Renewal Assessment Report

Cydia pomonella GV

Volume 3 – B.1 Identity

Rev. 0 – 16 October 2020

Rapporteur Member State: Germany

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Version history

When	What
16 October 2020	First version submitted to EFSA

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS.

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B Summary of the data and information

B.1 Identity of the micro-organism

B.1.1 Applicant

Applicant: CpGV AIR4 Task Force
Consisting of:
Andermatt Biocontrol GmbH
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Serbios srl
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Contact Point: [redacted]

Serbios srl has acquired all data and registrations concerning CpGV and formulated product from Sipcam S.p.A..

B.1.2 Producer

Confidential information, see Volume 4.

B.1.3 Name and species description, strain characterisation

B.1.3.1 Accession number in culture collection

In the DAR only CPGV Mexican isolate was evaluated. Several additional isolates were evaluated according to the "Guidance Document SANCO/0253/2008 on the assessment of new isolates of baculovirus species already included in Annex I of Council Directive 91/414" A Report was written for each isolate and the MS were given the opportunity to comment. After taking note at the SCFCAH the respective isolate was added to Appendix III of the Review Report. Studies submitted for these isolates are considered as new information for the renewal.
Additionally, two new isolates CpGV-V14 and CpGV-V45 were provided.

All isolates are deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ), Inhoffenstraße 7B, D-38124 Braunschweig, Germany.

References:
Winter (2011), The Granulosevirus preparation CpGV-Isolate V14 (BVL no 3714749)
Menzel (2017), The Granulosevirus preparation CpGV V45 (BVL no 3714797)

Overview of CpGV isolates

Applicant	CpGV Isolate (Virus Accession number) year included in culture collection	Plant protection product	Evaluation
Andermatt Biocontrol AG	Mexican isolate (GV-0001) 2005	MADEX	DAR
	CpGV-V01 (GV-0003) 2007	-	SANCO/0253/2008
	CpGV-V03 (GV-0006) 2008	-	SANCO/0253/2008
	CpGV-V15 (GV-0013) 2010	-	SANCO/0253/2008
	CpGV-V22 (GV-0014) 2010	MADEX TWIN	SANCO/0253/2008
	CpGV-V14 (GV-0015) 2011	-	new
	CpGV-V45 (GV-0017) 2017	-	new
Arysta LifeScience S.A.S.	Mexican isolate (GV-0002) 2005	Carpovirusine	DAR
	CpGV-R5 (GV-0007) 2009	-	SANCO/0253/2008
Serbios	No own isolate is produced.	Virgo	DAR

B.1.3.2 Scientific name and taxonomic grouping, i.e. family, genus, species, strain, serotype, pathovar or any other denomination relevant to the micro-organism

Information already provided in the DAR

References:

Evans, Harrap (1982), Persistence of insect viruses, published: Cambridge University Press (BWS 2006-13)
OECD (2002), Consensus document on information used in the assessment of environmental applications involving baculovirus, published: Series on Harmonization of Regulatory Oversight in Biotechnology, No. 20 (ENV/JM/MONO(2002)1) (BWS 2006-90)
Bilimoria S.L., 1986, Taxonomy and identification of Baculoviruses, published: The biology of baculoviruses, Vol. 1, p37-59 I, 37-59 (BWS 2006-88)
Gröner, A. (1986), Specificity and Safety of Baculoviruses, published: The Biology of Baculoviruses, Volume I, Biologiy Properties and Molecular Biologie, Chapter 9, 177-201 (BWS 2006-15)
ICTV (2000), 00.006.0.02.001. Cydia pomonella granulovirus, published: International Commitee of Taxonomy of Virus database (BWS 2006-123)
ICTV (2000), Cydia pomonella granulovirus -Comparison of single viral protein, 00.006.0.01 Nucleopolyhedrovirus, polyhedrin, 00.006.0.02. Granulovirus, granulin, published: International Commitee of Taxonomy of Virus database (BWS 2006-124)
Aupinel, P. (2005), Certificate of origin of CpGV isolate transmitted to NPP., Arysta LifeScience

S.A.S (BVL no. 2019054)
Tweeten K.A. et al. (1981), Applied and molecular aspects of insect granulosis viruses, published: Microbiological reviews, 45 (3), 379-408. , 379-408 (BWS 2006-89)

Cydia pomonella Granulovirus (CpGV), natural entomopathogen (not genetically modified), belongs to the family Baculoviridae. Baculoviruses are divided into two genera Nucleopolyhedroviruses (= NPV) (formerly nuclear polyhedrosis viruses) and Granuloviruses (= GV) (formerly granulosis viruses).
Baculoviridae are arthropod-specific, rod-shaped (baculum = rod), enveloped viruses with a circular double-stranded DNA genome. The most prominent characteristic is the formation of occlusion bodies (OB) which in case of NPV are polyhedra-like and in the case of GV are ovicylindrical (granule-like). Non-occluded viruses (NOV) are characterised by the absence of occlusion bodies.
The occlusion bodies of Granuloviruses are 120-300 nm in width and 300-500 nm in length. The matrix protein, called granulin, is genetically and serologically closely related to the protein of NPV-OBs, called polyhedrin. In contrast to NPVs, at GVs only one virion is incorporated in the OB, and each virion contains only one nucleocapsid.

Isolates used for the production of MADEX (Andermatt Biocontrol), Granupom (Probis GmbH), VIRGO (SipcamS.p.A.) and CARPOVIRUSINE (Arysta LifeScience S.A.S) derived from the Mexican isolate originally isolated in 1963.

Indigenous or non-indigenous:	The *Cydia pomonella* Granulovirus is naturally present in our environment.
Wild type:	yes

Species: *Cydia pomonella* Granulovirus (CpGV)

Genus: Betabaculovirus (formerly: Granulovirus)

Family: Baculoviridae

B.1.3.2.1 Additional isolates owned by Andermatt Biocontrol AG

These isolates were included in Annex III of the Review Report for CpGV.

References:
Kessler (2010), Declaration of Origin CpGV isolate ABC-V15 (DSMZ GV-00013), Andermatt Biocontrol GmbH (BVL no. 3306430)
Kessler (2010), Declaration of Origin CpGV isolate ABC-V22 (DSMZ GV-00014), Andermatt Biocontrol GmbH (BVL no. 3306431)
Kessler (2008), Declaration on the origin and characterization of the active ingredient of MADEX Plus, Andermatt Biocontrol GmbH (BVL no. 3306432)

CpGV-V15 (GV-0013)

The isolate CpGV-V15 has been isolated from *C. pomonella* larvae using classical selection methods (Kessler, BVL no. 3306430). The isolate does not have any characteristics differing from the typical description of the species and differs from CpGV-M only in the ability to break the resistance of *C. pomonella* populations that are resistant to CpGV-M.

The isolate CpGV-V15 was compared to the Mexican isolate using Restriction Fragment Analysis (see B.1.3.3.1: Jehle and Eberle, 2009, BVL no 3306433). CpGV-V15 was purified and DNA from CpGV-V15 and the reference isolate, CpGV-M was extracted. These DNA samples were digested separately with four different restriction endonucleases (*Bam*HI, *Eco*RI, *Sal*I, and *Eco*RV) and separated in gel electrophoresis. Differences were observed for *Bam*HI, *Eco*RI, and *Eco*RV restriction patterns, but not for *Sal*I.

CpGV-V15 consists of two different genotypes. When compared to CpGV-M, one genotype lacks a *Bam*HI site between fragments C and J. The resulting restriction fragment C is slightly larger than in CpGV-M, corresponding to the size of the A fragment in the genotype CpGV-E2 which apparently results from the lack of another *Bam*HI site. CpGV-V15 contains two additional *Eco*RI sites, and an additional *Eco*RV site when compared to CpGV-M. Taken together, one genotype corresponds to the genotype CpGV-E2 as described in the literature. The other one differs from CpGV-M and CpGV-E2. Changes in the presence of restriction sites result from point mutations. All genotypes clearly belong to the species CpGV and no insertions or deletions were detected in the CpGV-V15 genotypes when compared to CpGV-M.

CpGV-V22 (GV-0014)

The new isolate CpGV-V22 was obtained from infested *C. pomonella* larvae and does not contain genetic modifications (Kessler, BVL no. 3306431). Genetically, CpGV-V22 is closely related to CpGV-M and belongs to the same genome type A as CpGV-M (B.1.3.3.1: Jehle and Eberle, 2009, BVL no 3306434). In contrast to CpGV-M and other CpGV isolates, CpGV-V22 is infective to larvae of the oriental fruit moth, *Grapholita molesta* (Tortricidae). Like other CpGV isolates it is not infective to other tortricid species like *Adoxophyes orana*. The isolate does not have any other characteristics differing from the typical description of the species and the representative isolate CpGV-M.

The isolate CpGV-V22 was compared to the Mexican isolate using Restriction Fragment Analysis and single nucleotide polymorphism (SNP) analysis (see B.1.3.3.1: Jehle and Eberle, 2009, BVL no 3306434). CpGV-V22 was purified and DNA from CpGV-V22 and the reference isolate, CpGV-M was extracted. These DNA samples were digested separately with four different restriction endonucleases (*Bam*HI, *Eco*RI, *Sal*I, and *Eco*RV) and separated in gel electrophoresis. Differences were observed for *Eco*RI and *Eco*RV restriction patterns, but not for *Bam*HI and *Sal*I. The CpGV-V22 pattern contains an additional *Eco*RI fragment of 5.8 kb, and two additional bands of 6.5 kb and 13 kb after *Eco*RV digestion when compared to CpGV-M. These additional bands indicate that another very similar genotype is present at relatively low quantities in the isolate CpGV-V22 in addition to the dominating CpGV-M. Changes in the presence of restriction sites result from point mutations. CpGV-V22 and CpGV-M are highly similar and no insertions or deletions were detected in the CpGV-V22 genome when compared to CpGV-M.
Another method for the comparison of baculovirus genomes is the analysis of "single nucleotide polymorphisms" (SNP). To this end, the sequences of two highly conserved marker genes, encoding granulin (gran) and late expression factor 8 (lef-8) are determined and compared between CpGV-M and CpGV-V22. The differences detected between CpGV genomes analysed so far correspond to different restriction sites also detected by RFLP. No differences in these regions were found between CpGV-M and CpGV-V22, indicating that no SNPs are present and that CpGV-M and CpGV-V22 are highly similar. Accordingly, CpGV-V22 is thus assigned to the same genome type A like CpGV-M.

CpGV-V03 (GV-0006, Madex Max)

The new isolate CpGV-V03 does not contain genetic modifications. The isolate does not have any characteristics differing from the typical description of the species and differs from CpGV-M only in the ability to break the resistance of *C. pomonella* populations that are resistant to CpGV-M. For further information, see Vol. 4 Andermatt.

The Madex Max isolate CpGV-V03 was compared to the Mexican isolate using Restriction Fragment Analysis (see Vol. 4 V03, Jehle, 2007). CpGV-V03 was purified and DNA from CpGV-V03 and the reference isolate, CpGV-M was extracted. These DNA samples were digested separately with four different restriction endonucleases (*Bam*HI, *Eco*RI, *Sal*I, and *Eco*RV) and separated in gel electrophoresis. Differences were observed for *Eco*RI, *Bam*HI and *Eco*RV restriction patterns, but not for *Sal*I. CpGV-V03 contains an additional *Eco*RI site, but lacks a *Bam*HI site present in CpGV-M. Probably, also an additional *Eco*RV site is present in CpGV-V03 when compared to CpGV-M. Changes in the presence of restriction sites result from point mutations. No submolar bands were observed after re-

striction with these enzymes, indicating the absence of genotypes other than the typical CpGV-V03 isolate. CpGV-V-03 and CpGV-M are highly similar and no insertions or deletions were detected in the CpGV-V03 genome when compared to CpGV-M.

CpGV-V01 (GV-0003, Madex Plus)

The new isolate CpGV-V01 was selected from the genetic pool of the Mexican CpGV isolate CpGV-M (Kessler, 2008). The CpGV isolate Madex Plus was obtained without genetic modifications.

The Madex Plus isolate was compared to the Mexican isolate using Restriction Fragment Analysis (see B.1.3.3.1: Jehle, 2006, BVL no 3306435). Purified DNA from viruses extracted from MADEX (CpGV-M) and MADEX Plus (CpGV-Madex Plus) was digested separately with four different restriction endonucleases (*Bam*HI, *Eco*RI, *Sal*I, and *Eco*RV) and separated in gel electrophoresis. Slight differences were observed for *Bam*HI and *Eco*RI restriction patterns, but not for *Sal*I and *Eco*RV. Submolar bands were observed after restriction with *Eco*RI, indicating the presence of genotypes other than the typical CpGV-M in the CpGV-Madex Plus isolate. These genotypes correspond to CpGV-E and CpGV-R. The shift of one band in the *Bam*HI digest by 750 bp corresponds to an insertion present in CpGV-E, but not in CpGV-M. As the CpGV-Madex Plus isolate was selected from the original MADEX isolate (CpGV-M), a shift in the composition of the isolate has taken place. CpGV-Madex Plus and CpGV-M contain the same genotypes, but in different proportions.

B.1.3.2.2 New isolates owned by Andermatt Biocontrol AG

General

The species *Cydia pomonella* granulovirus is assigned to the genus *Betabaculovirus* in the family of *Baculoviridae* (Herniou et al., 2001). In general, baculoviruses comprise occluded dsDNA viruses with rod-shaped, enveloped virions infecting larval stages of the insect orders Lepidoptera, Diptera and Hymoptera. Virions of the genus *Betabaculovirus* are embedded singly into an ovocylindrical protein matrix termed granulin, which form together the occlusion bodies (OB). Granulin is highly similar to the protein matrix of the OB deriving from *Alphabaculovirus*, the icosahedral polyhedrin, in which few to numerous virions of this genus are embedded. The well described OB morphology was used previously in baculovirus taxonomy in which nucleopolyhedrovirus described the genus *Alphabaculovirus* and granulovirus the genus *Betabaculovirus*, respectively. However, nucleopolyhedroviruses now also describe the genera *Gammabaculovirus* infecting the insect order Hymoptera and *Deltabaculovirus* infecting the insect order Diptera, respectively. Infections of isolates from the genera *Alpha-* and *Betabaculovirus* are restricted to larvae of Lepidoptera only. Currently all know granuloviruses belong to genus *Betabaculovirus* (Herniou et al., 2001).
The morphology of the granule-like OB of *Cydia pomonella* granulovirus can be studied in light and electron microscopy. Similar to other granuloviruses, the dimensions of the nucleocapsids are 34 to 36 nm in diameter and 200 to 230 nm in length occluded in roughly 200 × 390 nm large OB. As all *Betabaculovirus* species share a common morphology, they cannot be distinguished by microscopic techniques.
The first molecular analysis of the CpGV-M genome was performed by Crook et al. (1997). Currently six full genome sequences of different CpGV isolates are deposited at GenBank[1] and the differences at genome level have been investigated. These six genome sequences correspond to five different CpGV lineages; termed CpGV genome types A - E. Different CpGV isolates, like the Mexican isolate (CpGV-M), the Russian isolate (CpGV-R), the English isolate (CpGV-E) and the Iranian isolates (CpGV-I07, CpGV-I12) can be assigned to these different CpGV genome types (Eberle et al., 2009). These assignments are described by comparative investigations of restriction fragments length polymorphisms (RFLP) and evaluations of single nucleotide polymorphisms (SNP) on the genome level.

1 CpGV-M1: GenBank Accession N° NC_002816, CpGV-S: GenBank Accession N° KM217573, CpGV-I07: GenBank Accession N° KM217574, CpGV-M: GenBank Accession N° KM217575, CpGV-I12: GenBank Accession N° KM217576, CpGV-E2: GenBank Accession N° KM217577

In contrast to SNP analysis, an RFLP analysis allows the comparison of genomic DNA with selected restriction endonucleases (restriction enzymes) and subsequent separation of the fragments by gel electrophoresis (Eberle et al., 2009; Wennmann et al. 2017). The genomes of all CpGV isolates range from 120816 bp to 124269 bp with almost equimolar %GC-content and they encode 137 to 143 putative genes. They share 73 homologous genes with the alphabaculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV), as well as 108 and 98 homologous genes with the betabaculoviruses *Xestia c-nigrum* granulovirus (XecnGV) and *Plutella xylostella* granulovirus (PlxyGV), respectively. The similarity to genes of XecnGV and PlxyGV confirms the assignment to the genus *Betabaculovirus*. The Mexican isolate, CpGV-M, is used in the formulated product Madex and other commercially available products. Although different CpGV isolates can be classified into the different lineages, baculoviruses are not genetically uniform. The presence of deletions, insertions and SNP represents a certain degree of heterogeneity, which can be visualized in restriction patterns of the viral genomes.

The isolates CpGV-V14 and CpGV-V45 are new and have not been evaluated before.

CpGV-V14 (GV-0015)

The novel isolate CpGV-V14 belongs alongside with the Mexican isolate CpGV-M to the baculovirus species *Cydia pomonella* granulovirus. CpGV-V14 is a natural entomopathogen to the Lepidopteran pests *Cydia pomonella*, derives from natural virus population and is not genetically modified (Crook et al., 1985).
In case of CpGV-V14, the restriction pattern was compared to the Mexican isolate CpGV-M by RFLP analysis (see B.1.3.3.2: Jehle and Eberle, 2010). CpGV-V14 was isolated from infected *C. pomonella* larvae under classical laboratory methods and has been deposited with the DSMZ GmbH Collection of Plant Viruses, 38124 Braunschweig, Germany, under the accession number GV-0015 (Winter, 2011). Viral DNA was extracted from OB of CpGV-V14 as well as from OB of the reference isolate CpGV-M. These DNA samples were digested separately with four different restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV and *Sal*I). Differences were observed for *Eco*RI and *Eco*RV, but not for *Bam*HI and *Sal*I.

CpGV-V45 (GV-0017)

The novel isolate CpGV-V45 was isolated from infected *C. pomonella* larvae under classical laboratory methods and has been deposited with the DSMZ GmbH Collection of Plant Viruses, 38124 Braunschweig, Germany, under the accession number GV-0017 (Menzel, 2017). As the Mexican isolate CpGV-M, it belongs to the baculovirus species *Cydia pomonella* granulovirus. CpGV-V45 is a natural entomopathogen to the Lepidopteran pests *Cydia pomonella* and *Grapholita molesta,* derives from natural virus population and is not genetically modified. The species *Cydia pomonella* granulovirus is assigned to the genus *Betabaculovirus* in the family of *Baculoviridae* (Crook et al., 1985; Herniou et al., 2001)
In case of CpGV-V45, the restriction pattern was compared to the isolates CpGV-M (genome type A), CpGV-E2 (genome type B), CpGV-I07 (genome type C), CpGV-I12 (genome type D), CpGV-S (genome type E), and other betabaculoviruses by RFLP analysis (see B.1.3.3.2: Brader, 2018). Viral DNA was extracted from OB of CpGV-V45 and compared to *in silico* deduced restricions of the other isolates. The DNA samples of CpGV-V45 were digested separately with six different restriction endonucleases (*Eco*RI, *Bam*HI, *Hind*III, *Pst*I, *Sal*I and *Xho*I). According to the restriction pattern CpGV-V45 was identified as a *Cydia pomonella* granulovsirus isolate, as it was clearly distinguished from the remaining betabaculoviruses evaluated in the *in silico* analyses.

B.1.3.2.3 Additional isolates owned by Arysta LifeScience S.A.S.

This isolate was included in Annex III of the Review Report for CpGV.

CpGV R5 (GV-0007)

CpGV isolate R1 of the company Arysta LifeScience S.A.S. was first selected from a virus collection of NPP (Natural Plant Protection, biological product subsidiary of Arysta LifeScience S.A.S.) as being the most pathogenic candidate to Cydia pomonella larvae that are resistant to the Mexican isolate. Then it was enhanced by serial passages through a laboratory colony of C. pomonella originating from field-collected resistant insects (RGV). The resulting isolate reaching a level of efficacy against resistant populations, similar to CpGV-M against susceptible populations (SV), also validated in field conditions, was CpGV R5.

The CpGV-R5 isolate from NPP was compared with the CpGV (Virosoft), CpGV-E2 and CpGV-M (Neustadt) (see B.1.3.3.3: Jehle and Eberle, 2009, BVL no 3306436). In this study, DNA of CARPOVIRUSINE R5 (Test Item) was isolated and purified and subjected to endonuclease restriction analysis using the endonucleases *Sal*I, *Bam*HI, *Eco*RI and *Eco*RV. The restriction fragments were separated in an agarose gel and the obtained restriction profiles were compared to the restriction profiles of CpGV (Virosoft), CpGV (Isolate E2), CpGV-M (Mexican Isolate, propagated in Neustadt) and to published profiles of CpGV-M. On the basis of DNA restriction analysis using *Sal*I, *Eco*RV, *Eco*RI and *Bam*HI, it can be concluded that Test Item CpGV CARPOVIRUSINE R5 is a CpGV isolate that is very similar to Reference Item CpGV (Virosoft). It differs slightly from the Reference Item CpGV-M (NW) and CpGV-E2. Additionally, few submolar bands were found, which could not be assigned to any of the Reference Items, indicating that there is a further genotype present at a low level in the preparation of CpGV CARPOVIRUSINE R5.

B.1.3.3 Test procedures and criteria used for identification at strain level

Information and studies already provided in the DAR

The morphology of CpGV can be studied under the electron microscope in ultrafine sections. The dimensions of the nucleocapsid are 180-200 x 390 nm.

References:
Crook et al. (1997), Comprehensive physical map of the Cydia pomonella granulovirus genome and sequence analysis of the granulin gene region, published: Journal of General Virology 78, 965-974 (BWS2006-93)
NCBI Sequence Viewer v2.0 (2001), U53466, Cydia Pomonella, published: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?va1=U53466 (BWS2006-17)
Luque et al. (2001), The complete sequence of the Cydia pomonella granulovirus genome, published: J. of General Virology, 82, 2531-2547 (BWS2006-94)

A first molecular analysis of the CpGV genome was done by Crook et al. (1997). The entire genome sequence of CpGV is available at GenBank under the number U53466 (NCBI Sequence Viewer v2.0, 2001, Luque et al., 2001). The nucleotide sequence of the DNA of the CpGV is made up of 123500 base pairs (Luque et al., 2001). Circa 143 putative genes have been identified, of which 73 are similar to genes of the *Autographa californica* Nucleopolyhedrovirus (AcMNPV), 108 are similar to genes of *Xestia c-nigrum* GV (XecnGV), and 98 are similar to genes from *Plutella xylostella* GV (PlxyGV). The similarity to genes from XecnGVand PlxyGV confirms the belonging of CpGV to the group of granuloviruses.

References:
Crook et al. (1985), Variation in Cydia pomonella Granulosis Virus isolates and physical maps of the DNA from three variants, published: J. gen. Virol., 66, 2423-2430 (BWS2006-91)
Crook et al. (1997), Comprehensive physical map of the Cydia pomonella granulovirus genome and sequence analysis of the granulin gene region, published: Journal of General Virology 78, 965-974 (BWS2006-93)
Jehle, J. (2006), Comparative Analysis CpGV (Neustadt Mexican isolate) with CpGV (Sipcam Mexican Isolate), SIP01, Serbios srl (BWS 2006-19)

Jehle, J. (2006), Comparative Analysis CpGV (Neustadt Mexican isolate) with CpGV (MADEX Mexican Isolate), Andermatt Biocontrol GmbH/Probis GmbH (BWS 2006-98)
Jehle, J. (2006), Comparative Restriction Analysis CpGV (Neustadt Mexican isolate) with CpGV (INRA Mexican isolate), ARY03, Arysta LifeScience S.A.S. (BWS 2006-87)

Recently, the isolates used for the production of MADEX and VIRGO and the CPGV isolate obtained from INRA (Institut National de la Recherche Agronomique) were compared in seperate studies to a reference isolate using DNA Restriction Endonuclease Analysis (REN).

"By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. On the other hand, a Baculovirus isolate has to be considered always as a mixture of different genotypes, which can slightly differ from each other by numerous small insertion and deletion mutations at different locations in the genome. Depending on the variations of genotypes some isolates appear to be highly homogenous, others are more heterogeneous. Discrimination between different isolates is thus always based on the differences in restriction patterns. However, this discrimination has to be considered as a continuous change in genotype composition, since they often reflect a quantitative prevalence of some genotypes compared to others rather than a qualitative difference (Jehle, 2006)".

Three different CpGV isolates are reported in the literature. The first isolate was found in Mexico in 1963 and named Mexican isolate (CpGV-M). Later, the English (CpGV-E) and Russian (CpGV-R) isolates were discovered. CpGV-E and CpGV-R are genotype variants, which are very similar to CpGV-M. They can be distinguished from CpGV-M by DNA endo-nuclease restriction analysis (REN) by small differences in their restriction patterns (Crook et al., 1985, BWS 2006-91).

The isolates used for the production of MADEX (Andermatt Biocontrol), GRANUPOM (Probis GmbH), VIRGO (SipcamS.p.A.) and the CPGV isolate obtained from INRA derived from the original Mexican isolate. The reference isolate used is as well derived from the original Mexican isolate and was multiplied in the DLR Rheinpfalz on *C. pomonella* (Neustadt Mexican isolate). DNA from the test isolate and the reference isolate was digested with four restriction enzymes (*Sal*I, *Bam*HI, *Eco*RV, *Eco*RI) and separated in gel electrophoresis.

Findings:
For all enzymes tested, DNA patterns did not show any difference between the MADEX and the INRA isolates and the reference isolate.

For three enzymes tested (*Sal*I, *Bam*HI, *Eco*RV), DNA patterns did not show any difference between the Sipcam isolate and the reference isolate. A very faint additional submolar band was observed in the DNA of the test item after digestion with *Eco*RI, indicating a minor variability.

Furthermore, comparison with the published restriction profiles of CpGV-M (Crook et al. 1985, BWS 2006-91; Crook et al. 1997 BWS 2006-93, BWS 2006-16) revealed that no differences exist between the production isolates and the originally described CpGV-M, proving that the MADEX, Sipcam and the INRA isolate is the Mexican isolate.

Reference:
Crook et al. (1985), Variation in Cydia pomonella Granulosis Virus isolates and physical maps of the DNA from three variants, published: J. gen. Virol., 66, 2423-2430 (BWS2006-91)
Crook et al. (1997), Comprehensive physical map of the Cydia pomonella granulovirus genome and sequence analysis of the granulin gene region, published: Journal of General Virology 78, 965-974 (BWS2006-93)
Jehle, J. (2006), Comparative Restriction Analysis CpGV (CARPOVIRUSINE, technical concentrate, batch 1456/SMT) with CpGV (INRA Mexican isolate), ARY02, Arysta LifeScience S.A.S. (BWS 2006-86)

The objective of this study was the comparison of the CpGV isolate used for the production of CARPOVIRUSINE with the INRA CpGV Mexican isolate. The INRA isolate is the virus stock conserved at INRA of which the industrial production of CpGV (CARPOVIRUSINE) is done.

As described in the previous studies the DNA from the two isolates was digested with four restriction enzymes (*Sal*I, *Bam*HI, *Eco*RV, *Eco*RI) and separated in gel electrophoresis. The DNA enzyme profile of CpGV (CARPOVIRUSINE, technical concentrate, batch 1461/SMT) was compared with the DNA enzyme profile of CpGV (INRA isolate). In addition, both pro-files were compared with the profile known from the literature for CpGV-M (Crook et al. 1985, BWS 2006-91; Crook et al. 1997, BWS 2006-93, BWS 2006-16).

It was found that the restriction profiles of the reference item CpGV (INRA Mexican isolate) and the test item CpGV (CARPOVIRUSINE, technical concentrate, batch 1461/SMT) did neither differ from each other nor to published restriction profiles of CpGV-M. It can be con-cluded that the test item CpGV (CARPOVIRUSINE technical concentrate, batch 1461/SMT) is identical to the originally described CpGV-M.

Conclusion:
The comparison studies showed no differences in the restriction profiles therefore and because the Baculoviruses are a very uniform group of biocontrol agents the different used isolates are seen as one active substance.

Conclusion RMS Renewal
The studies are still considered acceptable.

B.1.3.3.1 Additional isolates owned by Andermatt Biocontrol AG

These isolates were included in Annex III of the Review Report for CpGV.

CpGV-V15 (GV-0013)

Reference:
Jehle, Eberle (2009), Comparative Restriction Analysis of V15, Andermatt Biocontrol GmbH (BVL no 3306433)

Summary
For the identification of baculovirus isolates DNA endonuclease restriction (REN) analysis is usually used. By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. In this study, DNA of V15 (Test item) was isolated and purified and subjected to endonuclease restriction analysis using the endonucleases *Sal*I, *Bam*HI, *Eco*RI and *Eco*RV. The restriction fragments were separated in an agarose gel and the obtained restriction profiles were compared to the restriction profiles of CpGV-M (Mexican isolate, propagated in Neustadt) and to published profiles of CpGV-M. It was found that the test item (V15) was a CpGV isolate containing at least two genome types. One of these genome types showed similarity to the REN profile of CpGV-E2 (Crook at al., 1985, Eberle et al., 2009), the other genome type differed from CpGV-M and E2. The different genome types seemed to be present a similar level in the mixture.

Materials and Methods
Test Item V15 was purified by centrifugation in a 30-80% glycerol layer gradient. The purified CpGV OB pellet was resuspended in 2 mL sterile water. DNA was isolated from the purified CpGV pellet by phenol/chloroform/isoamylalcohol method.

The viral DNA of the Test Item (V15) and of the reference item (CpGV NW, Neustadt Mexican isolate) was digested with the restriction enzymes BamHI, EcoRI, SalI and EcoRV. These enzymes were chosen because they allow an easy differentiation of the previously described CpGV isolates. 17µL

isolated DNA were incubated in 2µL buffer and 1 µL enzyme at 37°C for 3h. Digested DNA was electrophoresed in a 0.8% agarose gel over night (25 V) using TAE as buffer system.

Findings
REN analysis:

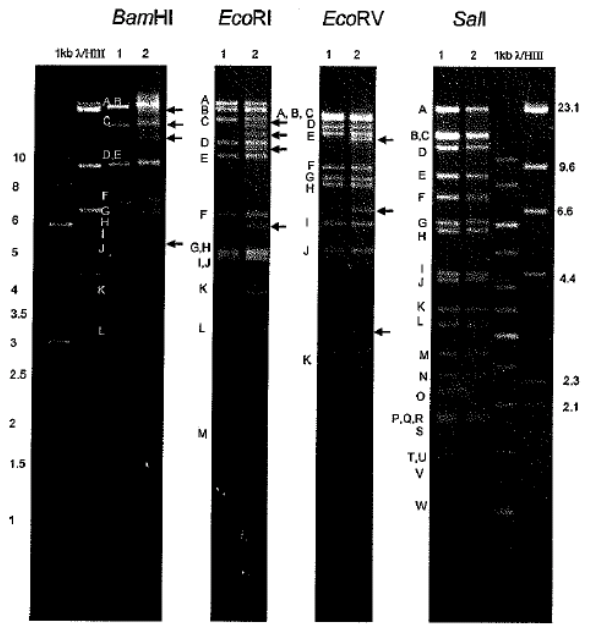


Figure B.1.3-1: Electrophoresis through 0.8% agarose gel of purified DNA from 1: Reference Item (Neustadt Mexican Isolate), 2: Test Item (V15). Restriction fragments are lettered on sequential order of their size. Size markers are given to the left and the right (kbp).

<u>*Bam*HI digest:</u> All *Bam*HI restriction fragments A to L of the reference Item CpGV-M (NW) could be identified at the expected position. In the Test Item (V15), the bands C (15.2 kb) and J (5.2 kb) were present as submolar bands. Beyond that, one additional submolar band of about 20 kb was present in Test Item V15. As the fragments C an J are adjacent in the CpGV restriction map (Crook et al., 1997), it is suggested that there is a genotype present in the Test Item (V1) carrying this additional band of about 20 kb due to a fusion of fragments C and J. This submolar fragment C migrated at a slightly higher position in the Test Item (V15) as in the reference item, as it was described for CpGV isolate E2 (Eberle et al., 2009). Another submolar band of about 13 kb was present in the REN profile (arrow). All other bands corresponded to the reference Item CpGV-M (NW).

<u>*Eco*RI digest:</u> Compared to the reference Item CpGV-M (NW), the Test Item V15 showed four additional bands (arrows) of lower intensity. Two fragments of about 11.5 kb and 5.8 kb (arrows) were present in Test item V15 as submolar bands. Fragment C was fainter than expected. It is most likely that these two fragments were generated by partial *Eco*RI restriction of fragment C due to a restriction site that is not present in the reference item CpGV-M (NW). Additionally, Test Item V15 showed two submolar bands of about 15.1 kb and 13.1 kb, which did not correspond to the profile of the reference item CpGV-M (NW). They correspond to bands described for the CpGV isolate E2 (Eberle et al., 2009) where they were due to an additional restriction site in fragment A. As the intensity of fragment A was also lower than expected, it is likely that these bands are due to a partial restriction of this fragment into two fragments A1 and A2 in one of the genotypes present in Test Item V15.

<u>EcoRV digest:</u> As shown in Figure 1.3-01, Reference item CpGV-M (NW) and Test Item V15 shared most of the *Eco*RV restriction fragments. In Test Item V15 two additional bands of 13 and 6.5 kb could be observed (arrows). These bands might be derived from an additional *Eco*RV site in one of the

fragments A, B or C. Since these bands are similarly large it is not clear from the picture which fragment is cut into two additional fragments. A further submolar band of about 3.5 kb was present in Test Item V15 which could not be attributed to one of the other fragments, as they were similar in size to reference Item CpGV-M (NW).

*Sal*I digest: All *Sal*I restriction fragments A to W could be identified at the expected position for the Reference Item CpGV-M (NW). Test Items V15 fragment J was slightly larger as in the Reference Item. However, it can be excluded that this increase in size is the result of a major genome insertion, since this would be observed in the other restriction profiles, too. One submolar band could be observed at about 2.1 kb.

Conclusion
On the basis of DNA restriction analysis using *Sal*I, *Eco*RV, *Eco*RI and *Bam*HI, it can be concluded that Test Item V15 is a CpGV isolate. There are at least two different genome types present: One shows similarity to the isolated CpGV-E2 (Eberle et al., 2009), the other differs from CpGV-M and -E2. These genome types are present in the mixture at a similar level.

Conclusion by RMS
The study is considered acceptable.

CpGV-V22 (GV-0014)

Reference:
Jehle, Eberle (2009), Comparative Restriction and Phylogenetic Analysis of V22, Andermatt Biocontrol GmbH (BVL no 3306434)

Summary
For the identification of baculovirus isolates DNA endonuclease restriction (REN) analysis is usually used. By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. In this study, DNA of V22 (Test item) was isolated and purified and subjected to endonuclease restriction analysis using the endonucleases *Sal*I, *Bam*HI, *Eco*RI and *Eco*RV. The restriction fragments were separated in an agarose gel and the obtained restriction profiles were compared to the restriction profiles of CpGV-M (Mexican isolate, propagated in Neustadt) and to published profiles of CpGV-M. It was found that the test item (V22) was a CpGV isolate with a predominant A type genome profile. Faint submolar bands could be observed in the REN profile obtained with *Eco*RI and *Eco*RV, suggesting there is another genome type present in Test Item V22 at low level. Phylogenetic analysis of baculoviruses can be based on the partial gene sequences of *late expression factor 8* (*lef-8*) and *polyhedrin/granulin* (*polh/gran*). Partial amplification of the *lef-8* and *polh/gran* followed by sequencing revealed no single nucleotide polymorphisms (SNPs) between V22 and CpGV-M. Phylogenetic analysis based on these partial sequences grouped V22 to GpGV-M. The main genome present in Test Item V22 can be attributed to A type genomes.

Material and Methods
Test Item V22 was purified by centrifugation in a 30-80% glycerol layer gradient. The purified CpGV OB pellet was resuspended in 2 mL sterile water. DNA was isolated from the purified CpGV pellet by phenol/chloroform/isoamylalcohol method.

The viral DNA of the Test Item (V22) and of the reference item (CpGV, Neustadt Mexican isolate) was digested with the restriction enzymes BamHI, EcoRI, SalI and EcoRV. These enzymes were chosen because they allow an easy differentiation of the previously described CpGV isolates. 17µL isolated DNA were incubated in 2µL buffer and 1 µL enzyme at 37°C for 3h. Digested DNA was electrophoresed in a 0.8% agarose gel over night (25 V) using TAE as buffer system.

The partial sequences of *late expression factor* (*lef-8*) and *granulin* (*polh/gran*) genes were amplified using the degenerate primer method described by Lange et al. (2004) and Jehle et al. (2006). PCR products used for direct sequencing were purified using the GFX PCR DNA and Gel Band Purifica-

tion Kit (Amersham, Freiburg, Germany), and both DNA strands were sequenced using M13 universal, M13 reverse and T7 standard primers (MWG, Germany). The sequences were aligned using BioEdit with the corresponding sequences of further CpGV isolates determined previousely and described in Eberle et al. (2009).

Partial *polh/gran* and *lef-8* sequences determined for Test Item V22 were concentrated and aligned with the corresponding sequences of *Cryptophlebia leucotreta* Granulovirus (*Crle*GV) (Lange & Jehle, 2003) as an outgroup using Clustal W (Thompson et al., 1994) implemented in Bioedit 7.0.5.3 (Hall, 1999). A phylogenetic analysis using Minimum Evolution algorithms was performed using MEGA 4.1 (Kumar et al., 2004).

Findings
REN Analysis:

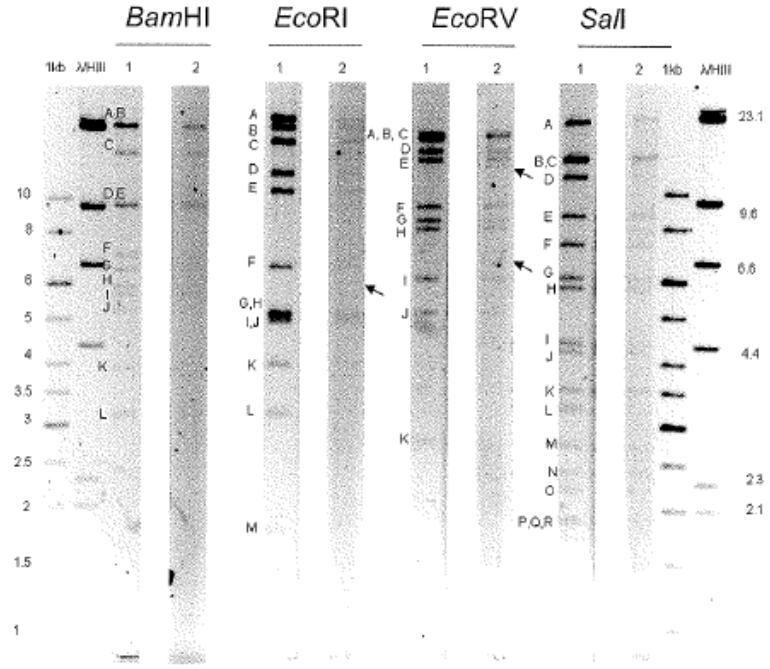


Figure B.1.3-2: Electrophoresis through a 0.8% agarose gel of purified DNA from 1: Reference Item (Neustadt Mexican Isolate), 2 Test Item (V22), Restriction fragments are lettered in sequential order of their size. Size markers are given to the left and to the right.

*Bam*HI digest: All *Bam*HI restriction fragments A to L of the reference Item CpGV-M (NW) could be identified at the expected position. DNA concentration of Test Item V22 was low. No additional or missing bands could be observed for the *Bam*HI digest, the V22 REN profile corresponded to CpGV-M (NW).

*Eco*RI digest: All restriction fragments present in the reference Item CpGV-M (NW) were present in the Test Item V22 at the expected position, no insertions or deletions were observed. One faint additional band could be observed at about 5.8 kb.

*Eco*RV digest: As shown in Figure 1.3-02, reference item CpGV-M (NW) and Test Item V22 shared most of the *Eco*RV restriction fragments. In Test Item V22 two additional bands of 13 and 6,5 kb could be observed (arrows). These bands might be derived from an additional *Eco*RV site in one of the fragments A, B, or C. Since these bands are similarly large it is not clear from the picture which fragment is cut into two additional fragments. However, it can be excluded that these bands are the result of a major genome insertion, since this would be observed in the other restriction profiles too.

*Sal*I digest: The *Sal*I restriction fragments A to R could be identified at the expected position for the Reference Item cpGV-M (NW) and for the Test Item V22. No additional, missing or submolar bands could be observed.

SNP analysis:

Sequence chromatograms obtained for the partial *lef-8* and *polh/gran* genes showed clear nucleotide peaks and no sequence heterogenesis. The sequences corresponded in all positions to CpGV-M; Test Item V22 showed no SNPs.

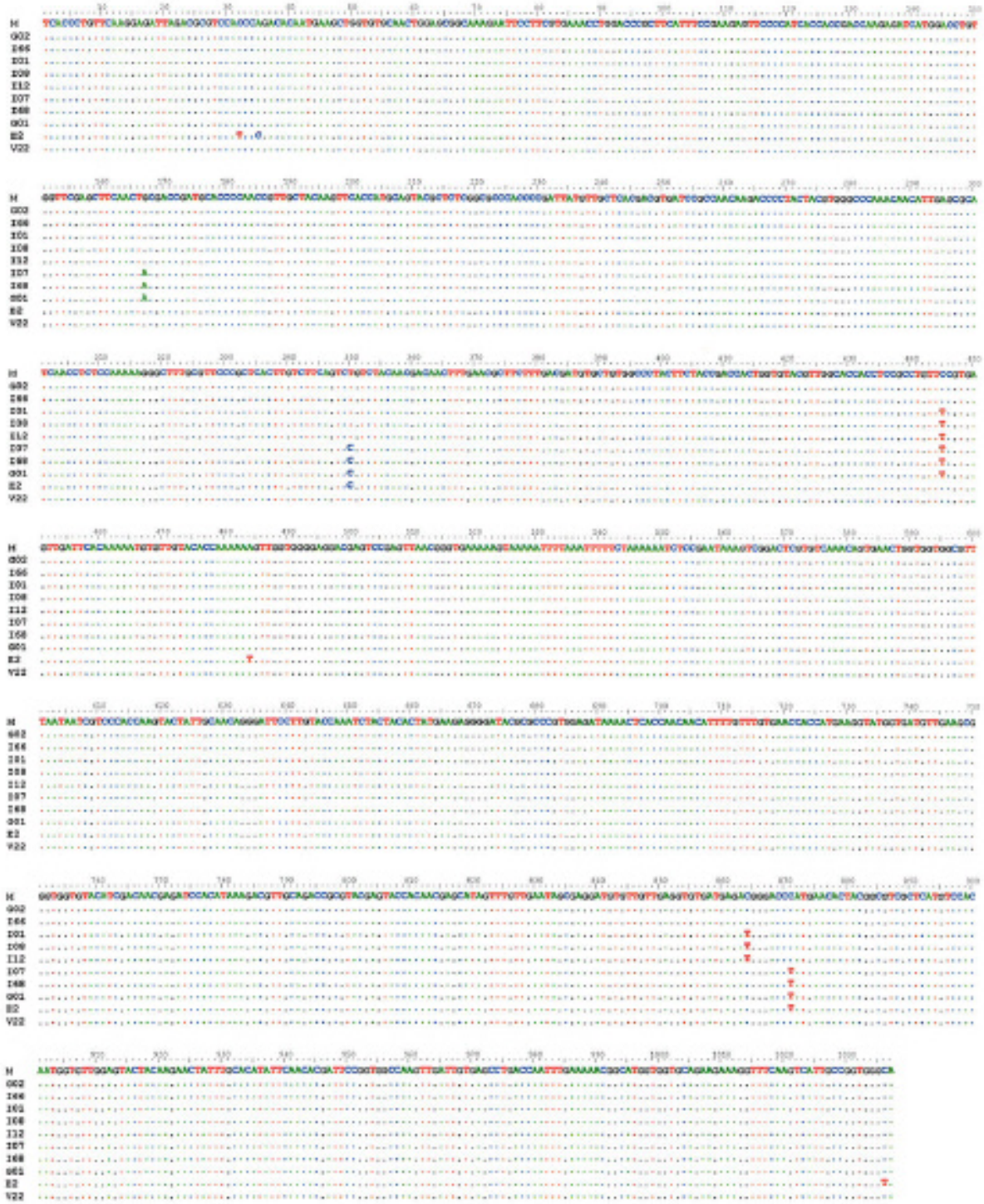


Figure B.1.3-3: Alignment of partial *polh/gran* sequences of Test Item V22, Reference Item CpGV-M and CpGV isolates described in Eberle et al. (2009).

Based on the alignment, a phylogenetic analysis was performed including the isolates described in Eberle et al. (2009) and using the corresponding sequences of CrleGV as an outgroup (Figure B.1.3-4).

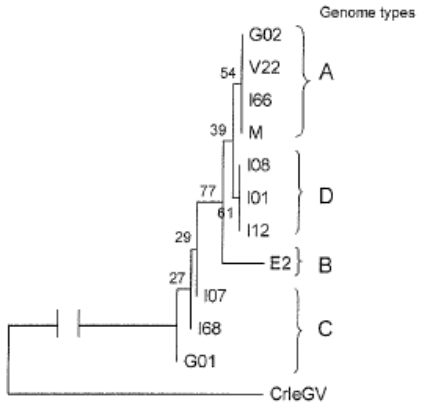


Figure B.1.3-4: **Minimum Evolution (ME) tree of Reference Item CpGV-M, Test Item V22 and nine further CpGV isolates. The analysis is based on 1037 nt derived from partial sequencing of the *polh/gran* and *lef-8* sequences (Jehle et al., 2006) using CrleGV as an outgroup. Numbers at the notes indicate bootstrap values of 500 bootstrap replicates. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analysis was conducted in MEGA4 (Tamura et al., 2007).**

Conclusion
REN analysis: On the basis of DNA restriction analysis using *Sal*I, *EcoR*I, *EcoR*V and *Bam*HI, it can be concluded that Test Item V22 is a CpGV isolate. The predominant genotype corresponds to CpGV-M (NW) and is therefore an A type genome isolate. However, there were some submolar bands observed in the *Eco*RI and *Eco*RV profiles, indicating that there is a second genome type present at aloe level.

Phylogenetic analysis: On the basis of the concatenated *polh/gran* and *lef-8* sequences, Test Item V22 did not differ in its predominant genome type from CpGV-M. V22 was found to contain a predominant A type genome.

Conclusion by RMS
The study is considered acceptable.

CpGV-V03 (GV-0006, Madex Max)

For evaluation of submitted study, see Vol. 4 Andermatt V03.

CpGV-V01 (GV-0003, Madex Plus)

Reference:
Jehle (2006), Comparative Restriction Analysis of CpGV (Neustadt Mexican isolate) with CpGV (Madex Plus) , Andermatt Biocontrol GmbH (BVL no 3306435)

Summary
For the identification of baculovirus isolates DNA endonuclease restriction (REN) analysis is usually used. By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. In this study, viral DNAs of CpGV (Mexican strain, (M-type), Neustadt) (Reference Item) and CpGV (Madex Plus) (Test Item) were isolated and purified and subjected to endonuclease restriction analysis using the endonucleases *Sal*I, *Bam*HI, *Eco*RI and *Eco*RV. The restriction fragments were separated in an agarose gel and the ob-

tained restriction profiles were compared to each other and to published profiles of CpGV-M. It was found that the restriction profiles of CpGV (MadexPlus) differed from CpGV (Mexican strain, (M-type), Neustadt) in two out of four restriction digests. The additional restriction fragments observed for CpGV (Madex Plus) correspond to restriction patterns suggest that Madex Plus contains variants of CpGV which resemble the E and R type.

Materials and Methods
Test Item (CpGV Madex Plus) was purified by centrifugation in a 60/70/80% glycerol layer gradient. The purified CpGV OB pellet was resuspended in 1 mL sterile water. DNA was isolated from the purified CpGV pellet by phenol/chloroform/isoamylalcohol method.

The viral DNA of the Test Item (CpGV Madex Plus) and of the reference item (CpGV, Neustadt Mexican isolate) was digested with the restriction enzymes BamHI, EcoRI, SalI and EcoRV. These enzymes were chosen because they allow an easy differentiation of the previously described CpGV isolates. About 650 ng DNA of isolated DNA were incubated in 2µL buffer and 2 µL enzyme and 6 µL water (bidest.) at 37°C for 3h. Digested DNA was electrophoresed in a 0.8% agarose gel over night (30 V) using TAE as buffer system.

Findings
REN Analysis:

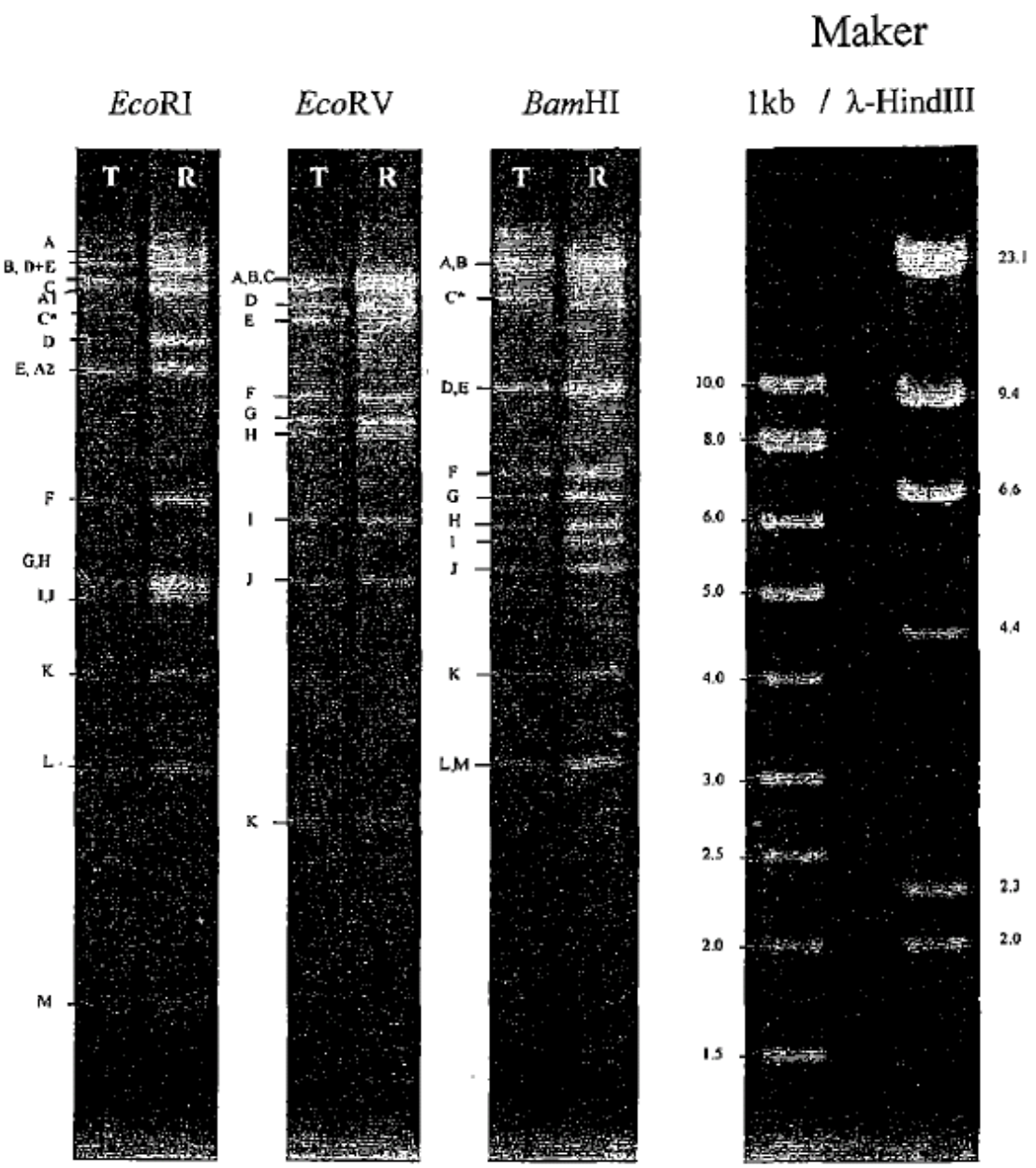


Figure B.1.3-5: Electrophoresis through a 0.8% agarose gel of purified DNA from Reference Item CpGV (Neustadt Mexican Isolate) (R), and Test Item CpGV (Madex Plus) (T), Restriction fragments are lettered in sequential order of their size. Size markers are given to the right.

<u>*Eco*RI digest:</u> The *Eco*RI digest of the Test Item (CpGV Madex Plus) showed a number of submolar bands as it typical for a mixture of genotypes. It is expected for a homogenous genotype that the intensity of restriction fragments is proportional to the size of the fragments, since each band consists of equimolar amounts of DNA fragments. In mixtures of genotypes the fragment intensity is not proportional to the size of the fragment. This can be seen, for example for fragments A and C*, which are much fainter than the following smaller fragments. Those bands, which differ from the reference Item (Neustadt Mexican Isolate) are indicated in red letters adjacent to the restriction lane (Fig. 1.3-05). Based on the known restriction patterns of other isolates (E type, R type) it is most likely that the test item (CpGV Madex Plus) contains genotypes corresponding to the E type and the R type.

*Eco*RV digest: All *Eco*RV restriction fragments A to L could be identified at the expected position. Fragments M and N were too small to be identified. No submolar bandy could be observed.

*Bam*HI digest: All *Bam*HI restriction fragments A to N of the Reference Item (CpGV Neustadt , Mexican isolate) could be identified at the expected position. For the Test Item (CpGV Madex Plus), all *Bam*HI restriction fragments A-B and D-N could be identified at the expected position. Only fragment C* is about 750 bp larger than the corresponding fragment C. This corroborates the finding that the test Item (CpGV Madex Plus) contains the E.type (CpGV-E).

*Sal*I digest (not shown): All *Sal*I restriction fragments A to W could be identified at the expected position. No submolar bands could be observed.

Conclusion
On the basis of DNA restriction analysis using *Sal*I, *Eco*RI, *Eco*RV and *Bam*HI, it can be concluded that the CpGV Madex Plus differs slightly from the reference Item CpGV (Mexican isolate, Neustadt). Two out of four analysed restriction profiles were identical. Two profiles (*Eco*RI and *Bam*HI) showed small variations, which indicate that the previously described genotypes of the E and R type of CpGV (Crook et al., 1985, Crook et al., 1997, Jehle, unpublished) are present in predominant concentration in CpGV (Madex Plus).

Conclusion by RMS
The study is considered acceptable.

B.1.3.3.2 New isolates owned by Andermatt Biocontrol AG

The isolates CpGV-V14 and –V45 are new and have not been evaluated before.

CpGV-V14 (GV-0015)

Reference:
Jehle, Eberle (2010), Comparative restriction analysis of C15, Andermatt Biocontrol AG (BVL no 3714752)

For the identification of baculovirus isolates DNA endonuclease restriction (REN) analysis is usually used (OECD, 2002). By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. On the other hand, a baculovirus isolate has to be considered always as a mixture of different genotypes, which can slightly differ from each other by numerous small insertion and deletion mutations at different locations in the genome. Depending on the variations of genotypes some isolates appear to be highly homogenous, others are more heterogeneous. Discrimination between different isolates is thus always based on the differences in restriction patterns. However, this discrimination has to be considered as a continuous change in genotype composition, since they often reflect a quantitative prevalence of some genotypes compared to others rather than a qualitative difference.

Materials and Methods
The viral DNA of the Test Item (C15) and of the Reference Item (CpGV, Neustadt Mexican isolate) was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Sal*I and *Eco*RV. For the Test Item C15 and the Reference Item (CpGV, Neustadt Mexican isolate) 17 µl of the isolated DNA were incubated with 2 µl buffer, 1 µl enzyme at 37°C for 3 h. One tenth of each sample was used to proof if the digestion was successful as well as to compare the DNA concentrations. Starting from this information 6 to 14 µl of the digestions of the Test Item (C15) were used for the gel electrophoresis. All digested DNAs were electrophoresed in a 0.8% agarose gel over night (25 V) using TAE as a buffer system.

The viral DNA isolated from purified occlusion bodies from the Test Item (C15) and Reference Item (CpGV, Neustadt Mexican isolate was subjected to DNA restriction analysis using the restriction enzymes SalI, EcoRI, EcoRV and BamHI. These enzymes have the following recognition sequences:

Enzyme	Recognition Sequence
BamHI	G'GATC_C
EcoRI	G'AATT_C
EcoRV	GAT'ATC
SalI	G'TCGA_C

These enzymes were chosen, since they allow an easy differentiation of the previously described CpGV isolates.

Findings
REN-Analysis

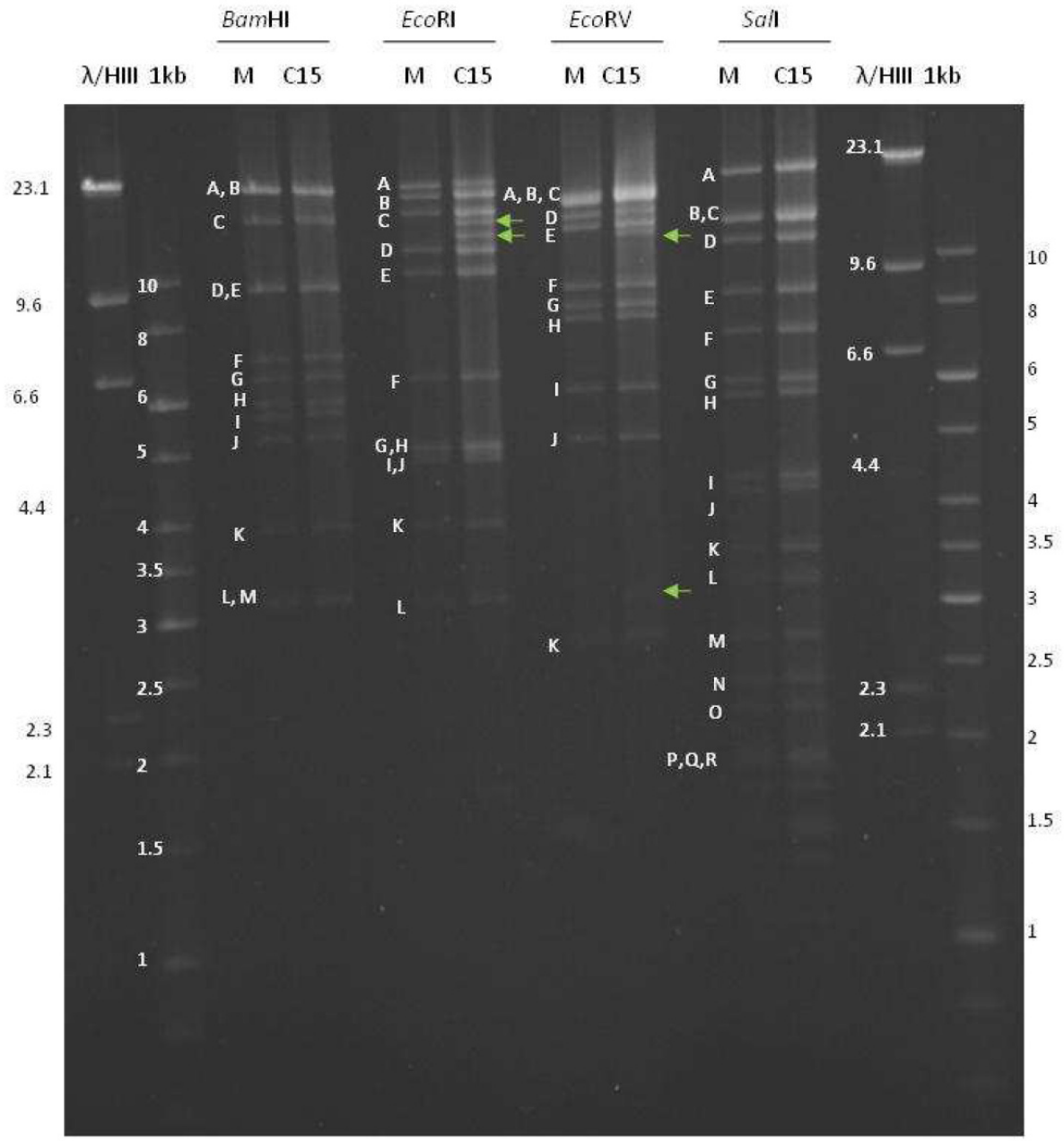


Figure B.1.3-6: **Electrophoresis through a 0.8% agarose gel of purified DNA from M: Neustadt Mexican Isolate, C15: Test Item (C15). Restriction fragments are lettered in sequential order of their size. Size markers are given to the left (kbp). λ/HIII = λ/HindIII DNA adder, 1 kb = 1 kbp DNA ladder.**

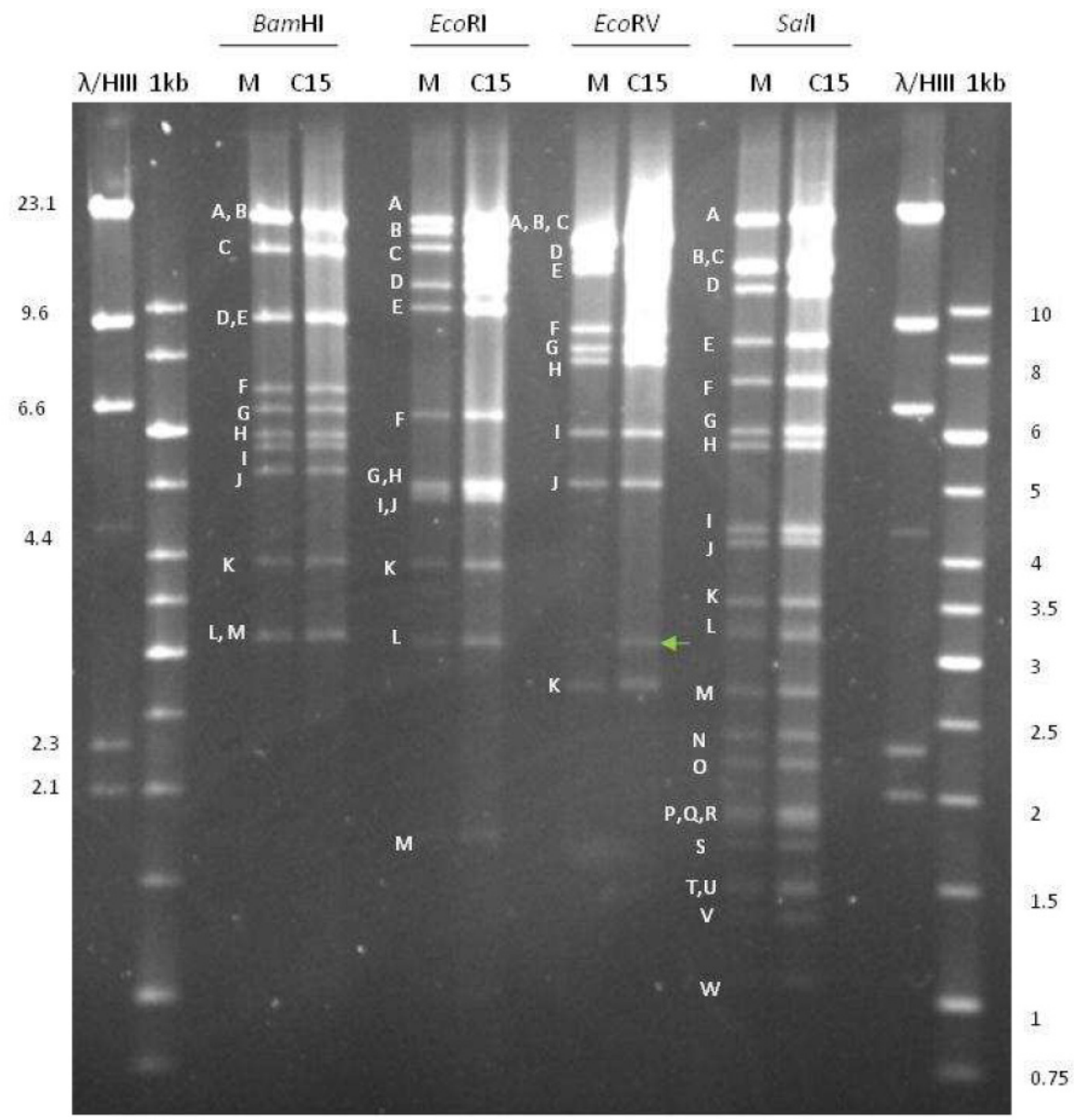


Figure B.1.3-7: Electrophoresis (modified exposure time) through a 0.8% agarose gel of purified DNA from M: Neustadt Mexican Isolate, C15: Test Item (C15). Restriction fragments are lettered in sequential order of their size. Size markers are given to the left (kbp). λ/HIII = λ/HindIII DNA adder, 1 kb = 1

*Bam*HI digest: All *Bam*HI restriction fragments A to M of the Reference Item CpGV-M (JKI) could be identified at the expected position. Test Item C15 corresponded in its DNA restriction profile to CpGV-M (JKI) and did not show any differences.

*Eco*RI digest: Compared to Reference Item CpGV-M (JKI), Test Item C15 showed two additional bands(green arrows) of lower intensity than the following bands, which indicates that they are not additional but submolar bands deriving from a second genotype. The two submolar bands were visible at about 15.1 kb and 13.1 kb and did not correspond to the profile of the Reference Item CpGV-M (JKI). They corresponded to bands described for the CpGV isolate E2 (Eberle et al., 2009) where they were due to an additional restriction site in fragment A, accompanied by an insertion of about 0.7 kb in this area.

*Eco*RV digest: As shown in the figures above, Reference Item CpGV-M (JKI) and Test Item C15

shared most of the *Eco*RV restriction fragments. In the Test Item C15 two additional bands of about 13 and 3.0 kb could be observed (green arrows). These two bands correspond to two bands visible in the profile of CpGV-E2, where they are attended by a third submolar band.
Fragment C15-D was fainter than expected. The two additional bands in C15 could be two parts of a submolar fragment D, which is cut in one genotype and present as one fragment in the other, CpGV-M like, genotype. As fragment D has a total size of 15 kb, the sum of the two additional fragments would be slightly larger (about 16 kb). If there was an insertion in this genotype resulting in an additional restriction site, this would be also visible in the *Eco*RI profile, where this region corresponds to fragment *Eco*RI-A. In *Eco*RI-A were also two genotypes visible, one of them corresponding to CpGV-E2 harbouring an insertion.
Therefore, the two additional C15/*Eco*RV bands derive most likely from a submolar genotype similar to CpGV-E2, where *Eco*RV-D is present as two fragments; the faint *Eco*RV-D derived from the genotype similar to CpGV-M.

*Sal*I digest: All *Sal*I restriction fragments A to W (Figures above) could be identified at the expected position for the Reference Item CpGV-M (JKI). Test Items C15 did not show any differences to the Reference Item`s profile.

Conclusion
On the basis of DNA restriction analysis using *Sal*I, *Eco*RV, *Eco*RI and *Bam*HI, it can be concluded that Test Item C15 is a CpGV isolate. There are at least two different genome types present: The main genotype corresponds to CpGV-M. A second genotype shows similarity to the isolated CpGV-E2 (Eberle et al., 2009) in the *Eco*RI and *Eco*RV profile. The genome types are present in the mixture at a similar level.

Conclusion by RMS
The study is considered acceptable.

CpGV-V45 (GV-0017)

Reference:
Brader (2018), Report AIT_KS_V45: Restriction enzyme analysis of CpGV-V45 and in silico comparison with sequenced CpGV isolates, Andermatt Biocontrol AG . (BVL no 3714804)

Summary
Isolation of DNA from a preparation of CpGV-V45 was perfomed to digest 880ng of *Cydia pomonella* granulovirus CpGV-V45 DNA with the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Pst*I and *Xho*I. The pattern was compared with virtual gels from 11 additional granuloviruses infecting Tortricidae and 9 granuloviruses infecting other Lepidoptera. Based on the restriction patterns CpGV-V45 can be clearly identified as CpGV strain, but the exact pattern is different to the previously published genome types A-E of CpGV.

Material and methods
DNA isolation
DNA was isolated from a virus preparation of CpGV-V45 provided by the sponsor Andermatt Biocontrol. 2 x 36 mL of the liquid virus preparation in 50 mL tubes were combined with 2 x 4 mL 1M Na2CO3 to a final concentration of 100 mM Na_2CO3 and incubated in a water bath for 1 h at 60 °C. The solution was adjusted to pH 8 with 1 M HCl, treated 10 min at 37 °C with RNaseA (90 µg/mL final concentration) and then 1 h with Proteinase K (250 µg/mL final concentration) and 1% SDS at 50 °C. DNA was separated by washing the solution with the same volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v), followed by chloroform washing and separated each time by 15min centrifugation at 4700rpm. Supernatants were combined with 10% volume of 2 M NaCl and 2.5-foldvolume of ethanol. DNA was precipitated overnight at -20 °C and washed with 70% ethanol. The phenol/chloroform/isoamylalcohol and precipitation steps were repeated and the DNA preparation was finally cleaned with the PowerClean DNA Clean-Up kit (Mo Bio, Qiagen, USA) ac-

cording to the protocol of the manufacturer.

DNA restriction and in silico *restrictions*

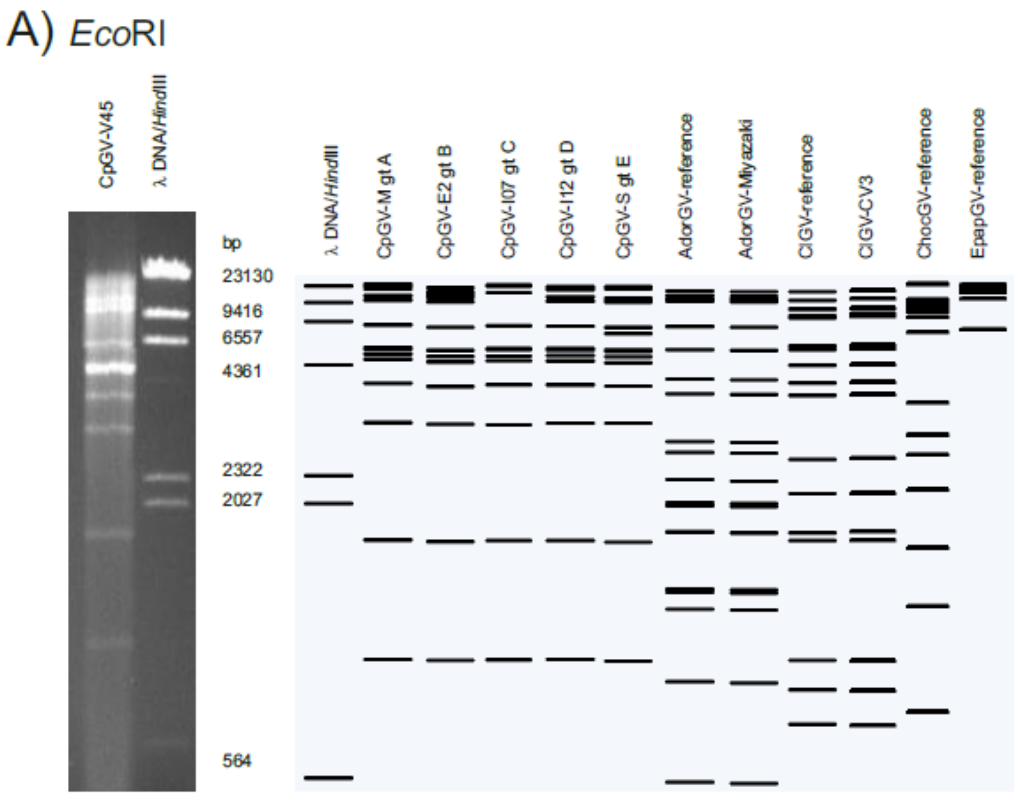
10 µL DNA (68 ng/µL) were loaded on a 0.8% Agarose gel in Tris-borate-EDTA (TBE) buffer to yield a high molecular band. Each 880 ng DNA were digested for 30 min at 37 °C with 2 µL Fast Digest *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Pst*I *and Xho*I (Thermo Fisher Scientific, USA) with FastDigest green buffer in a total volume of 20 µL and run on a 0.8% agarose gel in TBE for 1 h at 80 V and 1 h at 100 V together with λ/*Hind*III as marker ladder. DNA bands were stained with ethidium bromide solution (final concentration 0,5 µg/mL gel).

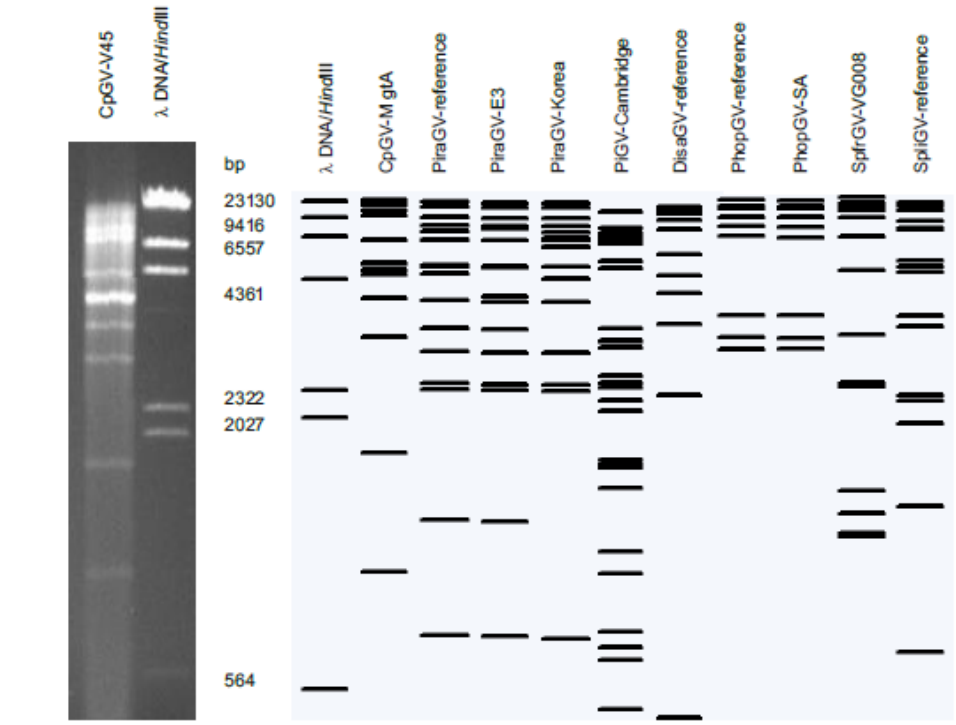
Genome sequences of the granulovirus isolates (see Table B.1.3-1) were downloaded from NCBI GenBank. Virtual gels with 0.8% agarose settings and λ/*Hind*III as marker ladder was performed with pDraw32 1.0 revision 1.1.134 (AcaClone, www.acaclone.com).

Table B.1.3-1: NCBI accession numbers and abbreviation of granulovirus isolates

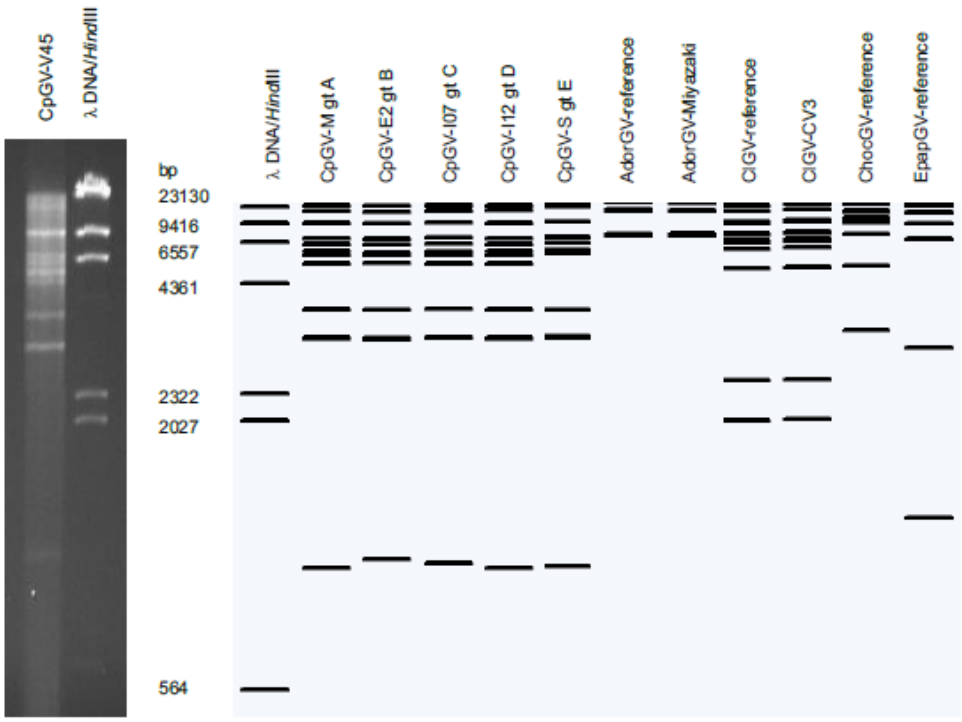
NCBI accession	*Granulovirus*	*Isolate abbreviation*	*Genome type*
KM217575	*Cydia pomonella* granulovirus	CpGV-M	Genome type A
KM217577	*Cydia pomonella* granulovirus	CpGV-E2	Genome type B
KM217574	*Cydia pomonella* granulovirus	CpGV-I07	Genome type C
KM217576	*Cydia pomonella* granulovirus	CpGV-I12	Genome type D
KM217573	*Cydia pomonella* granulovirus	CpGV-S	Genome type E
NC_005038	*Adoxophyes orana* granulovirus	AdorGV-genome reference	
KM226332	*Adoxophyes orana* granulovirus	AdorGV-Miyazaki	
NC_005068	*Cryptophlebia leucotreta* granulovirus	ClGV-genome reference	
AY229987	*Cryptophlebia leucotreta* granulovirus	ClGV-CV3	
NC_00816	*Choristoneura occidentalis* granulovirus	ChocGV-genome reference	
NC_018875	*Epinotia aporema* granulovirus	EpapGV-genome reference	
NC_013797	*Pieris rapae* granulovirus	PiraGV -genome reference	
GU111736	*Pieris rapae* granulovirus	PiraGV-E3	
JX968491	*Pieris rapae* granulovirus	PiraGV-Korea	
NC_032255	*Plodia interpunctella* granulovirus	PiGV-Cambridge	
NC_028491	*Diatraea saccharalis* granulovirus	DisaGV-genome reference	
NC_004062	*Phthorimaea operculella* granulovirus	PhopGV-genome reference	
KU666536	*Phthorimaea operculella* granulovirus	PhopGV-SA	
NC_026511	*Spodoptera frugiperda* granulovirus	SpfrGV-VG008	
NC_009503	*Spodoptera litura* granulovirus	SpliGV-genome reference	

Findings

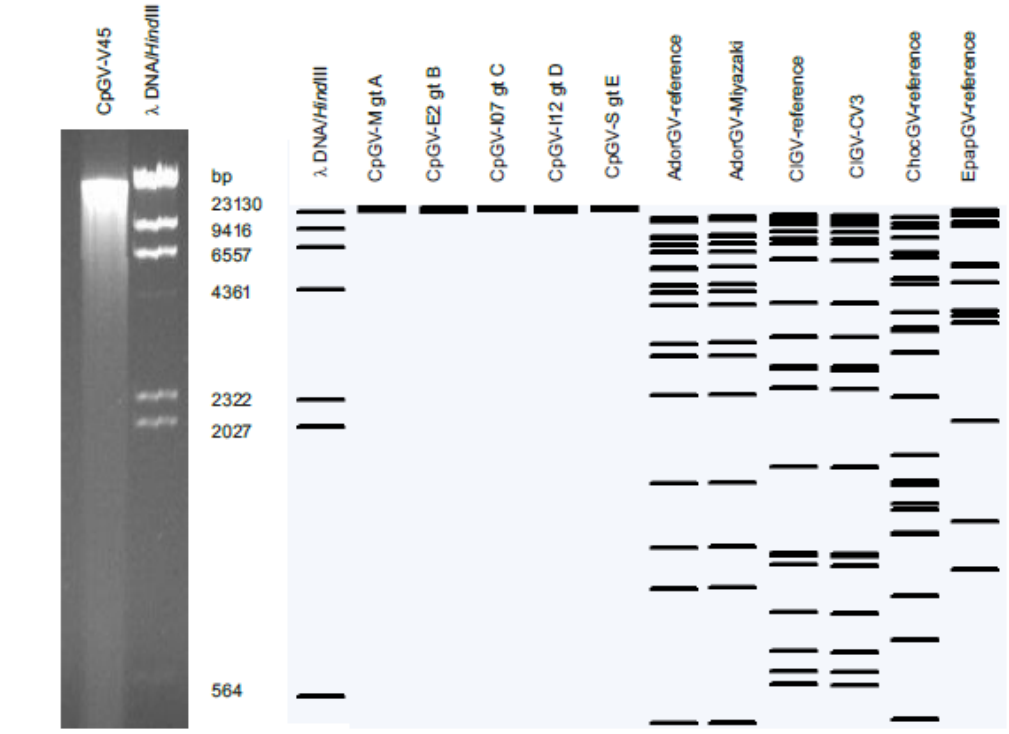

A) EcoRI
CpGV-V45
λ DNA/HindIII
bp
23130
9416
6557
4361
2322
2027
564
λ DNA/HindIII
CpGV-M gt A
CpGV-E2 gt B
CpGV-I07 gt C
CpGV-I12 gt D
CpGV-S gt E
AdorGV-reference
AdorGV-Miyazaki
ClGV-reference
ClGV-CV3
ChocGV-reference
EpapGV-reference


CpGV-V45
λ DNA/HindIII
bp
23130
9416
6557
4361
2322
2027
564
λ DNA/HindIII
CpGV-M gtA
PiraGV-reference
PiraGV-E3
PiraGV-Korea
PiGV-Cambridge
DisaGV-reference
PhopGV-reference
PhopGV-SA
SpfrGV-VG008
SpliGV-reference

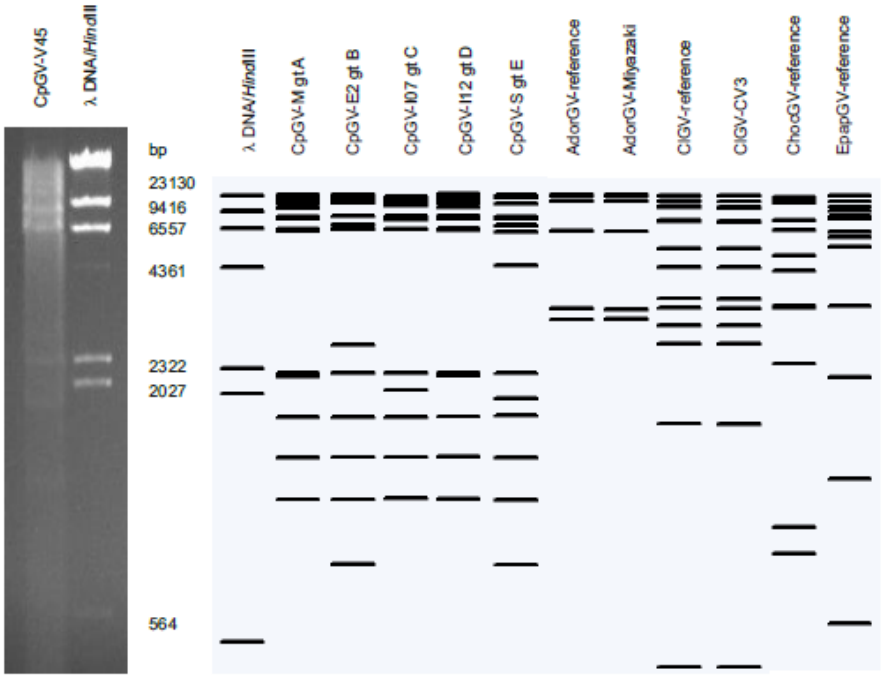
B) *Bam*HI



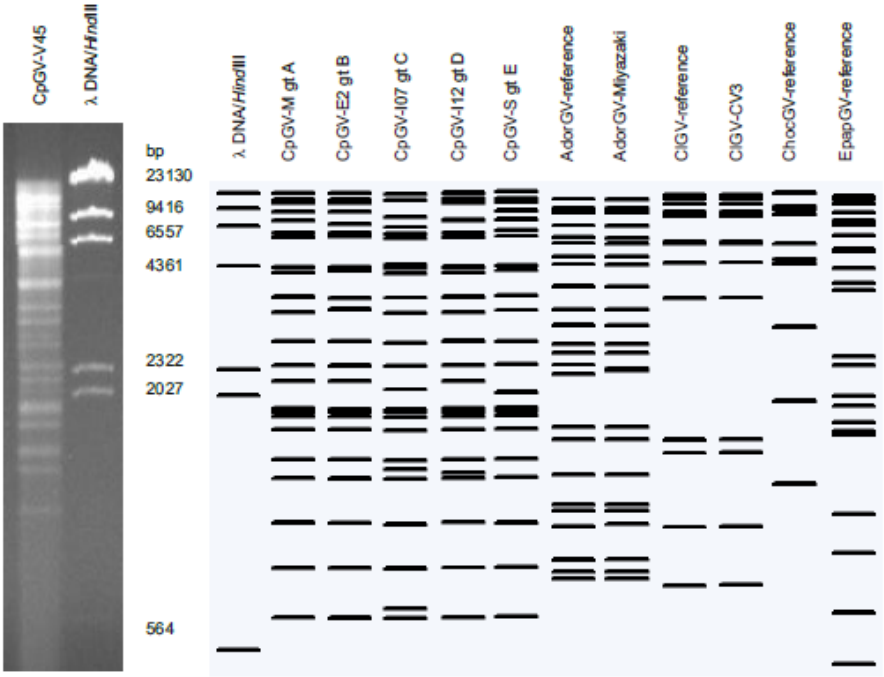
C) *Hind*III



D) *PstI*


CpGV-V45
λ DNA/HindIII
bp
23130
9416
6557
4361
2322
2027
564
λ DNA/HindIII
CpGV-M gt A
CpGV-E2 gt B
CpGV-I07 gt C
CpGV-I12 gt D
CpGV-S gt E
AdorGV-reference
AdorGV-Miyazaki
ClGV-reference
ClGV-CV3
ChocGV-reference
EpapGV-reference

E) *SalI*


CpGV-V45
λ DNA/HindIII
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4361
2322
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564
λ DNA/HindIII
CpGV-M gt A
CpGV-E2 gt B
CpGV-I07 gt C
CpGV-I12 gt D
CpGV-S gt E
AdorGV-reference
AdorGV-Miyazaki
ClGV-reference
ClGV-CV3
ChocGV-reference
EpapGV-reference

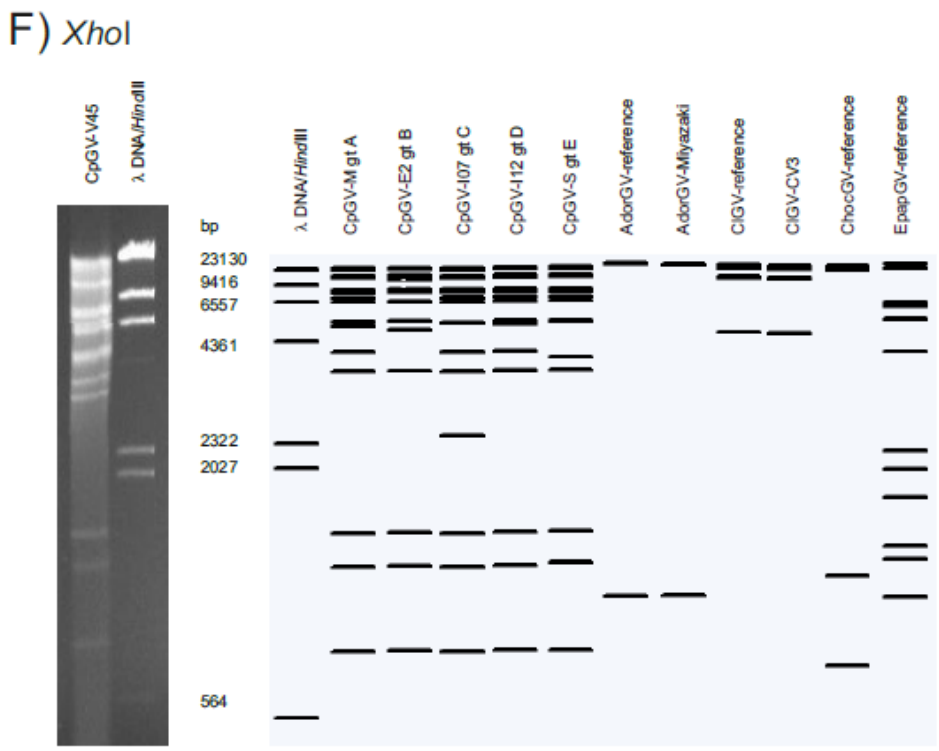

Figure B.1.3-8: **Restriction analysis of CpGV-V45 with *Eco*RI (A), *Bam*HI (B), *Hind*III (C), *Pst*I (D), *Sal*I (E) and *Xho*I (F) and comparision with virtual digestion with published genomes. Accession numbers are indicated. CpGV: *Cydia pomonella* granulovirus; AdorGV: *Adoxophyes orana* granulovirus; ClGV: *Cryptophlebia leucotreta* granulovirus; ChocGV: *Choristoneura occidentalis* granulovirus; EpapGV: *Epinotia aporema* granulovirus; PiraGV: Pieris rapae granulovirus; PiGV: *Plodia interpunctella* granulovirus; DisaGV: *Diatraea saccharalis* granulovirus; PhopGV: *Phthorimaea operculella* granulovirus; SpfrGV: *Spodoptera frugiperda* granulovirus; SpliGV *Spodoptera litura* granulovirus. CpGV genome types gt are indicated.**

Comparing the restriction patterns of the digestion of CpGV-V45 with the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Pst*I *and Xho*I to the *in silico* deduced restriction patters of the *Cydia pomonella* granulovirus reference genome sequence and to those of the remaining fully sequenced CpGV isolates CpGV-M (Mexican isolate, genome type A), CpGV-E2 (genome type B), CpGV-I07 (genome type C), CpGV-I12 (genome-type D) and CpGV-S (genome type E), CpGV-V45 was identified as a novel *Cydia pomonella* granulovirus isolate.

The *in silico* deduced restrictions obtained from the remaining granuloviruses infecting Tortricidae which were represented by complete genome sequences of two isolates of AdorGV, two isolates of ClGV, one isolate each of ChocGV and of EpapGV show clearly different restriction patterns to that of CpGVV45 (Fig. 1.3-08). Analogous, genome sequences of granuloviruses infecting other Lepidoptera which were represented by three isolates of PiraGV, two isolates of PhopGV and one isolate of PiGV, DisaGV, SpfrGV and SpliGV concluded in *in silico* deduced restriction patterns different to that of CpGV-V45.

These differences were in particular obtained when digesting with *Eco*RI (Fig. A). For means of redundancy, *in silico* digestions of PiraGV, PiGV, DisaGV, SpfrGV and SpliGV were not further displayed for *Bam*HI, *Hind*III, *Pst*I, *Sal*I and *Xho*I in Figure 1.3-08, as the differences to CpGV were as clearly distinguishable as those observed in the digestion with *Eco*RI.

*Eco*RI digest: In the restriction patterns obtained from the *in silico* deduced digestions with *Eco*RI, two specific fragments were generated for genome type E (CpGV-S) sized below 6.6 kb according to the λ/*Hind*III marker. These two fragments are missing in the remaining genome types A to D and were also not assessed in the restriction of CpGV-V45. Furthermore, genome type C (CpGV-I07) lacks two fragments with sizes larger than 10 kb that were detected in all other genome types and in CpGV-V45 (Fig A).
In conclusion, the CpGV-V45 restriction pattern with *Eco*RI is very similar to those of the genome types A, B and D.

*Bam*HI digest: In the *in silico Bam*HI restrictions of the CpGV genomes identical fragments were detected for all genome types except for genome type E (CpGV-S). In this genome type the two fragments at 5 and 15 kb (present in genome types A to D) are fused into one band at about 20 kb. The other genome types are very similar and the pattern of CpGV-V45 is similar to those of the genome types A, B, C and D and can be discriminated from genome type E by the two bands at 15kb and 5kb (Fig B).

*Hind*III digest: No discrimination between the CpGV genome types can be made by restrictions with *Hind*III, as all genome types and CpGV-V45 are similar and concluded in only very large fragments at 26 kb, 40 kb and 58 kb, which cannot be resolved in a 0.8% TBE agarose gel (Fig C).

Both *Bam*HI and *Hind*III restrictions resulted in clearly different restriction patterns compared to those of other granuloviruses infecting Tortricidae (Fig B and C).

*Pst*I digest: All CpGV genome types as well as CpGV-V45 could be effectively distinguished from other granuloviruses infecting Tortricidae by their restriction patterns in digestions *Pst*I and *Sal*I. (Fig D and E).
Compared to the genome types A and D one additional fragment was generated by *Pst*I within 2027 and 2322 bp for genome type C, two additional bands were generated for genome type B, one of >2322 bp and another one of small size and finally three additional fragments were generated for genome type E, the largest at about 4361 bp, one at about 2027 bp and one of identical size to the smallest of genome type B (Fig D). All additional fragments of genome type E were not identified in CpGV-V45. CpGV-V45 shows a number of fragments >10 kb, which can be found in all other genome types; differences could not be resolved here. Further, fragments at 2 kb typical for genome types C and E are lacking in CpGV-V45. Bands <1,8kb are only faintly visible in the digestion, but are consistent with showing a similar *Pst*I restriction pattern of CpGV-V45 and CpGV genome type B (isolate CpGV-E2).

*Sal*I digest: Digestion with *Sal*I generated by far the highest number of fragments, especially in sizes between 4.4 kb and 0.6 kb. In all *in silico* deduced *Sal*I digestions, several fragments below 2 kb could not be resolved in the genome types A to E. However, compared to genome type A, which generated roughly 20 fragments, the genome types distinguished from each other either by additional/missing fragments or size differences. In this case the two fragments from genome type A at ca. 4.4 kb fused to one thick band in genome type B. Genome type C generated two clearly distinguishable additional fragments, one at roughly 0.7 kb and one of intermediate size. In genome type D, this intermediate sized fragment was detected with a smaller size. The remaining fragments were more or less identical to genome type A. Finally, there was a size different detected in one fragment of genome type E at ca. 2 kb compared to genome type A. CpGV-45 shows three fragments from 2 to 2.4kb, while all described genome types show only two fragments in this region either at 2.2 and 2.4kb (genome types A, B, D) or at 2.1 and 2.4 kb (genome type C and E). Moreover, *Sal*I digested CpGV-V45 showed two fragments in the region of 2.7kb, while all characterized genome types A-E have only one band at 2.7kb.
CpGVV45 *Sal*I restriction pattern can therefore not be explained with a single type of the characterized genome types of CpGV.

*Xho*I digest: Virtual digestions of the CpGV genome types with *Xho*I concluded in similar patterns for genome types A and D (Fig F). Genome type B (isolate CpGV-E2) generated only one single fragment

below the λ/*Hind*III marker at 4.4 kb while all other types generated two fragments. Genome type C generated an additional fragment at 2.5 kb. Genome type E has two bands at 4 kb closer proximity to each other than the remaining genome types.
The pattern of *Xho*I restricted CpGV-V45 is similar to those of the genome types A (isolate CpGV-M) and D (isolate CpGV-I12).

Conclusion
Restriction analysis with 6 enzymes identified a pattern in CpGV-V45, which clearly resembles published CpGV restriction pattern confirming the identity as *Cydia pomonella* granulovirus. Albeit, similarities of CpGV-V45 and isolates from the genome types B and D were observed, CpGV-V45 could not be assigned to a single genome type. Furthermore, fragments were generated that were specific for CpGV-V45 only. Therefore, CpGV-V45 can be considered as a novel *Cydia pomonella* granulovirus isolate with high identity to mixtures of other CpGV isolates (from the genome types B and D) but with genomic traits that are exclusive to CpGV-V45.

Conclusion by RMS
The study is considered acceptable.

New cited references by Andermatt Biocontrol AG for KMA 1.3

Report: **KMA 1.3** – Herniou et al. (2001), Use of Whole Genome Sequence Data To Infer Baculovirus Phylogeny, Journal of Virology, 75(17), 8117-8126 (BVL no 3714747)
Published report
Abstract: Several phylogenetic methods based on whole genome sequence data were evaluated using data from nine complete baculovirus genomes. The utility of three independent character sets was assessed. The first data set comprised the sequences of the 63 genes common to these viruses. The second set of characters was based on gene order, and phylogenies were inferred using both breakpoint distance analysis and a novel method developed here, termed neighbor pair analysis. The third set recorded gene content by scoring gene presence or absence in each genome. All three data sets yielded phylogenies supporting the separation of the Nucleopolyhedrovirus (NPV) and Granulovirus (GV) genera, the division of the NPVs into groups I and II, and species relationships within group I NPVs. Generation of phylogenies based on the combined sequences of all 63 shared genes proved to be the most effective approach to resolving the relationships among the group II NPVs and the GVs. The history of gene acquisitions and losses that have accompanied baculovirus diversification was visualized by mapping the gene content data onto the phylogenetic tree. This analysis highlighted the fluid nature of baculovirus genomes, with evidence of frequent genome rearrangements and multiple gene content changes during their evolution. Of more than 416 genes identified in the genomes analyzed, only 63 are present in all nine genomes, and 200 genes are found only in a single genome. Despite this fluidity, the whole genome-based methods we describe are sufficiently powerful to recover the underlying phylogeny of the viruses.
Submitted for the new isolates CPGV-V14 and –V45
Evaluation by RMS: reliable considered as supplementary information.

Report: **KMA 1.3** – Jehle et al. (2006), On the classification and nomenclature of baculoviruses: A proposal for revision, Archives of Virology, 151, 1257-1266 (BVL no 3714748)
Published report

Abstract: Recent evidence from genome sequence analyses demands a substantial revision of the taxonomy and classification of the family *Baculoviridae*. Comparisons of 29 baculovirus genomes indicated that baculovirus phylogeny followed the classification of the hosts more closely than morphological traits that have previously been used for classification of this virus family. On this basis, dipteran- and hymenopteran-specific nucleopolyhedroviruses (NPV) should be separated from lepidopteran-specific NPVs and accommodated into different genera. We propose a new classification and nomenclature for the genera within the baculovirus family. According to this proposal the updated classification should include four genera: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific Granuloviruses), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV).
*
The taxonomy and classification of living organisms is itself a living and steadily evolving process. The same holds true for taxonomy and classification of viruses. In recent years, the nucleotide sequences of virus genes and genomes have become one of the most important tools for virus classification, not least because other distinguishing traits are often difficult to identify. Nucleotide sequence data have rationalised taxonomy in two ways. First, nucleotide sequences and deduced amino acid sequences can be compared and analysed using molecular phylogenetics, with methods based on objective mathematical models. Second, sequence data and molecular phylogenetic analysis can be transformed into quantifiable parameters, such as genetic distances or degrees of sequence identity, which may allow for defining taxon demarcation criteria. In the light of the increased knowledge of baculovirus genomes, the classification of the family *Baculoviridae* needs to be adapted to the emerging picture of baculovirus diversity.
Submitted for renewal
Evaluation by RMS: relevant and reliable

Report: **KMA 1.3** – Eberle et al. (2009), Diversity and Evolution of the Cydia pomonella granulovirus, Journal of General Virology, 90, 662-671 (BVL no 3714754)
Published report

Abstract: Eight new field isolates of *Cydia pomonella* granulovirus (CpGV) originating in Iran and Georgia and one English CpGV isolate were analysed for restriction fragment length polymorphisms (RFLPs) and by partial genome amplification and sequencing. According to the observed RFLPs, most of the predominant genotypes of these isolates could be assigned to those present in previously found isolates originating from Mexico (CpGV-M), England (CpGV-E) and Russia (CpGV-R). We suggest that these isolates should be designated genome A, B and C types, respectively. A fourth genome type was identified in three isolates and is designated D type. The isolates with A, B and D type genomes contained four open reading frames (ORFs) (ORF63– ORF66) not present in C type genomes. The lack of these ORFs in other granuloviruses suggests that the C type genome is evolutionarily ancestral to the other genome types. The B and D type genomes contained an additional insertion of a non-protein coding region of 0.7 kb, which was at different genome locations. Analysis of the partial gene sequences of late expression factor 8 (lef-8), lef-9 and polyhedrin/granulin (polh/gran) genes revealed single nucleotide polymorphisms (SNPs) that corresponded to the RFLP types. Phylogenetic analyses based on these SNPs corroborated the proposed ancestry of the C type genome. C type viruses were also less virulent to neonate codling moth larvae than the other virus types. In conclusion, the known diversity of CpGV isolates can be described by four major genome types, which appear to exist in different isolates as genotype mixtures.
Submitted for the new isolates CPGV-V14 and -V45
Evaluation by RMS: Relevant and reliable

Report: **KMA 1.3** – Wennmann et al. (2017), Deciphering Single Nucleotide Polymorphisms and Evolutionary Trends in Isolates of the Cydia pmonella granulovirus, Viruses, 9, 227, 1-12 (BVL no 3714755)
Published report

Abstract: Six complete genome sequences of *Cydia pomonella* granulovirus (CpGV) isolates from Mexico (CpGV-M and CpGV-M1), England (CpGV-E2), Iran (CpGV-I07 and CpGV-I12), and Canada (CpGV-S) were aligned and analyzed for genetic diversity and evolutionary processes. The selected CpGV isolates represented recently identified phylogenetic lineages of CpGV, namely, the genome groups A to E. The genomes ranged from 120,816 bp to 124,269 bp. Several common differences between CpGV-M, -E2, -I07, -I12 and -S to CpGV-M1, the first sequenced and published CpGV isolate, were highlighted. Phylogenetic analysis based on the aligned genome sequences grouped CpGV-M and CpGV-I12 as the most derived lineages, followed by CpGV-E2, CpGV-S and CpGV-I07, which represent the most basal lineages. All of the genomes shared a high degree of colinearity, with a common setup of 137 (CpGV-I07) to 142 (CpGV-M and -I12) open reading frames with no translocations. An overall trend of increasing genome size and a decrease in GC content was observed, from the most basal lineage (CpGV-I07) to the most derived (CpGV-I12). A total number of 788 positions of single nucleotide polymorphisms (SNPs) were determined and used to create a genome-wide SNP map of CpGV. Of the total amount of SNPs, 534 positions were specific for exactly one of either isolate CpGV-M, -E2, -I07, -I12 or -S, which allowed the SNP-based detection and identification of all known CpGV isolates.
Submitted for the new isolates CPGV-V14 and –V45
Evaluation by RMS: Relevant and reliable

B.1.3.3.3 Additional isolates owned by Arysta LifeScience S.A.S.

This isolate was included in Annex III of the Review Report for CpGV.

CpGV R5 (GV-0007)

Reference:
Jehle, Eberle (2009), Comparative Restriction Analysis of CpGV Carpovirusine-R5 with CpGV (Virosoft), CpGV-E2 and CpGV-M (Neustadt), Arysta LifeScience S.A.S. (BVL no 3306436)

Summary
For the identification of baculovirus isolates DNA endonuclease restriction (REN) analysis is usually used. By digesting viral DNA by different RENs specific restriction patterns can be identified and small genotypic variations can be located in a restriction map. In this study, DNA of CARPOVIRUSINE R5 (Test item) was isolated and purified and subjected to endonuclease restriction analysis using the endonucleases *Sal*I, *Bam*HI, *Eco*RI and *Eco*RV. The restriction fragments were separated in an agarose gel and the obtained restriction profiles were compared to the restriction profiles of CpGV (Virosoft, CpGV (Isolate E2), CpGV-M (Mexican isolate, propagated in Neustadt, NW) and two published profiles of CpGV-M. It was found that the Test Item CpGV CARPOVIRUSINE R5 was a CpGV isolate with a restriction pattern highly similar to the profile of Reference Item CpGV (Virosoft) in all four digests. The restriction profile of Test Item CpGV CARPOVIRUSINE R5 differed from the Reference Item CpGV-M (NW) in three out of four digests and from Reference Item CpGV-E2 in all four digests. Additionally, faint submolar bands were found in two profiles of Test Item CpGV CARPOVIRUSINE R5. They did not correspond to a genotype similar to the Reference Items CpGV-E2 or CpGV-M (NW), indicating that there is a further genotype at a low level present in Test Item CpGV CARPOVIRUSINE R5.

Material and Methods
Test Item CpGV R5 was purified by centrifugation in a 30-80% glycerol layer gradient. The purified CpGV OB pellet was resuspended in 2 mL sterile water. DNA was isolated from the purified CpGV pellet by phenol/chloroform/isoamylalcohol method.

The viral DNA of the Test Item (CpGV CARPOVIRUSINE R5) and of the reference items CpGV-M (NW), CpGV (Virosoft) and CpGV-E2 was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Sal*I and *Eco*RV. These enzymes were chosen because they allow an easy differentiation of the previously described CpGV isolates. 17 µL isolated DNA were incubated in 2 µL buffer and 1 µL enzyme at

37°C for 3h. Digested DNA was electrophoresed in a 0.8% agarose gel over night (25 V) using TAE as buffer system.

Findings

REN Analysis:

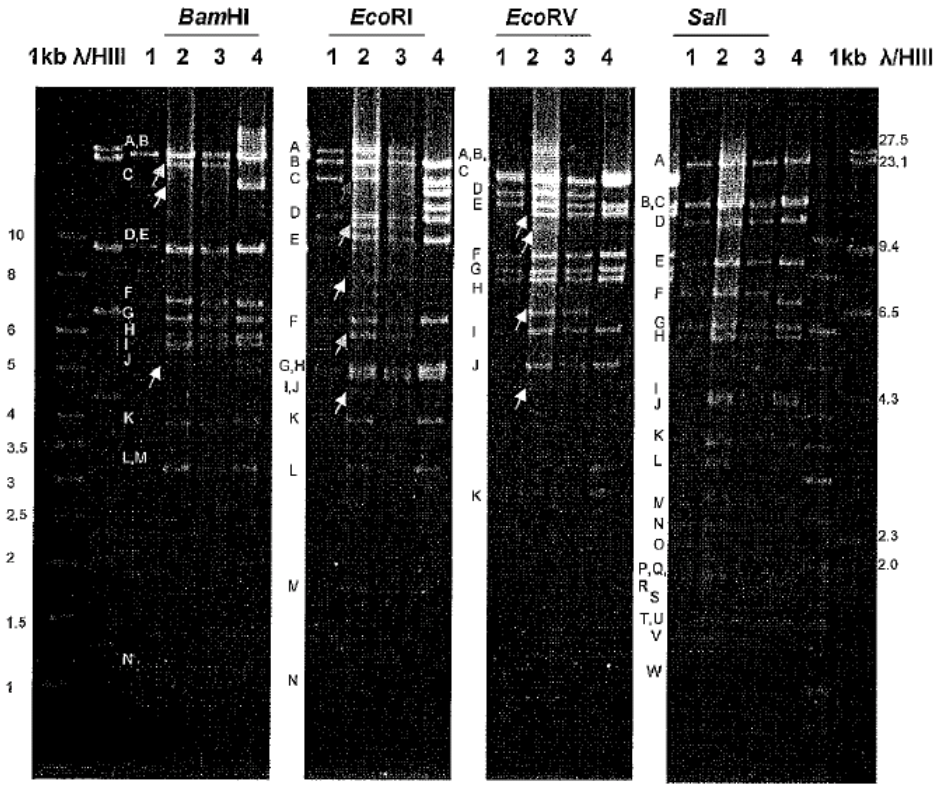

Figure B.1.3-9: Electrophoresis through a 0.8% agarose gel of purified DNA from 1 = CpGV-M (NW), 2 = CpGV (CARPOVIRUSINE R5), 3 = CpGV (Virosoft) and 4 = CpGV-E2. Restriction fragments are lettered in sequential order of their size. Size markers are given to the left (kbp).

*Bam*HI digest: All *Bam*HI restriction fragments A to N of the Reference Item CpGV-M (NW) could be identified at the expected position. Test Item CpGV CARPOVIRUSINE R5 corresponded in its *Bam*HI profile to Reference Item CpGV (Virosoft): For Test Item CpGV CARPOVIRUSINE R5 and for Reference Item CpGV (Virosoft) all *Bam*HI restriction fragments except C (15.2 kb) and J (5.2) could be identified at the appropriate sizes. Instead of fragment C and J (white arrows), one additional band of about 20 kb was present in Test Item CARPOVIRUSINE R5 and in Reference Item CpGV (Virosoft). Since the fragments C and J are adjacent in the map of CpGV M (Crook et al., 1997), it is suggested that this additional band of about 20 kb is a fusion fragment of C and J due to a missing *Bam*HI site.

*Eco*RI digest: Compared to Reference Item CpGV-M (NW), Test Item CpGV CARPOVIRUSINE R5 showed four additional bands (arrows), which had a different intensity. Two fragments of about 11.5 kb (C1) and 5.8 kb (C2) (arrows) were present in the Test Item and also in Reference Item CpGV (Virosoft). It is most likely that these fragments were generated by a partial *Eco*RI restriction of fragment C due to a restriction site that is not present in the Reference Item CpGV-M (NW). The intensity of *Eco*RI Fragment C (16.9 kb) was lower in Test Item CpGV CARPOVIRUSINE R5 and Reference Item CpGV (Virosoft) as expected.
Additionally, Test Item CpGV CARPOVIRUSINE R5 showed two faint submolar bands at about 4.8 and 7.8 kb, which did not correspond to the profiles of Reference Items CpGV-M (NW), CpGV (Virosoft) and CpGV-E2.

The Test Items (CARPOCIRUSINE R5) restriction profile was clearly different from Reference Item CpGV-E2. Fragment A (27.9) in the Reference Item CpGV-E2 is cut into two fragments A1 (15.1 kb) and A2 (13.2 kb) due to an insertion carrying one additional *Eco*RI restriction site. This was not the case for Test Item CpGV CARPOVIRUSINE R5.

*Eco*RV digest: As shown in Figure B.1.3-8. Reference Item CpGV-M (NW) and Test Item shared most of the *Eco*RV restriction fragments. In the Test Item CpGV CARPOVIRUSINE R5 and the Reference Item CpGV (Virosoft) two additional bands of 13 and 6.5 kb could be observed. These bands might be derived from an additional *Eco*RV site in one of the fragments A, B or C. Since these bands are similarly large it is not clear from the picture which fragment is cut into two additional fragments. However, it can be excluded that these additional bands are the result of a major genome insertion, since this would be observed in the other restriction profiles too. Submolar bands were observed in Test Item CpGV CARPOVIRUSINE R5 at about 11.8 and 4.4 kb. These bands were not present in the Reference item CpGV (Virosoft) and did not correspond to fragments present in Reference Item CpGV-E2.

*Sal*I digest: All *Sal*I restriction fragments A to W could be identified at the expected position for the Reference Item CpGV-M (NW). Test Item CpGV CARPOVIRUSINE R5 did not differ in its *Sal*I profile from Reference Items CpGV (NW) and CpGV (Virosoft). No submolar bands could be observed.

Conclusion
On the basis of DNA restriction analysis using *Sal*I, *Eco*RI, *Eco*RV and *Bam*HI, it can be concluded that Test Item CpGV CARPOVIRUSINE R5 is a CpGV isolate that is very similar to Reference Item CpGV (Virosoft). It differs slightly from the reference Item CpGV-M (NW) and CpGV (E2). Additionally, few submolar bands were found, which could not be assigned to any of the reference Items, indicating that there is a further genotype present at a low level in the preparation of CpGV CARPOVIRUSINE R5.

Conclusion by RMS
The study is considered acceptable.

B.1.3.4 Common name or alternative and superseded names and code names used during the development

Information already provided in the DAR

Scientific name:	*Cydia pomonella* Granulovirus (CpGV)
Synonymous virus names:	Codling moth granulovirus
	Granulosis virus of codling moth
	Apfelwickler-Granulosevirus
	Apfelwickler-Granulovirus
	Codling moth granulosis virus
	Laspeyresia pomonella GV
	Granulosis of *Laspeyresia pomonella*
	Carpocapsa pomonella GV
	CARPOVIRUSINE granulosis virus
	Virus de la Granulose du Carpocapse des Pommes et des Poires

B.1.3.5 Relationship to known pathogens

Information already provided in the DAR

CpGV as well as all other known baculoviruses have been exclusively isolated from arthropods and

not from other animals, humans or plants. They are not related to any known plant or human pathogen.

B.1.4 Specification of the material used for manufacturing of formulated products

B.1.4.1 Content of the micro-organism

Remark by RMS:
Different ways for expressing the content of CpGV were used by the applicants. In the case of betabaculoviruses occlusion bodies (OB) are granules. This is often shortened to GV. Therefore, OB/L, granules/L and GV/L can be used synonymously. In the identity part of the RAR only granules will be used. Text taken from the DAR will not be amended.

Information provided in the DAR

Andermatt Biocontrol GmbH

Only the active viruses are relevant. Therefore, the active ingredient has to be standardised by bioassays and not by weight. The CpGV aqueous virus slurry has a content of the active ingredient CpGV of 6.0 x 10^{13} granules/L. The calculated weight of the virus content in the technical material is approximately 13.08 g/L or 12 g/kg.

New information for RAR

Information is still valid.

Minimum content: 6 × 10^{13} granules/L, maximum content: 12 × 10^{13} granules/L.

Information provided in the DAR

Arysta LifeScience S.A.S.

The CpGV aqueous virus slurry has a content of the active ingredient CpGV of 4.0 x 10^{14} granules/L. Based on the volume of one virus granule the calculated amount of the active micro-organism in the technical material is approximately 8 g/L.

New information for RAR

Minimal CpGV concentration: 2.6 x 10^{13} granules/L
Nominal CpGV concentration: 3.2 x 10^{13} granules /L
Maximal CpGV concentration: 1.8 x 10^{14} granules /L

New information for RAR

Serbios srl

No own isolate is produced.

B.1.4.2 Identity and content of impurities, additives, contaminating micro-organisms

Content of contaminating micro-organism *Bacillus cereus*: < 1 × 10^{7} CFU/g in the formulated product.

B.1.4.3 Analytical profile of batches

Confidential information, see Volume 4

B.1.5 References relied on

Data point	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not BVL registration number	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMA 1.3	Evans, H.F., Harrap, K.A.	1982	PERSISTENCE OF INSECT VIRUSES not available, not applicable Virus Persistence, Publisher: Cambridge University Press, 58-96 GLP/GEP: no Published: yes 2098032 / BWS2006-13	no	no	not protected	-	Y KIIM 1.3.1
KMA 1.3	OECD	2002	CONSENSUS DOCUMENT ON INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING BACULOVIRUS not available, not applicable ENV/JM/MONO, 1, 1-90 GLP/GEP: no Published: yes 2019066 / BWS2006-90	no	no	not protected	-	Y KIIM 1.3.1
KMA 1.3	Bilimoria, S. L.	1986	TAXONOMY AND IDENTIFICATION OF BACULOVIRUSES not available, not applicable The Biology of Baculoviruses, Biological Properties and Molecular Biology, Publisher: CRC Press, 1, 37-59 GLP/GEP: no Published: yes 2019060 / BWS2006-88	no	no	not protected	-	Y KIIM 1.3.1
KMA 1.3	Gröner, A.	1986	SPECIFICITY AND SAFETY OF BACULOVIRUSES not available, not applicable The Biology of Baculoviruses, Volume I, Biological Properties and Molecular Biologie, Chapter 9, 177-201 GLP/GEP: no Published: yes 2098035 / BWS2006-15	no	no	not protected	-	Y KIIM 1.3.1

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KMA 1.3	ICTV (International Committee of Taxonomy of Virus database)	2000a	00.006.06.0.02.001 CYDIA POMONELLA GRANULOVIRUS Arysta LifeScience S.A.S., not stated not available GLP/GEP: no Published: no 2019057 / BWS2006-123	no	no	not protected	ALS	Y KIIM 1.3.3
KMA 1.3	ICTV (International Committee of Taxonomy of Virus database)	2000b	COMPARISON OF SINGLE VIRAL PROTEIN, 00.006.0.01 NUCLEOPOLYHEDROVIRUS, POLYHEDRON, 00.006.0.02. GRANULOVIRUS, GRANULIN Arysta LifeScience S.A.S., not stated not available GLP/GEP: no Published: no 2019064 / BWS2006-124	no	no	not protected	ALS	Y KIIM 1.3.3
KMA 1.3	Aupinel, P.	2005	CERTIFICATE OF ORIGIN OF CPGV ISOLATE TRANSMITTED TO NPP Arysta LifeScience S.A.S., not stated Institut National de la Recherche Agronomique, France GLP/GEP: no Published: no 2019054	no	no	not protected	ALS	Y KMII 1.3.1
KMA 1.3	Tweeten, K.A., Bulla, L.A., Consigli, R.A.	1981	APPLIED AND MOLECULAR ASPECTS OF INSECT GRANULOSIS VIRUSES not available, not applicable not available GLP/GEP: no Published: no 2019062 / BWS2006-89	no	no	not protected		Y KMII 1.3.3

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KMA 1.3	Crook, N.E., James, J.D., Smith, I.R.L., Winstanley, D.	1997	COMPREHENSIVE PHYSICAL MAP OF THE CYDIA POMONELLA GRANULOVIRUS GENOME AND SEQUENCE ANALYSIS OF THE GRANULIN GENE REGION not available, not applicable Journal of General Virology, 18, 965-974 GLP/GEP: no Published: yes 2019078 / BWS2006-93	no	no	not protected	-	Y KIIM 1.3.3
KMA 1.3	NCBI Sequence Viewer v2.0	2001	U53466, CYDIA POMONELLA not available, not applicable http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?va1=U53466 GLP/GEP: no Published: yes 1932501 / BWS2006-17	no	no	not protected	-	Y KIIM 1.3.3
KMA 1.3	Luque, T., Finch, R., Crook, N., O'Reilly, D.R., Winstanley, D.	2001	THE COMPLETE SEQUENCE OF THE CYDIA POMONELLA GRANULOVIRUS GENOME not available, not applicable Journal of General Microbiology, 82, 2531-2547 GLP/GEP: no Published: yes 2019080 / BWS2006-94	no	no	not protected	-	Y KIIM 1.3.3
KMA 1.3	Jehle, J.	2006a	COMPARATIVE RESTICTION ANALYSIS CPGV (NEUSTADT MEXICAN ISOLATE) WITH CPGV (SIPCAM MEXICAN ISOLATE) Sipcam S.p.A., SIP01 DLR-Rheinpfalz, Neustadt, Germany GLP: yes Published: no 3332644 / BWS2006-19	no	no	not protected	SIP	Y KIIM 1.3.3

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KMA 1.3	Jehle J.	2006	COMPARATIVE RESTRICTION ANALYSIS CPGV (NEUSTADT MEXICAN ISOLATE) WITH CPGV (MADEX MEXICAN ISOLATE) Andermatt Biocontrol GmbH / Probis GmbH, not applicable DLR-Rheinpfalz, Neustadt, Germany GLP/GEP: no Published: no 3431947 / BWS2006-98	no	no	not protected	PKA	Y KIIM 1.3.3
KMA 1.3	Jehle, J.	2005	COMPARATIVE RESTRICTION ANALYSIS CPGV (NEUSTADT MEXICAN ISOLATE) WITH CPGV (INRA MEXICAN ISOLATE) Arysta LifeScience S.A.S., ARY03 Dienstleistungszentrum Ländlicher Raum, Neustadt an der Weinstraße GLP/GEP: no Published: no 3332645 / BWS2006-87	no	no	not protected	ALS	Y KIIM 1.3.3
KMA 1.3	Jehle, J.	2006b	COMPARATIVE RESTRICTION ANALYSIS CPGV (CARPOVIRUSINE, TECHNICAL CONCENTRATE, BATCH 1461/SMT) WITH CPGV (INRA MEXICAN ISOLATE) Arysta LifeScience S.A.S., ARY02 not available GLP/GEP: no Published: no 3332646 / BWS2006-86	no	no	not protected	ALS	Y KIIM 1.4.3.1

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KMA 1.3	Crook, N.E., Spencer, R.A., Payne, C.C., Leisy, D.J.	1985	VARIATION IN CYDIA POMONELLA GRANULOSIS VIRUS ISOLATES AND PHYSICAL MAPS OF THE DNA FROM THREE VARIANTS not available, not applicable not available GLP/GEP: no Published: no 2019070 / BWS2006-91	no	no	not protected		Y KIIM 1.3.3
KMA 1.3/01	Kessler, P.	2010a	DECLARATION OF ORIGIN CPGV ISOLATE ABC-V15 (DSMZ GV-00013) Andermatt Biocontrol AG, CH, not stated Andermatt Biocontrol AG, Grossdietwil, Switzerland GLP/GEP: no Published: no 3306430	no	yes	protected	ABA	N
KMA 1.3/02	Kessler, P.	2010b	DECLARATION OF ORIGIN CPGV ISOLATE ABC-V22 (DSMZ GV-00014) Andermatt Biocontrol AG, CH, not stated Andermatt Biocontrol AG, Grossdietwil, Switzerland GLP/GEP: no Published: no 3306431	no	yes	protected	ABA	N
KMA 1.3/03	Kessler, P.	2008	DECLARATION ON THE ORIGIN AND CHARACTERIZATION OF THE ACTIVE INGREDIENT OF MADEX PLUS Andermatt Biocontrol AG, CH, not applicable Andermatt Biocontrol AG, Grossdietwil, Switzerland GLP/GEP: no Published: no 3306432	no	yes	protected	ABA	N

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KMA 1.3/04	Jehle, J., Eberle, K.	2009a	COMPARATIVE RESTRICTION ANALYSIS OF V15 Andermatt Biocontrol AG, CH, not stated DLR-Rheinpfalz, Neustadt, Germany GLP/GEP: no Published: no 3306433	no	yes	protected	ABA	N
KMA 1.3/05	Jehle, J., Eberle, K.	2009b	COMPARATIVE RESTRICTION AND PHYLOGENETIC ANALYSIS OF V22 Andermatt Biocontrol AG, CH, not stated DLR-Rheinpfalz, Neustadt, Germany GLP/GEP: no Published: no 3306434	no	yes	protected	ABA	N
KMA 1.3/06	Jehle, J.	2006c	COMPARATIVE RESTRICTION ANALYSIS OF CPGV (NEUSTADT MEXICAN ISOLATE) WITH CPGV (MADEX PLUS) Andermatt Biocontrol AG, CH, not applicable Dienstleistungszentrum Ländlicher Raum, Neustadt an der Weinstraße GLP/GEP: no Published: no 3306435	no	yes	protected	ABA	N
KMA 1.3/07	Jehle, J., Eberle, K.	2009c	COMPARATIVE RESTRICTION ANALYSIS OF CPGV CARPOVIRUSINE-R5 WITH CPGV (VIROSOFT), CPGV-E2 AND CPGV-M (NEUSTADT) Arysta LifeScience S.A.S., NPP_09.1 Dienstleistungszentrum Ländlicher Raum, Neustadt an der Weinstraße GLP/GEP: no Published: no 3306436	no	yes	protected	ALS	N

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KMA 1.3	Winter, S.	2011	The Granulosevirus preparation CpGV-Isolate V14 Andermatt Biocontrol AG, CH, GV-0015 DSMZ GmbH, Braunschweig, Germany GLP/GEP: no Published: no 3714749	no	yes	protected	ABA	N
KMA 1.3	Menzel, W.	2017	The Granulosevirus preparation CpGV V45 Andermatt Biocontrol AG, CH, GV-0017 DSMZ GmbH, Braunschweig, Germany GLP/GEP: no Published: no 3714797	no	yes	protected	ABA	N
KMA 1.3	Jehle, J.A.; Eberle, K.E.	2010	Comparative restriction analysis of C15 Certis Europe B.V., BE, not stated Julius Kühn-Institut GLP/GEP: no Published: no 3714752	no	yes	protected	ABA	N
KMA 1.3	Brader, G.	2018	Report AIT_KS_V45: Restriction enzyme analysis of CpGV-V45 and in silico comparison with sequenced CpGV isolates Andermatt Biocontrol AG, CH, ABA10256_AIT_KS_V45 Austrian Institute of Technology GmbH GLP/GEP: no Published: no 3714804	no	yes	protected	ABA	N
KMA 1.3	Herniou, E.A.; Luque, T.; Chen, X.; Vlak, J.M.; Winstanley, D.; Cory, J.S.; O'Reilly, D.R.	2001	Use of Whole Genome Sequence Data To Infer Baculovirus Phylogeny Journal of Virology, 75(17), 8117-8126 GLP/GEP: no Published: yes 3714747	no	no	not protected		

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KMA 1.3	Jehle, J.A.; Blissard, G.W.; Bonning, B.C.; Cory, J.S.; Herniou, E.A.; Rohrmann, G.F.; Theilmann, D.A.; Thiem, S. M.; Vlak, J.M.	2006	On the classification and nomenclature of baculoviruses: A proposal for revision Archives of Virology, 151, 1257-1266 GLP/GEP: no Published: yes 3714748	no	no	not protected		
KMA 1.3	Eberle, K. E.; Sayed, S.; Rezapanah, M.; Shojai-Estabragh, S.; Jehle, J. A.	2009	Diversity and Evolution of the Cydia pomonella granulovirus Journal of General Virology, 90, 662-671 GLP/GEP: no Published: yes 3714754	no	no	not protected		
KMA 1.3	Wennmann, J.T.; Radtke, P.; Eberle, K.E.; Gueli Alletti, G.; Jehle, J.A.	2017	Deciphering Single Nucleotide Polymorphisms and Evolutionary Trends in Isolates of the Cydia pmonella granulovirus Viruses, 9, 227, 1-12 GLP/GEP: no Published: yes 3714755	no	no	not protected		