8	34.2	47.1	39.1
APMV-8/Wakuya	44.7	47.7	39.4	40.6	25.2	26.7	42.0	28.7	43.4	33.8	47.3	39.1
APMV-9/NY	53.7	58.6	56.0	51.2	40.4	32.1	55.3	50.8	54.5	54.7	56.0	53.4
APMV-10/FLK	44.6	47.9	40.4	39.0	23.3	23.3	42.0	28.1	43.4	35.2	46.4	38.0
APMV-11/FRA	43.0	48.1	40.2	40.1	23.7	26.6	38.9	25.8	45.5	37.4	47.0	40.1
APMV/12/ITA/3920-1	62.2	67.6	74.4	61.4	51.3	45.2	65.1	73.6	61.3	60.5	63.1	65.5

a

```
Leader   3’- UGGUUUGUCUCUAAGACAUUCCAUGGAAUUUUAUGCUUAAUUAUUA
             |||||||| |||||  |  |   |  | | |         | |||
Trailer  5’- ACCAAACAAAGAUUUGGAGAACAAAAUAAUACCGACUUGUCAUAUA
```

b

```
                          3’-end
APMV/Shimane67            UGGUUUGUCUCUAAGACAUUCCAUGGAAUUUUAUGCUUAAUUAUUAACUUGAUCG
NDV/LaSota                ............U..G..C..A...CU.....CC....CC.CG.......C.G..
APMV-2/Yucaipa            ........UC..U.UC.....GU..C.U..AG.AU..AUU..GG..U...AGG.A
APMV-3/NLD                ..A.......U.U.AUU..GAACAAUC..CAGCG...A.U.AGGCGAU.AU..AA
APMV-4/HK/D3              ..C...U...UCUUAUUU.C.GUCUUCGGAAA..UU.CCU.GGGACC.GAC.G.A
APMV-5/Kunitachi          ........UC..U.CG.G...GUA...U...A.AUU.AUCGC.C..U.ACU..A.
APMV-6/HK/D199            ........UC..UU.GU..A.G.ACCCC.GAA.....CUCGCGAAC.U..UGG.A
APMV-7/TN                 ........UC..G.CGU....GUCAC.U...G.AAA...U..U...UUGAA.G.A
APMV-8/DE                 ........UC..U.CGUUC.GGU..CCC..GA.AUU.AUU..G...G...UG.AA
APMV-9/NY                 ........U...UUA......U...C.....CUG....C..CG..G...GA.G.A
APMV-11/FRA               .......GUC...GUGUGAC.GC..C.C.C.ACAU...UU...A..G...C...A
APMV-12/ITA/3920-1        ...AA.....UAG.....AA.....C...A..UCU..ACU....AU....U.U.U
```

c

```
                          5’-end
APMV/Shimane67            ACCAAACAAAGAUUUGGAGAACAAAAUAAUACCGACUUGUCAUAUAUCGACAUAU
NDV/LaSota                ..................U...UG.CGAG.CUA.ACUCAAGA...AU.GUG.GC.A
APMV-2/Yucaipa            ....G...UG..A.A.C.A.G...CG...CU.A.UGA.UAUGC.GUG.UUAUAU.
APMV-3/NLD                ..UAA.....A.G..AU.AUGAUUUU...AU.A.UC..CAACCU.U.GUU.U.UG
APMV-4/HK/D3              ..UAA.U...AGACAUA----------------------------------
APMV-5/Kunitachi          .........G....AC....GUU..G.U.AUUU..A.CA.AU.U.UGUUUACACC
APMV-6/HK/D199            .........G..AA.CAUA.G.UUUU.G.CUAU..UCCU.GUCGAC.UUC..GG.
APMV-7/TN                 .........G..C.GCAUA.G..GUG....A..UUUUAAUAA.A.AUAACUUUCG
APMV-8/DE                 ....G.A.UG..A.ACAG..AAU.CGGU.AUUUUCAC.AAU.C.AU.UUUU..UA
APMV-9/NY                 ............AA.U.UA.GAU.CG.U.A.GACCGAA..AGC.AC.G------
APMV-11/FRA               .......CAG....A.A.U.GG.GU.CU.AUAA.UUGA..A....UGU.UUUGUC
APMV-12/ITA/3920-1        ................UGG.UU.CG.CU.UAGU.G..UCTTCUCGGGUUAC.U.
```

Fig. 5

a

	Gene start	Gene stop	Intergenic
NP	UGCCCGUCUU	AAUUCUUUUUU	GUUUGACUGUCUUA
P	UGCCCGUCUU	AAUUCUUUUUU	A
M	UGCCCGUCUU	UUAUAUUUUUU	CA
F	UGCCCGUCUU	AAUUCUUUUU	CUAGGCCUAAGUUA
HN	UGCCCGUCUU	AAUUAUUUUUU	ACUGUUACAAUCACUGUUUUUCGGA
L	UCCCCGUCUU	AUCUCUUUUUU	
Consensus	UGCCCGUCUU	AAUUCU$_{5-6}$	

b

	Gene start	Gene stop
APMV/Shimane67	ACGGGCAGAA	TTAAGA$_{5-6}$
APMV-1/LaSota	ACGGGTAGAA	TTAAGA$_{6}$
APMV-2/Yucaipa	GGGGGCGAMA	TTAATA$_{6}$
APMV-3/NLD	AGGAGCGGAA	TTAATA$_{5-7}$
APMV-4/HK/D3	GTGGGGAAGG	TTAATTA$_{5}$
APMV-5/Kunitachi	GGGGGAANNN	TTAWNA$_{5-7}$
APMV-6/HK/D199	GAG$_{5-6}$AAG	TTAN$_{1-2}$TA$_{4-6}$
APMV-7/TN	GAGGGNGANN	TTANNAAANA$_{1-3}$
APMV-8/DE	GGGGGCGMN	TTAANA$_{6}$
APMV-9/NY	ACGGGTAGAA	TTAANA$_{6}$
APMV-10/FLK	GGGGCGACCC	TTAAGA$_{6}$
APMV-11/FRA	GCGGGCGAAG	TTAATA$_{6-7}$
APMV-12/ITA/3920-1	ACGGGCAGAA	TTAAGA$_{6-8}$

Fig. 6

a

	Regin 1	Region 2	Region 3
	V C	V LK AI	
Motif	QXWIXXXKAXT	FXXTIRΦGLXT	FXXXXYPXXΦSΦAMG
	171 181	267 277	322 336
APMV/Shimane67	QIWVTLAKAMT	FFLTLKYGINT	FAPAEYSLMYSFSMG
APMV-1/LaSota	.V...V.....	.....	......AQL...A..
APMV-2/Yucaipa	...IAAI.S..	.....RF..G.	....NF.TL..YA..
APMV-3/NLD	...ILV.....	.LT..R...G.	...GN...L..YA..
APMV-4/HK/D3	...ILV.....	.LT..R...G.	...GNFPH...YA..
APMV-5/Kunitachi	....SAM.S..	.....RF..G.	...GN.P.I..YA..
APMV-6/HK/D199	....AAM.S..	.....RF..G.	...GN.P....YA..
APMV-7/TN	.A..AAI.S..	.....RF.VG.	....N.T.L..YA..
APMV-8/DE	....SAI.S..	......F.VG.	....N..T...YA..
APMV-9/NY	.V..TV..S..	......F.VG.	....E.AQL..YA..
APMV-10/FLK	...*I*AAI.C..	.....RF..G.	....N..TL..YA.D
APMV-11/FRA	....AVI.S..	.....RF.LG.	...SN...L..YA..
APMV-12/ITA/3920-1	.L.....	.....	.....FA.....A..

b

	1 25
APMV/Shimane67	MASIFAEYDKLLDSQTKCNKGASPA
APMV-1/LaSota	.S.V.D..EQ..AA..RP.GAHGGG
APMV-2/Yucaipa	.S.V.S..QA.Q.QLV.PATRRADV
APMV-3/NLD	..G..NT.ELFVKD..CMH.R.ASL
APMV-4/HK/D3	..GV.SQ.ERFV.N.SQVSRKDHRS
APMV-5/Kunitachi	.S.V.TD....QEYLVTPC.KRVDG
APMV-6/HK/D199	.S.V.TD.A..Q.ALVAPS.RKVDS
APMV-7/TN	.S...TD.TN.QEQLVRPVGRKVDN
APMV-8/DE	.S.V.N..QA.QEQLV.PAVRRPDV
APMV-9/NY	.S...N..ES..E..L.PTGSNVLG
APMV-10/FLK	.....N..QS.Q.QLV.PAPRRVD.
APMV-11/FRA	..QALR..SS.Q.QLV.PSPRRQD.
APMV-12/ITA/3920-1	...V.E.....E...RPS..V...

Fig. 7

a

```
                          2331      2334
APMV/Shimane67            AAAAAAGGGCAGAT
APMV-1/LaSota             T.........CC..
APMV-2/Yucaipa            T........G.AGG
APMV-3/NLD                TTT......GGCCC
APMV-4/HK/D3              TTT......GG.CC
APMV-5/Kunitachi          ..........CC.G
APMV-6/HK/D199            .........G.A.G
APMV-7/TN                 .........TTAG.
APMV-8/DE                 T.........CCTC
APMV-9/NY                 ..........TC.A
APMV-10/FLK               T..........CTG
APMV-11/FRA               .G..GG...TC...
APMV-12/ITA/3920-1        .........ATC..
```

b

```
                          192   +   +           + +  +   +  + 230
APMV/Shimane67            IITTSWCNPDCAPITSTPQQFACRCGKCPKFCRLCSRDV
APMV-1/LaSota             VT.I.....S.S..KAE.R.YP.I..S..AT....AS.D
APMV-2/Yucaipa            LEV......V.S..R E.RREK.T..T..ES.I..RQPN
APMV-3/NLD                FQ.ET....A.S.V.AF.K.YK.A RQ..R..D..FNPA
APMV-4/HK/D3              .ELVE....G.TAVRIE.TRLD.V..H..TI.S..MY.D
APMV-5/Kunitachi          CSI.E....T.R...AI.SVQR.T..E..RR.SM.WN.S
APMV-6/HK/D199            C.LAE....V...V.PE.RT.K.I..R..RV.IN.RN.S
APMV-7/TN                 F.VS.....T....RPY.TVER....N.....PG.QSAL
APMV-8/DE                 TRVI.....Q.T..RAR.IYDE....E..TT.IM.RD.K
APMV-9/NY                 IV.I.....V.S.V.YE.RE.T.S..S..TE....AGSH
APMV-10/FLK               TLVE.....N.T..RAY.RREK.I.RR..TT.I..RD.N
APMV-11/FRA               Y.VE.....K....RAN.IREQ....Y..RA.TM.Y
APMV-12/ITA/3920-1        ..SLG....I...V.AE.RK.Q....E..PT....ARDA
```

Fig. 8

```
C                                249                                 293
  APMV/Shimane67                 LILKHVSCCPLIRTDIQQLKTAVAVMEGNISMMKLMDPGNANISS
  APMV-1/LaSota                  .V..QT.SI.MM.SE......S.....A.LG...IL...C.....
  APMV-2/Yucaipa                 EV.RLLGIL.G.KNE.S...AT..L.SNQ.ASIQIL.....GVK.
  APMV-3/NLD                     Q.Q.L..TL.Q.K...ASIRNMQ.AL..QL..IRIL....CSE..
  APMV-4/HK/D3                   ALVRNLAVL.QL.NEVAAIR.SQ.MI..TLNSI.IL....YQE..
  APMV-5/Kunitachi               ELV.SI.TTNQ.KS.T..I.ASC.LL..QMA.IQVLE..H.DV..
  APMV-6/HK/D199                 ELT.F..PIQQ.KA.M.IV..SC..I..QLATVQILE..HSS.R.
  APMV-7/TN                      K.TEL.ALI.IL.S...AV.GSC.LL.AQLASIRIL....IGV..
  APMV-8/DE                      D.A.V.NTT....N..N...ATT.LMSNQ.ASIQIL.....GVR.
  APMV-9/NY                      .V.R.L.SM.A..N....V...M..L.A..G...IL...S.H...
  APMV-10/FLK                    D.SRNAALF..V.N..N...ATT.L.STQLASIQVL.....GYK.
  APMV-11/FRA                    DVM.LTAQI.G.KN.VM...ATTGLLSTQMASIMVL...H.S...
  APMV-12/ITA/3920-1             LV...A.AV.I.KN.L..I..TL..L....G...I......T...
```

Fig. 8 (Continued)

a

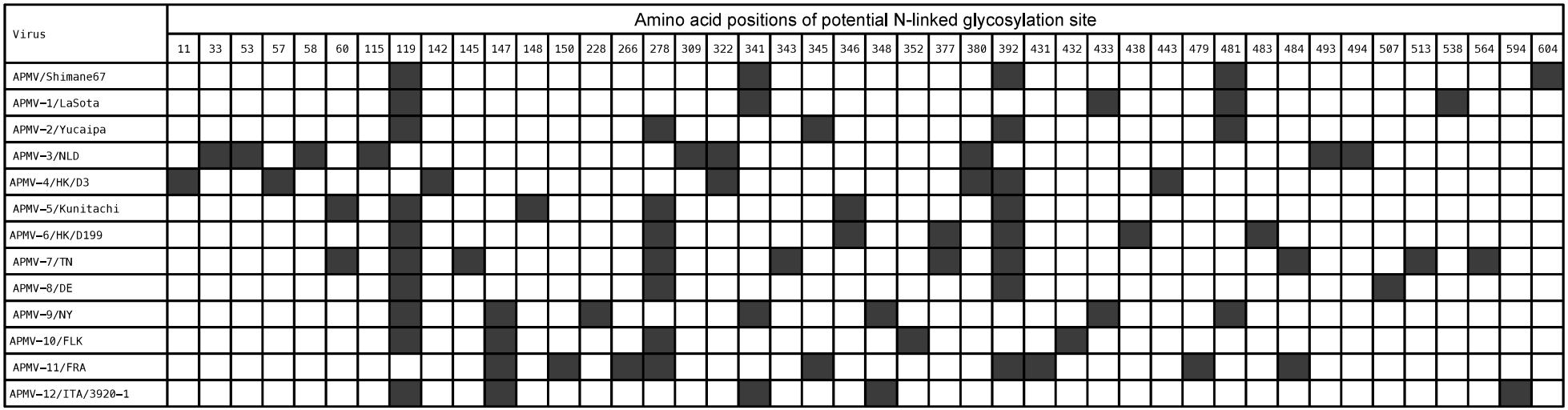
```
                         243                     267  346                 366
APMV/Shimane67           SCLDSKGNKISVDKLELKIRRMSIS   GSTKIEKSGRNGKFNPFKKTG*
APMV-1/LaSota            TTV.R..K.VTF....K...SLDL.   T...L..GHTLA.Y.....*
APMV-2/Yucaipa           KKTNA..ESRTISN..G.V.A.G.K   YGAVAKEKQAAKG.H..R.*
APMV-3/NLD               LKRTA.QRRRTPSEIMQ.V.K.GFR   SDAVAVNTS-IKSLS..RLFKKYKGKTTPRVCAYNLHILDYNH*
APMV-4/HK/D3             LKK.R...MRTLSQAAD.V...N.L   DPKFTV.KE-KARL...E.AA*
APMV-5/Kunitachi         KKTNR..ADR..LQIKE.V.K.GLK   KG.TSI.KSAVKE.SL.S.PAK*
APMV-6/HK/D199           KRT.RR.VDR..ENIRN.V.A.GLK   KGAISTDKKKTDG....I.TAK*
APMV-7/TN                KKTNAS.KPR.LEDMRK.V.D.G.K   S.AAKINKSGI.........*
APMV-8/DE                KKTSS..KPRTL.E.KT.VKN.GLK   RGAVAS.K..LSS.H....*
APMV-9/NY                .NI.KR.K.VTFE....K.....ELT   TT..LDQHAKIQS.....*
APMV-10/FLK              KKTNA..EARTLVN.QE.V.A.G.K   KGAVEVNKKSIPHL...R.*
APMV-11/FRA              AKKNM..TLR.IQDVTE.VK..ALA   P..SKIVNSGHKQ.FS...*
APMV-12/ITA/3920-1       .AI.KH...LAMER..N.....DM.   TT..LAHASK.........*
```

b

```
                         18          31
APMV/Shimane67           NTLLAFPVVLQEID
APMV-1/LaSota            SN.....I...GTG
APMV-2/Yucaipa           IE..SY.LIMKDTG
APMV-3/NLD               TQ.....LISEKTE
APMV-4/HK/D3             KE..S..LIPVTGP
APMV-5/Kunitachi         NR.....I.MK.SA
APMV-6/HK/D199           VR.....IIMKPKE
APMV-7/TN                CS.....IIM.TTS
APMV-8/DE                SS..S..L..KET.
APMV-9/NY                SSI....I.MEATG
APMV-10/FLK              LE..S..LIFRQQ.
APMV-11/FRA              LE.....IIVERGQ
APMV-12/ITA/3920-1       SA.........D.G
```

Fig. 9

a



b

```
                    547                                                                  610
APMV/Shimane67      EIGNTLFGEFRIVPLLLEVYSE-----KGKSLKSSFD-GWEDISINNPLRPLDN----HRVDPILISNYTSSWP*
APMV-1/415          ..S.............V.ILRDEGRSEARSA.TTQGHP..N.EVVDPIFCAVT.----QTDHRQKLEE.AQ...*
APMV-1/D26          ..S.............V.ILKDDGVREARSGRL.QLQE..K.DIVSPIFCDAK.----QTEYRRELES.AA...*
APMV-1/Ulster       ..S.............V.ILKDDGVREARAGRL.QLRE..K.DIVSPIFCDAK.----QTEYRRELES.AA...*
APMV-1/LaSota       ..S.............V.ILKDDGVREARSG*
APMV-9/NY           ..E.........T...S.IIFDPNLEPSDT.RN*
APMV-9/ITA/5709     ............T...S.IILDPSLETSD..HNRVKSAPEDGRWIDPASIA.TAGNASSLHSNATSAGPVD.Q.*
APMV-12/ITA/3920-1  ................I..QKTP--LTRRSE.RQQMPQPPI.L.ID..FCAPSG----NLSRKNA.DE.AN...*
```

Fig. 10

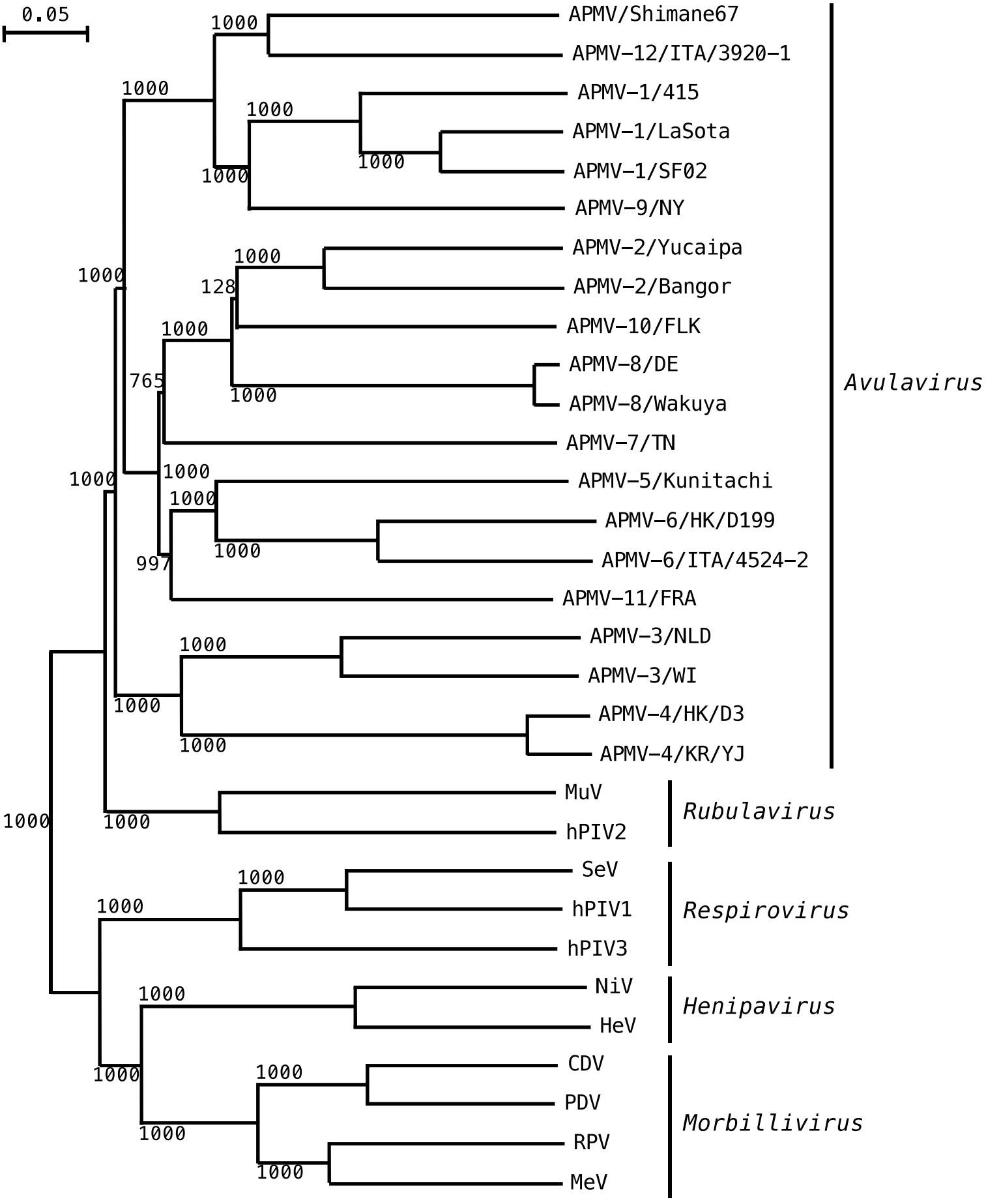
a

	Motif A	Motif B	Motif C	Motif D
	635 647	706 731	744 753	814 826
APMV/Shimane67	FITTDLSKYCLNW	IFIVSARGGIEGLCQKLWSMISIAAI	CMVQGDNQVI	KDGKILSQMLKNA
APMV-1/LaSota	......Q......	.Y.............T.......	....	...A....V...S
APMV-2/Yucaipa	.L....Q......	...............T....SI.	A.........	WE.R....A....
APMV-3/NLD	......Q......	.....P..A.......M.TI...S..	S.........	Y..RV.P.L...I
APMV-4/HK/D3	YM....K......	...I.........Q....TG....IA	ATL......L	FN.S....C...F
APMV-5/Kunitachi	.L....Q......	.....P..........M.T....S..	S.......A.	FE.R....G...A
APMV-6/HK/D199	.L....Q......	.....P..........M.T.......	S.......A.	FE......L....
APMV-7/TN	YL....Q......	.....P..........M.TL....L.	S.........	FE.RV...I....
APMV-8/DE	.L....E....Q.	.....P..........M.T....S..	A.........	FE.RL...V....
APMV-9/NY	Y.....Q......	.....G............TT...S..	....	........A....
APMV-10/FLK	.L....Q......	.....P..........M.T....SI.	A.........	WE.R....L....
APMV-11/FRA	.L....Q......	.....P..........M.T....S..	S.........	FE......S....
APMV-12/ITA/3920-1	.L..........	..........................S..	....	...R....L....

b

	1756 1782
APMV/Shimane67	KVGQIFSIPEVRQTKGGVSLFLGEGSG
APMV-1/LaSota	.ASHLL.V....CARH.N..Y.A....
APMV-2/Yucaipa	.YAVVGASV.KV.PTRST..YI.....
APMV-3/NLD	RQYRYVLSQLGHDQL..AT.Y......
APMV-4/HK/D3	RSAALLASGALSGLPE.S..Y....Y.
APMV-5/Kunitachi	.ISGLLAKGLCKGLPH.HG.YIA....
APMV-6/HK/D199	.AAG.LTT.GFLNLPK.NG.Y.A.S..
APMV-7/TN	.ASSLIASDILKGGPL.D..Y.C....
APMV-8/DE	.YAIAYAVS.KRSARL.G..YI.....
APMV-9/NY	.AANLL.L..VR.ARF.N..Y.A....
APMV-10/FLK	.YAVLYASERKTAKSS.D..YI.....
APMV-11/FRA	.YAGLY.SEFFRGMPQ.DA.Y......
APMV/12/ITA/3920-1	.I..L........SR.TNA.Y......

Fig. 11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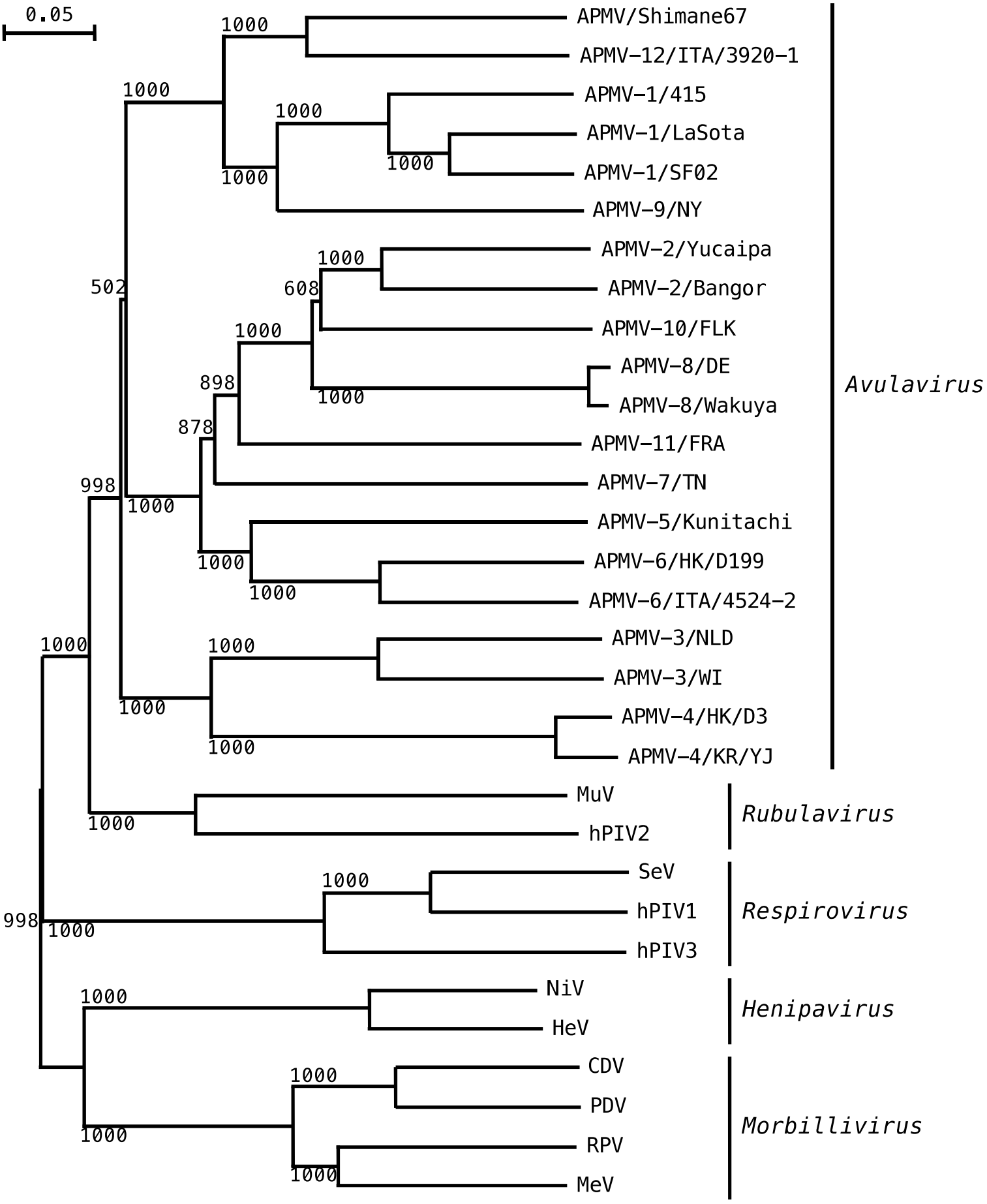
APMV-8/DE
APMV-8/Wakuya
APMV-7/TN
APMV-5/Kunitachi
APMV-6/HK/D199
APMV-6/ITA/4524-2
APMV-11/FRA
APMV-3/NLD
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
Avulavirus
MuV
hPIV2
Rubulavirus
SeV
hPIV1
hPIV3
Respirovirus
NiV
HeV
Henipavirus
CDV
PDV
RPV
MeV
Morbillivirus
1000
128
765
997

Fig. 12a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APMV-2/Bangor
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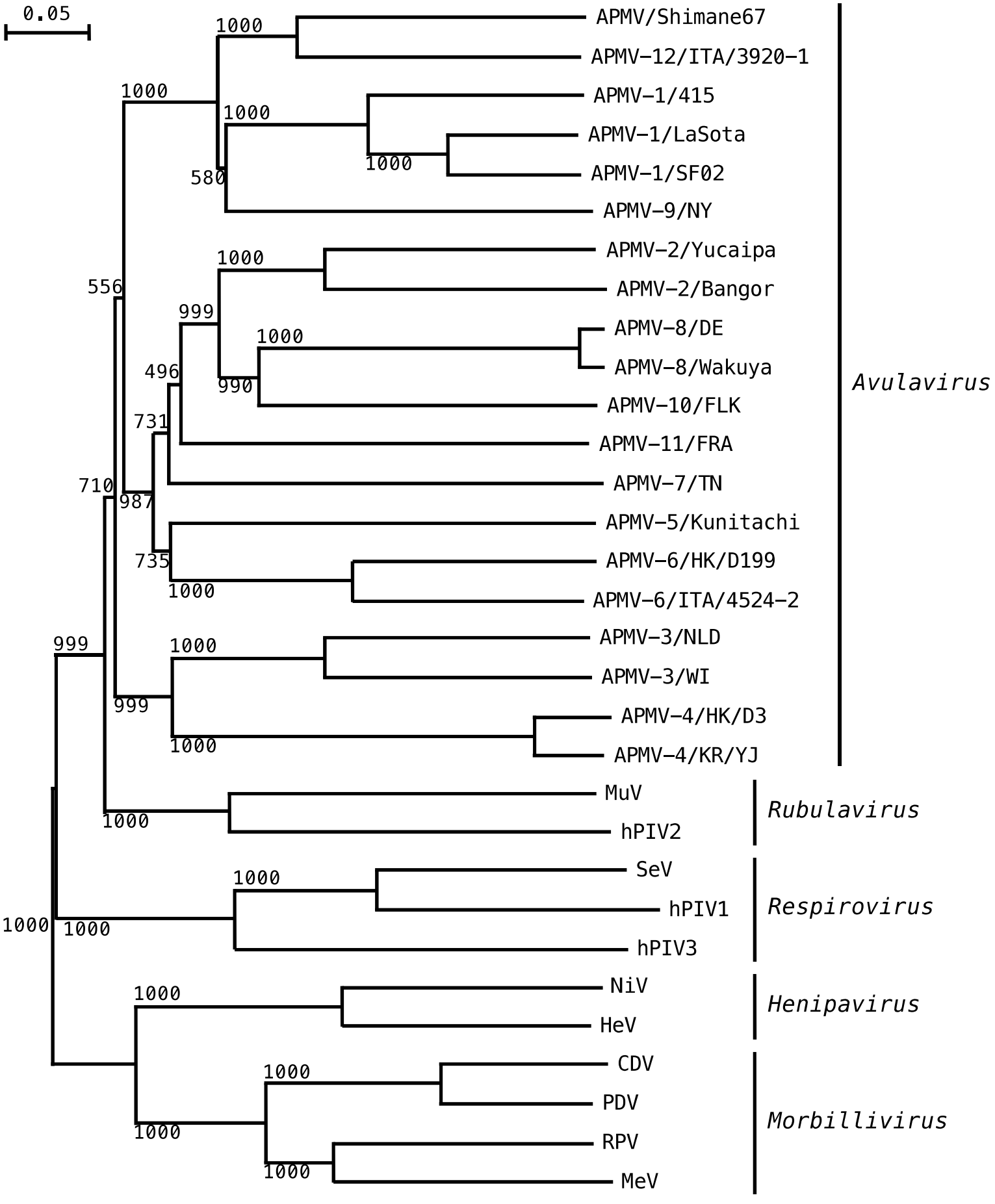
APMV-8/Wakuya
APMV-11/FRA
APMV-7/TN
APMV-5/Kunitachi
APMV-6/HK/D199
APMV-6/ITA/4524-2
APMV-3/NLD
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
MuV
hPIV2
SeV
hPIV1
hPIV3
NiV
HeV
CDV
PDV
RPV
MeV
1000
502
608
898
878
998
Avulavirus
Rubulavirus
Respirovirus
Henipavirus
Morbillivirus

Fig. 12b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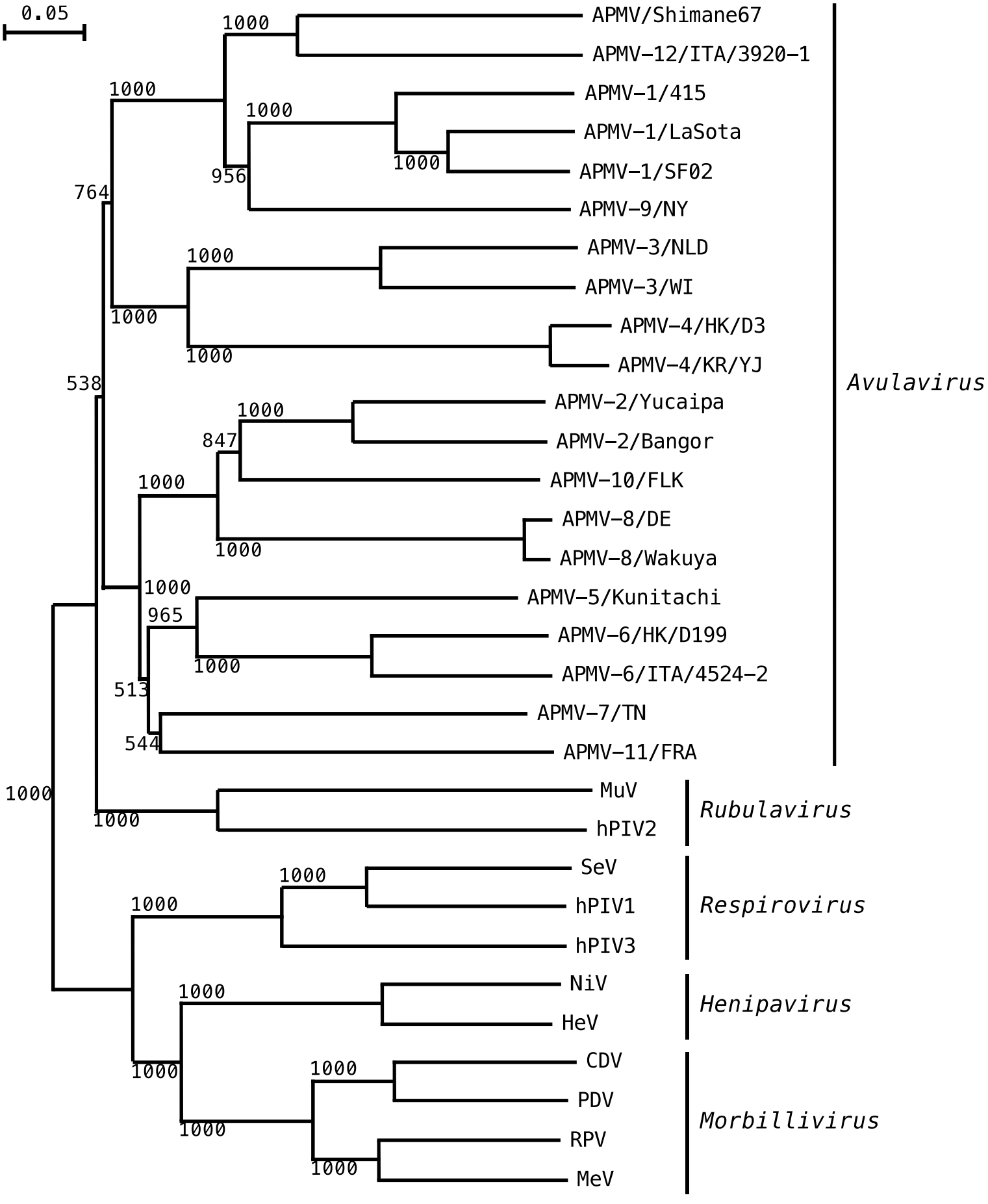
APMV-2/Bangor
APMV-8/DE
APMV-8/Wakuya
APMV-10/FLK
APMV-11/FRA
APMV-7/TN
APMV-5/Kunitachi
APMV-6/HK/D199
APMV-6/ITA/4524-2
APMV-3/NLD
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
MuV
hPIV2
SeV
hPIV1
hPIV3
NiV
HeV
CDV
PDV
RPV
MeV
1000
1000
1000
1000
580
1000
556
999
1000
496
990
731
710
987
735
1000
999
1000
999
1000
1000
1000
1000
1000
1000
1000
1000
Avulavirus
Rubulavirus
Respirovirus
Henipavirus
Morbillivirus

Fig. 12c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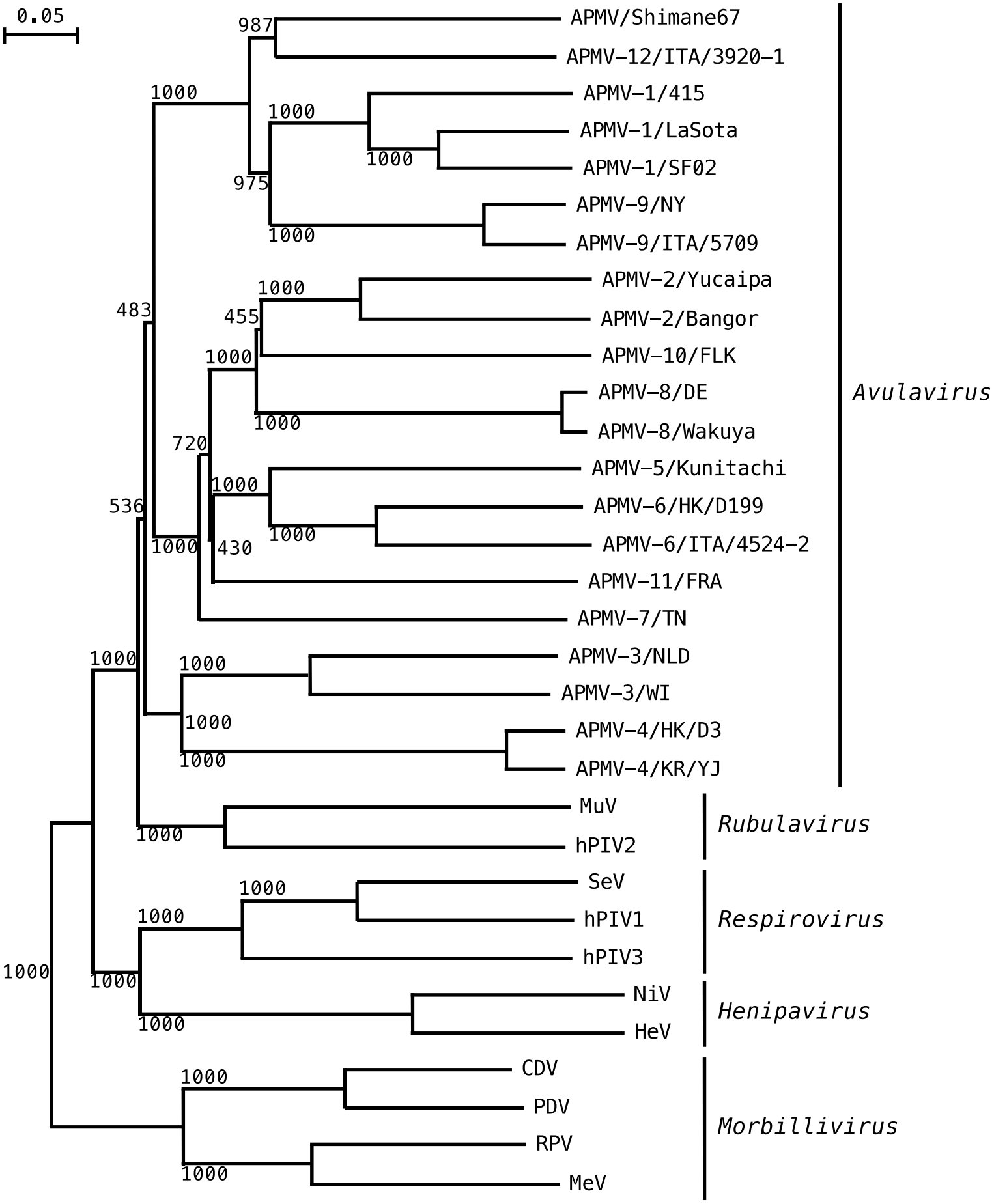
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
APMV-2/Yucaipa
APMV-2/Bangor
APMV-10/FLK
APMV-8/DE
APMV-8/Wakuya
APMV-5/Kunitachi
APMV-6/HK/D199
APMV-6/ITA/4524-2
APMV-7/TN
APMV-11/FRA
Avulavirus
MuV
hPIV2
Rubulavirus
SeV
hPIV1
hPIV3
Respirovirus
NiV
HeV
Henipavirus
CDV
PDV
RPV
MeV
Morbillivirus
1000
956
764
538
847
965
513
544

Fig. 12d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APMV-1/SF02
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APMV-9/ITA/5709
APMV-2/Yucaipa
APMV-2/Bangor
APMV-10/FLK
APMV-8/DE
APMV-8/Wakuya
APMV-5/Kunitachi
APMV-6/HK/D199
APMV-6/ITA/4524-2
APMV-11/FRA
APMV-7/TN
APMV-3/NLD
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
MuV
hPIV2
SeV
hPIV1
hPIV3
NiV
HeV
CDV
PDV
RPV
MeV
Avulavirus
Rubulavirus
Respirovirus
Henipavirus
Morbillivirus
987
1000
1000
1000
975
1000
483
455
1000
1000
720
1000
536
1000
430
1000
1000
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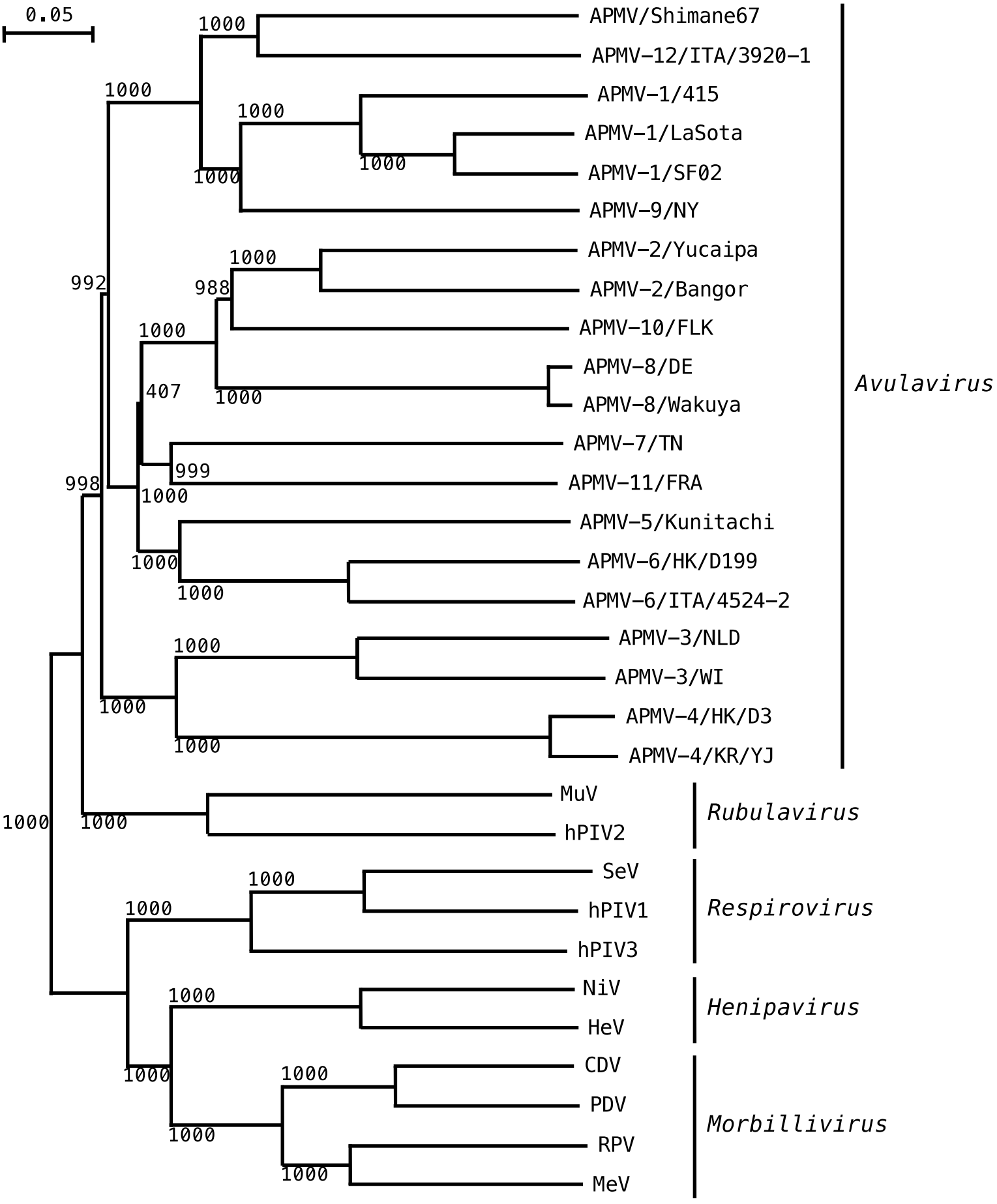
1000
1000
1000

Fig. 12e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APMV-2/Yucaipa
APMV-2/Bangor
APMV-10/FLK
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APMV-7/TN
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APMV-3/NLD
APMV-3/WI
APMV-4/HK/D3
APMV-4/KR/YJ
Avulavirus
MuV
hPIV2
Rubulavirus
SeV
hPIV1
hPIV3
Respirovirus
NiV
HeV
Henipavirus
CDV
PDV
RPV
MeV
Morbillivirus
1000
992
988
407
998
999

Fig. 12f

Figure legend

Fig. 5. Nucleotide sequences of the 3′ leader and 5′ trailer regions of the APMV/Shimane67 genome. (a) Complementary nucleotide pairs are indicated by the vertical bars. Alignment of the (b) 3′ leader and (c) 5′ trailer regions of the sequences from APMV/Shimane67 and other APMVs. Identical nucleotides with APMV/Shimane67 and gaps are shown by dots and dashes, respectively. Nucleotide sequences are shown in genomic sense.

Fig. 6. (a) The gene-start, gene-end, and intergenic sequences of APMV/Shimane67. (b) Alignment of the consensus gene-start and gene-end sequences from APMV/Shimane67 and other APMVs. Nucleotide sequence is shown in genomic sense.

Fig. 7. (a) Alignment of the conserved amino acid motif regions of APMV N proteins. The motifs are shown at the upper lines (X and Φ represent any amino acid and aromatic amino acid, respectively). (b) Alignment of N-terminal 25 amino acids of APMV N proteins. Identical amino acids between APMV/Shimane67 and other APMVs are shown by dots. Numbers indicate the amino acid positions of the APMV/Shimane67 N protein.

Fig. 8. Alignment of (a) nucleotide sequences of RNA editing sites (mRNA sense), (b) C-terminal cysteine-rich regions of V proteins, and (c) putative P-P and P-NP binding domains of P protein. Identical nucleotides or amino acids with APMV/Shimane67 are shown by dots. Conserved cysteine residues of V protein are

shown by cross. Numbers indicate the nucleotide or amino acid positions of the (a) genome, (b) V protein, and (c) P protein of APMV/Shimane67, respectively.

Fig. 9. Alignment of (a) putative bipartite nuclear localization signal and (b) late domain of APMV M protein. The arginine and lysine residues are underlined in (a). The putative late domains are shaded gray. Identical amino acids with APMV/Shimane67 are shown by dots. The stop codons are shown by asterisks. Numbers indicate the amino acid positions of the APMV/Shimane67 M protein.

Fig. 10. (a) Locations of potential N-linked glycosylation sites (gray squares) in the HN protein of APMVs. (b) Alignment of C-terminal 45 extension of APMV-1 HN protein and the corresponding regions of APMV/Shimane67, APMV-9, and -12. Identical amino acids with APMV/Shimane67 are shown by dots. Gaps, and the stop codons are shown by dashes and asterisks, respectively. Numbers indicate the amino acids positions of the APMV/Shimane67 HN protein. The GenBank accession of APMV-1/D26, Ulster and APMV-9/ITA/5709 are M19432, M19478 and GU068587, respectively.

Fig. 11. Alignment of (a) the conserved domain III of L protein of non-segmented negative strand RNA viruses and (b) the putative ATP-binding site of the L protein. The QGDNQ and K-X_{18-21}–G-X-G-X-G motifs are underscored. Identical amino acids with APMV/Shimane67 are shown by dots. Numbers indicate the amino acid positions of the APMV/Shimane67 L protein.

Fig. 12. Phylogenetic analyses of the complete genome (a), N (b), P (c), M (d), HN (e), and L (f) ORFs from members of the subfamily *Paramyxovirinae*. Phylogenetic trees are generated with the program ClustalX [Larkin *et al*., 2007] and viewed using NJplot [Perrière and Gouy, 1996]. The numbers at the branches represent bootstrap values from 1000 replicates. The number of nucleotide substitutions per site (scale bar) is shown.

CHAPTER 3

Complete genome sequence of pigeon circovirus detected in racing pigeons in western Japan

Introduction

Circoviruses are among the smallest pathogenic DNA viruses known; this class exhibits the potential to infect various animal species. Circovirus infection in pigeons was first documented in North America in 1993 [Woods *et al*., 1993], and subsequently reported in many countries throughout the world [Abadie *et al*., 2001; Duchatel *et al*., 2005; Franciosini *et al*., 2005; Gough and Drury, 1996; Raue *et al*., 2005; Todd *et al*., 2001]. The most commonly reported clinical signs of circovirus infection in pigeon lofts are poor performance, diarrhea, and ill thrift [Woods and Latimer, 2003].

Pigeon circovirus (PiCV) has a single-stranded circular DNA genome of approximately 2.0 kb, and consists of two major open reading frames (ORFs V1 and C1) and a third minor ORF (C2). ORF V1, located on the sense strand, encodes a putative replication-associated protein. ORF C1, located on the anti-sense strand, encodes a putative capsid protein [Todd *et al*., 2008].

In Japan, the first case of PiCV infection of racing pigeons was reported in 2000 [Sato *et al*., 2000]. However, genetic information on the PiCVs circulating in Japan is still limited. Therefore, in the present study we characterized a circovirus detected in pigeons in Japan.

Materials and Methods

In early summer of 2010, racing pigeons reared in a loft in the Shikoku district of western Japan began to exhibit symptoms of diarrhea. The disease was non-fatal but spread among loft-mates, with diarrheal symptoms observed in multiple animals

regardless of age. Treatments with antibiotics were not effective in resolving the diarrhea. In December of 2010, two pigeons in the loft were euthanized for pathological appraisal. At necropsy, the contents of the crops and intestinal tracts were collected for parasitological tests. A limited subset of organs (including heart, lung, liver, spleen, kidney, trachea, and rectum) was harvested, and samples of these organs were assessed by bacteriological and virological tests (see below). Remaining segments of these organs were submitted for histopathological evaluation; these tissues were fixed in 10% neutral buffered formalin and processed to paraffin, and the resulting sections were stained using hematoxylin-eosin. For bacteriology, samples were cultured on sheep blood agar and dextrose-hydrogen-sulfide-lactose (DHL) agar. For virology, homogenates of organs were pooled individually and inoculated into allantoic cavities and onto chorioallantoic membranes (CAMs) of 10-day-old embryonated chicken eggs. After three passages, embryos and CAMs were inspected for gross changes. Passaged allantoic fluid was collected and tested for hemagglutination activity using chicken erythrocytes. RNAs were extracted from the pooled homogenates using the QIAamp Viral RNA Mini Kit (QIAGEN K. K., Tokyo, Japan), according to the manufacturer's instruction. Polymerase chain reaction (PCR) assays were performed using the previously described primers specific for Avian Influenza Virus (AIV) and Newcastle Disease Virus (NDV) [Lee *et al*., 2001; Mase *et al*., 2009a] in combination with Superscript® VILO™ cDNA synthesis kit (Invitrogen Japan, Tokyo, Japan) and SapphireAmp® Fast PCR Master Mix (Takara, Shiga, Japan). DNAs also were extracted from the pooled homogenates using the DNeasy Blood & Tissue Kit (Qiagen K. K.), according to the manufacturer's instructions. PCR assays were performed using the previously described primers specific for Herpesvirus and Avian Adenovirus [Mase *et*

al., 2009b; VanDevanter *et al*., 1996] in combination with the SapphireAmp® Fast PCR Master Mix. To detect the conserved nucleotide sequences of PiCV, a primer pair (forward, 5'-CAAGGCTTTGTGCATYTRAA-3'; reverse, 5'- GACYTCSGTCATTGC TCTTC-3') was designed using the primer3 software [Rozen and Skaletsky, 2000]. To obtain the complete genome sequence of PiCV, we designed three further primer pairs, designated A (forward, 5'-GANACGTGGCTGGTGAGTGA-3'; reverse, 5'-CTGGCTT GGGWCCCTTTACTA-3'), B (forward, 5'-TTYARATGCACAAAGCCTTG-3'; reverse, 5'-AAGTTYGAAGATGCTCCKGAYTGT-3') and C (forward, 5'-TCACTCA CCAGCCAAGTGTC-3'; reverse, 5'-TCAGTGCATCCGCTAGTTTG-3'). PCR products were resolved on 2% agarose gels to confirm the presence of bands of expected size. The bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen K.K.), then directly sequenced at the Dragon Genomics Center (Takara Bio Inc., Mie, Japan). Sequence data was compared with sequences in the GenBank databases using the BLAST programs. ORFs in the sequence were predicted using the web program NCBI ORF Finder (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/projects/gorf/). Phylogenetic analysis and construction of the phylogenetic tree were performed using MEGA4 software with the neighbor-joining method [Tamura *et al*., 2007].

Results and Discussion

Obvious pathological changes were not observed by either gross or microscopic histological evaluation. Parasitological and bacteriological tests did not detect a causative agent associated with the birds' chronic diarrhea. Attempted virus isolation

using embryonated eggs did not yield apparent changes in embryos or CAMs. The results of hemagglutination activity tests of passaged allantoic fluids also were negative.

The results of PCR assays targeting AIV, NDV, Herpesvirus, and Avian Adenovirus all were negative. Products were detected only in the PCR targeting PiCV, with amplicons of approximately 220 bp recovered for both pigeons. The sequences of these two PCR products were identical to each other; BLAST comparison with GenBank sequences revealed similarity to portions of previously reported PiCV genomes. The sample from pigeon #2 was used for further genetic analysis, including recovery of further sequences by PCR walking. A complete sequence was obtained for the virus, which was tentatively designated PiCV/Japan/2/2010.

Sequence analysis revealed that the genome of this virus was circular and had a size of 2,041 bp (Accession No. LC035390). Three ORFs were predicted in the genome; these were designated ORF V1, C1 and C2, by analogy to the proposed structures of other PiCVs.

ORF C1 of PiCVs codes for the putative capsid protein; this ORF shows high sequence variability, and therefore can be used for phylogenetic grouping [Cságola *et al*., 2012]. In the present study, we compared sequence data for the ORF C1 sequences of 59 PiCV strains, including PiCV/Japan/2/2010. The corresponding phylogenetic tree was constructed using the sequence data of Beak and Feather Disease Virus, a member of the family *Circoviridae*, as the out-group (Fig. 13). In our analysis, three strains (PiCV/Japan/2/2010, SRK/US/01, and Dove) formed one branch that was clearly distinct from the other 56 strains. These 56 strains could be further divided into four groups (A to D), consistent with previously reported findings [Cságola *et al*., 2012]. In that previous analysis, SRK/US/01 and Dove comprised Group E; the present

phylogenetic analysis suggested that PiCV/Japan/2/2010 was an additional member of Group E.

We note, however, that on the basis of low identity values within the groups, Group E may be further divided into different genetic subgroups. The nucleotide (nt) and amino acid (aa) identity values between PiCV/Japan/2/2010 and SRK/US/01 or Dove were 81.7% (nt) and 62.8% (aa), and 81.2% (nt) and 60.2% (aa), respectively. In contrast, the nt and aa identity values between SRK/US/01 and Dove were 88.5% (nt) and 74.1% (aa). Evolutionary distances also were calculated using MEGA4 software (Table 6). Based on nt, the distance between PiCV/Japan/2/2010 and the average Group-E member was calculated as 0.190, a value similar to that between Groups A and B. Based on aa, the distance between PiCV/Japan/2/2010 and the average Group-E member was calculated as 0.422, a value greater than the Group A-B (0.368), A-C (0.411), B-C (0.394), B-D (0.390), and C-D (0.376). These findings suggest that strain PiCV/Japan/2/2010 likely will form a distinct genetic group or subgroup as more sequences become available.

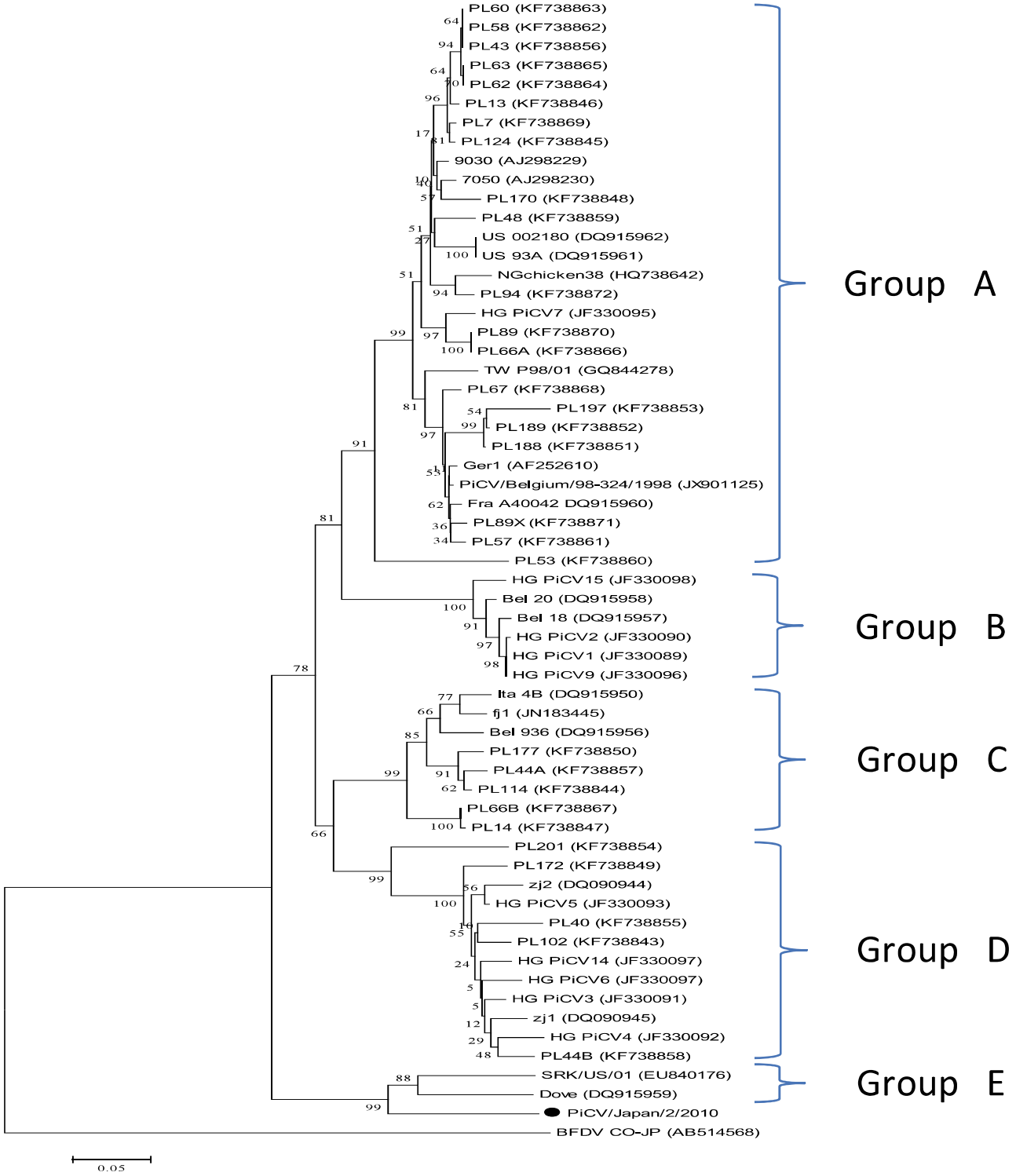
The Dove strain that shows similarity to strain PiCV/Japan/2/2010 was isolated from a juvenile dove in Senegal that presented with feather disorders [Raidal and Riddoch, 1997]. The symptom of feather disorders is not commonly associated with PiCV infection, but is typical of psittacine Beak and Feather Disease Virus infection. In the case of the present study, feather disorders were not observed among pigeons in the infected loft. Further experiments, including experimental infection of pigeons, would be necessary to clarify the pathogenicity of strain PiCV/Japan/2/2010.

To our knowledge, the present work represents the first report of a complete PiCV genome obtained in Japan. However, the epidemiological situation of PiCVs in

Japan remains unclear. Cságola *et al*. [2012] reported that Asian strains, for example, Taiwan (TW P98/01) and China (zj-1 and zj-2) strains, belong to Groups A and D, respectively. In the present study, PiCV/Japan/2/2010 sorts with Group E, making the new isolate markedly distinct from other Asian PiCVs and suggesting wider genetic diversity of PiCVs in Asia. In our recent surveillance study, PiCVs also were detected from the feces of apparently healthy free-living pigeons in Japan (T. Ito, personal communication, January 10, 2015). Preliminary genetic analysis suggests that the viruses from these asymptomatic animals clustered into Group A (data not shown). Collection of further molecular epidemiological information on PiCVs in Asia would facilitate the control and prevention of PiCV infection among reared and wild pigeons in Japan.

Table 6. Estimates of evolutionaly distance between PiCV/Japan/2/2010, Beak and Feather Disease Virus (BFDV), and five genotypes of PiCV

Amino acid (aa) Nucleotide (nt)	PiCV/Japan/2/2010	Genotype A	Genotype B	Genotype C	Genotype D	Genotype E	BFDV
PiCV/Japan/2/2010		0.585	0.578	0.578	0.580	0.422	1.163
Genotype A	0.299		0.368	0.411	0.426	0.556	1.147
Genotype B	0.351	0.190		0.394	0.390	0.554	1.149
Genotype C	0.302	0.207	0.230		0.376	0.574	1.037
Genotype D	0.331	0.234	0.254	0.205		0.574	1.152
Genotype E	0.190	0.299	0.324	0.319	0.316		1.149
BFDV	0.677	0.652	0.652	0.639	0.678	0.706	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PL43 (KF738856)
PL63 (KF738865)
PL62 (KF738864)
PL13 (KF738846)
PL7 (KF738869)
PL124 (KF738845)
9030 (AJ298229)
7050 (AJ298230)
PL170 (KF738848)
PL48 (KF738859)
US 002180 (DQ915962)
US 93A (DQ915961)
NGchicken38 (HQ738642)
PL94 (KF738872)
HG PiCV7 (JF330095)
PL89 (KF738870)
PL66A (KF738866)
TW P98/01 (GQ844278)
PL67 (KF738868)
PL197 (KF738853)
PL189 (KF738852)
PL188 (KF738851)
Ger1 (AF252610)
PiCV/Belgium/98-324/1998 (JX901125)
Fra A40042 DQ915960)
PL89X (KF738871)
PL57 (KF738861)
PL53 (KF738860)
HG PiCV15 (JF330098)
Bel 20 (DQ915958)
Bel 18 (DQ915957)
HG PiCV2 (JF330090)
HG PiCV1 (JF330089)
HG PiCV9 (JF330096)
Ita 4B (DQ915950)
fj1 (JN183445)
Bel 936 (DQ915956)
PL177 (KF738850)
PL44A (KF738857)
PL114 (KF738844)
PL66B (KF738867)
PL14 (KF738847)
PL201 (KF738854)
PL172 (KF738849)
zj2 (DQ090944)
HG PiCV5 (JF330093)
PL40 (KF738855)
PL102 (KF738843)
HG PiCV14 (JF330097)
HG PiCV6 (JF330097)
HG PiCV3 (JF330091)
zj1 (DQ090945)
HG PiCV4 (JF330092)
PL44B (KF738858)
SRK/US/01 (EU840176)
Dove (DQ915959)
PiCV/Japan/2/2010
BFDV CO-JP (AB514568)
0.05
Group A
Group B
Group C
Group D
Group E

Fig. 13

Figure legend

Fig. 13. Phylogenetic tree of ORF C1 nucleotide sequences of PiCVs. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strain described in this paper.

CHAPTER 4

Genetic Characterization of Avian Orthoreovirus Isolated from Tree Sparrow (*Passer montanus*) in Western Japan

Introduction

Avian reoviruses (ARVs) are members of the *Orthoreovirus* genus in the *Reoviridae* family and one of an important pathogen of chickens and turkeys. Viral arthritis/tenosynovitis, malabsorption syndrome, stynting/runting syndromes, enteric disease, immunosuppression and respiratory disease caused by ARVs are great problem of poultry industry [Jones, 2000]. Infection of ARVs to the birds other than chickens and turkeys also induces serious disease. ARV infection to psittaciformes with high mortality was observed [van den Brand *et al*., 2007]. ARV also causes die-off of American woodcock in 1989-90 [Docherty *et al*., 1994]. An African grey parrot infected with ARV showed subcutaneous hemorrhages, multiple foci, and necrosis in the liver, spleen, bone marrow, intestinal lamina propria, airsacculitis and epicarditis. In experimental infection of the ARV isolated from African grey parrot to African grey parrot, the ARV caused fatal hepatic necrosis to the inoculated birds similarly to the original bird [Graham, 1987]. Additionally, cases of various species of birds infected with ARVs were reported to date [Chen *et al*., 2012; Huhtamo *et al*., 2007; Palya *et al*., 2003; Perpiñán *et al*., 2010; Sakai *et al*., 2009; Senne *et al*., 1983; Yun *et al*., 2012]. Serological survey of ARVs in wild birds was reported and the results also indicate that ARVs were circulating in wild bird's population [Hlinak *et al*., 1998].

In the case of some avian disease, healthy wild birds are the reservoir of the pathogens and play important role in epidemiology of the disease [Fujimoto *et al*., 2010; Ito *et al*., 2001; Muradrasoli *et al*., 2010; Oaks *et al*., 2005]. Similar to these avian diseases, there is a possibility that healthy wild birds are also the reservoir of ARVs. Though there were some reports of isolation of ARVs from "diseased" or "dead" wild birds, the

isolation reports of ARVs from "healthy" wild birds were few [Takehara *et al*., 1989]. To estimate the epidemiological risk of wild birds in control of the avian disease caused by ARVs, information about ARVs carrying with healthy wild birds is important. To obtain more information about ARVs in healthy wild birds, we conducted the survey of ARVs by using fresh fecal samples dropped from wild birds living in Shikoku district of western japan.

Materials and Methods

Collection of materials

From April of 2006 to March of 2009, a total of 240 fresh fecal samples were collected from tree sparrow (*Passer montanus*), brown-eared bulbul (*Hypsipetesamaurotis*), pigeon (*Columbiformes*), gray starling (*Sturnuscineraceus*), japanese pied wagtail (*Motacilla alba lugens*), japanese white-eye (*Zosteropsjaponicus*), crow (*Corvidae*), thrush (*Turdusnaumanni*), swallow (*Hirundorustica*). Samples were collected at Kagawa prefecture in Shikoku district of western Japan. Samples were suspended at a concentration of approximately 30% in phosphate-buffered saline (PBS, pH7.2) containing penicillin at 5,000 units per ml and streptomycin at 5 mg per ml. The suspension was centrifuged at 1,000 x g for 5 min. The supernatants were stored at -80ºC until assayed.

PCR screening test of Orthoreovirus

Ten samples were pooled and Viral RNA was extracted by ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacture’s instruction. Reverse

transcription was performed using Superscript® VILO™ cDNA synthesis kit (Life Technologies Corporation, Carlsbad, CA USA) with initial denaturing of double-stranded viral RNA. A 9.0 µl of extracted RNA and 1.0µl of Dimethyl sulfoxide (DMSO) were mixed and incubated 5 min at 95ºC, then snap cooled on ice at least 5 min. 4.0 µl of 5x VILO™ Reaction Mix, 4.0 µl of DW and 2.0 µl of 10x Superscript® Enzyme Mix were added to the denatured RNA. Reverse transcription was performed with the following conditions: 10 min at 25ºC, 60 min at 42ºC and 5 min at 85ºC. PCR was performed using primers for Orthoreovirus consensus sequence encoding the RNA-dependent RNA polymerase (RdRP) gene, previously described [Wellehan *et al*., 2009]. Amplification of the genes were performed using SapphireAmp® Fast PCR Master Mix (Takara, Shiga, Japan) in a TaKaRa PCR Thermal Cycler Dice® Standard (Takara) with the following cycling conditions: 1min at 94ºC, 50 cycles of 5s at 98ºC, 5s at 45ºC, 15s (First PCR) or 5s (Nested PCR) at 72ºC and 10s at 72 ºC. 50 µl of reaction mixture contained 25 µl of 2x PCR Master Mix, 2.0 µM of each primer, 2.5 µl of DMSO and 5.0 µl of cDNA. PCR positive samples were retested individually to determine the positive samples. PCR products were resolved in 3% agarose gels. The bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen, Courtaboeuf, France) according to the manufacture's instruction. Purified PCR products were mixed with each nested PCR primers and directory sequenced by Dragon Genomics Center (Takara Bio Inc., Mie, Japan). Sequence data of the PCR products were compared with those in the GenBank databases by using the BLAST program. Phylogenetic analysis and constructing of phylogenetic tree were performed using MEGA4 software [Tamura *et al*., 2007]. The robustness of the clusters obtained by the neighbor joining algorithm was assessed using 1,000 bootstrap replicates.

Virus isolation

Orthoreovirus consensus PCR positive samples were used for virus isolation. Fore established cell lines (MDBK, Vero, HmLu1 and HRT-18) and two primary chicken embryo cells (fibroblast (CEF) and liver (CEL) cells) were used for virus isolation. All cells used in this study were maintained in Serum-Free medium GIT (NIHON PHARMACEUTICAL CO., LTD, Tokyo, Japan) with 5-10% of fetal bovine serum (FBS). Cells were seeded into 25 cm^2 tissue-culture treated flasks and incubated at 37 ºC to approximately 90% confluence of cell monolayer. Appropriate cells were washed twice with PBS and samples were inoculated onto the cells for one hour at 37 ºC. Then inoculated samples were removed and cells were washed twice again. The cells were incubated in Serum-Free medium GIT not adding FBS with flasks rolling. Infected cells were sub-cultured at least three times, once a week and observed daily for the occurrence of cytopathogenic effect (CPE). Samples were also inoculated into allantoic cavities and onto chorioallantoic membranes (CAM) of 10-day-old embryonated chicken eggs. Infected eggs were incubated 5 days at 37 ºC. Inoculated eggs were checked daily for the death of eggs. At egg death point or the end of incubation period, eggs were chilled overnight and then changes of embryos or CAMs were observed. Allantoic fluids or CAMs were collected and sub-cultured three times in each method. Uninfected cells or eggs were used for negative controls. From cell or egg passaged samples, RNA was extracted and checked the prevalence of orthoreovirus gene by PCR described above.

Genetic analysis of S-1 gene of Avian Orthoreovirus

Virus isolation confirmed sample was used for analysis of S-1 gene. RNA extraction from passaged sample and Reverse transcription were performed same as described above. PCR amplifications of S-1 gene were performed using KOD-Pius-Neo (TOYOBO CO., LTD, Osaka, Japan) with the following cycling conditions: 2min at 94ºC, 40 cycles of 10s at 98ºC, 30s at 50-55ºC, 60-90s at 68ºC and 60s at 68ºC. Annealing temperature and extension time were adjusted according to melting temperature of the primers and length of the PCR products. 50 µl of reaction mixture contained 5µl of 10x Buffer for KOD -Plus- Neo, 5µl of 2mM dNTPs, 3µl of 25mM MgSO4, 0.4µM of each primer, 2.5 µl of DMSO and 5.0 µl of cDNA. Both ends of the sequences of S-1 gene were determined by using 5'/3' RACE Kit, ^{2nd} Generation (Roche Diagnostics GmbH, Mannheim Germany) and E. coli Poly (A) Polymerase (New England Biolabs Inc., Massachusetts, U.S.A) according to the manufacturer's instructions. All primers used for analysis of S-1 gene in this study were shown in Table 7. PCR products were directory sequenced and analyzed as described above. Open reading flames (ORFs) in the sequence were predicted using web program NCBI ORF Finder (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/projects/gorf/). Constructing of phylogenetic trees was performed using MEGA4 software as described above [Tamura *et al*., 2007].

Results

PCR screening test of Orthoreovirus

Three samples collected from tree sparrows in 2006 were resulted PCR positive and total positive rate was 1.25% (Table 8). The results of sequence data of these PCR products (226bp, exclude primers) showed 100% homology with each other. These three viruses tentatively named as ARV/Sparrow/Kagawa/18/2006, ARV/Sparrow/Kagawa/20/2006 and ARV/ Sparrow/Kagawa/22/2006 respectively.

In phylogenetic analysis of partial RdRP gene generated in screening PCR, 20 strains of orthoreoviruses and one aquareovirus were analyzed with three detected genes in this study. An aquareovirus was used for outer group. In the phylogenetic tree, three genes detected from tree sparrow showed most close relationship to an isolate from aborted fetus of a Steller sea lion [Palacios *et al*., 2011]. These four strains rooted to the strains from psittacine and crow. These strains and chicken, water fowl and pteropine origins made a large cluster in the tree (Fig. 14).

Virus isolation

After inoculation and passaging two times in CEL cells, CPEs were clearly observed in all three PCR positive samples. The type of CPE was syncytium formation. The results of screening PCR of these passaged samples were positive and uninfected CEL cells for negative controls were negative. Sequence data of the PCR products obtained from passaged samples in CEL cells showed 100% homology compared to the data directly obtained from original fecal samples. In virus isolation using four established cell lines (MDBK, Vero, HmLu1 and HRT-18) and a CEF cells, CPEs were

not observed at all these cells and results of PCR using passaged samples were all negative. In virus isolation using embryonated chicken eggs, changes of CAM or embryo were not observed during passages, and results of PCR using passaged samples were also negative.

Genetic analysis of S-1 gene of ARV

One of the isolated viruses ARV/Sparrow/Kagawa/22/2006 was used for genetic analysis of S-1 gene. Total length of nucleotides of The S-1 gene was 1,650 bp. Three ORFs were predicted in the S-1 gene and considered p10, p17 and sigma C same as other ARVs and Pteropine orthoreoviruses (Table 9). The lengths of nucleotides of three predicted ORFs, p10, p17 and sigma C were 294 bp, 426 bp and 981 bp respectively. Phylogenetic trees of ORF p10, p17, sigma C and full length of S-1 gene were constructed and shown in Fig. 15, 16, 17, and 18, respectively. Total of 18 strains of orthoreoviruses were used to construct phylogenetic trees of p10 and p17 with the strain ARV/Sparrow/Kagawa/22/2006. In the analysis of sigma C and full length of S-1 gene, 22 and 21 strains of orthoreovirus were used with ARV/Sparrow/Kagawa/22/2006 respectively. In these analyses, two mammalian orthoreoviruses sigma 1 gene and segment S-1 were used for outer group.

In the phylogenetic tree of p10, ARV/Sparrow/Kagawa/22/2006 showed most close relationship with the strain isolated from diseased crow in Finland. These two strains were obviously distinguishable from four groups (Chicken, Duck/Goose, Psittacine/Steller sea lion and Pteropine) and a strain from turkey (Fig. 15).

In the phylogenetic tree of p17, ARV/Sparrow/Kagawa/22/2006 also showed most close relationship with the strain Crow. These two strains were clearly distinct from

four groups (Chicken, Duck/Goose, Psittacine/Steller sea lion and Pteropine) and a strain from turkey same as the phylogenetic tree of p10 (Fig. 16).

Phylogenetic tree of sigma C also showed that ARV/Sparrow/Kagawa/22/2006 have a close relationship with the strain Crow (Fig. 17). The bootstrap value of this cluster was 43. In phylogenetic tree constructed of full length of S-1 genes, the close relationship of ARV/Sparrow/Kagawa/22/2006 and strain of crow was also observed. Bootstrap value of this cluster was 98 (Fig. 18). These two strains showed relationship with the group Psittacine/Steller sea lion in both tree of sigma C and full length of S-1 genes.

Discussion

In PCR screening test of Orthoreovirus, three orthoreoviruses gene were detected from fecal samples of free living wild tree sparrows in Japan and total detection late was 1.25% (3/240) (Table 8). No PCR positive samples were detected from other species of birds than tree sparrows. These samples were collected on 14, 15 and 18 June 2006, at the same point under the nest of tree sparrows. The nest was made in a letter box of an apartment and the feces would be dropped from one of the parents sparrows in the nest. At that time, sparrows were in breeding season (normally from March to August in Japan) and baby birds were confirmed in their nest. Dean *et al*. [2006] reported the case of avian adenovirus infection in Falcon and they speculated that falcons may have been carriers of the adenovirus and may have shed the adenovirus during the breeding season. Likewise, there is a possibility that tree sparrows are the carriers of these orthoreoviruses and immunosuppression caused by the stress of breeding may concern with carrying the viruses. In this study, 14 samples were collected from tree sparrows at the same point during the breeding season of other years (in 2007, 2008 and 2009). The results of PCR screening tests of these samples were all negative. The relationship between carrying the virus and breeding season is still unclear.

In phylogenetic tree analysis based on the sequence of partial RdRP gene, Three ARVs from tree sparrow showed most close relationship with a strain isolated from steller sea lion. The strain steller sea lion is very unique virus that isolated from aborted fetus of a Steller sea lion [Palacios *et al*., 2011]. The report of complete genome analysis of the strain Steller sea lion revealed that the viral genes had similarities to other orthoreoviruses of birds, bats and mammals. Though the length of RdRP gene we

analyzed in this study was very short, the genes of ARVs isolated from tree sparrow may have a relationship with other orthoreoviruses isolated from other species like the strain Steller sea lion.

In genetic analysis of S-1 gene of ARV/Sparrow/Kagawa/22/2006, location of predicted three ORFs were resemble to other ARVs (Table 9). The length of these three ORFs, p10, p17 and sigma C were resemble to that of water fowl origins, pteropine origins and crow and chicken origins respectively (Table 9).

In phylogenetic tree analysis of p10 and p17, ARV/Sparrow/Kagawa/22/2006 showed closely relationship to the strain from crow. From these results, it was clear that the relationship between ARV/Sparrow/Kagawa/22/2006 and the strain from crow is very close compared to other strains.

Sigma C of ARVs is a homo-trimer displaying cell-binding activity and inducing type-specific neutralizing antibodies [Fujimoto *et al*., 2010; Muradrasoli *et al*., 2010]. Therefore, analysis of sigma C is benefit for epidemiological study of ARVs. In phylogenetic tree analysis of sigma C, ARV/Sparrow/Kagawa/22/2006 showed close relationship with the strain isolated from a wild crow in Finland same as the analysis of ORF p10 and p17 (Fig. 17). But the low bootstrap values (43) of these branches indicate the unreliability of this relationship. In the phylogenetic tree analysis of full length of S-1 genes, the relationship with ARV/Sparrow/Kagawa/22/2006 and ARV of crow was also observed. Compared to the sigma C, the bootstrap value of these branches is very high (98) and emphases the reliability of these branches. These results indicate that ARV/Sparrow/Kagawa/22/2006 have a common ancestor with the strain of crow but evolved apart especially in the part of sigma C gene. The nucleotides and amino acids identity of ARV/Sparrow/Kagawa/22/2006 and ARV isolated from crow in Finland

were 49.7 % and 38.0 % respectively. From these results of the analysis of S-1 gene, ARV/Sparrow/Kagawa/22/2006 would be considered a candidate for a new species among the strains of ARVs in the same way as the strain isolated from wild crow in Finland [Dandár *et al*., 2014; Huhtamo *et al*., 2007].

Tree sparrows are one of the common wild birds living in many countries and also in Japan. Tree sparrows are continually seen in poultry facilities and thought as a one of the source of pathogen of poultry disease [Gutiérrez *et al*., 2011]. All the time during we collected the samples, disease of poultry caused by ARV like the strain ARV/Sparrow/Kagawa/22/2006 was not reported in the area as far as we know. In virus isolation tests, these three ARVs from tree sparrows could replicate and induce CPEs only in CEL cells. Some strains of ARVs have been reported that they can replicate in some established cell lines and embryonated chicken eggs and sometimes cause death of embryos [Barta *et al*., 1984; Dandár *et al*., 2014; Sahu and Olson, 1975; Takehara *et al*., 1989]. But in the case of this study, virus isolation using CEL cells was the only effective method. These results might suggest that these viruses have few or no virulence to chickens. In the same way as the situation of poultry, abnormal death or apparently reduce of number of birds were not observed not only tree sparrows but also other species of wild birds routinely seen in the area. These results also suggest that these viruses have few or no virulence to not only chickens but also wild birds.

In the phylogenetic tree analysis, ARV/Sparrow/Kagawa/22/2006 showed relationship with a strain that isolated from a wild hooded crow in Finland. The crow showed symptoms of coordination problems, abnormal postures, cramps and paralysis [Dandár *et al*., 2014; Huhtamo *et al*., 2007]. It will be reliable that ARV/Sparrow/Kagawa/22/2006 has a common ancestor with the strain from crow by

the results of this study, the virulences of these strains are presumed to be very different. The distance of these viruses in sigma C gene may be concerning about the difference of their virulence. Nevertheless, the possibility that ARVs isolated from tree sparrow potentially have virulence to avian species is remaining. To measure the Epidemiological risks of these ARVs isolated from tree sparrow, more detailed experiments about pathogenicity are needed.

In conclusion, we conducted the survey of avian orthoreoviruses in healthy wild birds living in Japan. Three strains were isolated from tree sparrows and the total isolation rate was 1.25%. The results of genetic analysis indicate that the virus ARV/Sparrow/Kagawa/22/2006 have a common ancestor with the virulent ARV strain isolated from diseased crow in Finland. But the sequence identity indicated that ARV/Sparrow/Kagawa/22/2006 could be considered a candidate for a new species among the strains of ARVs. These results will be benefit for taxonomy and epidemiology of ARVs.

Table 7. Primers for analysis of S-1 gene

Code	Sequence(5'-3')	Sense	Location
A OrthoreoV FA1	TCKTGTAACGGWGCGACTKCTGTATT	+	44-69
A OrthoreoV RA1	GCCACACCTTADGTGTCGATGCC	-	1603-1625
Seq AF	GGTGGTGAATCTCCAGGCTA	+	107-126
Seq A-B F	AGGCTCAGCATGAACCTCCTCCGAT	+	285-309
Seq BF	GATTGGCTGCTCTGGAAGAC	+	740-759
Seq CF	AGCTCTAGAGAGCGCTGGTG	+	1083-1102
Seq DFN	AATCATCAATCCACCATCTGACGT	+	1392-1415
Seq AR	TAGCCTGGAGATTCACCACC	-	126-107
Seq A-B R	ATCGGAGGAGGTTCATGCTGAG	-	309-285
Seq BR	GTCTTCCAGAGCAGCCAATC	-	759-740
Seq CR	CACCAGCGCTCTCTAGAGCT	-	1102-1083
5'RACE1	GATAGTGAGCAAGATGATACCACCAG	-	190-165
5'RACE2	GATAGAGGTTCTTAGCGATATCCGAC	-	254-229
5'RACE3	GTAAATCGGAGGAGGTTCATGCTGAG	-	313-288

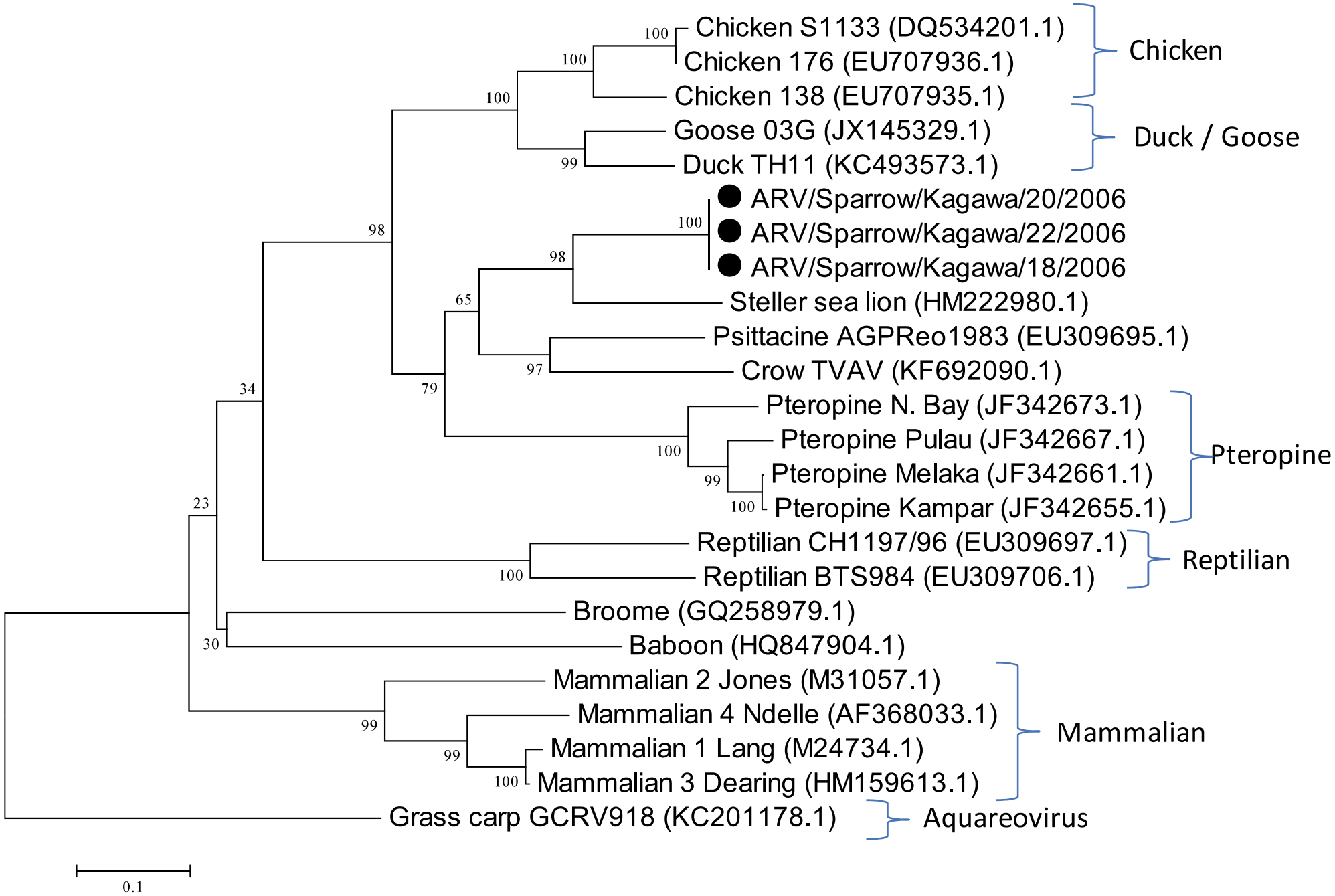
Table 8. Results of screening PCR

Species	Orthoreovirus detected in each fiscal year				Total	detection rate (%)
	2006	2007	2008	2009		
Tree sparrow	3/36*	0/43	0/28	0/20	3/127	2.36
brown-eared bulbul	0/4	0/10			0/14	
pigeon	0/7	0/5	0/1		0/13	
gray starling	0/8	0/2	0/3		0/13	
japanese pied wagtail		0/5			0/5	
japanese white-eye		0/2			0/2	
crow			0/2		0/2	
thrush		0/1			0/1	
swallow	0/1				0/1	
Other	0/12	0/28	0/16	0/6	0/62	
Total	3/36	0/96	0/50	0/26	3/240	1.25
detection rate (%)	4.41				1.25	

* Number of Orthoreovirus detection / total tested sample

Table 9. Sequence characteristics of S-1 gene

Strain	Total Length (nts)	Gene						accession No.
		P10		P17		Sigma C		
		Location (nt)	Lemgth (nts)	Location (nt)	Lemgth (nts)	Location (nt)	Lemgth (nts)	
ARV/Sparrow/Kagawa/22/2006	1650	29-322	294	294-719	426	637-1617	981	This study
Crow Tvärminne avian virus	1659	23-319	297	291-809	519	646-1626	981	KF692095.1
Psittacine SRK/Germany/2007	1636	25-321	297	293-736	444	630-1604	975	EU252582.1
Steller sea lion	1637	25-321	297	293-736	444	630-1604	975	HM222974.1
Chicken S1133	1643	25-321	297	293-733	441	630-1610	981	AF330703.1
Chicken 138	1643	25-321	297	293-733	441	630-1610	981	AF218359.1
Chicken 1733	1643	25-321	297	293-733	441	630-1610	981	AF004857.1
Goose 03G	1568	20-313	294	273-761	489	571-1536	966	JX145334.1
Duck NP03/CHN/2009	1568	20-313	294	273-761	489	571-1536	966	KC312699.1
Turkey NC/SEP-R44/03	1614	45-341	297	304-699	396	593-1582	990	DQ525419.1
Pteropine N. Bay	1617	27-314	288	277-699	423	611-1582	972	AF218360.1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Chicken
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Duck TH11 (KC493573.1)
Duck / Goose
ARV/Sparrow/Kagawa/20/2006
ARV/Sparrow/Kagawa/22/2006
ARV/Sparrow/Kagawa/18/2006
Steller sea lion (HM222980.1)
Psittacine AGPReo1983 (EU309695.1)
Crow TVAV (KF692090.1)
Pteropine N. Bay (JF342673.1)
Pteropine Pulau (JF342667.1)
Pteropine Melaka (JF342661.1)
Pteropine Kampar (JF342655.1)
Pteropine
Reptilian CH1197/96 (EU309697.1)
Reptilian BTS984 (EU309706.1)
Reptilian
Broome (GQ258979.1)
Baboon (HQ847904.1)
Mammalian 2 Jones (M31057.1)
Mammalian 4 Ndelle (AF368033.1)
Mammalian 1 Lang (M24734.1)
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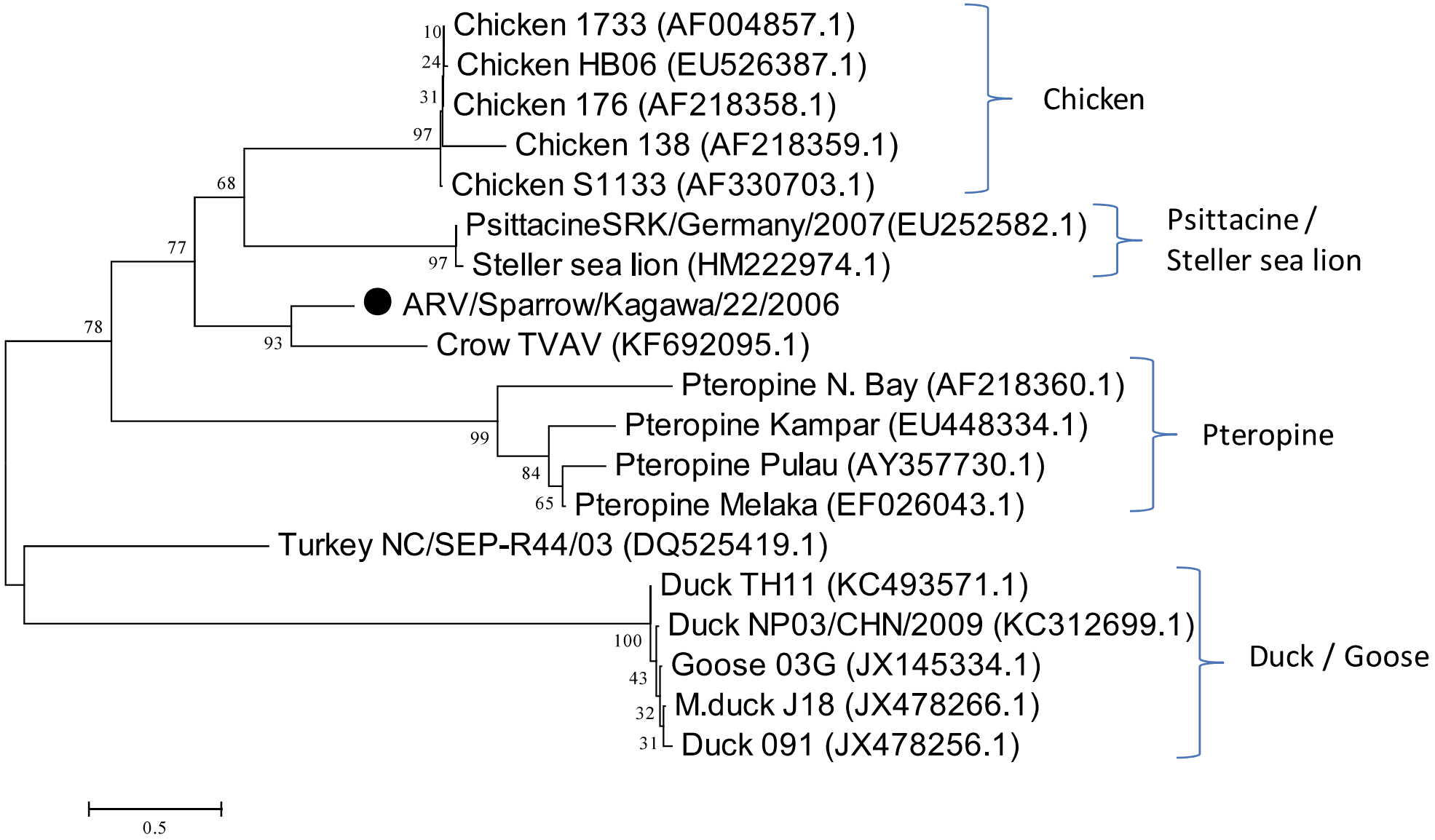
Mammalian
Grass carp GCRV918 (KC201178.1)
Aquareovirus
0.1

Fig. 14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Chicken
PsittacineSRK/Germany/2007(EU252582.1)
Steller sea lion (HM222974.1)
Psittacine / Steller sea lion
ARV/Sparrow/Kagawa/22/2006
Crow TVAV (KF692095.1)
Pteropine N. Bay (AF218360.1)
Pteropine Kampar (EU448334.1)
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Pteropine
Turkey NC/SEP-R44/03 (DQ525419.1)
Duck TH11 (KC493571.1)
Duck NP03/CHN/2009 (KC312699.1)
Goose 03G (JX145334.1)
M.duck J18 (JX478266.1)
Duck 091 (JX478256.1)
Duck / Goose
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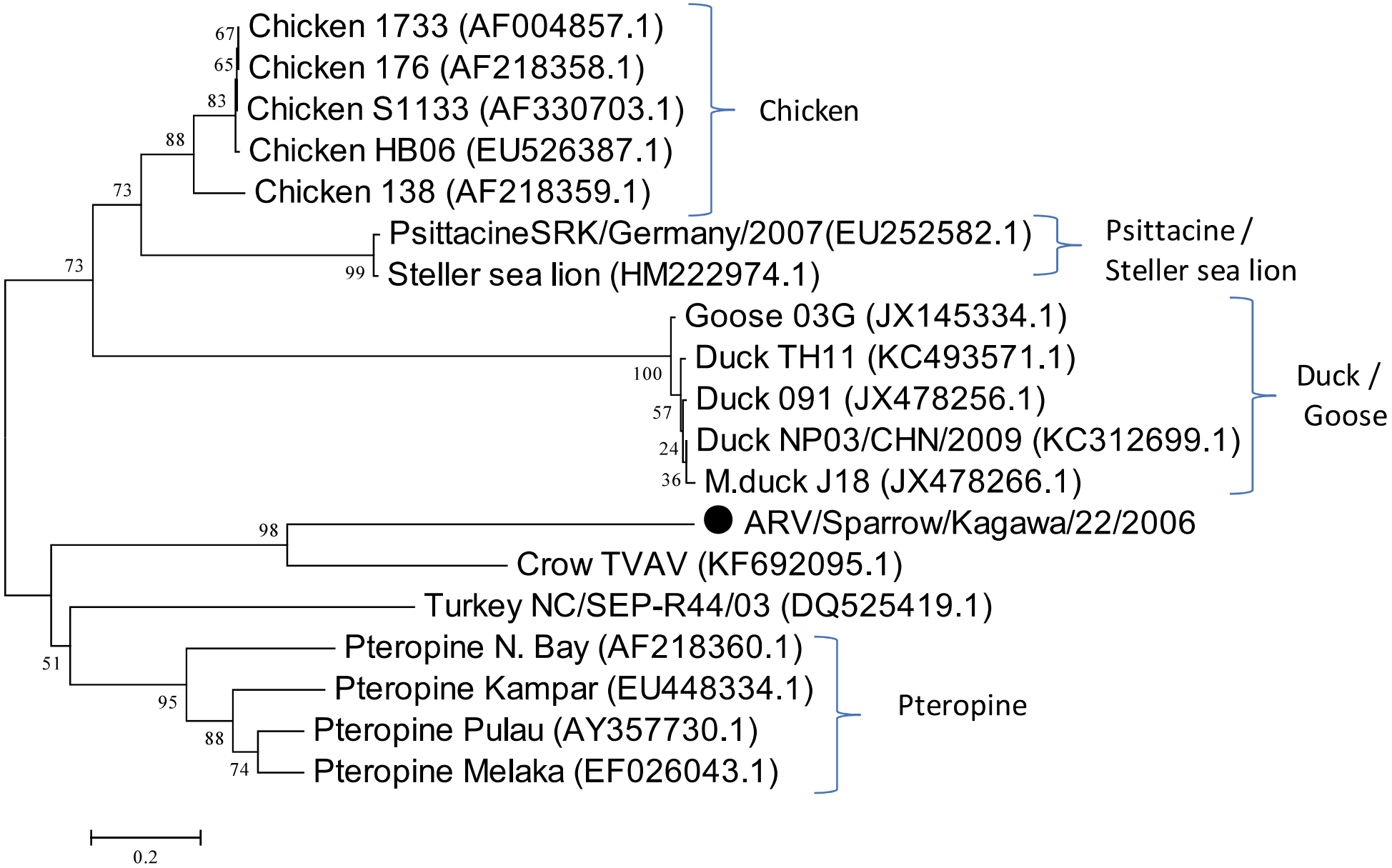
Fig. 15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Chicken
PsittacineSRK/Germany/2007(EU252582.1)
Steller sea lion (HM222974.1)
Psittacine / Steller sea lion
Goose 03G (JX145334.1)
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Duck NP03/CHN/2009 (KC312699.1)
M.duck J18 (JX478266.1)
Duck / Goose
ARV/Sparrow/Kagawa/22/2006
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Pteropine Melaka (EF026043.1)
Pteropine
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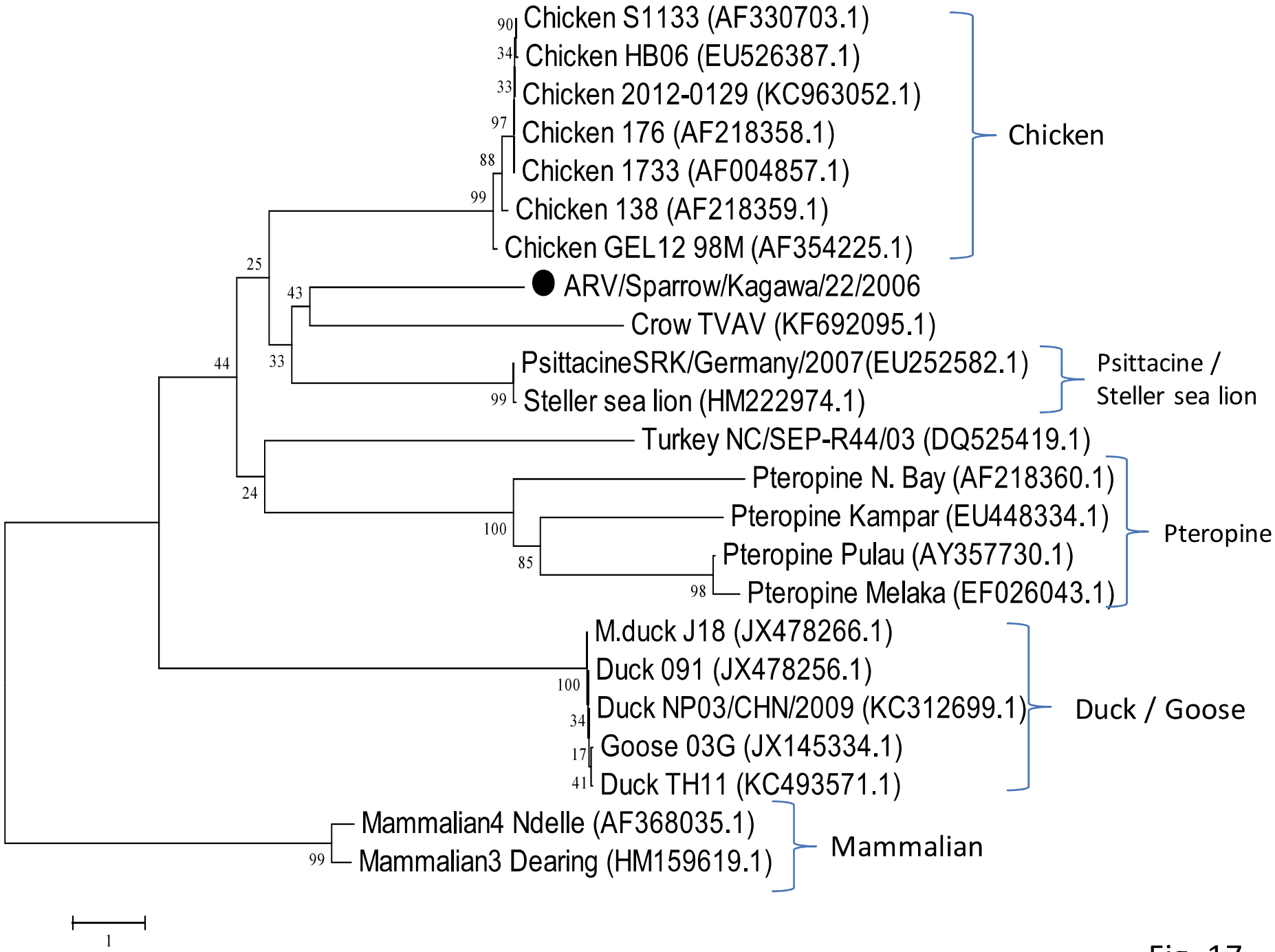
Fig. 16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Pteropine
M.duck J18 (JX478266.1)
Duck 091 (JX478256.1)
Duck NP03/CHN/2009 (KC312699.1)
Goose 03G (JX145334.1)
Duck TH11 (KC493571.1)
Duck / Goose
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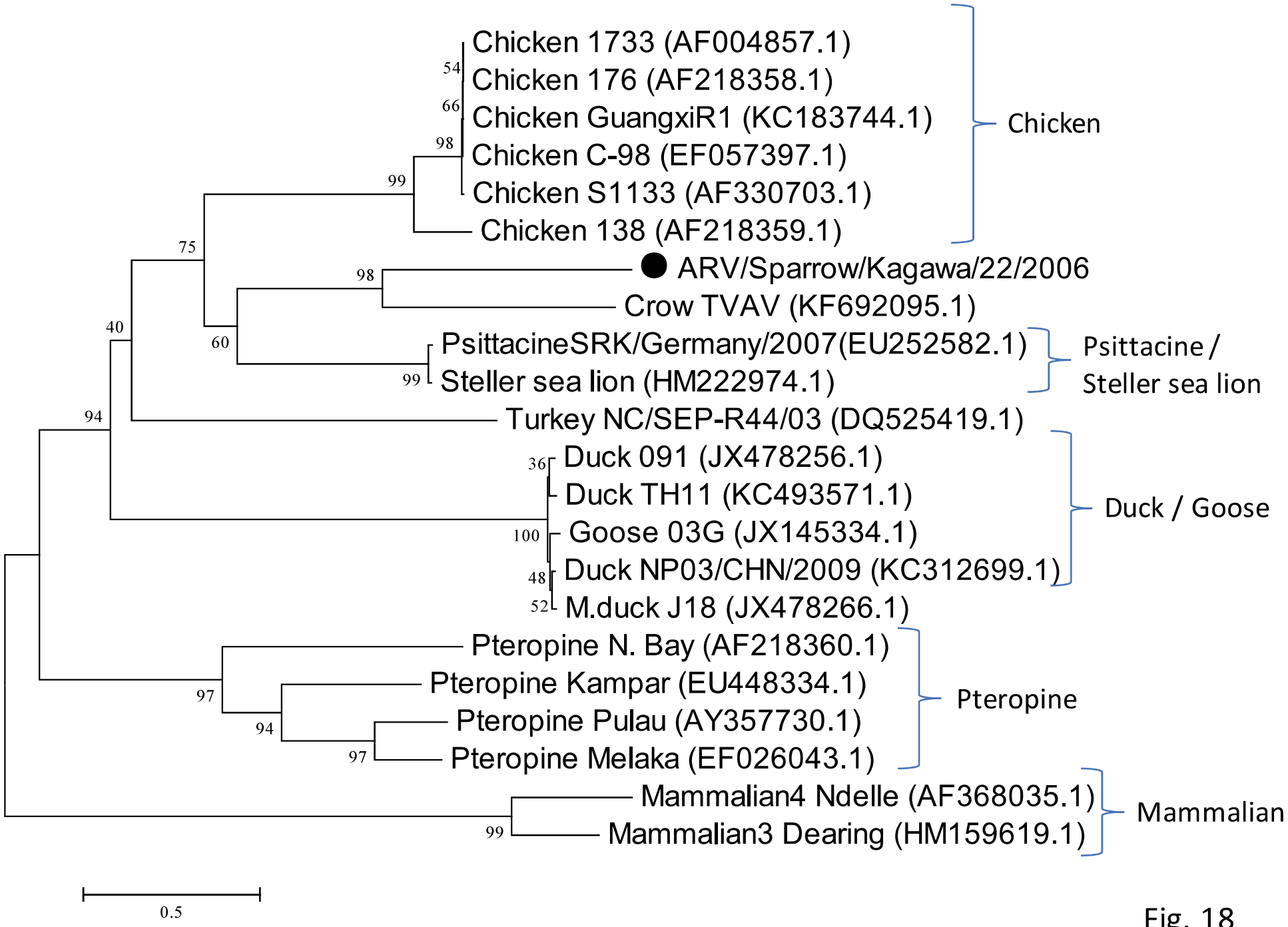
Mammalian3 Dearing (HM159619.1)
Mammalian
1

Fig. 17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Chicken 176 (AF218358.1)
Chicken GuangxiR1 (KC183744.1)
Chicken C-98 (EF057397.1)
Chicken S1133 (AF330703.1)
Chicken 138 (AF218359.1)
Chicken
ARV/Sparrow/Kagawa/22/2006
Crow TVAV (KF692095.1)
PsittacineSRK/Germany/2007(EU252582.1)
Steller sea lion (HM222974.1)
Psittacine / Steller sea lion
Turkey NC/SEP-R44/03 (DQ525419.1)
Duck 091 (JX478256.1)
Duck TH11 (KC493571.1)
Goose 03G (JX145334.1)
Duck NP03/CHN/2009 (KC312699.1)
M.duck J18 (JX478266.1)
Duck / Goose
Pteropine N. Bay (AF218360.1)
Pteropine Kampar (EU448334.1)
Pteropine Pulau (AY357730.1)
Pteropine Melaka (EF026043.1)
Pteropine
Mammalian4 Ndelle (AF368035.1)
Mammalian3 Dearing (HM159619.1)
Mammalian
54
66
98
99
75
98
40
60
99
94
36
100
48
52
97
94
97
99
0.5

Fig. 18

Figure Legends

Fig. 14. Phylogenetic tree of orthoreovirus strains based on the nucleotide sequence of partial RNA-dependent RNA polymerase gene. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strains described in this paper.

Fig. 15. Phylogenetic tree of orthoreovirus strains based on the nucleotide sequence of complete ORF p10 gene. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strain described in this paper.

Fig. 16. Phylogenetic tree of orthoreovirus strains based on the nucleotide sequence of complete ORF p17 gene. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strain described in this paper.

Fig. 17. Phylogenetic tree of orthoreovirus strains based on the nucleotide sequence of complete Sigma C gene. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strain described in this paper.

Fig. 18. Phylogenetic tree of orthoreovirus strains based on the nucleotide sequence of full length of S-1 gene. The tree was constructed using the neighbor-joining method with bootstrap values calculated for 1000 replicates. Black dot shows the strain described in this paper.

GENERAL CONCLUSION

In this thesis, the author described the virological, serological and genetical analyses of avian pathogenic virus isolates originated from wild or free-ranging birds in western Japan.

In Chapter 1, an avian paramyxovirus (APMV) isolated from goose feces (APMV/Shimane67) was biologically, serologically and genetically characterized. APMV/Shimane67 showed typical paramyxovirus morphology on electron microscopy. On hemagglutination inhibition test, antiserum against APMV/Shimane67 revealed low reactivity with other APMV serotypes and *vice versa*. The fusion (F) protein gene of APMV/Shimane67 contained 1,638 nucleotides in a single open reading frame encoding a protein of 545 amino acids. The cleavage site of F protein contained a pair of single basic amino acid (VRENR/L). The nucleotide and deduced amino acid sequences of the F gene of APMV/Shimane67 had relatively low identities (42.9-62.7% and 28.9-67.3%, respectively) with those of other APMVs. Phylogenetic analysis showed that APMV/Shimane67 was related to NDV, APMV-9 and APMV-12, but was distinct from those APMV serotypes. These results suggest that APMV/Shimane67 is a new APMV serotype, APMV-13.

In Chapter 2, the nucleotide sequences of nucleocapsid protein (N); phosphoprotein (P); matrix protein (M); hemagglutinin-neuraminidase (HN); and large polymerase protein (L) genes, 3′-end leader, 5′-end trailer, and intergenic regions of the avian paramyxovirus (APMV) strain goose/Shimane/67/2000 (APMV/Shimane67) were determined. Together with previously reported data on fusion protein (F) gene sequence [52], the determination of the genome sequence of APMV/Shimane67 has

been completed in this study. The genome of APMV/Shimane67 comprised 16,146 nucleotides in length and contains six genes in the order of 3′-N-P-M-F-HN-L-5′. The features of the APMV/Shimane67 genome (e.g., nucleotide length of whole genome and each of the six genes, and predicted amino acid length of each of the six genes) were distinct from those of other APMV serotypes. Phylogenetic analysis indicated that although APMV/Shimane67 was grouped with APMV-1, -9, and -12, the evolutionary distance between APMV/Shimane67 and these viruses was longer than that observed between intra-serotype viruses. These results show that the genome sequence of APMV/Shimane67 contains specific characteristics and is distinguishable from other types of APMV.

In Chapter 3, the genetic analysis of a pigeon circovirus (PiCV/Japan/2/2010) detected in a racing pigeon with diarrheal symptoms in western Japan was reported. Sequencing revealed a genome size of 2,041 bp that contained 3 open reading frames, designated V1, C1, and C2, a structure consistent with to those of other pigeon circoviruses. Phylogenetic analyses of the open reading frame C1 revealed that PiCV/Japan/2/2010 diverges from other Asian pigeon circoviruses, suggesting genetic diversity of pigeon circoviruses in Asia.

In Chapter 4, to investigate the ecology of avian reovirus (ARV) in wild birds, the surveillance study was conducted in Shikoku district of western Japan. From 240 fresh fecal samples of wild birds such as sparrow, pigeon, crow, swallow and so on, collected from April of 2006 to March of 2009, three avian reoviruses were isolated by using primary chicken embryo liver cells. Phylogenetic analysis of S-1 gene was conducted. The total length of the isolate was 1650 bp and three open reading frames (ORFs) (p10, p17 and sigma C) were predicted same as other ARVs. In phylogenetic analysis of three

ORFs, an ARV isolated from diseased wild crow in Finland showed close relationship with ARV/Sparrow/Kagawa/22/2006. An ARV isolated from healthy wild tree sparrow living in Japan showed unique feature in genetic analysis and these results suggest that the isolate could be considered a candidate for a new species among the strains of ARVs and these results will be benefit for epidemiology of ARVs.

As described above, in the present study, some unique characteristics of avian viruses, i.e. avian paramyxoviruses, circoviruses and orthoreoviruses, originated from wild or free-ranging birds were demonstrated. However, there are still numerous unknown factors in these avian pathogenic virus ecology. Therefore, continued surveillance over multiple years will be necessary for better understanding of the role of wild birds in the introduction and dissemination of these viruses in the field.

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学位論文要旨

渡り鳥を含む野生鳥類がある種の鳥由来病原ウイルスの拡散伝播に重要な役割を果たしている可能性は以前から指摘されている。特に鳥インフルエンザウイルスにとっては、野生水禽類が自然宿主であり、ウイルスの家禽への流行伝播に関わっていることはよく知られている。しかしながら、鳥インフルエンザウイルス以外の鳥由来病原ウイルスの野生鳥類における生態はほとんど明らかとなっておらず、野生鳥類を対象とした広範囲な疫学調査も未だ十分には行われていない。鳥由来病原ウイルスの拡散や維持に野鳥が如何に関わっているのかを明らかにすることは当該ウイルスが野鳥から家禽に伝播する要因を理解し、それを防疫対策に役立てる上で極めて重要と考えられる。

そこで本研究では西日本において、野生および半野生（放し飼い）鳥類を対象とした疫学調査を実施し、そこで分離・検出された鳥パラミクソウイルス、ハトサーコウイルスおよび鳥レオウイルスについて、ウイルス学的、血清学的および遺伝学的解析を実施した。

第 1 章では、野生のガンの糞便から分離された鳥パラミクソウイルス（APMV/Shimane67 株）の生物学的、血清学的及び遺伝学的性状を解析した結果、APMV/Shimane67 株は電子顕微鏡下で、典型的なパラミクソウイルスの粒子形態を持つことが確認された。また、赤血球凝集阻止試験において、本ウイルス株は他のいずれの鳥パラミクソウイルスとも交差反応性が低いことが明らかとなった。本ウイルス株の F 遺伝子は 1,638 塩基からなり、545 アミノ酸からなる F 蛋白をコードしていた。また、その F 蛋白の開裂部位には一対の塩基性アミノ酸が存在する典型的な弱毒型の配列（VRENR/L）が認められた。F 蛋白遺伝子の塩基配列およびそこから推定されるアミノ酸配列は他の鳥パラミクソウイルスのそれらと比較的低い相同性（それぞれ 42.9-62.7% および 28.9-67.3%）を示した。さらに、F 遺伝子の進化系統樹解析において本ウイルス株は NDV、鳥パラミクソウイルス 9 型および 12 型と比較的近縁であったが、異なる系統に属することが明らかとなった。これらの成績からわが国

で野生のガンの糞便から分離された鳥パラミクソウイルス APMV/Shimane67 株は新たな血清型（13 型）である可能性が示唆された。

第 2 章では、APMV/Shimane67 株の遺伝子性状をさらに詳細に解析するため、当該ウイルス遺伝子の全塩基配列を決定した。 その結果、APMV/Shimane67 株の全ゲノムは 16,146 塩基からなり、６つの遺伝子が他の鳥パラミクソウイルス同様 3′-N-P-M-F-HN-L-5′ の順で構成されていた。しかし、全ゲノムサイズおよび６つのウイルス蛋白遺伝子の推定アミノ酸残基数等は他の鳥パラミクソウイルスのそれらと異なっていた。全ゲノム配列情報に基づく進化系統樹解析においても、本ウイルス株と他の既存の血清型のウイルスとの遺伝的距離が同一血清型内の株間の遺伝的距離よりも長かったことから、本ウイルス株が新たな血清型に属する可能性を支持した。

次に第３章では、西日本において下痢症状を呈したレース鳩から検出されたハトサーコウイルス（PiCV/Japan/2/2010）の遺伝学的性状を解析した。その結果、本ウイルスのゲノムサイズは 2,041bp であり、海外で検出されている他のハトサーコウイルスと同様、V1, C1 および C２の３つのオープンリーディングフレームを含んでいた。C１遺伝子の進化系統樹解析結果は、本ウイルスがアジア地域における他のハトサーコウイルスと異なる系統に属することを示しており、アジアにおけるハトサーコウイルス株間の遺伝的多様性が示唆された。

さらに第４章では野生鳥類における鳥レオウイルスの生態を明らかにする目的で陸生野鳥を対象とした疫学調査を実施した。その結果、四国地域で採取された一見健康なスズメの糞便から、３株の鳥レオウイルスが分離された。分離ウイルス株 S1 遺伝子の性状を解析した結果、ゲノムサイズは 1650bp であり、他のレオウイルス同様、p10, p17 および sigma C の３つのオープンリーディングフレームが含まれていた。これらの遺伝子について進化系統学的解析を行った結果、本ウイルスはフィンランドにおいて神経症状を呈したカラスから分離された株と最も近縁であり、他の既存の鳥レオウイルスとは明らかに異なるグループに属することが判明した。これらの成績から、スズメなどの養鶏場周辺に多く生息する

陸生野鳥の間にも鳥レオウイルスが循環、維持されている可能性が考えられた。