

Phylogenetic analysis of the RNA-dependent RNA polymerase (RdRp) and a predicted structural protein (pSP) of the Chronic bee paralysis virus (CBPV) isolated from various geographic regions.

Philippe Blanchard, Frank Schurr, Violaine Olivier, Olivier Celle, Karina Antùnez, Tamàs Bakonyi, H el ene Berthoud, Eric Haubruge, Mariano Higes, Sylwia Kasprzak, et al.

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1 **PHYLOGENETIC ANALYSIS OF THE RNA-DEPENDENT RNA**
2 **POLYMERASE (RdRp) AND A PREDICTED STRUCTURAL PROTEIN**
3 **(pSP) OF THE CHRONIC BEE PARALYSIS VIRUS (CBPV) ISOLATED**
4 **FROM VARIOUS GEOGRAPHIC REGIONS.**

5

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10

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35 **Summary**

36

37 Chronic bee paralysis virus (CBPV) is responsible for chronic paralysis, an infectious and
38 contagious disease of adult honey bees (*Apis mellifera* L.). The full-length nucleotide
39 sequences of the two major RNAs of CBPV have previously been characterized. The Orf3 of
40 RNA1 has shown significant similarities to the RNA-dependent RNA polymerase (RdRp) of
41 positive single-stranded RNA viruses, whereas the Orf3 of RNA2 encodes a putative
42 structural protein (pSP). In the present study, honey bees originating from 9 different
43 countries (Austria, Poland, Hungary, Spain, Belgium, Denmark, Switzerland, Uruguay and
44 France) were analysed for the presence of CBPV genome. The complete genomic nucleotide
45 sequence of the RdRp (1947bp) and of the pSP (543bp) from 24 honey bee positive samples
46 was determined and the phylogenetic relationship among isolates was investigated. Four
47 distinct genotypes of CBPV were observed.

48

49 Keywords: Chronic bee paralysis virus (CBPV), RNA-dependent RNA polymerase, Predicted
50 structural protein, Phylogenetic analysis, *Apis mellifera*.

51

52 **Short communication**

53

54 Chronic bee paralysis virus (CBPV) is the aetiological agent of an infectious and contagious
55 disease of adult honey bees (*Apis mellifera* L.) known as Chronic paralysis (Ball & Bailey,
56 1997). This pathology is known to induce significant losses in honey bee colonies (Allen &
57 Ball, 1996), characterized by clusters of trembling, flightless, crawling bees and by individual
58 black, hairless bees standing at the hive entrance (Bailey et al., 1983; Ball & Bailey, 1997).
59 Although the complete sequences of the two major genomic RNAs of CBPV have recently
60 been determined (Olivier et al., 2008), its taxonomic position is currently unassigned (Mayo et
61 al., 2005). RNA1 (3674 nt) and RNA2 (2305 nt) are positive single-stranded RNAs that are
62 capped but not polyadenylated. Although CBPV shares several characteristics with viruses of
63 the *Nodaviridae* and *Tombusviridae* families, CBPV could be considered as the type species
64 of a new family of positive single-stranded RNA viruses (Olivier et al., 2008).

65 The distribution of CBPV is worldwide (Allen & Ball, 1996; Ribière et al., 2008). The
66 prevalence of this virus has been investigated in various countries, such as Great Britain
67 (Bailey et al., 1981), Poland (Topolska et al., 1995), France (Ribière et al., 2000; Tentcheva et
68 al., 2004), Uruguay (Antunez et al., 2005), Austria (Berenyi et al., 2006), New Zealand (Todd
69 et al., 2007), Hungary (Forgach et al., 2008), Denmark (Nielsen et al., 2008), Brazil (Teixeira
70 et al., 2008), Spain (unpublished data) and Belgium (unpublished data).

71 Among the many viruses able to infect honey bees, some have been studied by phylogenetic
72 analysis, including *Acute bee paralysis virus* (ABPV) (Bakonyi et al., 2002), *Sacbrood virus*
73 (SBV) (Grabensteiner et al., 2001), *Kashmir bee virus* (KBV) (Hung et al., 2000), *Deformed*
74 *wing virus* (DWV) (Berenyi et al., 2007) and more recently, *Israeli acute paralysis virus* (IAPV)
75 (Blanchard et al., 2008b; Palacios et al., 2008). While the phylogenetic analysis of SBV and
76 ABPV revealed clustering of the strains according to their geographic origins (Bakonyi et al.,
77 2002; Grabensteiner et al., 2001), the phylogenetic analysis of DWV revealed a monophyletic
78 cluster, despite the various geographic origins of the isolates (Berenyi et al., 2007). The
79 preliminary results on the phylogenetic analysis of IAPV also seem to reveal distinct lineages
80 according to their geographic origins (Blanchard et al., 2008b; Palacios et al., 2008).

81 Up to date, no study on the genetic diversity of CBPV could be carried out, because of the
82 unavailability of complete nucleotide sequence of CBPV. Recently, we have reported genomic
83 variability between partial sequences of CBPV from different bee samples that led us to
84 develop improved molecular diagnostic methods and quantitative assays (Blanchard et al.,
85 2007, 2008a). By using these assays, 40% of the CBPV isolates that were not detected with
86 the first CBPV RT-PCR test published by our laboratory (Ribière et al., 2002) were
87 successfully detected.

88 The aim of this study was to assess the genetic relationship among CBPV isolates from
89 various geographic origins. We have chosen to perform the phylogenetic analysis on the Orf3
90 of CBPV RNA1, reported as the only amino acid sequence sharing significant similarities with
91 the conserved sequence domains of the RNA-dependent RNA polymerase (RdRp) of single-
92 stranded RNA viruses (Olivier et al., 2008). Moreover, Baker and Schroeder (2008) have
93 recently demonstrated the possibility of using the RdRp as a taxonomic marker for the
94 classification of Picorna-like viruses infecting honey bees. To complete our study, we also
95 performed a phylogenetic analysis on the Orf3 of CBPV RNA2, coding for a predicted
96 structural protein (pSP), as suggested by Olivier et al, (2008).

97 Samples of adult worker bees were collected from nine countries (Austria, Belgium, Denmark,
98 France, Hungary, Poland, Spain, Switzerland, Uruguay). The samples were collected
99 between 2003 and 2007, from honey bee colonies presenting symptoms of chronic paralysis
100 or abnormal mortalities, except for the Swiss samples and an Austrian sample, where no
101 specific symptoms were identified. Samples tested CBPV-positive, either by the AGID test
102 (Agarose Gel ImmunoDiffusion) (Ribière et al., 2000), or by the RT-PCR test (Ribière et al.,
103 2002) were sent to the bee pathology unit (French Food Safety Agency, Sophia Antipolis,
104 France) for phylogenetic analysis. A total of 101 bee samples were analysed. Sample
105 preparation, RNA extraction and cDNA synthesis were performed as described previously
106 (Blanchard et al., 2007; Ribière et al., 2002). Bee samples were first analysed using the newly
107 developed RT-PCR CBPV test (Blanchard et al., 2008a), completed by the recently upgraded
108 real-time RT-PCR test (Blanchard et al., 2007; Celle et al., 2008). cDNAs of each CBPV
109 positive sample were subjected to supplementary PCRs, allowing to amplify the entire RdRp
110 and the predicted structural protein of CBPV. Sixty-four out of the 101 investigated bee

111 samples from the different countries were found CBPV positive with high viral load (over 10^{10}
112 CBPV copies per bee), enabling to amplify the entire RdRp and the pSP.

113 Three different primer pairs were designed by Primer3 Output for the amplification of
114 overlapping fragments which cover the entire RdRp of CBPV (Table 1), based on the
115 sequence of CBPV RNA1 (GenBank accession no. **EU122229**) described by Olivier et al.
116 (2008). Amplifications were carried out in a total reaction volume of 50 μ l using the Platinum[®]
117 *Pfx* DNA Polymerase (Invitrogen). This polymerase has a proofreading 3'-5' exonuclease
118 activity, and therefore provides higher fidelity. The final PCR reaction contained 2X Platinum[®]
119 *Pfx* Amplification Buffer, 1mM of MgSO₄, 0.3 mM of dNTPs mix PCR grade, 0.3 μ M of each
120 primer, 2.5 U of Platinum[®] *Pfx* DNA Polymerase and 5 μ l of cDNA. The thermal cycling
121 conditions were 5 min at 94°C (denaturing of the template and activation of the enzyme),
122 followed by 35 cycles consisting of denaturing at 94°C for 15 s, annealing at 55°C for 30 s
123 and extension at 68°C for 30 s to 2 min (depending on the primer pair), completed by a final
124 extension at 68°C for 10 min. The PCR products were then electrophoresed in 1% agarose
125 gel in TAE buffer, stained with ethidium bromide, and visualised under UV light. The PCR
126 products were then purified using the Qiaquick PCR purification kit (Qiagen) and sequenced
127 in both orientations by using primers described above (Millegen, France). The complete
128 genomic nucleotide sequence of the RdRp of different CBPV isolates (1947bp) was
129 determined by overlapping the three sequences using the Seqman™ II program of the
130 DNASTAR software package (Lasergene). A primer pair was designed, covering the entire
131 Orf3 (543bp), coding for a predicted structural protein (pSP) (Table 1), based on the
132 sequence of CBPV RNA2 (GenBank accession no. **EU122230**) described by Olivier et al.
133 (2008). Amplification, PCR product purification, sequencing and nucleotide sequence
134 determination were carried out as described for the RdRp gene.

135 Preliminary comparison of the sequences from isolates from the same geographical origin
136 revealed that most frequently they shared near 100% identity. The analysis was thus
137 restricted to the divergent sequences (5 from France and 17 from different countries, Table
138 2). The nucleotide sequences of the RdRp and the pSP from the 22 new isolates were
139 submitted to the GenBank database under accession numbers **FJ345306** to **FJ345349**. The
140 nucleotide sequence of RdRp and pSP of CBPV reference isolates A and B, were determined

141 respectively from RNA1 (GenBank accession nos. EU122229 and EU122231) and RNA2
142 sequences (GenBank accession nos. EU122230 and EU122232), described previously by
143 Olivier et al. (2008). In this study, CBPV reference isolates A and B were respectively named
144 Fr 1 and Fr 2.

145 Nucleotide sequences of RdRp and predicted structural protein were aligned by using the
146 MegAlign™ program of the DNASTAR software package (Lasergene) and the CLUSTAL_X
147 program (Thompson et al., 1997). The final alignment was checked by visual inspection.
148 Phylogenetic analysis was performed by using the neighbour-joining (NJ), maximum
149 likelihood (ML) and maximum parsimony (MP) methods implemented in the PHYLOWIN
150 program (Galtier et al., 1996), and by using the NJ and MP methods implemented in the
151 Molecular Evolutionary Genetics Analysis (MEGA) program version 3.1 (Kumar et al., 2004).
152 All methods gave similar profiles. Results using the MEGA software are shown. Branches
153 were condensed when bootstrap values were less than 70 %.

154 The analysis of the sequence pair distances using the Clustal W method revealed 0.3-9.6%
155 divergence between the 24 RdRp nucleotide sequences and 0.2-4.6% divergence between
156 the 24 RdRp amino acid sequences. The phylogenetic relationship among the 24 CBPV
157 RdRp sequences was assessed and revealed four main clusters, supported by high bootstrap
158 values (Figure 1) using NJ, ML and MP methods of analysis. Lineage A contained most of
159 French isolates (5/7) and the isolates from Spain and Belgium. Lineage B contained a French
160 isolate, two Swiss isolates and all the Polish, Austrian, Danish and Hungarian isolates.
161 Lineage C contained two Uruguayan isolates and lineage D contained 3 isolates obtained
162 from different countries (France, Switzerland and Uruguay). Since isolates from Uruguay,
163 Switzerland and France were present in several clusters, no clear geographical segregation
164 was observed. However, lineage A mainly contains isolates from South and Western Europe,
165 compared to lineage B clustering isolates from North and Eastern Europe, while lineage C
166 contains isolates from South America. The clustering of a French, a Swiss and an Uruguayan
167 isolates (Lineage D) remains unexplained and raises the question of the possible role of
168 commercial exchange of honey bees. However, the presence of French isolates in three of
169 four major lineages (5 in lineage A, 1 in lineage B and 1 in lineage D) could actually results
170 from the over representation of isolates from France. Studying more isolates originating from

171 a broader range of countries could help to definitively identify geographical trends. In this
172 study, no correlation between sequence variation and occurrence of clinical symptoms was
173 observed. The branching of the phylogenetic tree did not enable the segregation of CBPV
174 isolates from symptomatic (83%) and asymptomatic bee colonies (17%).

175 The phylogenetic relationship among the 24 CBPV pSP sequences revealed a similar profile
176 to that obtained with the RdRp sequences (Figure 2), except for an Uruguayan isolate (Ur 3).
177 The pSP of this isolate clustered in lineage B, compared to the RdRp of this isolate, which
178 clustered in lineage C. Moreover, lineage C and D were clustered together. While the
179 alignment of the 24 pSP nucleotide sequence revealed 0-6.6% divergence, the alignment of
180 the 24 pSP amino acid sequences showed a very low degree of divergence (0-2.3%
181 corresponding to only 2 amino acids), unexpectedly suggesting a highly conserved protein.
182 Due to its low variability, pSP sequence seems not to be indicated for phylogenetic study,
183 while RdRp sequences are more discriminating. Whereas it is very likely that the Orf3 of
184 CBPV RNA1 encodes the viral RdRp (Olivier et al., 2008), it is necessary to further
185 characterize the proteins encoded by the different ORFs of the CBPV genome (work in
186 progress in our laboratory).

187 In conclusion, this study underlines the presence of chronic paralysis outbreaks in several
188 countries and points to the need to assess the impact of this disease in colony weakness
189 worldwide. Further investigations on CBPV isolates from a broader origin are necessary to
190 confirm their geographical distribution, to assess the global distribution of this virus and to
191 further investigate the CBPV role in disease outbreaks.

192

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200

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- 287

288 **Captions to figures**

289 Table 1

290 Oligonucleotide primers used for the amplification of the RNA-dependent RNA polymerase
291 (RdRp, position 1643-3589 on RNA1) and for the amplification of the predicted structural
292 protein (pSP, position 303-845 on RNA2).

293

294 Table 2

295 Origin of the studied CBPV isolates used for the phylogenetic analysis: isolate reference, year
296 of sampling, presence of CBPV symptoms, country of origin, identification of sequence and
297 GenBank accession number.

298

299 Figure 1

300 Phylogenetic tree constructed using the neighbour-joining (NJ) method with Kimura
301 parameters, included in the MEGA version 3.1 program (Kumar et al., 2004), on the alignment
302 of the 1947bp of RdRp sequence of 24 CBPV isolates. The number of each node represents
303 the bootstrap values as the result of 1000 replicates. Branches were condensed when
304 bootstrap values were less than 70 %.

305

306 Figure 2

307 Phylogenetic tree constructed using the neighbour-joining (NJ) method with Kimura
308 parameters, included in the MEGA version 3.1 program (Kumar et al., 2004), on the alignment
309 of the 543bp of predicted structural protein sequence of 24 CBPV isolates. The number of
310 each node represents the bootstrap values as the result of 1000 replicates. Branches were
311 condensed when bootstrap values were less than 70 %.

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Table 1.

Target	Primer Name	Sequence (5' – 3')	Position	PCR product size (bp)
CBPV RNA 1 RdRp	CBPV A1	TGAGGCTTGCTTCTGACAAA	1589-1608*	902
	CBPV A2	ACTACTAGAAACTCGTCGCTTCG	2490-2468*	
	CBPV A3	TCAGACACCGAATCTGATTATTG	1921-1933*	1525
	CBPV A4	CCGGAGACAAAGGTCATCAT	3445-3426*	
	CBPV A5	GGCCCATCATGCAGAAGTAT	3264-3283*	404
	CBPV A6	ACCAGTGCCTGACGGACTTA	3667-3646*	
CBPV RNA 2 pSP	CBPV A7	ACTCCCGTCGTTGTGTTCTC	109-128°	895
	CBPV A8	GGCGATTGGTATTTGTTTGG	1003-984°	

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* Nucleotide positions refer to the CBPV RNA1 sequence ([EU122229](#)) described by Olivier et al., (2008).

° Nucleotide positions refer to the CBPV RNA2 sequence ([EU122230](#)) described by Olivier et al., (2008).

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Table 2

Isolate ¹ / Year	Country	Symptoms ²	Identification	GenBank accession no. CBPV RdRp	GenBank accession no. CBPV pSP [°]
A* / 2004	France	Yes	Fr 1	EU122229	EU122230
B* / 2005	France	Yes	Fr 2	EU122231	EU122232
198 / 2007	France	Yes	Fr 6	FJ345313	FJ345334
596 / 2007	France	Yes	Fr 7	FJ345314	FJ345335
351 / 2007	France	Yes	Fr 8	FJ345315	FJ345336
363 / 2007	France	Yes	Fr 9	FJ345316	FJ345337
274 / 2007	France	Yes	Fr 10	FJ345312	FJ345338
8-C / 2006	Uruguay	Yes	Ur 1	FJ345325	FJ345347
6-M / 2006	Uruguay	Yes	Ur 2	FJ345326	FJ345348
13-F / 2006	Uruguay	Yes	Ur 3	FJ345327	FJ345349
R1-C6 / 2004	Switzerland	No	Sw 1	FJ345322	FJ345344
R2-C102 / 2004	Switzerland	No	Sw 2	FJ345323	FJ345345
R3-C10 / 2004	Switzerland	No	Sw 3	FJ345324	FJ345346
BE 104 / 2003	Austria	Yes	Au 1	FJ345306	FJ345328
BE 78 / 2006	Austria	No	Au 5	FJ345308	FJ345329
AT 34 / 2004	Austria	Yes	Au 19	FJ345307	FJ345330
H4 300 / 2005	Hungary	Yes	Hu 15	FJ345317	FJ345339
1 M / 2006	Poland	Yes	Po 1	FJ345318	FJ345340
3 NZ / 2006	Poland	Yes	Po 3	FJ345319	FJ345341
4 RZ / 2006	Poland	Yes	Po 4	FJ345320	FJ345342
1 W / 2006	Poland	Yes	Po 6	FJ345321	FJ345343
L – 4 / 2007	Denmark	Yes	De 4	FJ345310	FJ345332
B4 V / 2006	Spain	Yes	Sp 1	FJ345311	FJ345333
23 / 2006	Belgium	Yes	Be 23	FJ345309	FJ345331

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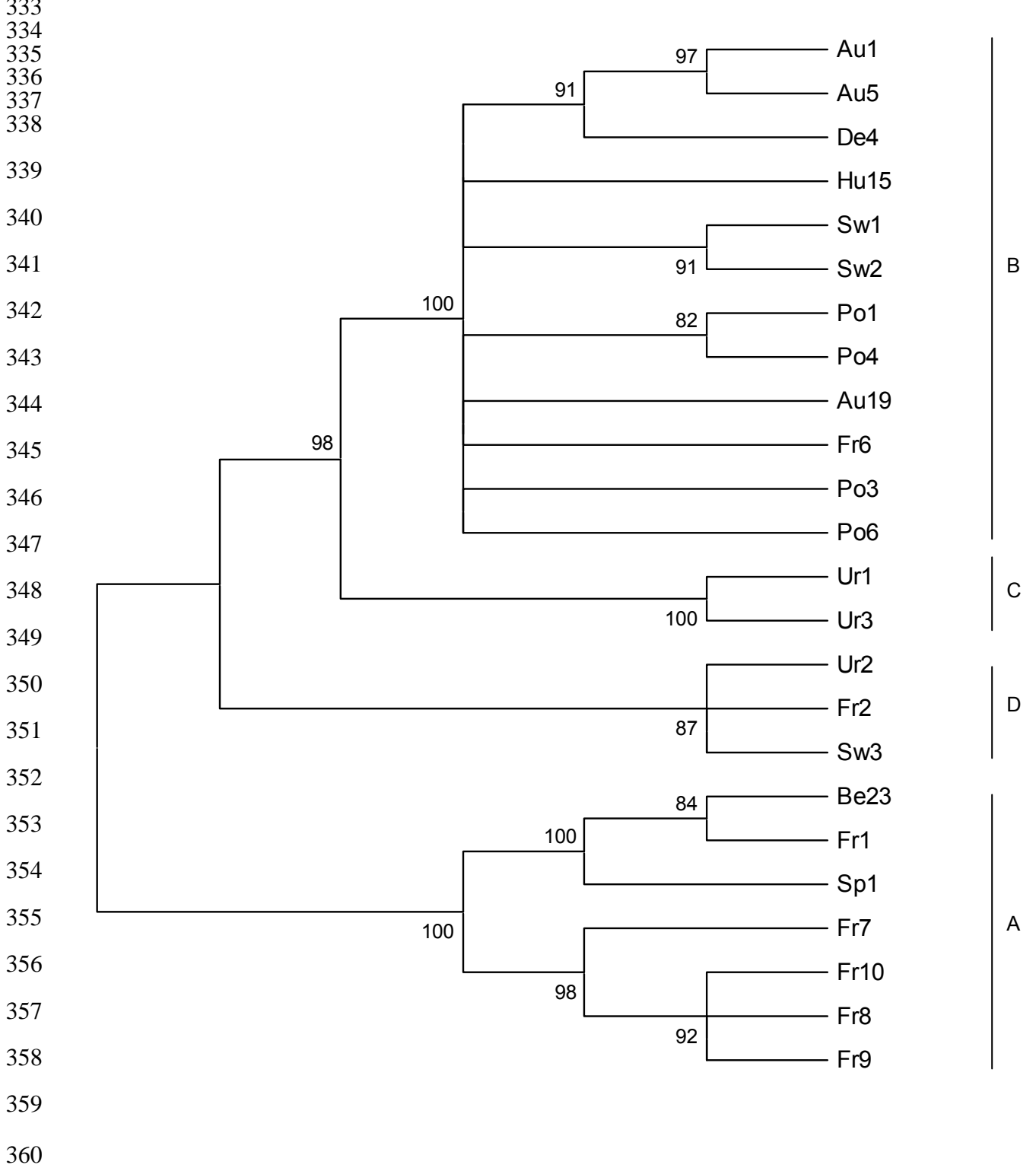
¹ Isolate reference given by our colleagues

²: Presence of symptoms like chronic paralysis or abnormal mortalities

*: CBPV reference isolates A and B (Olivier et al., 2008)

°: pSP, predicted structural protein

332 Figure 1
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361 Figure 2
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