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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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Graziosi G., Mescolini G., Silveira F., Lupini C., Tucciarone C.M., Franzo G., et al. (2022). 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. *AVIAN PATHOLOGY*, 51(3), 283-290 [10.1080/03079457.2022.2051429].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/905450> since: 2022-11-22

*Published:*

DOI: <http://doi.org/10.1080/03079457.2022.2051429>

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6 **Giulia Graziosi, Giulia Mescolini, Flavio Silveira, Caterina Lupini, Claudia M. Tucciarone,**  
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23 **First detection of Avian metapneumovirus subtype C Eurasian**  
24 **Lineage in a Eurasian wigeon (*Mareca penelope*) wintering in**  
25 **Northeastern Italy: an additional hint on the role of migrating birds**  
26 **in the viral epidemiology**

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37

38 **Acknowledgments:** The authors thank Professor Marco Martini (University of Padua,  
39 Department of Animal Medicine, Production and Health) for the statistical support.

40

41 **Disclosure statement:** The authors declare no conflict of interest.

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44

45 **Abstract**

46

47 Avian metapneumovirus (aMPV) economically affects the global poultry industry causing  
48 respiratory and reproductive disorders. Considering the paucity of data on the aMPV occurrence  
49 in European free-ranging avifauna, a molecular survey was conducted on wild birds of 23  
50 species belonging to the orders Anseriformes, Charadriiformes or Passeriformes, captured alive  
51 and sampled in Northeast Italy as part of the national Avian influenza virus (AIV) surveillance  
52 activities. A total of 492 oropharyngeal swabs, collected from 2007 to 2010, all AIV negative,  
53 were screened from aMPV by subtype-specific qRT-PCR. An aMPV-C strain, named  
54 aMPV/C/IT/Wigeon/758/07, was found in a wintering young Eurasian wigeon (*Mareca*  
55 *penelope*) sampled in November 2007. The matrix, fusion, and attachment glycoprotein genes  
56 of the detected strain were subsequently amplified by specific independent RT-PCRs, then  
57 sequenced, and compared in a phylogenetic framework with known aMPV homologous  
58 sequences retrieved from GenBank. Close genetic relationships were found between the  
59 aMPV/C/IT/Wigeon/758/07 strain and subtype C Eurasian lineage strains isolated in the late  
60 1990s in French domestic ducks, suggesting epidemiological links. Eurasian wigeons are  
61 indeed medium to long-range migrant dabbling ducks that move along the Black  
62 Sea/Mediterranean flyway, our finding might therefore be related to migratory bridges between  
63 countries. To our knowledge, this is the first molecular evidence of the occurrence of a subtype  
64 C in Italy and backdates the aMPV-C circulation to 2007. Moreover, results suggest the  
65 susceptibility of Eurasian wigeons to aMPV. Broader investigations are needed to assess the  
66 role of wild ducks and the significance of the wildfowl/poultry interface in the aMPV-C  
67 epidemiology.

68

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

71

72 **Research Highlights:**

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

76

## 77 **Introduction**

78

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

85           To date, different aMPV-subtypes have been recognized based on genetic and antigenic  
86 profiles (aMPV-A, -B, -C, -D) further showing diverse spatial distribution (Rautenschlein,  
87 2020) and host range (Brown *et al.*, 2019). aMPV-A and -B subtypes have been detected in  
88 Asia, Africa, Europe, and South America, whereas subtype D it was only detected once in  
89 France (Bäyon-Auboyer *et al.*, 2000). Subtype C, firstly reported in the US (Senne *et al.*, 1997),  
90 genetically diverges from aMPV-A, -B and -D, and is closely related to hMPV, suggesting a  
91 possible common origin (Yunus *et al.*, 2003). Two aMPV-C lineages have been recognized so  
92 far, according to the attachment glycoprotein (G) gene sequence and named North American or  
93 Eurasian lineages (Toquin *et al.*, 2006). Moreover, two divergent aMPV strains have been  
94 recently detected in North America, one in a great black-backed gull (*Larus marinus* LINNAEUS,  
95 1758) (Canuti *et al.*, 2019), and another in a monk parakeet chick (*Myiopsitta monachus*  
96 BODDAERT 1783) (Retallack *et al.*, 2019), tentatively increasing aMPV known subtypes to six.

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

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

103         Since aMPV first appearance in South Africa in 1978 (Buys & du Preez, 1980) followed  
104 by an initial spread to Europe and Israel (Jones, 1996), migratory birds and respective flyways  
105 have been hypothetically linked to aMPV global transmission. Repeated detections of aMPV  
106 subtype C in free-living birds have been reported in North America (Shin *et al.*, 2000; Bennett  
107 *et al.*, 2002, 2004; Turpin *et al.*, 2008; Cha *et al.*, 2013; Jardine *et al.*, 2018) and the periodic  
108 pattern of aMPV outbreaks in poultry has been linked to migratory movements (Shin *et al.*,  
109 2000). As regards to previous aMPV detections in European countries, aMPV has been detected  
110 in wild mallards (*Anas platyrhynchos* LINNAEUS, 1758), graylag geese (*Anser anser* (LINNAEUS,  
111 1758)) and common gulls (*Larus canus* LINNAEUS, 1758) in the Netherlands and characterized  
112 as subtype C according to partial polymerase gene (L) sequence analysis (van Boheemen *et al.*,  
113 2012). Serological (Catelli *et al.*, 2001; Gethöffer *et al.*, 2021) and molecular evidence (Curland  
114 *et al.*, 2018) of aMPV in free-living pheasants (*Phasianus colchicus* LINNAEUS, 1758) has been  
115 reported in Italy and Germany, without providing further indication regarding the subtype  
116 involved. Taken as a whole, all this evidence increasingly supports the possible role of wild  
117 birds as aMPV carriers or reservoir hosts.

118         To enlighten the status of migratory and resident free-living birds with respect to the  
119 aMPV epidemiology, a molecular survey was performed in wild species sampled from 2007 to  
120 2010 in northeast Italy for the Avian influenza (AI) National Surveillance Plan.

121

## 122 **Materials and methods**

123

### 124 ***Ethical Statement***

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

135

### 136 ***Background and sample collection***

137

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

146 Of the birds studied, 53.9% (265/492) belonged to the order Anseriformes; 19.5%  
147 (96/492) to the order Charadriiformes, and 26.6% (131/492) to the order Passeriformes.  
148 According to age classes as defined by trained ornithologists, 27.8% (137/492) of the birds  
149 were aged as adults, 40% (197/492) as juveniles (1<sup>st</sup> calendar year), and 32.1% (158/492) were



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

154

#### 155 ***Sample processing and RNA extraction***

156

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

161

#### 162 ***aMPV detection and subtyping by qRT-PCR***

163

164 A total of 99 pools was screened by qRT-PCR, with minor modifications of the method  
165 described by Lemaitre *et al.* (2018), for aMPV detection and subtyping. The reaction was  
166 performed with SuperScript™ III Platinum™ SYBR™ Green One-Step (Invitrogen, Waltham,  
167 MA, USA) on Lightcycler® 96 Instrument (Roche, Basel, Switzerland), using the primer pair  
168 PanMPV/N1fwdA and PanMPV/N1RevB (Lemaitre *et al.*, 2018) targeting the nucleoprotein  
169 (N) gene. For each reaction, 2 µl of extracted RNA were added to a standard reaction mix  
170 composed of 1X SYBR® Green Reaction Mix, 0.2 µl of SuperScript III RT/Platinum Taq Mix,  
171 and 0.6 µM of each primer. Molecular biology grade water was added up to a final volume of  
172 10 µl. The thermal protocol was set as follows: 50° C for 3 minutes for the reverse transcription  
173 phase, 95° C for 5 minutes for the initial activation step, 45 cycles at 95° C for 15 seconds for  
174 the denaturation phase and 60° C for 30 seconds for annealing and extension. Melting curve

175 analysis was performed by progressively increasing the temperature from 40° C to 90° C and  
176 continuously monitoring the fluorescence data. Tentative strain subtyping was performed by  
177 melting temperature (T<sub>m</sub>) evaluation and comparison with reference strains.

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

180

### 181 *aMPV subtype C molecular characterization*

182

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

193 The following reaction mix was used in each PCR: 0.25 µL of GoTaq® DNA  
194 Polymerase (Promega, Madison, WI, United States), 10 pmol of the appropriate primer pair, 1  
195 µL of dNTPs (Promega, Madison, WI, United States), 10 µL of PCR GoTaq® 5x reaction  
196 buffer, 3.5 µL MgCl<sub>2</sub> (3 mM), 31.25 µL of nuclease free water (Promega, Madison, WI, United  
197 States) and 3 µL of cDNA. The PCR cycling parameters for both F and G gene PCR assays  
198 were as follows. A pre-cycle step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s,  
199 50°C for 40 s and 72°C for 65 s, followed by a final extension at 72°C for 5 min.

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

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

214

## 215 **Results**

216

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

224 (2160 bases) were obtained by RT-PCR and sequencing. Sequence data were submitted to the  
225 NCBI GenBank database under accession numbers OM021855, OM021856, and OM021857.

226 The phylogenetic tree constructed on the G gene sequence (Figure 1) demonstrated the  
227 clustering of the aMPV/C/IT/Wigeon/758/07 strain within the Eurasian lineage of aMPV-C,  
228 which includes French and Chinese subtype C field strains isolated from domestic duck flocks  
229 (Toquin *et al.*, 1999, 2006; Sun *et al.*, 2014). Specifically, the aMPV/C/IT/Wigeon/758/07  
230 strain was closely related to a clade including strains isolated from Muscovy ducks (99178 and  
231 99350 strains) and White Pekin ducks (00094 strain) in France (Toquin *et al.*, 2006). Between  
232 group mean distance, measured among the G gene sequence obtained in the present study and  
233 the French clade, was 0.042.

234 M and F genes phylogenetic trees (Figure 2 and Figure 3, respectively), confirming the  
235 results obtained with the G gene, showed a close relationship between the  
236 aMPV/C/IT/Wigeon/758/07 strain and the 99178 strain (Brown *et al.*, 2014). Pairwise p-  
237 distance values obtained comparing the above-mentioned strains were of 0.020 in M gene and  
238 0.014 in F gene, respectively.

239

## 240 **Discussion**

241

242 We hereby report the detection of an aMPV subtype C in a juvenile Eurasian wigeon  
243 (*M. penelope*) wintering in northeast Italy. To our knowledge, this is the first molecular  
244 evidence of the occurrence of a subtype C in Italy and it backdates the aMPV-C circulation to  
245 2007. Eventually, our finding suggests for the first time the susceptibility to aMPV infection of  
246 Eurasian wigeons.

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

249 domestic ducks. This might be related to migratory bridges between countries, along the Black  
250 Sea/Mediterranean flyway. Eurasian wigeons are indeed considered medium to long-range  
251 migrant dabbling ducks. Whilst major concentrations of wintering individuals are found in  
252 Northwestern Europe and Eastern Asia, remarkable numbers are also reported for France and  
253 other Mediterranean countries, including Italy (Atkinson *et al.*, 2006). Furthermore, ringed  
254 birds' recoveries showed a direct connection between Italian and French wetlands (Atkinson *et*  
255 *al.*, 2007; Spina & Volponi, 2008). Diverse factors such as migratory movements, dense birds'  
256 congregations, and presence of immunologically naïve juveniles are considered relevant drivers  
257 in disease dynamics as already known for a major avian viral disease such as AI (Gaidet *et al.*,  
258 2010; van Dijk *et al.*, 2014). Our finding suggests that these factors might also be relevant for  
259 the aMPV-C epidemiology. Considering that the geographical separation of wild hosts  
260 undertaking seasonal movements along different migration routes was linked to the initial  
261 diversification of AI viruses into Eurasian and American clades (Krauss *et al.*, 2007), the  
262 presence of two distinct genetic lineages of aMPV-C could also be associated to wild hosts'  
263 migratory movements.

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

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

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

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## 282 **References**

283

284 Atkinson, P.W., Clark, J.A., Delany, S., Diagona, C.H., du Feu, C., Fiedler, W., Fransson,  
285 T., Gauthier-Clerc, M., Grantham, M.J., Gschweg, M., Hagemeyer, W., Helmink, T.,  
286 Johnson, A., Khomenko, S., Martakis, G., Overdijk, O., Robinson, R.A., Solokha, A., Spina,  
287 F., Sylla, S.I., Veen, J. & Visser, D. (2006). *Urgent preliminary assessment of ornithological*  
288 *data relevant to the spread of Avian Influenza in Europe*. Retrieved from  
289 [https://ec.europa.eu/environment/nature/conservation/wildbirds/birdflue/docs/rep\\_spread\\_avian\\_influenza\\_report.pdf](https://ec.europa.eu/environment/nature/conservation/wildbirds/birdflue/docs/rep_spread_avian_influenza_report.pdf)

291 Atkinson, P.W., Robinson, R.A., Clark, J.A., Miyar, T., Downie, I.S., du Feu, C.R.,  
292 Fiedler, W., Fransson, T., Grantham, M.J., Gschweg, M., Spina, F. & Crick, H.Q.P. (2007)  
293 Migratory movements of waterfowl: a web-based mapping tool. EURING report to the EU  
294 Commission. Retrieved from <http://blx1.bto.org/ai-eu/>

295 Bâyon-Auboyer, M. H., Arnauld, C., Toquin, D. & Eterradossi, N. (2000). Nucleotide  
296 sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV)

297 reveal a novel APV subtype. *The Journal of general virology*, 81, 2723–2733.  
298 <https://doi.org/10.1099/0022-1317-81-11-2723>

299 Bennett, R.S., McComb, B., Shin, H.J., Njenga, M.K., Nagaraja, K.V. & Halvorson,  
300 D.A. (2002). Detection of avian pneumovirus in wild Canada (*Branta canadensis*) and blue-  
301 winged teal (*Anas discors*) geese. *Avian diseases*, 46, 1025–1029.  
302 [https://doi.org/10.1637/0005-2086\(2002\)046\[1025:DOAPIW\]2.0.CO;2](https://doi.org/10.1637/0005-2086(2002)046[1025:DOAPIW]2.0.CO;2)

303 Bennett, R.S., Nezworski, J., Velayudhan, B.T., Nagaraja, K.V., Zeman, D.H., Dyer,  
304 N., Graham, T., Lauer, D.C., Njenga, M.K. & Halvorson, D.A. (2004). Evidence of avian  
305 pneumovirus spread beyond Minnesota among wild and domestic birds in central North  
306 America. *Avian diseases*, 48, 902–908. <https://doi.org/10.1637/7208-051804R>

307 Brown, P.A., Lemaitre, E., Briand, F. X., Courtillon, C., Guionie, O., Allée, C., Toquin,  
308 D., Bayon-Auboyer, M-H., Jestin, V. & Eterradossi, N. (2014). Molecular Comparisons of Full  
309 Length Metapneumovirus (MPV) Genomes, Including Newly Determined French AMPV-C  
310 and –D Isolates, Further Supports Possible Subclassification within the MPV Genus. *PLoS*  
311 *ONE*, 9, e102740. <https://doi.org/10.1371/journal.pone.0102740>

312 Brown, P.A., Allée, C., Courtillon, C., Szerman, N., Lemaitre, E., Toquin, D., Mangart,  
313 J.M., Amelot, M. & Eterradossi, N. (2019). Host specificity of avian  
314 metapneumoviruses. *Avian pathology*, 48, 311–318.  
315 <https://doi.org/10.1080/03079457.2019.1584390>

316 Buys, S.B. & du Preez, J.H. (1980). A preliminary report on the isolation of a virus  
317 causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, 36.

318 Cannon, R.M. & Roe, R.T. (1982). *Livestock Disease Surveys. A Field Manual for*  
319 *Veterinarians*. Canberra: Australian Government Publishing Service.

320           Catelli, E., De Marco, M.A., Delogu, M., Terregino, C. & Guberti, V. (2001).  
321 Serological evidence of avian pneumovirus infection in reared and free-living pheasants. The  
322 *Veterinary Record*, 149, 56-57.

323           Catelli, E., Cecchinato, M., Delogu, M., De Matteo, P., Ortali, G., Franciosi, C., De  
324 Marco, M.A. & Naylor, C.J. (2004). Avian Pneumovirus infection in turkey and broiler farms  
325 in Italy: A virological, molecular, and serological field survey. *Italian Journal of Animal*  
326 *Science*, 3, 287–292. <https://doi.org/10.4081/ijas.2004.287>

327           Catelli, E., Cecchinato, M., Savage, C.E., Jones, R.C. & Naylor, C.J. (2006).  
328 Demonstration of loss of attenuation and extended field persistence of a live avian  
329 metapneumovirus vaccine. *Vaccine*, 24, 6476–6482.  
330 <https://doi.org/10.1016/j.vaccine.2006.06.076>

331           Cecchinato, M., Lupini, C., Silveira, F., Listorti, V., Mescolini, G., Morandini, E.,  
332 Franzo, G. & Catelli, E. (2018). Molecular characterization of avian metapneumovirus from  
333 guinea fowls (*Numida meleagris*). *Pakistan Veterinary Journal*, 38, 419-423.  
334 <http://dx.doi.org/10.29261/pakvetj/2018.088>

335           Canuti, M., Kroyer, A., Ojkic, D., Whitney, H.G., Robertson, G.J. & Lang, A.S. (2019).  
336 Discovery and Characterization of Novel RNA Viruses in Aquatic North American Wild  
337 Birds. *Viruses*, 11, 768. <https://doi.org/10.3390/v11090768>

338           Cha, R.M., Qingzhong, Y. & Zsak, L. (2013). The pathogenicity of avian  
339 metapneumovirus subtype C wild bird isolates in domestic turkeys. *Virology journal*, 38.  
340 <https://doi.org/10.1186/1743-422X-10-38>

341           Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid  
342 guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, 162, 156–  
343 159. <https://doi.org/10.1006/abio.1987.9999>



344 Curland, N., Gethöffer, F., van Neer, A., Ziegler, L., Heffels-Redmann, U., Lierz, M.,  
345 Baumgärtner, W., Wohlsein, P., Völker, I., Lapp, S., Bello, A., Pfankuche, V.M., Braune, S.,  
346 Runge, M., Moss, A., Rautenschlein, S., Jung, A., Teske, L., Strube, C., Schulz, J., Bodewes,  
347 R., Osterhaus, A.D.M.E. & Siebert, U. (2018). Investigation into diseases in free-ranging ring-  
348 necked pheasants (*Phasianus colchicus*) in northwestern Germany during population decline  
349 with special reference to infectious pathogens. *European Journal of Wildlife Resources*, 64.  
350 <https://doi.org/10.1007/s10344-018-1173-2>

351 Fair, J., Paul, E. & Jones, J. (2010). *Guidelines to the Use of Wild Birds in Research*.  
352 Washington: Ornithological Council. Retrieved from [https://birdnet.org/info-for-](https://birdnet.org/info-for-ornithologists/guidelines-english-3rd-edition-2010/)  
353 [ornithologists/guidelines-english-3rd-edition-2010/](https://birdnet.org/info-for-ornithologists/guidelines-english-3rd-edition-2010/)

354 Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, J., Iverson, S.A., Douglas, D.C., Perry,  
355 W.M., Mundkur, T. & Newman, S.H. (2010). Potential spread of highly pathogenic avian  
356 influenza H5N1 by wildfowl: dispersal ranges and rates determined from large-scale satellite  
357 telemetry. *Journal of Applied Ecology*, 47, 1147–1157.

358 Gethöffer, F., Curland, N., Voigt, U., Woelfing, B., Ludwig, T., Heffels-Redmann, U.,  
359 Hafez, H. M., Lierz, M. & Siebert, U. (2021). Seroprevalences of specific antibodies against  
360 avian pathogens in free-ranging ring-necked pheasants (*Phasianus colchicus*) in Northwestern  
361 Germany. *PloS one*, 16, e0255434. <https://doi.org/10.1371/journal.pone.0255434>

362 Hall, T., Ibis Biosciences & Carlsbad, C. (2011). BioEdit: an important software for  
363 molecular biology. *GERF Bulletin of Biosciences*, 2, 60-61.

364 Jardine, C.M., Parmley, E.J., Buchanan, T., Nituch, L. & Ojkic, D. (2018). Avian  
365 metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. *Transboundary and*  
366 *emerging diseases*, 65, 1098–1102. <https://doi.org/10.1111/tbed.12832>

367 Jones, R.C. (1996). Avian pneumovirus infection: Questions still unanswered. *Avian*  
368 *pathology*, 25, 639–648. <https://doi.org/10.1080/03079459608419171>

369 Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A. & Jermin, L.S.  
370 (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature*  
371 *Methods*, 14, 587–589. <https://doi.org/10.1038/nmeth.4285>

372 Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002). MAFFT: a novel method for  
373 rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*,  
374 30, 3059–3066. <https://doi.org/10.1093/nar/gkf436>

375 Krauss, S., Obert, C. A., Franks, J., Walker, D., Jones, K., Seiler, P., Niles, L., Pryor,  
376 S.P., Obenauer, J.C., Naeve, C.W., Widjaja, L., Webby, R.J. & Webster, R.G. (2007). Influenza  
377 in migratory birds and evidence of limited intercontinental virus exchange. *PLoS pathogens*, 3,  
378 e167. <https://doi.org/10.1371/journal.ppat.0030167>

379 Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular  
380 Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and*  
381 *Evolution*, 35, 1547-1549.

382 Legnardi, M., Allée, C., Franzo, G., Cecchinato, M. & Brown, P. (2021). Research Note:  
383 Detection of Avian metapneumovirus subtype C specific antibodies in a mallard flock in  
384 Italy. *Poultry science*, 100, 101186. <https://doi.org/10.1016/j.psj.2021.101186>

385 Lemaitre, E., Allée, C., Vabret, A., Etteradossi, N. & Brown, P.A. (2018). Single  
386 reaction, real time RT-PCR detection of all known avian and human  
387 metapneumoviruses. *Journal of virological methods*, 251, 61–68.  
388 <https://doi.org/10.1016/j.jviromet.2017.10.010>

389 Listorti, V., Lupini, C., Cecchinato, M., Pesente, P., Rossi, G., Giovanardi, D., Naylor,  
390 C.J. & Catelli, E. (2014). Rapid detection of subtype B avian metapneumoviruses using RT-  
391 PCR restriction endonuclease digestion indicates field circulation of vaccine-derived viruses in  
392 older turkeys. *Avian pathology*, 43, 51–56. <https://doi.org/10.1080/03079457.2013.866212>

393 Lupini, C., Cecchinato, M., Ricchizzi, E., Naylor, C.J. & Catelli, E. (2011). A turkey  
394 rhinotracheitis outbreak caused by the environmental spread of a vaccine-derived avian  
395 metapneumovirus. *Avian pathology*, 40, 525–530.  
396 <https://doi.org/10.1080/03079457.2011.607428>

397 Mescolini, G., Lupini, C., Franzo, G., Quaglia, G., Legnardi, M., Cecchinato, M.,  
398 Tucciarone, C. M., Blanco, A., Turblin, V., Biarnés, M., Tatone, F., Falchieri, M. & Catelli, E.  
399 (2021). What is new on molecular characteristics of Avian metapneumovirus strains circulating  
400 in Europe? *Transboundary and Emerging Diseases*, 68, 1314–1322.  
401 <https://doi.org/10.1111/tbed.13788>

402 <https://doi-org.ezproxy.unibo.it/10.4081/ijas.2004.287>Qu, L., Liu, W., Yang, F., Hou,  
403 Z., Zheng, J., Xu, G. & Yang, N. (2009). Origin and domestication history of Peking ducks  
404 determined through microsatellite and mitochondrial marker analysis. *Science in China. Series*  
405 *C, Life sciences*, 52, 1030–1035. <https://doi.org/10.1007/s11427-009-0145-x>

406 Rautenschlein, S. (2020). Avian metapneumovirus. In D.E. Swayne, M.  
407 Boulianne, C.M. Logue, L.R. McDougald, V. Nair, D.L. Suarez, S. de Wit, T. Grimes, D.  
408 Johnson, M. Kromm, T.Y. Prajitno, I. Rubinoff & G. Zavala (Eds.). *Diseases of Poultry* 14th  
409 edn (pp. 135–143). Hoboken: Wiley Blackwell.

410 Retallack, H., Clubb, S. & DeRisi, J.L. (2019). Genome Sequence of a Divergent Avian  
411 Metapneumovirus from a Monk Parakeet (*Myiopsitta monachus*). *Microbiology resource*  
412 *announcements*, 8, e00284–19. <https://doi.org/10.1128/MRA.00284-19>

413 Rima, B., Collins, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R.A., Lee, B.,  
414 Maisner, A., Rota, P., Wang, L. & ICTV Report Consortium. (2017). ICTV Virus Taxonomy  
415 Profile: Pneumoviridae. *The journal of general virology*, 98, 2912–2913.  
416 <https://doi.org/10.1099/jgv.0.000959>

417 Seal, B.S. (1998). Matrix protein gene nucleotide and predicted amino acid sequence  
418 demonstrate that the first US avian pneumovirus isolate is distinct from European strains. *Virus*  
419 *research*, 58, 45–52. [https://doi.org/10.1016/s0168-1702\(98\)00098-7](https://doi.org/10.1016/s0168-1702(98)00098-7)

420 Senne, D., Edson, J.C. & Pederson, B.P. (1997). Avian pneumovirus update. In  
421 Proceedings of the *134th annual convention of the American Veterinary Medical Association*  
422 (p. 190). Reno, Nevada.

423 Shin, H.J., Njenga, M.K., McComb, B., Halvorson, D.A. & Nagaraja, K.V. (2000).  
424 Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic  
425 homology with RNA from APV isolates from domestic turkeys. *Journal of clinical*  
426 *microbiology*, 38, 4282–4284. <https://doi.org/10.1128/JCM.38.11.4282-4284.2000>

427 Spina, F. & Volponi, S. (2008). *Atlante della Migrazione Degli Uccelli in Italia: Non-*  
428 *Passeriformi*. Roma: Ministero dell’Ambiente e della Tutela del Territorio e del Mare, Istituto  
429 Superiore per la Protezione e la Ricerca Ambientale (ISPRA).

430 Sun, S., Chen, F., Cao, S., Liu, J., Lei, W., Li, G., Song, Y., Lu, J., Liu, C., Qin, J. & Li,  
431 H. (2014). Isolation and characterization of a subtype C avian metapneumovirus circulating in  
432 Muscovy ducks in China. *Veterinary research*, 45. <https://doi.org/10.1186/s13567-014-0074-y>

433 Trifinopoulos, J., Nguyen, L.T., von Haeseler, A. & Minh, B.Q. (2016). W-IQ-TREE:  
434 a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic acids research*, 44.  
435 <https://doi.org/10.1093/nar/gkw256>

436 Toquin, D., Băyon-Auboyer, M.H., Eterradossi, N., Jestin, V. & Morin, H. (1999).  
437 Isolation of a pneumovirus from a Muscovy duck. *The Veterinary Record*, 145, 680.

438 Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C. & Eterradossi, N. (2006).  
439 European and American subtype C isolates of avian metapneumovirus belong to different  
440 genetic lineages. *Virus genes*, 32, 97–103. <https://doi.org/10.1007/s11262-005-5850-3>

441 Tucciarone, C.M., Franzo, G., Lupini, C., Torres Alejo, C., Listorti, V., Mescolini, G.,  
442 Branão, P.E., Martini, M., Catelli, E. & Cecchinato, M. (2018). *Avian*  
443 *Metapneumovirus* circulation in Italian broiler farms. *Poultry Science*, 97, 503-509.  
444 <https://doi.org/10.3382/ps/pex350>.

445 Turpin, E.A., Stallknecht, D.E., Slemons, R.D., Zsak, L. & Swayne, D.E. (2008).  
446 Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South  
447 Carolina, Arkansas and Ohio, USA. *Avian pathology*, 37, 343–351.  
448 <https://doi.org/10.1080/03079450802068566>

449 van Boheemen, S., Bestebroer, T. M., Verhagen, J. H., Osterhaus, A.D.M.E., Pas, S.D.,  
450 Herfst, S. & Fouchier, R.A. (2012). A family-wide RT-PCR assay for detection of  
451 paramyxoviruses and application to a large-scale surveillance study. *PloS*  
452 *one*, 7. <https://doi.org/10.1371/journal.pone.0034961>

453 van Dijk, J.G., Hoyer, B.J., Verhagen, J.H., Nolet, B.A., Fouchier, R.A. & Klaassen, M.  
454 (2014). Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus. *The*  
455 *Journal of animal ecology*, 83, 266–275. **Errore. Riferimento a collegamento ipertestuale non**  
456 **valido.**<https://doi.org/10.1111/1365-2656.12131>

457 Yunus, A.S., Govindarajan, D., Huang, Z. & Samal, S.K. (2003). Deduced amino acid  
458 sequence of the small hydrophobic protein of US avian pneumovirus has greater identity with  
459 that of human metapneumovirus than those of non-US avian pneumoviruses. *Virus*  
460 *research*, 93, 91–97. [https://doi.org/10.1016/s0168-1702\(03\)00074-1](https://doi.org/10.1016/s0168-1702(03)00074-1)

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

470 Table 1. *Cont.*  
 471

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



European greenfinch ( <i>Carduelis chloris</i> )	-	-	1	-	-	1	-	1	W; B; R
<b>Total</b>	197	137	158	61	64	302	65	492	

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474 <sup>a</sup>Age classes: juv. = juvenile; ad. = adult; n.s. = not specified.

475 <sup>b</sup>Migratory status in Italy: W = wintering; B = breeding; R = resident.

476

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

478 C.

479

<b>Primer name</b>	<b>Sequence (5' – 3')</b>	<b>Gene</b>	<b>Position<sup>a</sup></b>	<b>Product size (bp)</b>
FC3+	GGTATACAAATGTGTTCAC	F	3130	591
FC3-	GTCCTGAAGATGTGGGCATG	F	3721	
FC5+	GACAGTTTTCTGACAATGC	F	3616	597
FC5-	CCAAAGGTCTGATTATCC	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	

480 <sup>a</sup> Anti-genome position of primer 5' terminus based on subtype C sequence of the 99178 viral

481 strain (GenBank accession number: HG934338).

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485 **Figure captions**

486

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

493

494 **Figure 2.** Phylogenetic tree based on partial M gene nucleotide sequences (from nucleotide  
495 2514 to 2913 in the genome) of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red)  
496 and aMPV-C strains obtained from NCBI GenBank database. Only bootstrap values  $\geq 70$  are  
497 shown. The partial M sequence of the Human respiratory syncytial virus strain  
498 RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup.  
499 Sequence data are reported in Table S2 (Supplemental Material).

500

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

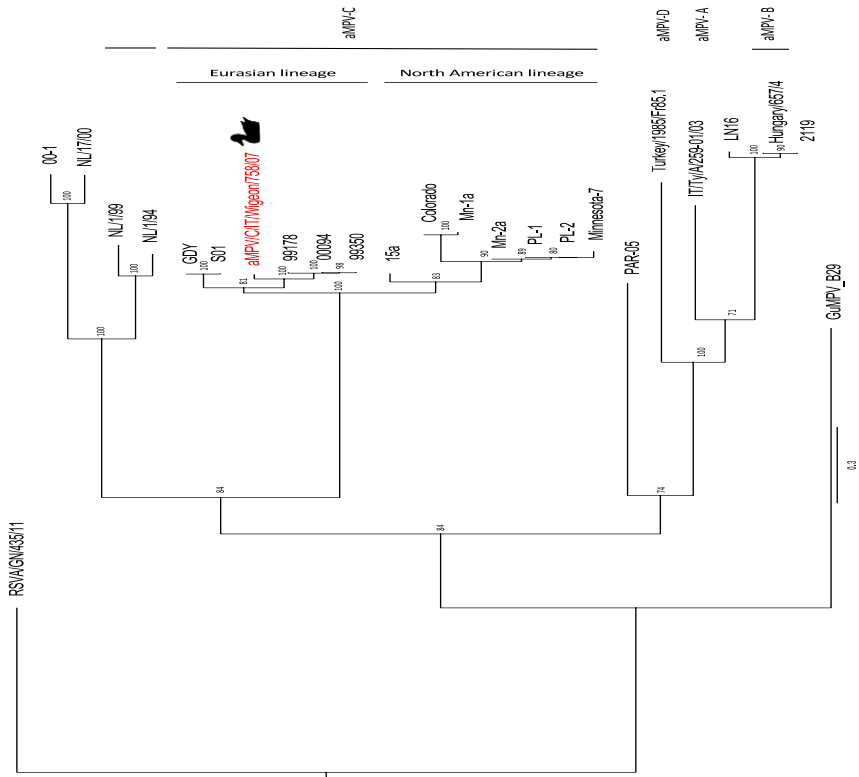
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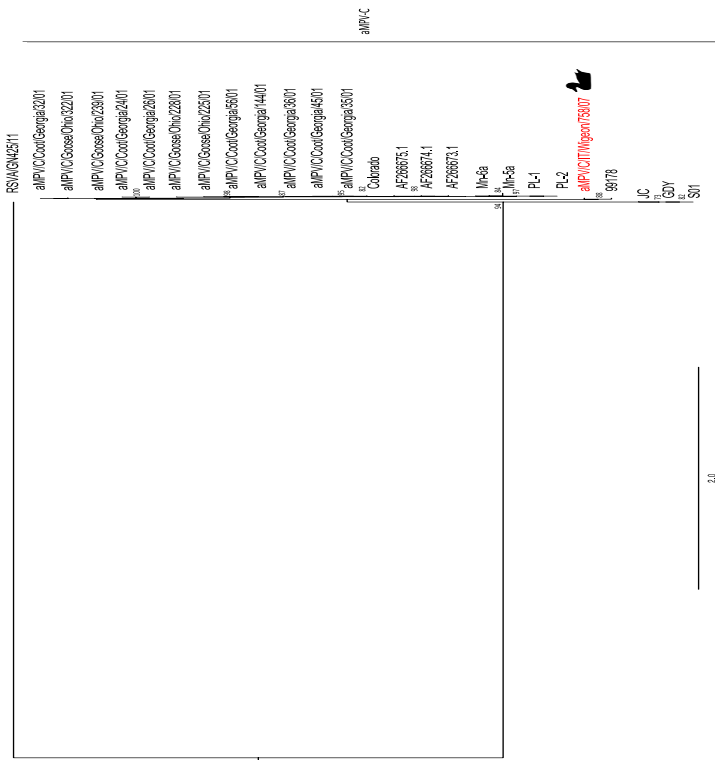
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510 **Figure 1.**

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