Virus Genes (2011) 43:471–475
DOI 10.1007/s11262-011-0640-6

Identification and sequence analysis of the *Condylorrhiza vestigialis* MNPV *p74* gene

Maria Elita B. Castro · Débora P. Paula · Geraldo F. Almeida · Zilda Maria A. Ribeiro · Marlinda L. Souza · Peter W. Inglis · Bergmann M. Ribeiro

Received: 10 February 2011 / Accepted: 20 June 2011 / Published online: 1 July 2011

Abstract The baculovirus *Condylorrhiza vestigialis multiple nucleopolyhedrovirus* (CoveMNPV), isolated from *C. vestigialis* infected larvae in Paraná (Brazil), was identified in our laboratory. A full-length clone was obtained from the CoveMNPV genome, of the gene that encodes the homolog to baculoviral p74, essential for oral infectivity which was then sequenced and characterized. The CoveMNPV *p74* gene (GenBank accession number EU919397) contains an ORF of 1935 bp that encodes a deduced protein of 73.61 kDa. The phylogenetic affiliations of the CoveMNPV gene were determined by a heuristic search of 40 aligned baculovirus *p74* nucleotide sequences using maximum parsimony (PAUP 4.0b4a). The phylogenetic analysis placed CoveMNPV within lepidopteran nucleopolyhedrovirus (NPV) Group I, Clade A, as being the closest to *Choristoneura fumiferana defective* NPV.

Keywords *Alphabaculovirus* · *Condylorrhiza vestigialis* MNPV · *p74* gene · Phylogenetic analysis

Baculoviruses are widely used as bioinsecticides for the control of agricultural and forest insect pests. In most cases, these naturally occurring viruses are obtained from field-collected larvae that have been isolated and selected based on their infectivity and virulence. They comprise a large family of insect-specific DNA viruses, divided into four genera: *Alphabaculovirus* (subdivided into Group I and Group II nucleopolyhedroviruses (NPVs)), *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* [1–4], (www.ictvonline.org).

The baculovirus *Condylorrhiza vestigialis multiple nucleopolyhedrovirus* (CoveMNPV), recently identified by Castro et al. [5], was isolated from the defoliating caterpillar *C. vestigialis* (Guenée) (Lepidoptera: Crambidae), an important pest of Poplar (*Populus* spp. Salicaceae). Initial field and laboratory tests to evaluate the infectivity of CoveMNPV to *C. vestigialis* have revealed a considerable potential for its use in the control of this important insect pest [6].

The genome of CoveMNPV has not yet been sequenced, and very little is known about the host infection process and proteins related to its infectivity. Identification of structural proteins associated with occlusion-derived virus (ODV), the viral form responsible for horizontal transmission between insect hosts, has facilitated the study of the functional role of these proteins in viral virulence and host specificity. In addition, studies based on sequences of individual genes have provided additional information on their molecular characteristics and phylogenetic relationships [7].

The *p74* gene encodes a protein that is conserved among all the sequenced baculoviruses and is essential for *per os* infection [8–10]. P74 was the first of the *per os infectivity factor* (PIF) family to be identified among the baculoviruses. To date, this family is represented by six genes (the *pif*s): *pif1*, *pif2*, *pif3, pif4*, *pif5*, and *p74* [11–14].

In order to investigate the taxonomic and phylogenetic statuses of the CoveMNPV, the viral *p74* gene was cloned, sequenced, and characterized. Occlusion bodies (OB) were

M. E. B. Castro (✉) · D. P. Paula · G. F. Almeida · Z. M. A. Ribeiro · M. L. Souza · P. W. Inglis
Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica W5 Norte Final, Brasília, DF 70770-917, Brazil
e-mail: elita@cenargen.embrapa.br

G. F. Almeida · B. M. Ribeiro
Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, Brazil

isolated and purified from CoveMNPV-infected larvae. All DNA manipulations described above, including restriction enzyme digestion, agarose gel electrophoresis, *Escherichia coli* transformation, and plasmid purification, were performed essentially as described by Sambrook et al. [15].

Primers for PCR-based probe construction and p74 ORF sequencing were designed based on the conserved regions of a *p74* sequence alignment of representatives from each of the baculovirus groups obtained from GenBank/EMBL and aligned by CLUSTALX 1.81 [16]. The following primers were used: *p74*/Cove-GI-Forward: 5′-gtgtacagcgagctgctggc-3′ (+348 to +367 nucleotides relative to the start codon), *p74*/Cove-GI-Reverse: 5′-tacaccttgcgtcccgcgtc-3′ (+1905 to +1924), *p74*/Cove-GII-Forward: 5′-agattgcgtttcatacccaaat-3′ (+185 to +207), *p74*/Cove-GII-Reverse: 5′-agatgagtgtacaga gcgctgg-3′ (+1835 to +1857), *p74*-Forward: 5′-aataccctgggc aaaccaat-3′ (+1951 to +1931), and *p74*-Reverse: 5′-aattttgtc gtttagtta-3′ (+1380 to +1362).

The polymerase chain reaction (PCR) was performed using 10–20 ηg of CoveMNPV DNA, 10 μM of forward and reverse primers, and one unit of *Taq* DNA polymerase (Invitrogen®) in 1× PCR buffer supplemented with 200 μM of each dNTP and 2.0 mM $MgCl_2$ was performed using a MJ Research PTC-100 thermal cycler according to the following steps: an initial denaturation of 94°C for 5 min, 30 cycles of 94°C, 1 min; 64°C (56°C), 1 min; and 72°C, 1 min, followed by a final extension step of 72°C for 10 min.

After obtaining the *p74* [α-^{32}P] dCTP labeled probe, Southern blots of restriction enzyme-digested CoveMNPV DNA were hybridized overnight at 68°C, and the membranes were washed three times in 2× SSC (standard saline citrate), 0.1% SDS (sodium dodecyl sulfate) for 30 min each before the exposure of Kodak T-MAT™ G/RA film for 48 h at −80°C.

Restriction fragments corresponding to the hybridized bands were cloned in pBlueScript II KS$^+$ (Stratagene®) and the PCR amplicons cloned in pGEM®-T Easy (Promega®), and then employed to transform *E. coli* XL-1-Blue. Clones were sequenced using the BigDye 3.1 kit and the ABI 3130 XL automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The detection of the *p74* locus in the CoveMNPV genome consisted of the hybridization of a *p74* consensus baculovirus probe (1,600 bp), generated by PCR amplification, and with the CoveMNPV DNA digested by the restriction enzymes *Hin*dIII, *Pst*I, and *Eco*RI (Fig. 1a). A single, strongly hybridized band was detected in the three digests respectively: a 1-kb fragment from *Hin*dIII digestion, a 4.2–4.3-kb fragment from *Pst*I digestion, and a 3.1-kb fragment from *Eco*RI digestion (Fig. 1b). To confirm the identity of the gene, the restriction fragments were eluted from the 0.8% low-melting agarose gel, and cloned into pBlueScript II KS$^+$ for DNA sequencing. We found that the 1.0-kb *Hin*dIII fragment (pBS-CoveHindIII) had part of the *p74* gene and was present on the *Eco*RI 3.1 kb fragment, which contained 75% of the gene (about 1,500 bp). Since 25% of the *p74* gene sequence was still missing, we performed a PCR amplification of this region from the CoveMNPV genome using the specific primers previously cited. The amplified fragment was cloned into pGEM®-T Easy for DNA sequencing. After the combination of both strategies, the full length of the CoveMNPV *p74* gene was successfully obtained.

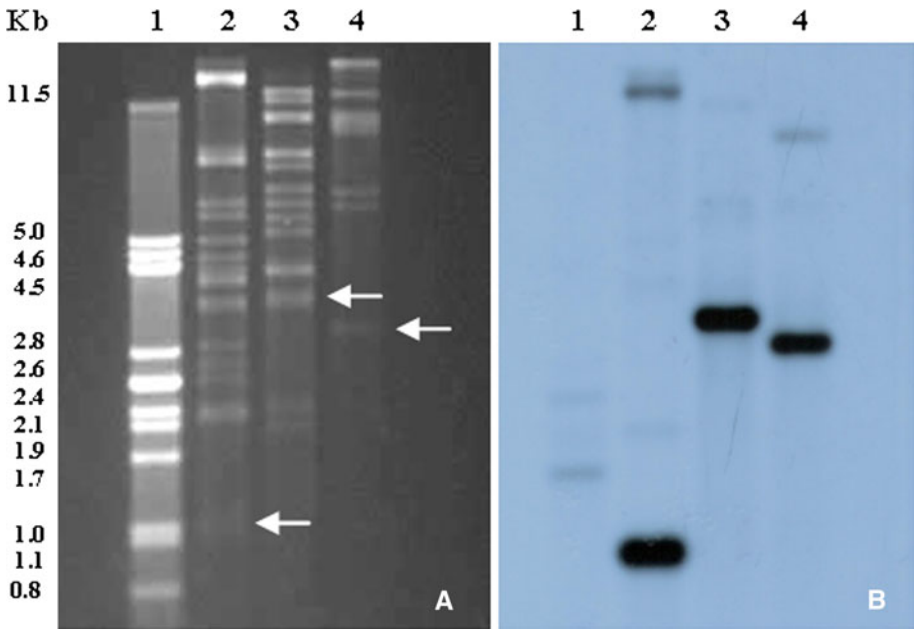

Fig. 1 **a** Agarose gel (0.8%) electrophoretic profile of the CoveMNPV genome digested with *Hin*dIII, *Pst*I and *Eco*RI, respectively (*lanes 2–4*). The molecular mass marker DNA*λ*/*Pst*I is contained in *lane 1*. **b** Autoradiogram of the Southern blot assay to locate the *p74 locus* in the CoveMNPV genome digested as mentioned in **a**, using a radioactive *p74* probe (^{32}P). The *white arrows* indicate the corresponding intensely hybridizing regions

The analysis of the nucleotide sequence of the CoveMNPV *p74* gene revealed the presence of an ORF of 1,935 bp that was deposited in GenBank under the accession number EU919397. The CoveMNPV p74 ORF potentially encodes a protein of 644 amino acids of molecular mass 73613.3 Da (pI 5.12) containing P74 domains related to oral infectivity, and transmembrane domains (C-terminal region) potentially related to anchoring inside of ODV envelope (data not shown). The deduced protein alignment revealed a high similarity to

Table 1 *p74* baculovirus sequences used for phylogenetic analyses

GenBank accession number	Baculovirus	Genome position	ORF	Size (bp)
NP_818674.1	*Adoxophyes honmai* NPV	23,773–25,788	27	2,016
NP_872507.1	*Adoxophyes orana* GV	37,367–39,295	54	1,929
YP_006288.1	*Agrotis segetum* GV	57,392–59,419	56	2,028
YP_529814.1	*Agrotis segetum* NPV	135,968–137,902	144	1,935
YP_610989.1	*Antheraea pernyi* NPV	107,641–109,563	129	1,923
AAY19516.1	*Anticarsia gemmatalis* MNPV–2D	112,268–114,202	134	1,935
AAY19519.1	*Anticarsia gemmatalis* NPV-SF	419–2,353	–	1,935
AAA46729.1	*Autographa californica* MNPV	65–2,002	138	1,938
NP_047536.1	*Bombyx mori* NPV	108,796–110,733	138	1,938
NP_932741.1	*Choristoneura fumiferana* DEFMNPV	111,392–113,398	132	2,007
AAL13071.2	*Choristoneura fumiferana* GV	337–2,331	–	1,995
AF512031	*Choristoneura fumiferana* MNPV	110,746–112,683	130	1,938
YP_249621.1	*Chrysodeixis chalcites* NPV	18,970–20,946	17	1,977
YP_717552.1	*Clanis bilineata* NPV	18,543–20,522	14	1,980
NP_891905.1	*Cryptophlebia leucotreta* GV	47,165–49,177	58	2,013
NP_203378.1	*Culex nigripalpus* NPV	64,492–66,537	73	2,046
NP_148844.1	*Cydia pomonella* GV	48,578–50,644	60	2,067
YP_874207.1	*Ecotropis obliqua* NPV	16,243–18,201	14	1,959
NP_203290.1	*Epiphyas postvittana* NPV	101,271–103,205	121	1,935
NP_203576.1	*Helicoverpa armigera* NPV	16,224–18,290	20	2,067
AF334030_89	*Helicoverpa zea* SNPV	16,195–18,261	19	2,067
YP_473207.1	*Hyphantria cunea* NPV	19,839–21,773	19	1,935
YP_758321.1	*Leucania separata* NPV	24,273–26,249	–	1,977
NP_047663.1	*Lymantria dispar* MNPV	26,645–28,663	27	2,019
NP_613243.1	*Mamestra configurata* NPV-A	144,165–146,138	160	1,974
NP_689333.1	*Mamestra configurata* NPV-B	147,569–149,542	159	1,974
ABM05422.1	*Maruca vitrata* MNPV	94,724–96,661	106	1,938
YP_025247.1	*Neodiprion lecontei* NPV	41,772–43,673	47	1,902
YP_025157.1	*Neodiprion sertifer* NPV	49,560–51,464	50	1,905
YP_001650925.1	*Orgyia leucostigma* NPV	16,694–18,652	15	1,959
O10365.1	*Orgyia pseudotsugata* MNPV	112,559–114,493	134	1,935
NP_663220.1	*Phthorimaea operculella* GV	46,284–48,260	55	1,977
NP_068268.1	*Plutella xylostella* GV	37,776–39,512	48	1,737
YP_758602.1	*Plutella xylostella* MNPV (CL3)	119,644–121,581	134	1,938
NP_703125.1	*Rachiplusia* MNPV	116,861–118,798	138	1,938
NP_037891.1	*Spodoptera exigua* MNPV	124,099–126,060	131	1,962
AAO45529	*Spodoptera frugiperda* MNPV	166–2,106	134	1,941
CAA67755.1	*Spodoptera littoralis* NPV	148–2,121	–	1,974
NP_258289.1	*Spodoptera litura* NPV	19,706–21,679	21	1,974
NP_059225.1	*Xestia c-nigrum* GV	71,928–74,060	77	2,133

several other baculoviruses. The highest similarity values were observed among the P74 of CfDEFNPV (94% identity) AgMNPV (90%), AnpeNPV (81%), OpMNPV (79%), and CfMNPV (79%).

The CoveMNPV *p74* sequence along with 39 other baculovirus p74 DNA sequences (Table 1) was aligned using MUSCLE [17]. Phylogenetic analysis was performed under the maximum parsimony criterion using PAUP 4.0b4a [18]. Heuristic searches comprised 1,000 cycles of random taxon addition, with TBR branch swapping, holding five trees per cycle. The three most parsimonious trees were obtained, one of which is depicted in Fig. 2. The phylogenetic tree confirmed the classification of the *Baculoviridae* family into four moderately to well-supported clades representing genera: *Alpha*-(lepidopteran NPVs), *Beta*-(lepidopteran GVs), *Gamma*-(hymenopteran NPVs) and *Deltabaculovirus* (dipteran NPVs) [2]. Within the lepidopteran NPVs, there is a division comprising two groups: Group I and Group II were observed (Fig. 2).

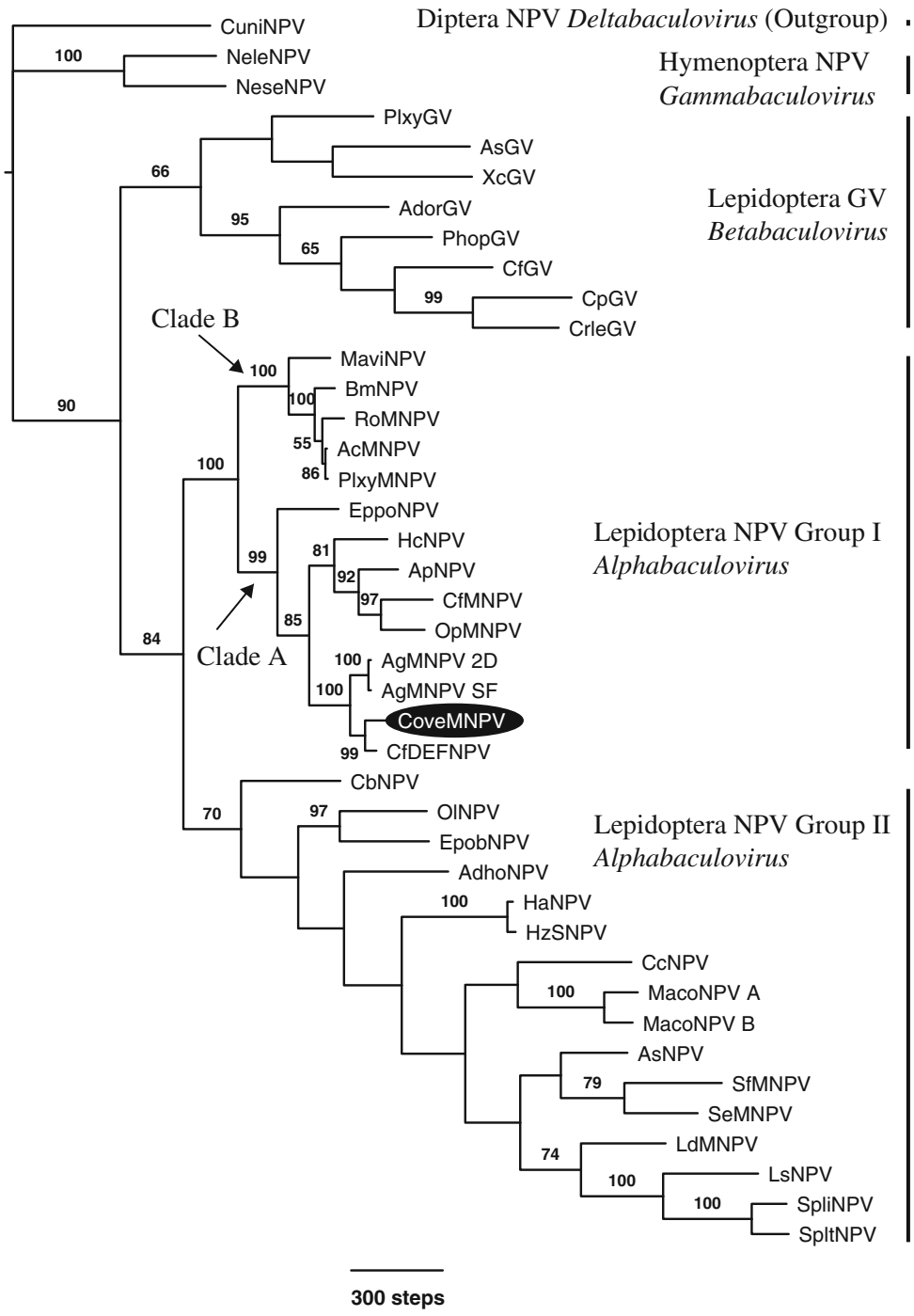

Fig. 2 One of three most parsimonious trees resulting from a heuristic search of 40 aligned baculovirus p74 DNA sequences using PAUP 4.0b4a [18]. The tree was rooted using the sequence from CuniNPV, and the branch support is given as a percentage of 1,000 bootstrap pseudoreplicates. The *circle* indicates the CoveMNPV baculovirus

The Group I *Alphabaculovirus* presented a topology that favors its division into two subgroups: Clade A and Clade B, with the CoveMNPV baculovirus appearing in Clade A. The Group II *Alphabaculovirus* possessed a largely linear hierarchy in the tree, where many internal branches possessed poor bootstrap support. Compared with Group I, Group II has a greater and more ancient diversification among the species from their common ancestor [19].

Herniou et al. [20] found that the most topological variations of the family *Baculoviridae* were within the GV and NPV Group II. These variations may be because some representatives of these groups have very large genomes, creating an imbalance when phylogenetic analyses are based on gene content and gene order. Another explanation, discussed in their study, suggests that some species were very similar and others, more divergent, thus complicating the choice of appropriate markers to establish their phylogenetic relationships.

According to the topology shown for the clades belonging to Group I, Clade A, and Clade B, CoveMNPV is the most closely related to CfDEFNPV (*Choristoneura fumiferana defective* NPV), but is also closely related to AgMNPV (*Anticarsia gemmatalis* MNPV), both belonging to Clade A. These relationships were supported by high bootstrap values. The phylogenetic data reported in this study indicate that the CoveMNPV baculovirus belongs to Group I, which was previously classified only in terms of genus.

The *p74* sequence is the first gene of the baculovirus CoveMNPV to be described until now, and provides a first approach for CoveMNPV phylogeny. Our findings are in agreement with the current classification and phylogenetic relationships within the family, *Baculoviridae* [2] and provide new insights on CoveMNPV taxonomy reinforcing the view that this virus is a distinct baculovirus species.

Acknowledgments The authors thank Dr. Filipe R. da Silva (EMBRAPA/CENARGEN) for his comments and suggestions. To Dr. Nilton J. Sousa (Universidade Federal do Paraná, Curitiba-PR) and Edilene B. Machado (Swedish Match do Brasil S.A., Curitiba-PR) for providing the CoveMNPV infected larvae. The authors also thank the Plataforma de Sequenciamento de DNA of Embrapa Recursos Genéticos e Biotecnologia (CENARGEN), especially to Luciana B.D. Labuto. This study was supported by EMBRAPA/CENARGEN, and Geraldo F. Almeida was supported by an MSc fellowship from CAPES-Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

References

1. D.A. Theilmann, G.W. Blissard, B. Bonning, J.A. Jehle, D.R. O'Reilly, G.F. Rohrmann, S. Thiem, J.M. Vlak in *Virus Taxonomy Classification and Nomenclature of Viruses*, 8th ICTV Report, ed. by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball, (Elsevier, New York, 2005), pp. 177–185
2. J.A. Jehle, G.W. Blissard, B.C. Bonning, J.S. Cory, E.A. Herniou, G.F. Rohrmann, D.A. Theilmann, S.M. Thiem, J.M. Vlak, Arch. Virol. **151**, 1257–1266 (2006)
3. E.A. Herniou, J.A. Jehle, Curr. Drug Targets **8**, 1043–1050 (2007)
4. E.B. Carstens, L.A. Ball, Arch. Virol. **154**, 1181–1188 (2009)
5. M.E.B. Castro, Z.M.A. Ribeiro, A.C.B. Santos, M.L. Souza, E.B. Machado, N.J. Sousa, F. Moscardi, J. Invertebr. Pathol. **102**, 149–154 (2009)
6. E.B. Machado, Dissertação de Mestrado (Curitiba: UFPR, 2006), p. 76
7. E.A. Herniou, J.A. Olszewski, D.R. O'Reilly, J.S. Cory, J. Virol. **78**, 3244–3251 (2004)
8. J. Kuzio, R. Jaques, P. Faulkner, Virology **173**, 759–763 (1989)
9. Y.J. Wang, R.G. Kleespies, A.M. Huger, J.A. Jehle, J. Virol. **81**, 5395–5406 (2007)
10. J.M. Slack, S.D. Lawrence, P.J. Krell, B.M. Arif, J. Gen. Virol. **91**, 915–918 (2010)
11. E.J. Haas-Stapleton, J.O. Washburn, L.E. Volkman, J. Virol. **78**, 6786–6789 (2004)
12. W. Zhou, L. Yao, H. Xu, F. Yan, Y. Qi, Virus Genes **30**, 139–150 (2005)
13. M. Fang, Y. Nie, S. Harris, M.A. Erlandson, D.A. Theilmann, J. Virol. **83**, 12569–12578 (2009)
14. W.O. Sparks, R.L. Harrison, B.C. Bonning, Virology **409**, 69–76 (2011)
15. J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd edn. (Cold Spring Harbor Laboratory Press, New York, 1989)
16. J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, Nucleic Acids Res. **24**, 4876–4882 (1997)
17. R.C. Edgar, Nucleic Acids Res. **32**, 1792–1797 (2004)
18. D.L. Swofford, *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)* (Sinauer Associates, Sunderland, 2002)
19. Z.H. Hu, B.M. Arif, J.W.M. Martens, X.W. Chen, J.S. Sun, D. Zuidema, R.W. Goldbach, J.M. Vlak, J. Gen. Virol. **79**, 2841–2851 (1998)
20. E.A. Herniou, T. Luque, X. Chen, J.M. Vlak, D. Winstanley, J.S. Cory, D.R. O'Reilly, J. Virol. **75**, 8117–8126 (2001)