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First detection of Avian metapneumovirus subtype C Eurasian Lineage in a Eurasian wigeon (*Mareca penelope*) wintering in Northeastern Italy: an additional hint on the role of migrating birds in the viral epidemiology

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Abstract

Avian metapneumovirus (aMPV) economically affects the global poultry industry causing respiratory and reproductive disorders. Considering the paucity of data on the aMPV occurrence in European free-ranging avifauna, a molecular survey was conducted on wild birds of 23 species belonging to the orders Anseriformes, Charadriiformes or Passeriformes, captured alive and sampled in Northeast Italy as part of the national Avian influenza virus (AIV) surveillance activities. A total of 492 oropharyngeal swabs, collected from 2007 to 2010, all AIV negative, were screened from aMPV by subtype-specific qRT-PCR. An aMPV-C strain, named aMPV/C/IT/Wigeon/758/07, was found in a wintering young Eurasian wigeon (*Mareca penelope*) sampled in November 2007. The matrix, fusion, and attachment glycoprotein genes of the detected strain were subsequently amplified by specific independent RT-PCRs, then sequenced, and compared in a phylogenetic framework with known aMPV homologous sequences retrieved from GenBank. Close genetic relationships were found between the aMPV/C/IT/Wigeon/758/07 strain and subtype C Eurasian lineage strains isolated in the late 1990s in French domestic ducks, suggesting epidemiological links. Eurasian wigeons are indeed medium to long-range migrant dabbling ducks that move along the Black Sea/Mediterranean flyway, our finding might therefore be related to migratory bridges between countries. To our knowledge, this is the first molecular evidence of the occurrence of a subtype C in Italy and backdates the aMPV-C circulation to 2007. Moreover, results suggest the susceptibility of Eurasian wigeons to aMPV. Broader investigations are needed to assess the role of wild ducks and the significance of the wildfowl/poultry interface in the aMPV-C epidemiology.

Keywords: Avian Metapneumovirus subtype C; Eurasian wigeon; *Mareca penelope*; waterfowl; molecular epidemiology; migratory birds.

Research Highlights:

- Wild birds live-captured in Italy were tested for aMPV detection and characterization
- aMPV-C Eurasian lineage was found for the first time in a wintering Eurasian wigeon
- Migratory birds could be involved in the aMPV epidemiology

Introduction

The *Metapneumovirus* (MPV) genus belongs to the *Pneumoviridae* family, and includes non-segmented, single-stranded, negative-sense RNA viruses. To date, two MPVs species are recognized: *Avian metapneumovirus* (aMPV) and *Human metapneumovirus* (hMPV) (Rima *et al*., 2017). *Avian metapneumovirus* is an emerging poultry pathogen mainly associated to respiratory and reproductive disorders in turkeys and chickens, resulting in severe economic losses to domestic poultry industry.

To date, different aMPV-subtypes have been recognized based on genetic and antigenic profiles (aMPV-A, -B, -C, -D) further showing diverse spatial distribution (Rautenschlein, 2020) and host range (Brown *et al*., 2019). aMPV-A and -B subtypes have been detected in Asia, Africa, Europe, and South America, whereas subtype D it was only detected once in France (Bäyon-Auboyer *et al*., 2000). Subtype C, firstly reported in the US (Senne *et al*., 1997), genetically diverges from aMPV-A, -B and -D, and is closely related to hMPV, suggesting a possible common origin (Yunus *et al*., 2003). Two aMPV-C lineages have been recognized so far, according to the attachment glycoprotein (G) gene sequence and named North American or Eurasian lineages (Toquin *et al*., 2006). Moreover, two divergent aMPV strains have been recently detected in North America, one in a great black-backed gull (*Larus marinus* LINNAEUS, 1758) (Canuti *et al*., 2019), and another in a monk parakeet chick (*Myiopsitta monachus* BODDAERT 1783) (Retallack *et al*., 2019), tentatively increasing aMPV known subtypes to six.

In Italy, field studies revealed an aMPV widespread circulation in poultry mainly from densely populated poultry areas in the Northeastern part of the country. Even though the infection is mostly sustained by subtype B (Catelli *et al*., 2004; Listorti *et al*., 2014; Cecchinato *et al*., 2018; Tucciarone *et al*., 2018), aMPV-A has also been sporadically detected (Catelli *et*

al, 2006; Lupini *et al*., 2011). Eventually, aMPV-C specific antibodies were demonstrated in intensively raised domestic mallards (Legnardi *et al*., 2021).

Since aMPV first appearance in South Africa in 1978 (Buys & du Preez, 1980) followed by an initial spread to Europe and Israel (Jones, 1996), migratory birds and respective flyways have been hypothetically linked to aMPV global transmission. Repeated detections of aMPV subtype C in free-living birds have been reported in North America (Shin *et al*., 2000; Bennett *et al*., 2002, 2004; Turpin *et al*., 2008; Cha *et al*., 2013; Jardine *et al*., 2018) and the periodic pattern of aMPV outbreaks in poultry has been linked to migratory movements (Shin *et al*., 2000). As regards to previous aMPV detections in European countries, aMPV has been detected in wild mallards (*Anas platyrhynchos* LINNAEUS, 1758), graylag geese (*Anser anser* (LINNAEUS, 1758)) and common gulls (*Larus canus* LINNAEUS, 1758) in the Netherlands and characterized as subtype C according to partial polymerase gene (L) sequence analysis (van Boheemen *et al*., 2012). Serological (Catelli *et al*., 2001; Gethöffer *et al*., 2021) and molecular evidence (Curland *et al*., 2018) of aMPV in free-living pheasants (*Phasianus colchicus* LINNAEUS, 1758) has been reported in Italy and Germany, without providing further indication regarding the subtype involved. Taken as a whole, all this evidence increasingly supports the possible role of wild birds as aMPV carriers or reservoir hosts.

To enlighten the status of migratory and resident free-living birds with respect to the aMPV epidemiology, a molecular survey was performed in wild species sampled from 2007 to 2010 in northeast Italy for the Avian influenza (AI) National Surveillance Plan.

Materials and methods

Ethical Statement

All the samples were collected as part of Italy's live wild bird Avian Influenza (AI) surveillance activities carried out by the Istituto Zooprofilattico delle Venezie, the Italian National Reference Centre and European Union Reference Laboratory for AI and Newcastle disease. Particularly, wild birds were captured alive during the authorized ringing activities conducted from 2007 to 2010 in accordance with the Italian Institute for Environmental Protection and Research (ISPRA—Higher Institute for Environmental Protection and Research). No supplementary permits or approvals were needed for sampling from wild birds captured alive for ringing activities, the sampling was indeed conducted as part of the National Avian influenza surveillance program. All birds were handled in accordance with "Guidelines to the Use of Wild Birds in Research" (Fair *et al*., 2010).

Background and sample collection

Oropharyngeal swabs were obtained from 23 species of wild birds (Table 1) sampled within the National Surveillance Plan for AI performed from spring to autumn 2007-2010. Swabs were individually collected and immersed in 1 ml of phosphate-buffered saline (PBS) with antibiotics and stored at -80°C until processing. Sample size was defined according to a previously published aMPV survey on wild birds (van Boheemen *et al*., 2012). Setting the average aMPV prevalence at 1%, a minimum of 299 samples was required to find at least one positive sample in an infinite population with a confidence of 95% (Cannon & Roe, 1982). A total number of 492 samples was eventually analyzed.

Of the birds studied, 53.9% (265/492) belonged to the order Anseriformes; 19.5% (96/492) to the order Charadriiformes, and 26.6% (131/492) to the order Passeriformes. According to age classes as defined by trained ornithologists, 27.8% (137/492) of the birds were aged as adults, 40% (197/492) as juveniles (1st calendar year), and 32.1% (158/492) were

not categorized. The study area comprised wetlands of the Veneto and Emilia-Romagna regions located in the Po river delta, considered as strategic resting, wintering, and breeding sites for Anseriformes, Charadriiformes and Passeriformes species. All the samples screened for aMPV molecular detection resulted negative for AI.

Sample processing and RNA extraction

For elution, each sample was centrifuged at 1.500 g for 5 minutes at 4°C and processed in pools of five. Samples were pooled, whenever possible, according to the species. Total RNA was extracted from each pool using a protocol based on guanidine-thiocyanate method (Chomczynski & Sacchi, 1987).

aMPV detection and subtyping by qRT-PCR

A total of 99 pools was screened by qRT-PCR, with minor modifications of the method described by Lemaitre *et al*. (2018), for aMPV detection and subtyping. The reaction was performed with SuperScript™ III Platinum™ SYBR™ Green One-Step (Invitrogen, Waltham, MA, USA) on Lightcycler® 96 Instrument (Roche, Basel, Switzerland), using the primer pair PanMPV/N1fwdA and PanMPV/N1RevB (Lemaitre *et al*., 2018) targeting the nucleoprotein (N) gene. For each reaction, 2 µl of extracted RNA were added to a standard reaction mix composed of 1X SYBR® Green Reaction Mix, 0.2 µl of SuperScript III RT/Platinum Taq Mix, and 0.6 µM of each primer. Molecular biology grade water was added up to a final volume of 10 µl. The thermal protocol was set as follows: 50° C for 3 minutes for the reverse transcription phase, 95° C for 5 minutes for the initial activation step, 45 cycles at 95° C for 15 seconds for the denaturation phase and 60° C for 30 seconds for annealing and extension. Melting curve

analysis was performed by progressively increasing the temperature from 40° C to 90° C and continuously monitoring the fluorescence data. Tentative strain subtyping was performed by melting temperature (Tm) evaluation and comparison with reference strains.

Pools positive for aMPV were traced back to the original samples, which underwent individual RNA extraction and subsequent qRT-PCR analysis.

aMPV subtype C molecular characterization

For molecular characterization of the aMPV subtype C positive samples, a RT-PCR targeting partial matrix (M) viral gene was performed as previously described (Seal, 1998). Furthermore, additional primers were designed on a previously published aMPV-C complete sequence (Brown *et al*., 2014) targeting the fusion (F) and attachment glycoprotein (G) viral genes (Table 2). F and G genes were amplified using one reverse transcription (RT) and 3 overlapping independent PCRs for each gene. RT reactions were performed using a OneScript® Plus cDNA Synthesis Kit according to manufacturer instructions (Applied Biological Materials Inc., Richmond, BC, Canada). Random primers (1 µM) were used for total RNA transcription. The reactions were performed at 50°C for 15 min followed by RT inactivation at 85°C for 5 min.

The following reaction mix was used in each PCR: 0.25 µL of GoTaq® DNA Polymerase (Promega, Madison, WI, United States), 10 pmol of the appropriate primer pair, 1 µL of dNTPs (Promega, Madison, WI, United States), 10 µL of PCR GoTaq® 5x reaction buffer, 3.5 µL MgCl2 (3 mM), 31.25 µL of nuclease free water (Promega, Madison, WI, United States) and 3 µL of cDNA. The PCR cycling parameters for both F and G gene PCR assays were as follows. A pre-cycle step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 50°C for 40 s and 72°C for 65 s, followed by a final extension at 72°C for 5 min.

PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). Sequencing was performed by a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands).

Nucleotide sequences were edited and assembled using BioEdit Sequence Alignment Editor version 7.2 (Hall *et al*., 2011), then aligned against and compared with previously published aMPV sequences available on NCBI GenBank database (Table S1, S2 and S3 in Supplemental Material), using MAFFT version 7.397 online service which applied automatic detection of the parameter set (Katoh *et al*., 2002). Best partition scheme, substitution model selection according to Bayesian information criterion (BIC), and maximum likelihood phylogenetic reconstruction for M, F and G genes were performed separately on the IQ-TREE web server (Trifinopoulos *et al*. 2016; Kalyaanamoorthy *et al*., 2017). The robustness of inferred clades was evaluated using 1000 ultrafast bootstrap replicates. Branches with bootstrap values ≥70 were considered reliable. Pairwise genetic p-distance and between group mean distance were estimated using MEGA software version 11.0.10 (Kumar *et al*. 2018).

Results

Among all the tested samples, a young male Eurasian wigeon (*Mareca penelope* (LINNAEUS, 1758)) captured alive in November 2007 in the Po river delta area (Rovigo province, Veneto) was positive at qRT-PCR for aMPV subtype C (qRT-PCR cycle threshold value of the positive pooled sample: 35; qRT-PCR cycle threshold value of the positive individual sample: 33.5). The strain, named aMPV/C/IT/Wigeon/758/07 following the nomenclature reported by Mescolini *et al*. (2021), was molecularly characterized. Partial M and F gene sequences of 400 and 1620 bases respectively, and the complete sequence of the G gene

(2160 bases) were obtained by RT-PCR and sequencing. Sequence data were submitted to the NCBI GenBank database under accession numbers OM021855, OM021856, and OM021857.

The phylogenetic tree constructed on the G gene sequence (Figure 1) demonstrated the clustering of the aMPV/C/IT/Wigeon/758/07 strain within the Eurasian lineage of aMPV-C, which includes French and Chinese subtype C field strains isolated from domestic duck flocks (Toquin *et al*., 1999, 2006; Sun *et al*., 2014). Specifically, the aMPV/C/IT/Wigeon/758/07 strain was closely related to a clade including strains isolated from Muscovy ducks (99178 and 99350 strains) and White Pekin ducks (00094 strain) in France (Toquin *et al*., 2006). Between group mean distance, measured among the G gene sequence obtained in the present study and the French clade, was 0.042.

M and F genes phylogenetic trees (Figure 2 and Figure 3, respectively), confirming the results obtained with the G gene, showed a close relationship between the aMPV/C/IT/Wigeon/758/07 strain and the 99178 strain (Brown *et al*., 2014). Pairwise p-distance values obtained comparing the above-mentioned strains were of 0.020 in M gene and 0.014 in F gene, respectively.

Discussion

We hereby report the detection of an aMPV subtype C in a juvenile Eurasian wigeon (*M. penelope*) wintering in northeast Italy. To our knowledge, this is the first molecular evidence of the occurrence of a subtype C in Italy and it backdates the aMPV-C circulation to 2007. Eventually, our finding suggests for the first time the susceptibility to aMPV infection of Eurasian wigeons.

Close genetic relationships were found between the detected strain and strains of the subtype C Eurasian genetic lineage, particularly with those isolated in the late 1990s in French

domestic ducks. This might be related to migratory bridges between countries, along the Black Sea/Mediterranean flyway. Eurasian wigeons are indeed considered medium to long-range migrant dabbling ducks. Whilst major concentrations of wintering individuals are found in Northwestern Europe and Eastern Asia, remarkable numbers are also reported for France and other Mediterranean countries, including Italy (Atkinson *et al*., 2006). Furthermore, ringed birds' recoveries showed a direct connection between Italian and French wetlands (Atkinson *et al*., 2007; Spina & Volponi, 2008). Diverse factors such as migratory movements, dense birds' congregations, and presence of immunologically naïve juveniles are considered relevant drivers in disease dynamics as already known for a major avian viral disease such as AI (Gaidet *et al*., 2010; van Dijk *et al*., 2014). Our finding suggests that these factors might also be relevant for the aMPV-C epidemiology. Considering that the geographical separation of wild hosts undertaking seasonal movements along different migration routes was linked to the initial diversification of AI viruses into Eurasian and American clades (Krauss *et al*., 2007), the presence of two distinct genetic lineages of aMPV-C could also be associated to wild hosts' migratory movements.

Recent serological findings of aMPV-C infection in Italian asymptomatic intensively raised domestic mallards (Legnardi *et al*., 2021) further underline the potential epidemiological role of ducks for aMPV-C circulation. Considering that the Eurasian lineage of aMPV-C is well adapted to domestic ducks as demonstrated by *in vivo* experimental trials (Brown *et al*., 2019), and that domestic duck breeds were domesticated from wild mallards (Qu *et al*., 2009), it is plausible that wild duck species might act as carrier and reservoir hosts for this viral lineage. Our detection of aMPV-C in an additional wild anatid species further supports the latter hypothesis. However, given the paucity of aMPV-C Eurasian-origin strains found, the inferred relationship might be affected by a sampling bias and only partially represents the actual

scenario. Thus, broader investigations are needed to assess the role of wild ducks and the significance of the wildfowl/poultry interface in the aMPV-C epidemiology.

Eventually, to evaluate the biological features of aMPV strains circulating in wild species through *in vitro* and *in vivo* studies and to facilitate whole genome sequencing, viral isolation, hereby not performed, would be essential.

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467 Table 1. Wild birds sampled from 2007 to 2010 during national Avian Influenza surveillance efforts in northeastern Italian wetlands and tested for

468 aMPV detection. Age classes were assessed by trained ornithologists attending sampling sessions.

469

Taxonomic identification	**Age classes[a]**			**Sampling period**				**Total**	**Migratory status[b]**
	juv.	**ad.**	**n.s.**	**2007**	**2008**	**2009**	**2010**		
Anseriformes									
Common teal (*Anas crecca*)	21	9	-	6	3	21	-	30	W; B
Northern pintail (*Anas acuta*)	3	3	-	-	-	6	-	6	W
Mallard (*Anas platyrhynchos*)	40	44	-	10	40	21	13	84	W; B; R
Garganey (*Anas querquedula*)	9	9	-	-	-	18	-	18	B
Common pochard (*Aythya ferina*)	17	4	-	-	7	14	-	21	W; B
Eurasian wigeon (*Mareca penelope*)	36	44	-	42	5	9	24	80	W
Gadwall (*Mareca strepera*)	12	1	-	-	5	6	2	13	W; B
Northern shoveler (*Spatula clypeata*)	11	2	-	3	4	4	2	13	W; B
Charadriiformes									
Yellow-legged gull (*Larus michahellis*)	-	-	96	-	-	96	-	96	W; B; R

470 Table 1. *Cont.*
471

Taxonomic identification	Age classes[a]			Sampling period				Total	Migratory status[b]
	juv.	ad.	n.s.	2007	2008	2009	2010		
Passeriformes									
Eurasian blackcap (*Sylvia atricapilla*)	7	1	1	-	-	9	-	9	W; B
European goldfinch (*Carduelis carduelis*)	-	1	-	-	-	1	-	1	W; B; R
Great tit (*Parus major*)	11	3	1	-	-	15	-	15	W; B; R
Common chaffinch (*Fringilla coelebs*)	-	-	4	-	-	4	-	4	W; B; R
Common blackbird (*Turdus merula*)	-	12	14	-	-	20	6	26	W; B; R
Dunnock (*Prunella modularis*)	2	2	4	-	-	8	-	8	W; B
European robin (*Erithacus rubecula*)	25	1	1	-	-	27	-	27	W; B; R
Eurasian wren (*Troglodytes troglodytes*)	1	-	-	-	-	1	-	1	W; B
Song trush (*Turdus philomelos*)	1	-	35	-	-	20	16	36	W; B
Redwing (*Turdus iliacus*)	1	1	-	-	-	-	2	2	W
Cetti's warbler (*Cettia cetti*)	-	-	1	-	-	1	-	1	B

European greenfinch (*Carduelis chloris*)	-	-	1	-	-	1	-	1	W; B; R
Total	197	137	158	61	64	302	65	492	

[a]Age classes: juv. = juvenile; ad. = adult; n.s. = not specified.

[b]Migratory status in Italy: W = wintering; B = breeding; R = resident.

Table 2. Sequence of primers used for PCR amplifications of F and G genes of aMPV subtype C.

Primer name	**Sequence (5' – 3')**	**Gene**	**Position[a]**	**Product size (bp)**
FC3+	GGTATACAAATGTGTTCAC	F	3130	591
FC3-	GTCCTGAAGATGTGGGCATG	F	3721	
FC5+	GACAGTTTTCTGACAATGC	F	3616	597
FC5-	CCAAAGGTCTGATTATTCC	F	4213	
FC7+	CGTCACCCAATAAGCATGG	F	4102	691
FC7-	CTCAGTAATTGATTCAGCAGG	M2	4793	
SHC2+	GGGATTGTGGTTATGCTTGAG	SH	5910	615
GC2-	CTGTCTGTCCTTGGGTGCTG	G	6525	
GC2+	GAGCAACAACAGGGGCAGAGC	G	6505	981
GC4-	TTGCTCTGGGCTGTTTTGG	G	7486	
GC4+	GAGGCAGACACGAGAACACCC	G	7314	820
LC2-	GTTCTTCTCCTTGATCCTAGTC	L	8134	

[a] Anti-genome position of primer 5' terminus based on subtype C sequence of the 99178 viral strain (GenBank accession number: HG934338).

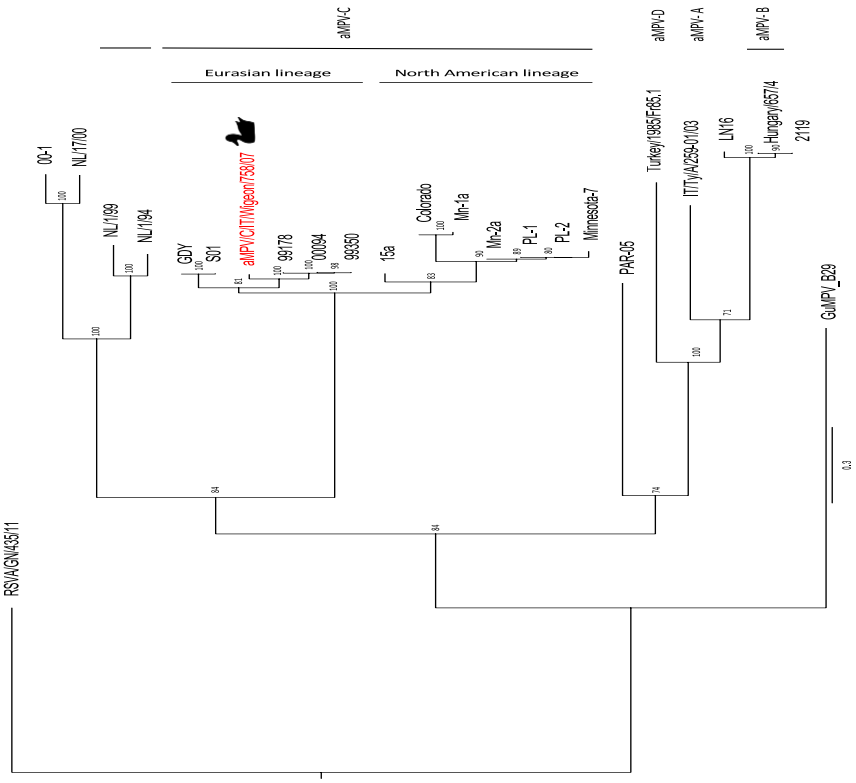
Figure captions

Figure 1. Phylogenetic tree based on G gene nucleotide sequences of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red), aMPV and hMPV reference strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The G sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup. Sequence data are reported in Table S1 (Supplemental Material).

Figure 2. Phylogenetic tree based on partial M gene nucleotide sequences (from nucleotide 2514 to 2913 in the genome) of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red) and aMPV-C strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The partial M sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup. Sequence data are reported in Table S2 (Supplemental Material).

Figure 3. Phylogenetic tree based on partial F gene nucleotide sequences (from nucleotide 3319 to 4650 in the genome) of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red), aMPV and hMPV reference strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The partial F sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup. Sequence data are reported in Table S3 (Supplemental Material).

Figure 1.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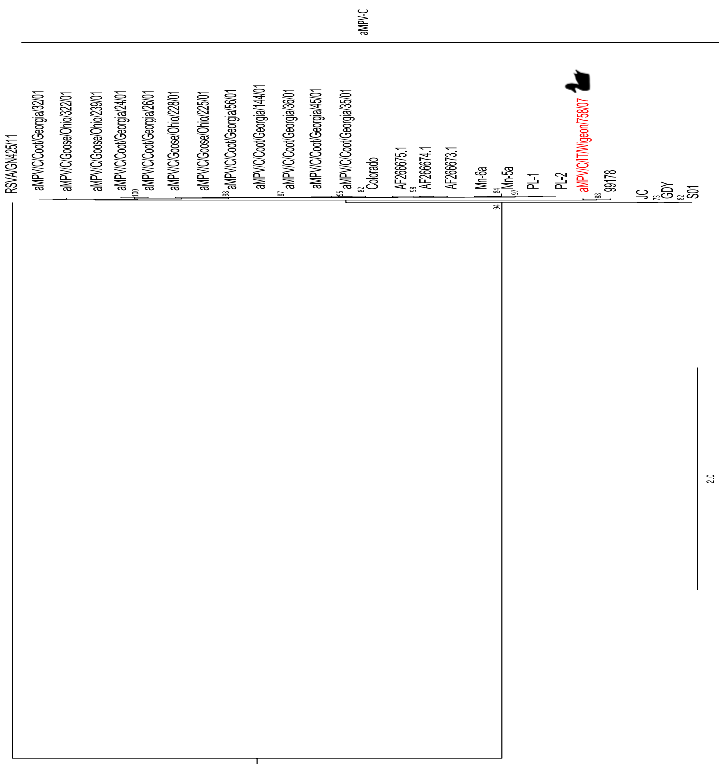
Eurasian lineage
North American lineage
aMPV-D
aMPV-A
aMPV-B
00-1
NL/17/00
NL/1/99
NL/1/94
GDY
SD1
aMPV/CHTM/goose/758/07
99178
000/04
99/350
15a
Colorado
Mn-1a
Mn-2a
PL-1
PL-2
Minnesota-7
PAR-05
Turkey/1985/F/65.1
ITT1/A/259-01/03
LN/16
Hungary/65/14
2119
GdMPV_B29
RSV/A/GN/435/11
0.3

Figure 2.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RSVA/GN425/11
aMPV/C/Coot/Georgia/32/01
aMPV/C/Goose/Ohio/322/01
aMPV/C/Goose/Ohio/239/01
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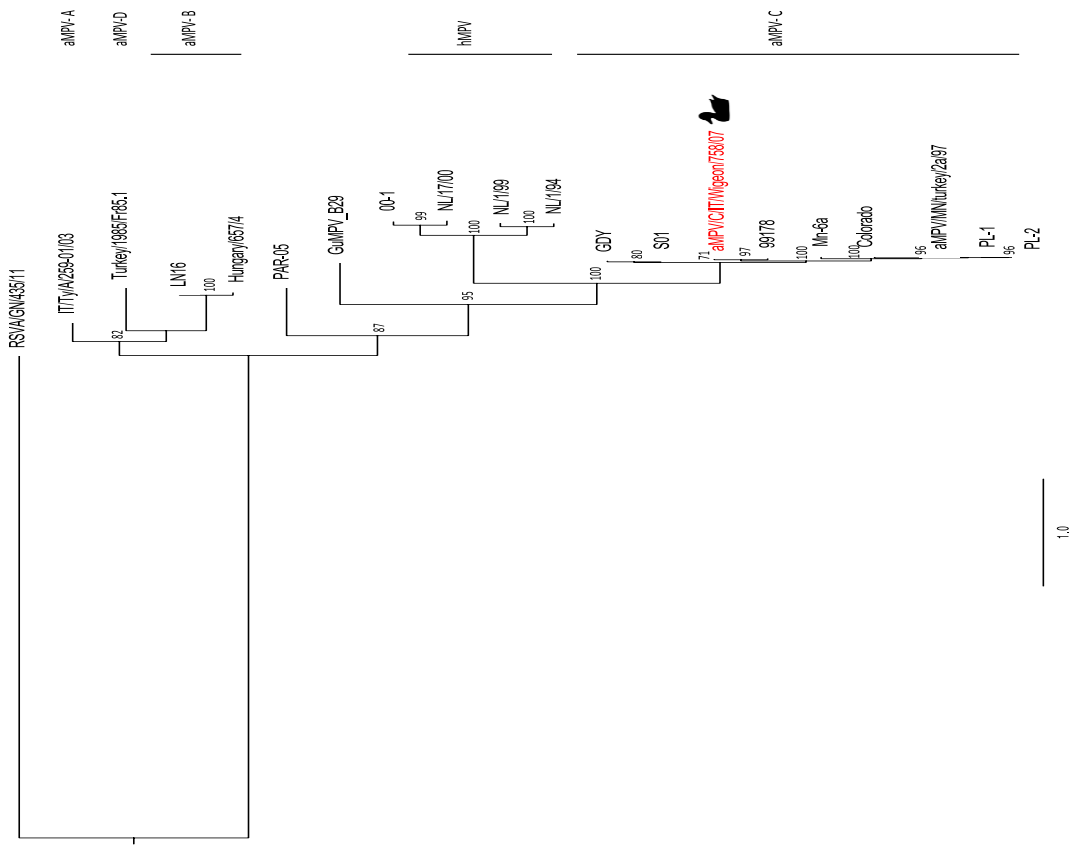
aMPV/C/Coot/Georgia/26/01
aMPV/C/Goose/Ohio/228/01
aMPV/C/Goose/Ohio/225/01
aMPV/C/Coot/Georgia/56/01
aMPV/C/Coot/Georgia/144/01
aMPV/C/Coot/Georgia/36/01
aMPV/C/Coot/Georgia/45/01
aMPV/C/Coot/Georgia/35/01
Colorado
AF266675.1
AF266674.1
AF266673.1
Mn-6a
Mn-5a
PL-1
PL-2
aMPV/C/IT/Wigeon/758/07
99178
JC
GDY
S01
100
98
87
95
82
98
84
97
94
88
73
82
2.0

532 **Figure 3.**


aMPV-A
aMPV-D
aMPV-B
hMPV
aMPV-C
RSV/AGN/435/11
ITT/TyA/2258-01/03
Turkey/1985/F/85.1
LN/16
Hungary/657/4
PAR-05
GuMPV_B29
00-1
NL/17/00
NL/1/99
NL/1/94
GDY
SD1
aMPV/CHT/Wigeon/7538/07
99178
Mn-6a
Colorado
aMPV/MN/turkey/2a/97
PL-1
PL-2
82
87
95
100
99
100
100
80
71
97
100
100
96
96
1.0