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.

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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.

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Summary

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

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

Short communication

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

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

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

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

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

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

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

Three different primer pairs were designed by Primer3 Output for the amplification of overlapping fragments which cover the entire RdRp of CBPV (Table 1), based on the sequence of CBPV RNA1 (GenBank accession no. **EU122229**) described by Olivier et al. (2008). Amplifications were carried out in a total reaction volume of 50µl using the Platinum® *Pfx* DNA Polymerase (Invitrogen). This polymerase has a proofreading 3'-5' exonuclease activity, and therefore provides higher fidelity. The final PCR reaction contained 2X Platinum® *Pfx* Amplification Buffer, 1mM of $MgSO_4$, 0.3 mM of dNTPs mix PCR grade, 0.3 µM of each primer, 2.5 U of Platinum® *Pfx* DNA Polymerase and 5 µl of cDNA. The thermal cycling conditions were 5 min at 94°C (denaturing of the template and activation of the enzyme), followed by 35 cycles consisting of denaturing at 94°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 30 s to 2 min (depending on the primer pair), completed by a final extension at 68°C for 10 min. The PCR products were then electrophoresed in 1% agarose gel in TAE buffer, stained with ethidium bromide, and visualised under UV light. The PCR products were then purified using the Qiaquick PCR purification kit (Qiagen) and sequenced in both orientations by using primers described above (Millegen, France). The complete genomic nucleotide sequence of the RdRp of different CBPV isolates (1947bp) was determined by overlapping the three sequences using the Seqman™ II program of the DNASTAR software package (Lasergene). A primer pair was designed, covering the entire Orf3 (543bp), coding for a predicted structural protein (pSP) (Table 1), based on the sequence of CBPV RNA2 (GenBank accession no. **EU122230**) described by Olivier et al. (2008). Amplification, PCR product purification, sequencing and nucleotide sequence determination were carried out as described for the RdRp gene.

Preliminary comparison of the sequences from isolates from the same geographical origin revealed that most frequently they shared near 100% identity. The analysis was thus restricted to the divergent sequences (5 from France and 17 from different countries, Table 2). The nucleotide sequences of the RdRp and the pSP from the 22 new isolates were submitted to the GenBank database under accession numbers **FJ345306** to **FJ345349**. The 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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Captions to figures

Table 1

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

Table 2

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

Figure 1

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

Figure 2

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

313
314 Table 1.
315
316

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°	

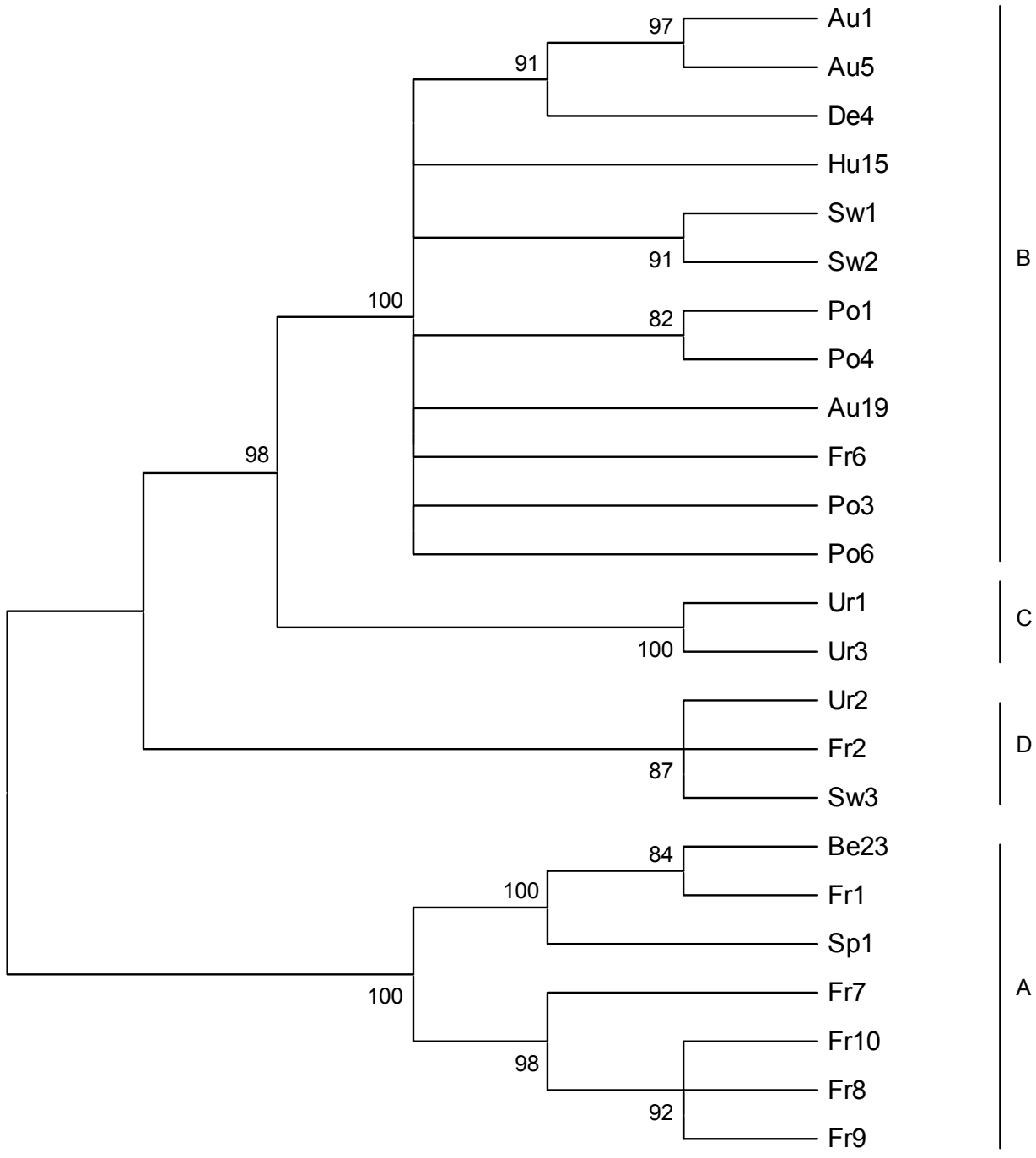
317
318 * Nucleotide positions refer to the CBPV RNA1 sequence (**EU122229**) described by Olivier et
319 al., (2008).
320 ° Nucleotide positions refer to the CBPV RNA2 sequence (**EU122230**) described by Olivier et
321 al., (2008).
322

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**

[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

Figure 1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Ur3
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361 Figure 2
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