Completion of full length genome sequence of novel avian paramyxovirus strain APMV/Shimane67 isolated from migratory wild geese in Japan

Eiji YAMAMOTO¹, Toshihiro ITO¹–³ and Hiroshi ITO¹–³)*

¹United Graduate School of Veterinary Sciences, Yamaguchi University, Yamaguchi 753–8515, Japan
²Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, 4–101 Koyama-Minami, Tottori 680–8553, Japan
³Avian Zoonoses Research Center, Faculty of Agriculture, Tottori University, 4–101 Koyama-Minami, Tottori 680–8553, Japan

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ABSTRACT. 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 [46], 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.

KEY WORDS: APMV, avian paramyxovirus, avulavirus, complete genome sequence, phylogenetic tree

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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 [23]. 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 was reported [23]. 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) [3, 22, 41]. 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 chicks suggested that APMV-10 and -12 revealed little or no virulence in chickens, resembling the low or non-virulent

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 [3, 22, 35, 41]. 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 [5]. APMV genome contains nucleocapsid protein (N); phosphoprotein (P); matrix protein (M); fusion protein (F); hemagglutinin-neuraminidase (HN); and large polymerase protein (L) genes, similar to other members of the family Paramyxoviridae [35]. APMV-6 contains an additional gene that encodes the small hydrophobic protein (SH) [6]. Each gene encodes a single viral structural protein with the exception of P gene. mRNA transcribed from P gene has a potential to translate an additional nonstructural protein, termed V protein [38]. V proteins are translated from mRNA containing one 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 antigenicomic RNAs [21].

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 [17, 29, 30, 36, 37, 40, 44, 45]. Moreover, the number of reports on complete
genome sequences from APMVs other than APMV-1 has been increasing in recent years [1, 3, 4, 6, 15, 18, 22, 33, 39, 41, 42]. 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 a goose collected in 2000 [46]. The F gene of APMV/Shimane67 shared 42.9–62.7% and 28.9–67.3% identities at nt and deduced aa levels, respectively, with those of other APMVs. The deduced aa sequence at the F protein cleavage site of APMV/Shimane67 was QVRENIR/LVG, which resembles the motifs of lentogenic NDV. Phylogenetic analysis revealed that APMV/Shimane67 had a relationship with NDV, APMV-9 and APMV-12, but was distinct from those APMVs. These results and serological 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 and compare with other APMVs genome.

MATERIALS AND METHODS

Extraction of viral RNA, RT-PCR and nucleotide sequencing: The isolation and characterization of APMV/Shimane67 have been previously described [46]. Viral genomic RNA was extracted from infected allantoic fluid using QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo, Japan), according to the manufacturer’s instructions manual. The cDNA of the APMV/Shimane67 genome was synthesized using PromerScript Reverse Transcriptase (TaKaRa Bio, Otsu, Japan) 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, Foster City, CA, U.S.A.) and analyzed using the 3130 xl Genetic Analyzer (Applied Biosystems).

Determination of 3′- and 5′-ends of the viral genome sequence: The 3′-end of the viral genome (LC region) sequence of APMV/Shimane67 was determined by a method previously described [43]. 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, Palo Alto, CA, U.S.A.) 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/) [11]. The transmembrane region of HN protein was predicted by the SOSUI system (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) [13]. 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 [19]. Phylogenetic trees were generated by Neighbor-Joining method [34] with 1,000 bootstraps using Clustal X program and then visualized with NJPlot [31]. The nucleotide sequence data reported in this study have been deposited in the DDBJ database under the accession number 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/Yucaipa, EU338441); APMV-2/finch/Northern Ireland/Bangor/73 (APMV-2/Bangor, HM159995); APMV-3/parakeet/Netherlands/449/75 (APMV-3/NLD, EU403085); APMV-3/turkey/Wisconsin/68 (APMV-3/WI, EU782025); APMV-4/duck/Hong Kong/D3/75 (APMV-4/D3, FJ177514); APMV-4/4/05 (APMV-4/KR/YJ/06 (APMV-4/KR/YJ, EU877976); APMV-5/duckerigar/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, QQ406232); APMV-7/duck/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/Falkland islands/324/2007, AM147142); 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

Genomic features of APMV/Shimane67: The genome characteristics of APMV/Shimane67 and some other APMVs are summarized in Table 1. The genome of APMV/Shimane67 comprised 16,146 nt that was slightly AU-rich (A 25.9%, C 22.1%, G 20.5% and U 31.5%). 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 other APMVs, except for APMV-6. The SH protein gene existing in the APMV-6 genome was not present in the APMV/Shimane67. Whole genome sequences of other APMVs for comparison with APMV/Shimane67 were from the following sources (abbreviation and accession number): APMV-1/strain SF02 (APMV-1/SF02, AF473851); APMV-2/chicken/California/Yucaipa/56 (APMV-2/Yucaipa, EU338441); APMV-2/finch/Northern Ireland/Bangor/73 (APMV-2/Bangor, HM159995); APMV-3/parakeet/Netherlands/449/75 (APMV-3/NLD, EU403085); APMV-3/turkey/Wisconsin/68 (APMV-3/WI, EU782025); APMV-4/duck/Hong Kong/D3/75 (APMV-4/D3, FJ177514); APMV-4/4/05 (APMV-4/KR/YJ/06 (APMV-4/KR/YJ, EU877976); APMV-5/duckerigar/Kunitachi/74 (APMV-5/Kunitachi, GU206351); APMV-6/duck/Italy/4524-2/07 (APMV-6/ITA/4524-2, QQ406232); APMV-7/duck/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/Falkland islands/324/2007, AM147142); 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).
tioned as promoters of viral genome replication. Moreover, similar sequence motifs were reported in APMV-2 genome [40]. 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. 1a). 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. 1b and 1c). The three times repeated motifs, 3′-GGUGGC-5′, 3′-ACAAAGC-5′ and 3′-UCAAAGC-5′, and 3′-AUUUCGC-5′, 3′-UCCAGCC-5′ and 3′-UUGACGC-5′ were found in 73–90 nt from the 3′-terminus of the genome and antigenome of APMV/Shimane67, respectively. The GS signal of APMV/Shimane67 was well preserved, and its consensus sequence was “ACGGGCAGAA” (Fig. 2a). In contrast, the preservation of GE signal sequences of APMV/Shimane67 was relatively low. The consensus sequence of the APMV/Shimane67 GE signal was TTAAGA, 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. APMV/Shimane67 GS and GE sequences had similarities with those of APMV-1, -9 and -12 (Fig. 2b). The IGS of APMV/Shimane67 varied in nucleotide sequence and length (Fig. 2a). The IGS length of NP-P, P-M, M-F, F-HN and HN-L junctions was 14 nt, one nt, two nt, 14 nt and 25 nt, respectively. 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

| 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        | 210         | 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           | 1          | 30                  |
| 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          | 31         | 31                  |
| 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          | 2,002      | 2,002       | 2,130      | 2,072               |
| Amino acid      | 610           | 577            | 616        | 571         | 579        | 614                 |
| HN-L intergenic (nt) | 25           | 47             | 48         | 47          | 40         | 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 1. Comparison of nucleotide and amino acid length between APMV/Shimane67, APMV-1, -9 and-12

a) The nucleotide sequence of APMV/Shimane67 F gene was reported by Yamamoto et al. [46].
Table 2. Nucleotide (nt) and deduced amino acid (aa) sequence identities between APMV/Shimane67 and other APMVs (%)

| Virus          | Full genome | N  | P  | V  | M  | HN | L  |
|----------------|-------------|----|----|----|----|----|----|
| 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/SD02    | 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 | 42.1 | 31.7 | 44.4 | 36.1 | 43.8 | 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 | 38.7 | 32.2 | 46.3 | 33.2 |
| APMV-3/MI      | 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 | 46.2 | 35.4 | 43.2 | 32.5 |
| APMV-4/KR/YJ   | 42.3        | 48.4 | 37.0 | 39.2 | 22.5 | 20.4 | 41.0 | 24.4 | 43.6 | 32.7 | 45.3 | 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-7/7N      | 44.8        | 49.1 | 39.1 | 38.7 | 20.0 | 26.7 | 44.2 | 30.3 | 45.2 | 37.0 | 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        | 49.0 | 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 | 26.8 | 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 | 61.3 | 45.2 | 65.1 | 73.6 | 61.3 | 60.5 | 63.1 | 65.5 |

Fig. 1. 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.
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 2). There are 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 [25]. Among these regions, region 3 is particularly important, because this region is thought to be involved in the N–N protein self-assembly [26]. The N protein of APMV/Shimane67 contained aa sequences similar to these motifs: 171QIQWVTAKAMT181, 266FFI_LTKYGINT277 and 322FAPAEYSLMYFSFG336 (Fig. 3). The 7th (P) and 13th (A) aa of region 3 motif were replaced with 328S and 334S, respectively. The aa sequence of region 2 was completely identical among APMV/Shimane67, APMV-1 and APMV-12.

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 2). 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 and -9, and 38.7%–42.9% with the other remaining APMVs (Table 2).
Fig. 3. 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). 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.

| Motif                  | V       | C       | LK     | AI     | V       |
|------------------------|---------|---------|--------|--------|---------|
| APMV/Shimane67         | QIWTLKAMT| FLYTIVG | FXXTIR°| GLXT   | FXXXYPXXSΦAMG |
| APMV-1/LaSota          | .V...   | .V...... | .V...... | .V...... | .V...... |
| APMV-2/Yucaipa         | .IAI..  | .S...... | .RF...  | .G..... | .NF..  | .TL.. | .YA.. |
| APMV-3/NLD             | .ILV... | .LT...  | .R...... | .G..... | .GN..  | .L...... | .YA.. |
| APMV-4/HK/03           | .ILV... | .LT...  | .R...... | .G..... | .GNF... | .PH... | .YA.. |
| APMV-5/Kunitachi       | .SAM... | .S...... | .RF...  | .G..... | .GN...  | .P... | .YA.. |
| APMV-6/HK/D199         | .AAM... | .S...... | .RF...  | .G..... | .GN...  | .P... | .YA.. |
| APMV-7/TN              | .A...AI | .S...... | .RF...  | .VG...  | .N...T | .L... | .YA.. |
| APMV-8/DE              | .S...AI | .S...... | .F...VG | .N...T | .L... | YA.. |
| APMV-9/NY              | .V...TV | .S...... | .F...VG | .E...AQL | .YA.. |
| APMV-10/FLK            | .ZAI... | .C...... | .RF...  | .G..... | .N...T | .L... | Y.A.D |
| APMV-11/FRA            | .AVI... | .S...... | .RF...  | .LG...  | .SN... | .L... | YA.. |
| APMV-12/ITA/3920-1     | .L...... | .L...... | .F...VG | .N...T | .L... | YA.. |

Fig. 4. Alignment of (a) nucleotide sequences of RNA editing sites (mRNA sense) and (b) C-terminal regions of V protein. Identical nucleotides or amino acids with APMV/Shimane67 are shown by dots. Conserved cysteine residues of V protein are shown by cross. Conserved HRRE and WCNP motifs are shaded gray. Numbers indicate the nucleotide or amino acid positions of the (a) P gene ORF and (b) V protein of APMV/Shimane67, respectively.

| Motif                  | ATC..   | ATC..   | ATC..   |
|------------------------|---------|---------|---------|
| APMV/Shimane67         | PGR...  | HSIS... | STW... |
| APMV-1/LaSota          | .TMG... | .VT.I... | .S...S... | .KAE.R.YP.I... | .S...AT... | AS.D |
| APMV-2/Yucaipa         | .Y.FIS... | .RLEV. | .V...S.R | .E.RREK.T...T. | .ES.I... | .RQP... |
| APMV-3/NLD             | .S...Y... | .PG...GF | .ET...A.S.V...AF.K.YK.A | RQ...R...D... | .FNPA |
| APMV-4/HK/03           | .FP...MAI... | .VDTK... | .ATGD.E | .ELVE... | G.TA...VRI...E.TRLD.V...H...TI...S... | .MY...D |
| APMV-5/Kunitachi       | .Y.LF...DG... | .RCSI.E | .T...R... | .AI.SVQR.T....E.RR.SM...WN...S |
| APMV-6/HK/D199         | .RR...F...RH...G... | .OC...LAE...V...V...| .V...V...PE.RT.K....I.R...V... | .IN...R...N...S |
| APMV-7/TN              | .Y...FA... | .AGT...NKF... | .VS... | .T...RPY...TVER... | .N... | .PG...QSAL |
| APMV-8/DE              | .Y...FTTFH... | .G...TTRV... | .I.T...R...AR...IYDE... | .E...TT...IM...RD...K |
| APMV-9/NY              | .G...M...YV...NAN... | .V...I...V...S.V...VE...RE...T.S...S... | .TE... | .AGSH |
| APMV-10/FLK            | .Y...S.INE...H... | .STLVE... | .N...T...RAY.RREK.I.RR... | .TT...T...R...N...D...N |
| APMV-11/FRA            | .V...YIRL... | .RSE...G... | .NQY...V.E... | .K...RAN...IREQ... | .Y...RA...TM...Y... |
| APMV-12/ITA/3920-1     | .V... | .Y... | .E...K... | .---... | .SLG... | .I...V...AE...RK...Q...E... | .PT... | .ARDA |

Fig. 3. 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). 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. 4. Alignment of (a) nucleotide sequences of RNA editing sites (mRNA sense) and (b) C-terminal regions of V protein. Identical nucleotides or amino acids with APMV/Shimane67 are shown by dots. Conserved cysteine residues of V protein are shown by cross. Conserved HRRE and WCNP motifs are shaded gray. Numbers indicate the nucleotide or amino acid positions of the (a) P gene ORF and (b) V protein of APMV/Shimane67, respectively.
[7, 10]. NLS comprised a bipartite clustering of basic amino acids (e.g., BKGGKVTDFKLKLEKKIR of APMV-1/LaSota M protein). In the APMV/Shimane67 M protein, the putative bipartite NLS motif (258KGKKVTDKLEKLKIR263) was found at positions 248–263 aa (Fig. 3a). The FPV late domain that contributes to efficient viral release and replication was at positions 23–26 aa of the APMV-1 M protein [10]. Furthermore, a comparable sequence motif in other APMV M proteins was reported [37, 44, 45]. A similar aa sequence motif (FPVV) was found at positions 23–26 aa in the M protein of APMV/Shimane67 (Fig. 3b).

**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 pl 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 2). The HN protein of paramyxoviruses is a type II transmembrane protein. The transmembrane region of APMV/Shimane67 HN protein was predicted at positions 25–47 by the SOSUI system [13]. Five N-glycosylation motifs (N-X-S/T) were found at positions 119, 341, 392, 481 and 604 aa of APMV/Shimane67 HN protein (Table 3). 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. The sialic acid-binding motif NRKCS was identified at positions 234–239 of the HN protein of APMV/Shimane67 (Table 3). Twelve aa (174E, 186R, 265K, 288E, 299Y, 317Y, 401E, 408R, 489R, 525Y and 547E) engaged in sialic acid-binding and neuraminidase activity.

![Fig. 5. 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. Numbers indicate the amino acid positions of the APMV/Shimane67 M protein.](image)

**Table 3. Comparison of amino acid position and sequences of functional domains of the HN protein between APMV/Shimane67 and other APMVs**

| Virus               | Amino acid positions of potential N-linked glycosylation site | Sialic acid-binding motif | Sialic acid-binding and neuraminidase activity |
|---------------------|-------------------------------------------------------------|---------------------------|------------------------------------------------|
| APMV/Shimane67      | 119, 341, 392, 481, 604                                     |                           |                                                |
| APMV-1/LaSota       | 119, 341, 433, 481, 528                                      |                           |                                                |
| APMV-2/Yucaipa      | 119, 278, 345, 392, 481                                     |                           |                                                |
| APMV-3/NLD          | 33, 53, 58, 115, 309, 322, 380, 493, 494                     |                           |                                                |
| APMV-4/HK/D3        | 11, 57, 142, 322, 380, 392, 443                             |                           |                                                |
| APMV-5/Kunitachi    | 60, 119, 148, 278, 346, 392                                 |                           |                                                |
| APMV-6/HK/D199      | 119, 278, 346, 377, 392, 438, 483                           |                           |                                                |
| APMV-7/TN           | 60, 119, 145, 278, 343, 377, 392, 483, 513, 564             |                           |                                                |
| APMV-8/DE           | 119, 278, 392, 507                                          |                           |                                                |
| APMV-9/NY           | 119, 147, 228, 341, 348, 433, 483                           |                           |                                                |
| APMV-10/FLK         | 119, 147, 278, 352, 432, 483                                |                           |                                                |
| APMV-11/FRA         | 147, 150, 266, 278, 345, 392, 431, 479, 484                |                           |                                                |
| APMV-12/ITA/3920-1  | 119, 147, 341, 348, 594                                     |                           |                                                |

a) Numbering of amino acid residues corresponds to APMV/Shimane67 HN protein sequence. b) The identical amino acids between APMV/Shimane67 and other APMVs are shown by dot.
Avulavirus, but distinct from other APMVs. The APMV/Shimane67 was classified as a member of the genus

DISCUSSION

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 [46], whole genome sequencing of APMV/Shimane67 was completed.

The genome of APMV/Shimane67 basically had following common features with other APMVs: agreement with the "rule of six"; gene order (3'-N-P-M-F-HN-L-5') and existence of GS and GE signal, and IGS; complementation of 3' and 5'-terminus sequence; existence of three times repeated motif; existence of the putative RNA editing site of P gene. In addition, the coding proteins of APMV/Shimane67 genome contained following conserved aa sequence motifs with other APMVs: N protein self-assembly motif; cysteine-rich region and HRRE and WCNP motifs of V protein; putative bipartite NLS motif and the late domain of M protein; amino acids constituting the sialic acid binding site of HN protein; putative active site for nucleotide polymerization and ATP-binding site of L protein. These nt and aa characteristics of APMV/Shimane67 can contribute to the efficient replication and transcription of viral genome, and viral growth.

By contrast, the genome of APMV/Shimane67 had differences with other 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 (Table 1). The genome size of APMV/Shimane67 was 16,146 nt long. This genome size is different from any other known APMVs genome and was the fifth longest among APMVs. With the exception of APMV/Shimane67, there are four APMVs, which have genome 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 [3, 6, 17, 18, 37, 42, 45]. The relatively large genome size of APMV/Shimane67 is attributable to its long Tr sequence (776 nt). The Tr sequence longer than 700 nt was also found in APMV-3/NLD genome [17]. However, the significance of these long Tr sequence is unclear, and a further study is needed to clarify this issue. The phylogenetic trees indicated that APMV/Shimane67 had the closest relationship with APMV-12/ITA/3920-1. 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/TWN/Y1 and APMV-6/ITA/4524-2 at 0.265. These strains were antigenically and genetically divided into subgroups in each serotype [2, 18, 39, 45]. 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,
The complete genome sequence of APMV/Shimane67

-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 [3, 22, 35, 41]. Our previous serological analysis demonstrated that APMV/Shimane67 was distinct from APMV-1, -2, -3, -4, -6 and -7 [46]. 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. Quite recently, Karamendin et al. [16] reported the whole genome sequence of a novel APMV isolated from a white-fronted goose in northern Kazakhstan (GenBank accession number KU64513). The genome length of APMV/Shimane67 is 150 nt longer than that of Kazakhstan APMV, while genome nt identity of these strains was very high (approximately 96%). The deduced aa sequence identities of the N, P, M, F, HN and L proteins between APMV/Shimane67 and Kazakhstan APMV are 99.6%, 99.2%, 98.9%, 99.6%, 99.1% and 99.6%, respectively. Although there is no serological evidence for the close relationship between APMV/Shimane67 and
Fig. 8. Phylogenetic analyses of the complete genome from members of the subfamily Paramyxovirinae. Phylogenetic trees are generated with the program ClustalX [19] and viewed using NJplot [31]. The numbers at the branches represent bootstrap values from 1,000 replicates. The number of nucleotide substitutions per site (scale bar) is shown.
Kazakhstan APMV, the genomic similarities of APMV/Shimane67 and Kazakhstan APMV suggest that these two strains would be classified as the same serotype, APMV-13.

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