Empirical estimates of the mutation rate for an alphabaculovirus

Dieke Boezen[1a], Ghulam Ali[2a,b], Manli Wang[3], Xi Wang[3], Wopke van der Werf[4], Just M. Vlak[2], Mark P. Zwart[1*]

[1] Department of Microbial Ecology, The Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands

[2] Laboratory of Virology, Wageningen University and Research, Wageningen, The Netherlands

[3] Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, PR China

[4] Centre for Crop Systems Analysis, Wageningen University and Research, Wageningen, The Netherlands

[a] Equal contribution

[b] Current affiliation: Air University Multan Campus, Abdali Road, Multan, Pakistan

* Correspondence: M.Zwart@nioo.knaw.nl; Tel.: +31 317 473431

Abstract

Mutation rates are of key importance for understanding evolutionary processes and predicting their outcomes. Empirical estimates of mutation rate are available for a number of RNA viruses, but few are available for DNA viruses, which tend to have larger genomes. Whilst some viruses have very high mutation rates, lower mutation rates are expected for viruses with large genomes to ensure genome integrity. Alphabaculoviruses are insect viruses with large genomes and often have high levels of polymorphism, suggesting high mutation rates despite evidence of proofreading activity by the replication machinery. Here, we report an empirical estimate of the mutation rate per base per strand copying (s/n/r) of Autographa californica multiple nucleopolyhedrovirus (AcMNPV). To avoid biases due to selection, we analyzed mutations that occurred in a stable, non-functional genomic insert after five serial passages in *Spodoptera exigua* larvae. Population bottlenecks, viral mode of replication and thresholds for mutation detection likely affect mutation rate estimates, and we therefore used population genetic models that account for these processes to infer the mutation rate. We estimated a mutation rate of 1×10^{-7} s/n/r. This estimate was not sensitive to different model assumptions or including whole genome data. The rates at which different classes of mutations accumulate provide good evidence for neutrality of mutations occurring within the inserted region. We therefore present a robust approach for mutation rate estimation for viruses with stable genomes, and strong evidence of a much lower alphabaculovirus mutation rate than supposed based on the high levels of polymorphism observed.

Author Summary

Virus populations can evolve rapidly, driven by the large number of mutations that occur during virus replication. It is challenging to measure mutation rates because selection will affect which mutations are observed: beneficial mutations are overrepresented in virus populations, while deleterious mutations are selected against and therefore underrepresented. Few mutation rates have been estimated for viruses with large DNA genomes, and there are no estimates for any insect virus. Here, we estimate the mutation rate for an alphabaculovirus, a virus that infects caterpillars and has a large, 134 kilobase pair DNA genome. To ensure that selection did not bias our estimate of mutation rate, we studied which mutations occurred in a large artificial region inserted into the virus genome, where mutations did not affect viral fitness. We deep sequenced evolved virus populations, and compared the distribution of observed mutants to predictions from a simulation model to estimate mutation rate. We found evidence for a relatively low mutation rate, of one mutation in every 10 million bases replicated. This estimate is in line with expectations for a virus with self-correcting replication machinery and a large genome.

35 # Introduction

36 Mutation rates are of key importance for understanding and predicting evolutionary patterns, as
37 the mutation rate modulates the mutation supply of a population [1]. Large mutation supplies can
38 fuel rapid and repeatable adaptation [2,3], but also increase the mutational load on a population
39 [4]. By contrast, low mutation supplies can limit the rate of adaptation [5], but also result in a lower
40 mutational load [4]. The impact of mutational supply also depends on the topography of the fitness
41 landscape. Small mutational supplies can have advantages for evolution on rugged fitness
42 landscapes: although adaptation will be slower and in most cases less fit genotypes will be
43 selected, some populations can avoid becoming trapped on local fitness peaks [6]. Mutation rates
44 are not only relevant to understanding basic evolutionary processes, but they also impinge on
45 real world outcomes, such as the efficacy of prophylactic or therapeutic interventions to infectious
46 diseases [7,8].

47 Viruses have high mutation rates [7,9,10], with estimates of mutations per site per strand
48 copying (s/n/r) ranging from 2×10^{-8} for Enterobacteria phage T2 [11] to 2×10^{-4} for Influenza A virus
49 [12]. Whilst these high mutation rates are thought to contribute to the rapid adaptation of viruses,
50 the majority of mutations are typically deleterious [13,14]. Many viruses with large genomes
51 belong to Group I of the Baltimore classification (dsDNA viruses) [15], and typically have
52 polymerases with proofreading activity, which should enhance the fidelity of replication [16]. A
53 general expectation is therefore that viruses with relatively large genomes have lower mutation
54 rates [9]. An inverse relationship between genome size and mutation rate indeed has been found.
55 Small genomes can tolerate higher mutation rates as a larger proportion of mutation-free
56 genomes are generated in each round of replication, due to their small size [9,17].

57 The alphabaculoviruses are a large group of insect baculoviruses that have been studied
58 because of their biocontrol and biotechnological potential [18,19]. Alphabaculoviruses have

59 relatively large dsDNA genomes compared to other viruses [20], and high levels of within-host
60 genetic diversity have been documented from wild [21–23] and captive [24] insect populations. It
61 has been suggested that baculoviruses might therefore have high mutation rates despite their
62 large genome sizes [24]. To our knowledge, no empirical estimates of the mutation rate have
63 been reported for any baculovirus or insect virus to date, and there are only a few estimates for
64 other large dsDNA viruses [9].

65 A major challenge for making empirical estimates of mutation rates is the need to account
66 for biases due to selection [9]. Studies on the distribution of mutational fitness effects (DMFE)
67 have shown that most mutations in viral genomes are deleterious [13,14], and selection will
68 remove these mutations from populations. By contrast, beneficial mutations will increase in
69 frequency due to selection. These opposing effects of purifying and directional selection make it
70 problematic to derive information on mutation rates directly from mutation accumulation in a viral
71 genome. Many different approaches have been developed to remove the bias introduced by
72 selection [7,9]. For example, some studies considered the frequency of lethal mutations in a
73 population, since these variants cannot replicate autonomously and therefore represent a
74 snapshot of genetic variation [25]. Others have evolved viruses in hosts expressing a viral gene,
75 and then restricting their analysis to the sequence of this redundant viral gene [26]. Another
76 strategy reported recently has been to incorporate fluorescent markers with inactivating mutations
77 into a viral genome, and then performing fluctuation tests based on recovering fluorescence [12].

78 In the current study, we report the first empirical estimate of mutation rate for a large
79 dsDNA virus, the alphabaculovirus Autographa californica multiple nucleopolyhedrovirus
80 (AcMNPV). To ensure selection did not bias our estimates, we analyzed virus populations carrying
81 a large, nonfunctional genomic insert that was stably maintained [27], exploiting the genomic
82 stability of the Group I viruses [28]. For our analysis, we assumed that mutations in this region
83 are neutral due to the absence of known viral genes and regulatory sequences, and verified this

84 assumption. We also developed a population genetics model to estimate mutation rates from
85 empirical data, that incorporates the effects of population bottlenecks and different modes of virus
86 replication on the occurrence and maintenance of mutations. Using this approach, we made
87 robust estimates of the mutation rate for a baculovirus for the first time.

88 # Results and Discussion

89 **Serial passage and detection of mutations**

90 To estimate the mutation rate of a large dsDNA virus, we experimentally evolved a variant of
91 alphabaculovirus AcMNPV containing a stable, non-functional genomic region. The AcMNPV
92 variant used was a so-called bacmid: an infectious clone that also contains the AcMNPV genome
93 (~134 kb). It also contains bacterial sequences that enable propagation as a low copy number
94 plasmid in *Escherichia coli* (~12.5 kb) and the acceptance of expression cassettes by
95 transposition [27]. The specific variant used here contains an expression cassette from the
96 pFastBac-Dual vector to restore expression of complete and functional polyhedrin (Figure 1). We
97 consider the inserted bacterial sequences to be non-functional and therefore neutral in insects,
98 except for the polyhedrin promoter and open reading frame (ORF) sequences derived from the
99 pFastBac Dual vector. This renders an 11,646 bp neutral region for a mutation accumulation
100 experiment (Figure 1). By contrast, the remainder of the AcMNPV genome is intact and unaltered,
101 making this system representative for this virus. We will hereafter refer to this bacmid-derived
102 AcMNPV variant with restored polyhedrin expression as “BAC”. The virus could be reconstituted
103 from the infectious clone by transfection of the BAC genome into fourth instar (L4) *Spodoptera*
104 *exigua* (Hübner) (see Materials and Methods). Mutations were allowed to accumulate across the
105 BAC genome by experimentally evolving five replicate BAC lineages (referred to as lineages A,
106 B, C, D and E) for five passages in *S. exigua* L2. For each replicate lineage, passaging was
107 performed in five larvae exposed to a high viral dose of occlusion bodies (OBs) sufficient to kill all
108 larvae. Upon death larval cadavers were collected and pooled prior to the isolation of OBs, which
109 were used to inoculate larvae orally for the next passage. Further details on the experimental
110 evolution experimental setup can be found in the Materials and Methods.

111 Evolved lineages A - E, as well as the ancestral BAC were sequenced using Illumina

112 HiSeq to detect mutations in both the non-functional genomic insert, as well as across the whole
113 baculovirus genome. When mapping reads to the BAC reference genome we noticed a correlation
114 between low sequencing coverage and the calling of mutations, and we therefore removed
115 regions with relatively low coverage (Figures S1-S4, see Materials and Methods). We show that
116 mutations indeed accumulated across lineages and passages (Table 1). When analyzing the
117 sequence data, the number of mutations detected is dependent on the minimum threshold value
118 (τ) for mutation frequency (e.g. τ values of 0.5, 1, 2% were used). Regardless of the chosen
119 mutation frequency threshold, the number of mutations detected is low for both the bacmid insert
120 and genome after five passages in *S. exigua* L2.

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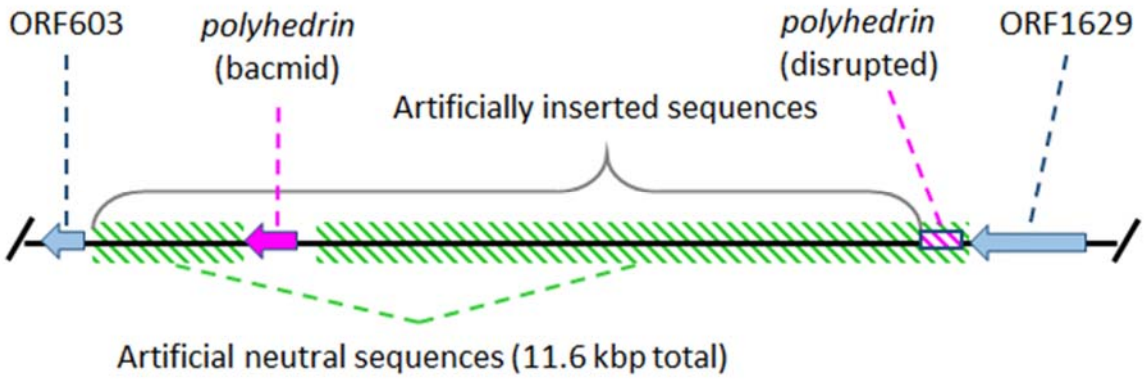

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123 **Figure 1:** Illustration of the neutral region (green diagonal bars) used for estinating mutation rate
124 . ORF603 and ORF1629 (light blue) are native AcMNPV genes, between which lies the *polyhedrin*
125 gene which was disrupted (box with magenta bars) by the insertion of the bacmid sequences in
126 its 5' end. To restore *polyhedrin* expression the gene has been reinserted under control of its
127 native promotor within the bacmid insert. We consider the sequences of bacterial origin in the
128 insert and the remnants of the pseudogenized *polyhedrin* gene copy as neutral sequences.

Table 1: Mutations called per lineage, where τ is the threshold frequency for detecting mutations.

	Neutral region only			Whole genome		
Lineage	τ = 0.5%	τ = 1.0%	τ = 2.0%	τ = 0.5%	τ = 1.0%	τ = 2.0%
BAC	1	-	-	1	-	-
A	2	-	-	20	7	1
B	2	-	-	14	3	2
C	1	1	-	9	2	1
D	1	1	-	7	6	2
E	2	1	1	18	2	2

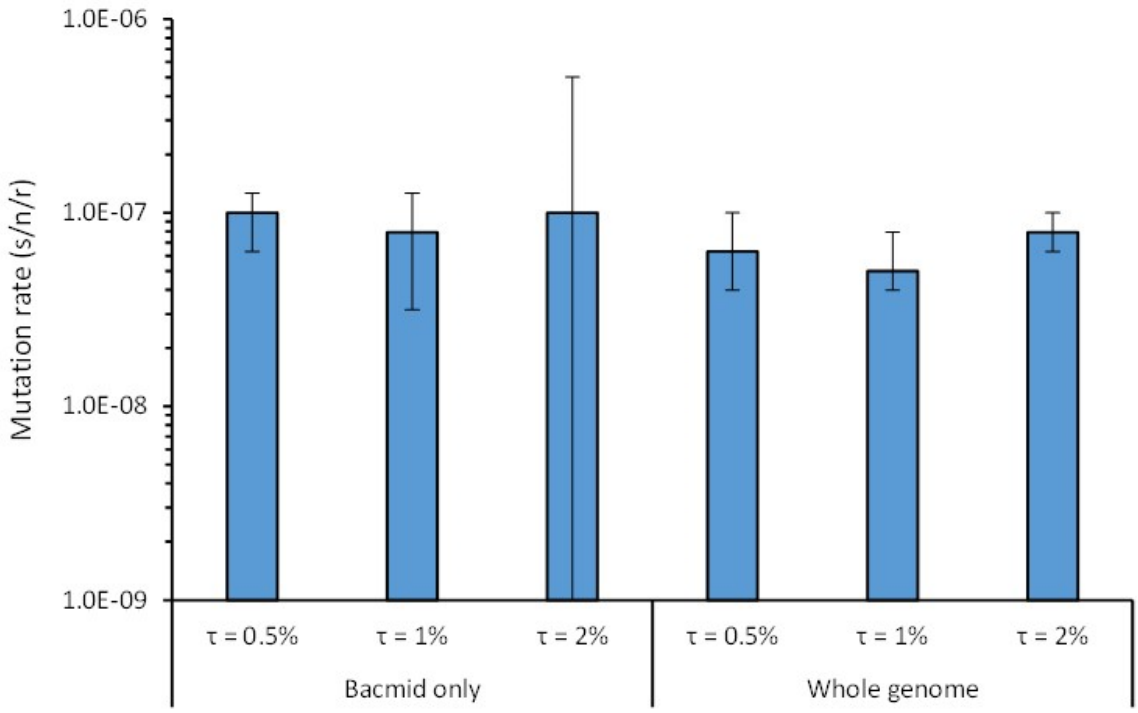

Figure 2: Mutation rate estimates (s/n/r, mutations per site per strand copying) derived with the model are given based on the neutral bacmid region or the whole genome. Different values for the threshold frequency for detecting mutations (τ) were used, noted as percentages here. Error bars represent the 95% fiducial limits, as determined by bootstrapping. Note that for the neutral region and τ = 2.0%, the lower fiducial limit extends to zero due to the low number of mutations detected.

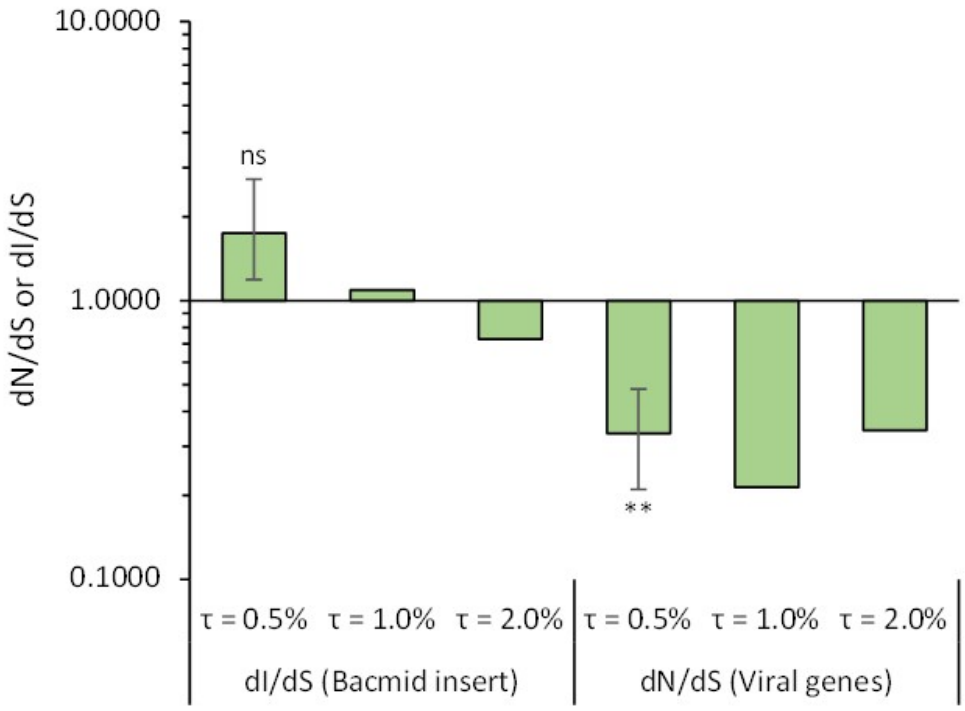

Figure 3: The rate of mutations in the artificial neutral regions (dI), normalized by the rate of synonymous mutations in native viral genes (dS), is shown by the three bars on the left. The values are close to 1, indicating neutral evolution. The three bars on the right show the rate of non-synonymous mutations in viral genes (dN), normalized by dS. These values are less than 1, indicating purifying selection. dI/dS and dN/dS values were determined for different values for the threshold frequency for detecting mutations (τ), noted as a percentage in the figure. Only for the lowest threshold value for mutation detection (τ = 0.5%) were there synonymous mutations detected in each sample, and therefore we could perform statistical analysis only for this condition. Error bars indicate the 95% confidence interval of the estimate as determined by bootstrapping, and results of a one-sample *t*-test are indicated by ns (non-significant, $p > 0.05$) and ** ($P < 0.01$).

150 **Low mutation rate for AcMNPV with bacmid and whole-genome mutation data**

151 To make robust inferences on the mutation rate (μ) from these experimental data, we developed
152 a population genetic model. Briefly, we generated a stochastic model simulating neutral evolution
153 in a virus genome, modelled as mutation frequencies per base per viral generation. We fitted this
154 model to the experimental data by considering the number of bases with a frequency of mutations
155 above the threshold τ, using a maximum likelihood approach. We estimated the viral mutation
156 rate to be ~1×10^{-7} s/n/r, with all estimates for the neutral bacmid region and the whole genome
157 data being similar (Figure 2). Below we describe these results in more detail.

158 The simulation model was run for a range of model parameter values for mutation rate,
159 viral replication modes (ρ values of 1, 3 and 10; see also Materials and Methods section),
160 threshold values for mutation detection (τ values of 0.5%, 1% and 2%), and lengths corresponding
161 to the neutral bacmid region only and the whole virus genome (see Materials and Methods section
162 for details). Strikingly, our mutation rate estimates were robust relative to the replication mode ρ
163 value and to the choice of experimental data used (neutral region or whole genome), with
164 estimates of approximately 1×10^{-7} s/n/r in all scenarios (Figure 2, Figure S5-S6). We also
165 estimated the mutation rate with established models for sequencing data from clones [9], adapting
166 these methods to use the frequency of mutations determined by high-throughput sequencing data
167 instead of Sanger sequences from clones (see Materials and Methods). These estimates were
168 similar to those obtained with the first approach, although they tended to be about a factor 2.5
169 smaller ($\mu \leq 4 \times10^{-8}$ s/n/r, Figure S7). This difference was expected, as this alternative approach
170 does not take into consideration the effects of the mutation detection threshold τ, but rather
171 assumes all mutations will be detected irrespective of their frequency. As the sensitivity limits of
172 the deep-sequencing data is not taken into account, this approach will underestimate the mutation
173 rate.

174 One of the purported strengths of the first approach is that we have a large region (11.6
175 kb) in which point mutations will not affect fitness, given there are no known viral genes or
176 regulatory sequences (Figure 1). Although large genomic deletions in this region would
177 presumably be beneficial as they could speed up viral replication [28], none were detected:
178 Sequence coverage was similar to the rest of the genome for the evolved populations (Figures
179 S1-S4), ruling out the occurrence of large deletions at high frequencies. To test if point mutations
180 in this region were indeed neutral, we considered the rate at which mutations occurred in the
181 neutral intergenic region (dI) normalized by the rate of synonymous substitutions in viral genes
182 (dS) (see Materials and Methods). If mutations in the inserted region are neutral, we expect dI/dS
183 ~ 1. For the different values of τ used, dI/dS estimates were indeed approximately 1 (Figure 3),
184 ranging from 0.72 to 1.96. For the condition under which we could perform a formal test ($\tau > 0.5\%$),
185 dI/dS was not significantly different from 1 (One-sample *t*-test: $t_4 = 1.685$, $p = 0.167$), providing
186 support to the idea that mutations in the bacmid region are neutral. By contrast, when the same
187 analysis was performed for the rate of nonsynonymous mutations (dN) in viral genes, all dN/dS
188 values were ≤ 0.34 (Figure 3). For the condition under which we could perform a formal test ($\tau >$
189 0.5%), dN/dS was significantly lower than 1 ($t_4 = -5.717$, $p = 0.005$). This underrepresentation of
190 nonsynonymous mutations in the viral genome presumably occurs because most
191 nonsynonymous mutations will be deleterious [13,14], and therefore are removed by purifying
192 selection. Despite evidence for purifying selection acting on viral genes, mutation rate estimates
193 were similar for the neutral region and the whole genome (Figure 2).

194 The accumulation of mutations in a virus population is affected by the viral replication
195 mode (ρ) [9,10,29–31], and the distribution of mutation frequencies is linked to the mode of
196 replication [31]. Here we considered this effect by fitting the genome evolution model with values
197 $\rho = 1$ (the "geometric growth" scenario), $\rho = 3$ (mixed replication scenario) and $\rho = 10$ ("stamping
198 machine" scenario). We found that viral mode of replication had a consistent but small effect on

199 the estimated mutation rate (Figure S6), with higher values of ρ corresponding to higher mutation
200 rate estimates, as expected. The effect on model fit was minimal (Figure S5), and we therefore
201 cannot make inferences on the mode of replication from these data. This result is not surprising,
202 given that the number of mutations we detected was small and that the model fitting only considers
203 the number of sites with a mutation frequency above τ, and not the frequency of individual
204 mutations. For baculoviruses, the mode of replication has not been described formally, to the best
205 of or our knowledge. However, as these viruses probably employ rolling circle amplification [32],
206 replication is likely to follow the "stamping machine" scenario and to be described best by high
207 values of ρ.

208 **AcMNPV mutation rate estimate is congruent with estimates for other viruses**

209 Mutation rates (s/n/r) have been estimated for a number of viruses, allowing for a comparison with
210 our baculovirus estimate. For this comparison, we included a collection of mutation rate data
211 [9,33], with updated mutation rates for the RNA viruses influenza A virus [12] and poliovirus [31]
212 due to the availability of better estimates. When multiple mutation rates were available for one
213 virus, we used only the most recent estimate because methodological advances make estimates
214 that are more recent more reliable. Our estimate clearly is congruent with mutation rate estimates
215 for other dsDNA viruses, as it is close to the predicted relationship between genome size and
216 mutation rate (Figure 4). As AcMNPV has a relatively large genome, it is also one of the lowest
217 estimates of mutation rate in DNA viruses reported in the literature, similar to that of Escherichia
218 virus λ, and with only Enterobacteria phage T2 (170 kbp) being lower.

219 Besides being consistent with trends in other viruses, our estimate of mutation rate for
220 AcMNPV is congruent with what is known about its polymerase. AcMNPV codes for a DNA-
221 dependent DNA polymerase (DNApol) that belongs to the family B DNA polymerases and
222 contains the exonuclease domain, thought to be responsible for proofreading and editing of

223 mismatches [16,34,35]. The 3' to 5' exonuclease activity of this domain - essential for repairing
224 errors - has been confirmed [36], and hence a low mutation rate is expected for AcMNPV. The
225 other viruses with low mutation rates are both dsDNA bacteriophages with relatively large
226 genomes (Figure 4). Proofreading activity also has been demonstrated in the case of T2 [37].

227 **Analysis of the mutational spectrum: a low transition to transversion ratio?**

228 We considered whether our data shed light on AcMNPV's mutation spectrum, the occurrence of
229 the different kinds of single-nucleotide mutations, focusing on the transition to transversion ratio
230 (Table 2; see also Table S1). For the neutral region, the number of observed mutations is too
231 small to be informative, even for the lowest mutation detection threshold of τ = 0.5%. For the
232 whole genome and for the lowest mutation detection threshold, transitions are close to the
233 expected ratio in the absence of mutation bias (0.5), which may indicate a low transition to
234 transversion ratio. However, this trend does not hold for higher values of τ, and selection may
235 bias the mutations detected outside of the neutral region. Due to the small number of mutations
236 detected in the neutral bacmid region and the possible effects of selection on the whole-genome
237 data, we therefore cannot draw any firm conclusions on AcMNPV's mutational spectrum. Analysis
238 of a larger number of neutral mutational events will be necessary to draw conclusions on mutation
239 spectrum. These larger numbers could be achieved by analyzing a larger number of replicate
240 populations or populations evolved over a longer period of time, although improved sequencing
241 methods with much lower error rates [38] would probably suffice to analyze mutation bias for the
242 evolved populations described here.

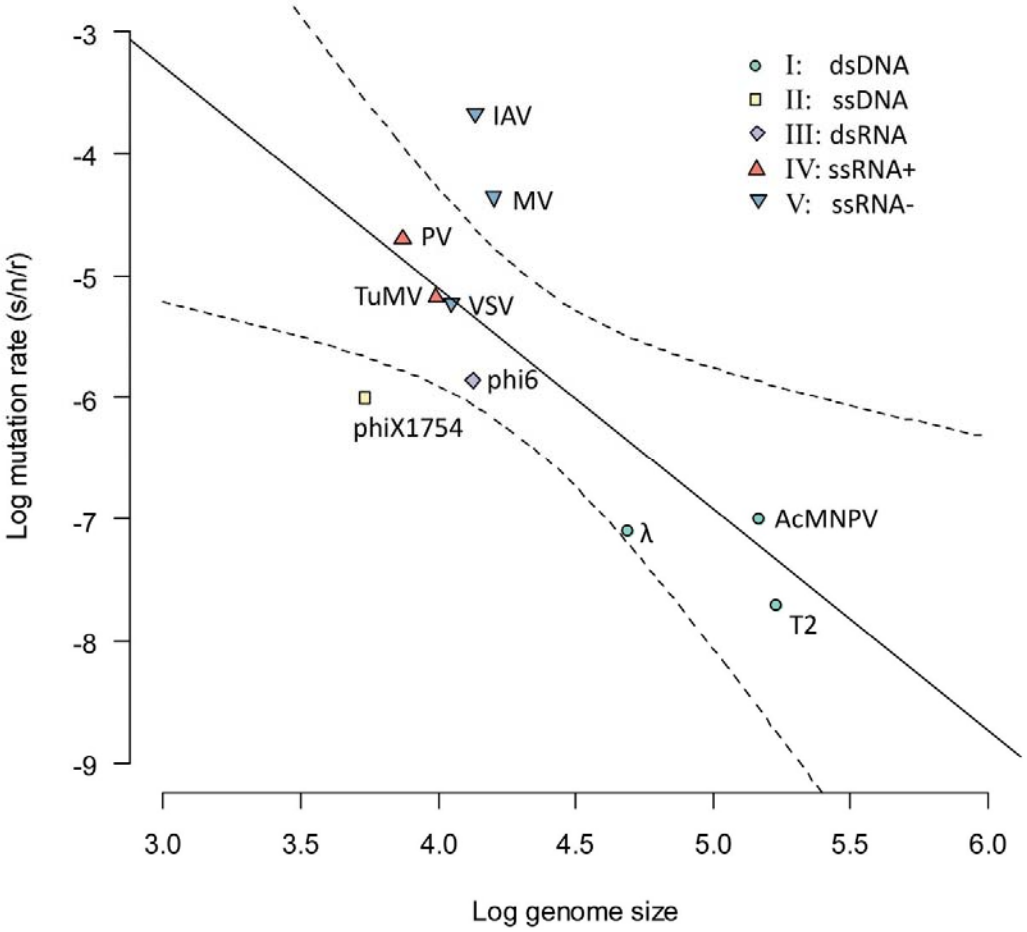

Figure 4: An overview of known mutation rate estimates (s/n/r, ordinate) for viruses with different genome sizes (abscissa) is given. The color and shapes of symbols indicate the Baltimore classification group to which each virus belongs, as indicated by the legend in the top right of the figure. The solid black line marks the regression line, with dotted lines marking the 95% confidence interval. The slope of the fitted relationship is significantly less than zero (t_8 = -3.243, p = 0.012), and the coefficient of determination (r^2) is 0.568. Our mutation rate estimate for AcMNPV is in good agreement with the fitted relationship between genome size and mutation rate. Other virus names in the figure are Enterobacteria phage T2 (T2), Escherichia virus λ (λ), Escherichia virus ΦX174 (phiX174), Influenza A virus (IAH), Measles virus (MV), Poliovirus (PV), Pseudomonas virus Φ6 (Phi6), Turnip mosaic virus (TuMV), and Vesicular stomatitis virus (VSV).

254 **Table 2:** Overview mutation spectrum

	Relative frequency of mutation			
	Whole genome			Bacmid only
Observation or index	$\tau = 0.5$	$\tau = 1.0$	$\tau = 2.0$	$\tau = 0.5$
Transitions / Transversions	28/50	9/11	6/2	4/5
Ratio[a] [95% confidence interval]	0.56 [0.34-0.91]	0.82 [0.30-2.17]	3.00 [0.54-30.3]	0.80 [0.15-3.72]

255 [a] The ratio of transitions to transversions

256 **Concluding remarks**

257 Our low mutation rate estimate is congruent with the large genome size of AcMNPV and its known
258 proofreading activity [36]. By contrast, the high genetic variation often observed within
259 alphabaculovirus populations [21-24] remains a conundrum. In one case frequency-dependent
260 selection has been observed and may account for stable polymorphisms [39,40]. Whether
261 intricate relationships between genetic variants that complement each other are common or
262 evolutionarily stable remains to be seen, but such relationships would help to explain the genetic
263 diversity often seen within alphabaculovirus populations.

264 We estimated the mutation rate for the alphabaculovirus AcMNPV, using a novel approach
265 that depends on the insertion of an artificial neutral region in the viral genome, and which starts
266 with a single genotype and exploits the genome stability of group I dsDNA viruses. Such an
267 approach requires mutations in this 'artificial' region to be neutral, and dI/dS results suggest this
268 assumption is met. Whereas others have employed rolling circle amplification of sequencing
269 templates to reduce sequencing error [38], here we developed an approach to analyzing regular
270 Illumina sequencing data that were not gathered specifically with the intent of determining
271 mutation rates. Our approach relies on a detailed analysis of the sequencing data to eliminate
272 obvious sequencing biases and a comparison of sequencing data to simulation-model predictions.
273 The use of models allows us to take into consideration the effects of viral demography on mutation
274 rate estimates, and also limit the impact of choosing thresholds for mutation detection, as the
275 same threshold is applied in the model.

276 # Materials and Methods

277 *Experimental evolution of AcMNPV*

278 pBac-E2 (BAC) was evolved experimentally in *S. exigua* larvae in five replicate lineages (A, B, C,
279 D and E). To reconstitute the virus from the bacmid, the haemocoel of *S. exigua* L4 was injected
280 with a total volume of 20 µl (i.e., 2 × 10 µl), containing a 4:1:1 mixture of Lipofectin transfection
281 reagent (ThermoFisher Scientific), water and BAC DNA (~ 15 µg DNA per larva). Upon larval
282 death, OBs were harvested from cadavers. From a single infected larva, OBs were isolated and
283 diluted to $2x10^7$ OBs/ml. Serial passage of BAC was performed five times for each replicate
284 lineage, with five larvae used for each replicate lineage. For each passage, newly molted L2 were
285 starved for 12 h and then inoculated by droplet feeding with an OB suspension exceeding 10 x
286 LC_{99} (≥ $2x10^7$ OBs/ml), to avoid narrow transmission bottlenecks. Per replicate lineage, five
287 inoculated larvae were transferred to 6-well tissue culture plates with artificial diet plugs. Larvae
288 were incubated at 26°C and with a 14 h:10 h day-night photoperiod. Upon death, larval cadavers
289 were collected, pooled and used to inoculate the next passage. After five passages, lineages A -
290 E were amplified using 100 *S. exigua* L3, and 1.5×10^9 OBs were used to extract viral genomic
291 DNA. Briefly OBs were dissolved with DAS buffer (0.1 M Na_2CO_3, 0.15 M NaCl, 10 mM EDTA,
292 pH 11), and DNA was then extracted from the liberated occlusion-derived virus particles using a
293 DNA isolation kit (Omega Bio-tek) following the manufacturer's instructions. For the BAC, DNA
294 was extracted from 50 ml LB from an overnight culture using a plasmid midi kit (Qiagen).
295 Successful genomic DNA extraction was confirmed by PCR with primers gp41 inner F (5'-
296 CAAGAGCAAAGAACCGACG-3') and inner R (5'-TTATGCAGTGCGCCCTTTCGT-3'), and
297 contamination of SeMNPV was ruled out by PCR with primers Se F (5'-
298 GACGACGAATTATGTTGTGACCGAC-3') and R (5'- AGATGGATGGAAAGGCAACGCT-3').
299 Purified DNA (~ 1.5 ug) from the evolved AcMNPV lineages A, B, C, D and E, as well as the

300 ancestral BAC, was sequenced by Illumina HiSeq PE150 (Beijing Novogene Bioinformatics

301 Technology Co., Ltd).

302 **Table 3:** Parameters for the models for estimating mutation rates

Fixed or fitted parameter	Parameter	Value	Explanation
Fixed	g	11,646	The length of the neutral bacmid region in bases.
		145,465	The length of the full genome, including the neutral bacmid region.
	λ	46	The bottleneck size for the viral founding population in each insect larvae. Following [44], the bottleneck size is related to the host survival (S) for AcMNPV infection of *S. exigua:* $\lambda = \ln(S)$. For each insect exposed to 10 x LD_{99} dose: $\lambda = 10 * \ln(1 - 0.99) \sim 46$.
	κ	5.32×10^{8}	The final size of the virus population within a single host insect. As AcMNPV generates OBs with multiple occlusion derived viruses (ODVs), and ODVs with multiple nucleocapids, each containing single copy of the genome. The mean OB yield per larvae during the experiment was $1.33*10^{6}$, based on the OB concentrations measured for each pool of five insects made after each round of passaging, assuming 100 ODV per OB (we are not aware of any empirical estimates) and 4 nucleocapsids per ODV [45].
	σ	1001	Parameter value minus one indicates the max. number of mutations per genome allowed in the simulations.
	φ	10^{4}	Threshold value of population growth for switching from stochastic to deterministic mutation.
	τ	0.5%, 1%, 2%	Minimum frequency for the detection of mutations, indicated as a percentage.
Fitted	ρ	1, 3, 10	Parameter that describes the viral mode of replication, with 1 being equivalent to equivalent to “geometric growth” by fission and large values (i.e., 10) representing “stamping machine” replication kinetics.
	$\log_{10}\mu$	-10, -9.9, -9.8, [...], -5	Mutation rate (s/n/r)

303 *Mutation calling and filtering*

304 Because the number of viral reads was not equal across samples, fastq files were subsampled to
305 ensure an approximately equal mean coverage across the reference genome for each isolate
306 using seqtk sample [41]. NGS data was analyzed using CLC Genomics Workbench 20.0 [42].
307 Reads were trimmed (quality limit = 0.05) and mapped to the reference genome (see Online
308 Materials for sequence and annotation files). Mutations were called using the “low frequency
309 variant detection tool” (minimum frequency = 0.5%). An overview of parameter settings can be
310 found in the Supplementary Online Materials (Appendix 1).

311 Due to the small likelihood of the same mutation occurring independently in two or more
312 populations, we assume mutations found in two or more evolved populations were present in the
313 ancestral population (having been present at low frequency in the BAC DNA or occurring *de novo*
314 after reconstitution of the virus. Therefore, mutations called in multiple lineages and/or in the
315 ancestral BAC isolate were filtered out. Additional filtering criteria were: forward-reverse balance
316 > 0.05, read count > 10, and the type of mutation is "SNV" (single nucleotide variant). Additionally,
317 positions with extreme coverage values were excluded. To this end, we ranked the coverage
318 value per position for each lineage and excluded the upper and lower 1%. Analyses were done
319 with three thresholds (τ) for mutation frequency: 0.5%, 1% and 2% (see Supplementary Online
320 materials, Appendices 2-4). Finally, mutations were tallied per isolate, both across the whole
321 genome and for the neutral bacmid insert.

322 *Mutation model and mutation rate estimation*

323 We generated a stochastic model that predicts the distribution of mutation frequencies per base
324 in an evolving virus genome, and then fitted this model to our empirical data with a maximum
325 likelihood approach to obtain mutation rate estimates. The model was implemented in R 4.0.3
326 [43] and all code is available (see Supplementary Online Material, Appendices 5-6). We model

327 the genome region under consideration as a vector with *g* elements for each nucleotide position,
328 with each element representing the total frequency *f* of mutated bases at position *i*. For simplicity,
329 we do not consider the identity of the mutated bases and we do not allow for reversions, as we
330 are considering scenarios in which mutations are rare and the probability of a reversion occurring
331 and reaching high frequency is very low. We assume that all mutations are strictly neutral, and
332 that all changes in mutation frequency result from the occurrence of *de novo* mutations or neutral
333 processes like stochastic changes in allele frequencies due to population bottlenecks (i.e. genetic
334 drift). All model parameter values are given in Table 3, together with additional explanation and
335 justification.

336 At the start of the infection of an individual host, there is a bottleneck with λ virus genomes
337 initiating infection. For each position in the genome, we draw the number of genomes containing
338 a mutation at this position from a binomial distribution, where for the ith position: $P(X_i = x_i) =$
339 $\binom{\lambda}{x_i} f_i^{x_i}(1 - f_i)^{\lambda - x_i}$, where X is the number of mutant genomes added to the population at a
340 particular step in the infection process (and *f* is the frequency of mutated bases at position *i*). We
341 obtained an estimate of λ = 46 by considering the relationship between host mortality and the
342 number of viral founders [44] (see Table 3). The virus population then expands within the host
343 exponentially with a replication factor ρ per cycle of virus replication within the host, such that $N_t =$
344 $\lambda(1 + \rho)^t$ where *N* is the number of genomes present at a time *t*, measured in generations of viral
345 replication within the host. It is unknown what the mode of replication [30,31] is for a baculovirus.
346 We therefore used values of 1 ("geometric" or "symmetric" replication with a doubling of the
347 number of copies per cycle – one original genome copy and one replicated copy), 3 ("mixed"
348 replication – one original genome copy and three replicated copies) and 10 ("stamping machine"
349 or "asymmetric" replication) for ρ. Replication proceeds until the carrying capacity κ of a host is
350 reached, with an expansion to exactly κ virus genomes allowed in the final round of replication.
351 During each round of replication, the number of new mutants that occur at each position follows

352 a binomial distribution, such that the mutation rate μ is the probability of success and η, the number
353 of genomes generated during that round of replication which are not mutated at this nucleotide
354 position (i.e., $\eta_{i,t} = \rho N_{t-1}(1 - f_{i,t-1})$), is the number of events: $P(X_{i,t} = x_{i,t}) = \binom{\eta_{i,t}}{x_{i,t}} \mu^{x_{i,t}}(1 -$
355 $\mu)^{\eta_{i,t} - x_{i,t}}$. However, to make the model computationally tractable for large population sizes, we
356 switched to deterministic mutation (I.e., $X_{i,t} = \eta_{i,t}\mu$) once a large number of virus genomes was
357 being generated relative to the mutation rate ($N_t\varphi > \mu^{-1}$), where φ is a constant. Note that all
358 mutations are assumed to be neutral and that the bottleneck at the start of infection is narrow (λ
359 = 46). Mutations occurring late in infection when the viral census population size is large therefore
360 will rarely be sampled during the bottleneck events at the start of the next round of infection (i.e.
361 serial transfer). To model the infection of five host larvae per replicate, we simulated five separate
362 infections and pooled the viral progeny by taking the mean mutation frequency per site over the
363 five larvae for each position in the genome, and using these f_i values for the next round of infection.
364 To fit the model to the data, we first ran 1000 simulations for each combination of
365 parameter values (i.e., μ, ρ and τ) to generate model predictions. We compared the observed
366 number of experimental replicates (q_j) with $j - 1$ mutated nucleotide positions with a frequency f_i
367 higher than the threshold value τ, to the frequency predicted by the model (β_j). (I.e., q_1 is the
368 number of experimental replicates with 0 nucleotide positions for which $f_i > \tau$, q_2 is the number of
369 replicates with 1 nucleotide positions for which $f_i > \tau$, etc.) The multinomial pseudo-likelihood of
370 any realization is then: $P(\beta_1 = q_1, \beta_2 = q_2, [\ldots], \beta_\sigma = q_\sigma) = \frac{q!}{\prod_{j-1}^{\sigma} q_j!} \prod_{j-1}^{\sigma} \beta_j{}^{q_j}$, where σ-1 = 1000 is
371 the maximum number of mutated bases that were tracked in the simulations. If any values of β
372 exceeded σ-1 in any simulation, results from that set of parameter values were excluded from
373 further analysis. We fitted the model to 1000 bootstrapped datasets to determine the 95%
374 confidence interval of the parameter estimates.
375

376 *Mutation rate estimates with established approaches*

377 We estimated the mutation rate with an established approach, to compare to the estimates made
378 with our simulation-model approach. A canonical approach [9] for calculating mutation rate (s/n/r)
379 is $\mu = \frac{3m_s}{T_s c\alpha}$, where m_s is the number of observed mutations observed in sequenced clones, T_s is
380 the mutational target size, c is the number of viral generations, and α is a correction for the effects
381 of selection. To obtain m_s from our deep sequencing data, we sum the frequency of all observed
382 mutations (f_i) above the threshold for mutation selection τ in all lineages. If these sequencing data
383 are accurate and mutations are neutral, the frequency of each mutation is also the probability that
384 this mutation would be detected in a randomly selected clone by sequencing. This approach is a
385 simple approximation, as here we do not consider the effect of the threshold for mutation detection
386 (τ) on mutation rate estimates. (Lowering τ will lead to a larger number of mutations and
387 consequently a higher mutation rate estimate. To keep this method as simple as possible and
388 free of additional assumptions, we choose not to incorporate any corrections.) Note that because
389 we sum the frequency of all possible mutations over each site, we can drop the three in the
390 numerator. To obtain T_s, we multiply the length of the neutral region by the number of replicates.
391 To obtain c we estimate the number of generations assuming different values for ρ (i.e., 1, 3 and
392 10 as for the simulation-based model fitting), such that $c = \theta \cdot ln(\kappa/\lambda)/ln(1+\rho)$, where θ is the
393 number of passages. Finally, we can drop α because we only consider mutations in the neutral
394 bacmid region. One-thousand bootstrapped datasets were used to obtain fiducial limits for the
395 mutation rate estimates.

396 *dI/dS and dN/dS analyses*

397 Estimates of dN/dS (i.e., the normalized rate of nonsynonymous mutations, here made for
398 authentic viral genes) were made using standard methods [46,47]. As we are not aware of any

399 estimates of the mutation spectrum for insect DNA viruses and our own data suggest these biases
400 may not be very strong (Table 2), we assume no mutation bias is present (i.e., a transition to
401 transversion ratio of 1). For the dI/dS [3], the dS term is the same as for the dN/dS analysis,
402 derived from the results for authentic viral genes. The dI term is determined for mutations in the
403 bacmid neutral region. Ninety-five percent confidence intervals of the dI/dS and dN/dS were
404 obtained using 1000 bootstrapped datasets, and data were tested for significance with a one-
405 sample *t*-test on the dN/dS values calculated for individual samples compared to a test value of
406 1. However, when $\tau > 0.5\%$ for one or more samples the number of synonymous samples was 0,
407 and hence these analyses could only be performed for $\tau = 0.5\%$. Full results and R code are
408 available in the Supplementary Online Information (Appendices 7-9).

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Appendix

Table S1: Relative frequencies of mutations observed per type, for the whole genome and bacmid only datasets, across mutation frequency threshold values (τ), given as a percentage.

	Relative frequency of mutation				
	Whole genome			Bacmid only	
Mutation	τ = 0.5 (n=69)	τ = 1 (n=20)	τ = 2 (n=8)	τ = 0.5 (n=9)	Mutation type
CA	0.174	0.250	0.125	0.111	Transversion
CG	0.014	0.000	0.000	0.000	Transversion
TA	0.043	0.000	0.000	0.000	Transversion
TG	0.014	0.000	0.000	0.111	Transversion
AC	0.043	0.000	0.000	0.111	Transversion
AT	0.058	0.000	0.000	0.111	Transversion
GC	0.029	0.000	0.000	0.000	Transversion
GT	0.217	0.300	0.125	0.111	Transversion
GA	0.130	0.200	0.250	0.222	Transition
CT	0.101	0.150	0.250	0.111	Transition
TC	0.072	0.050	0.125	0.000	Transition
AG	0.101	0.050	0.125	0.111	Transition

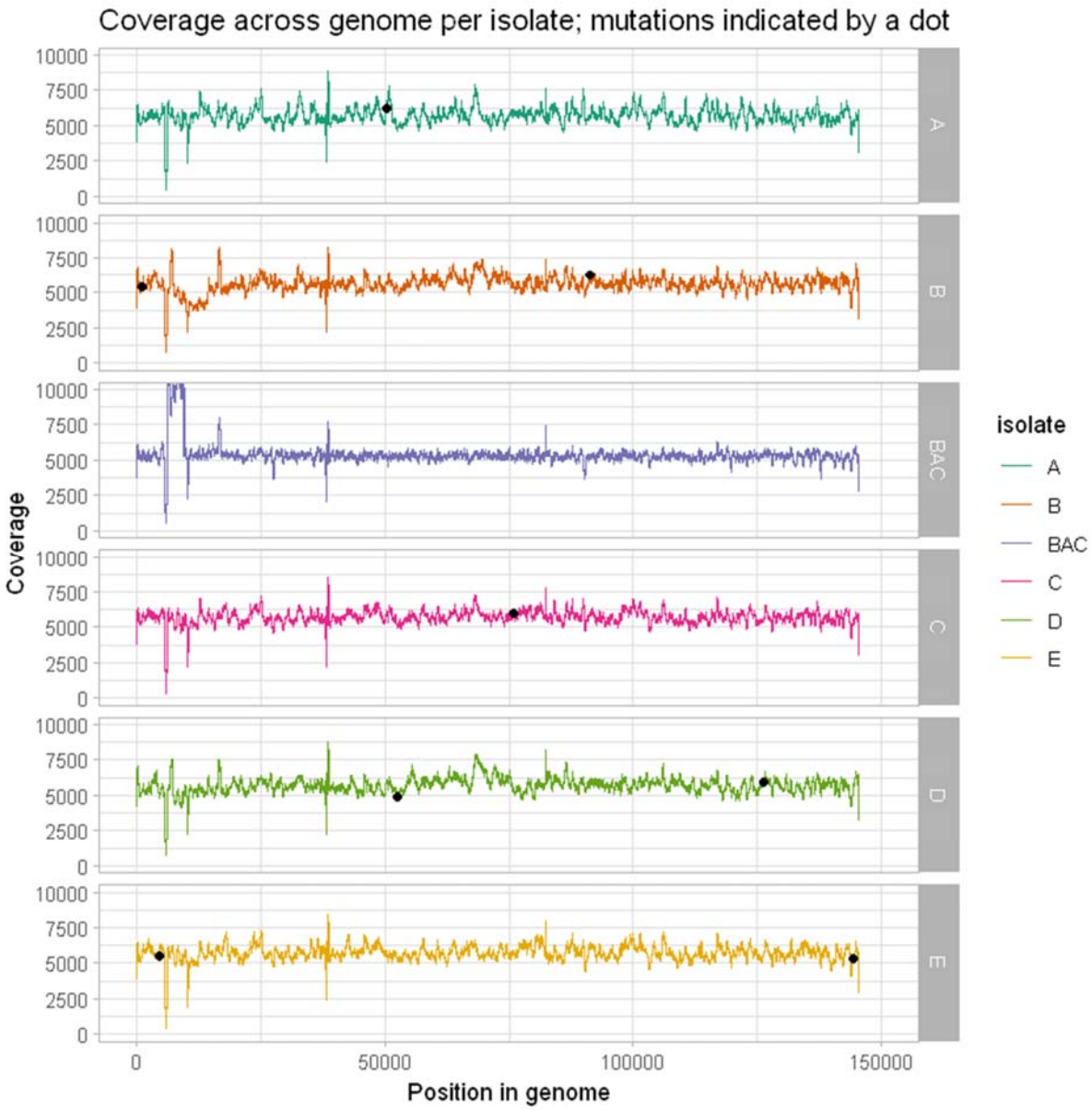

Figure S1: We show coverage along the genome for each evolved line (A, B, C, D and E) as well as ancestral strain BAC. Position of mutations observed at mutation frequency threshold value (τ) = 2 are shown as black dots. Coverage patterns are similar between the different isolates. The peak observed at around 10000 bp for the BAC isolate is due to the presence of empty bacmid vectors in sequencing data and is omitted from mutation calling.

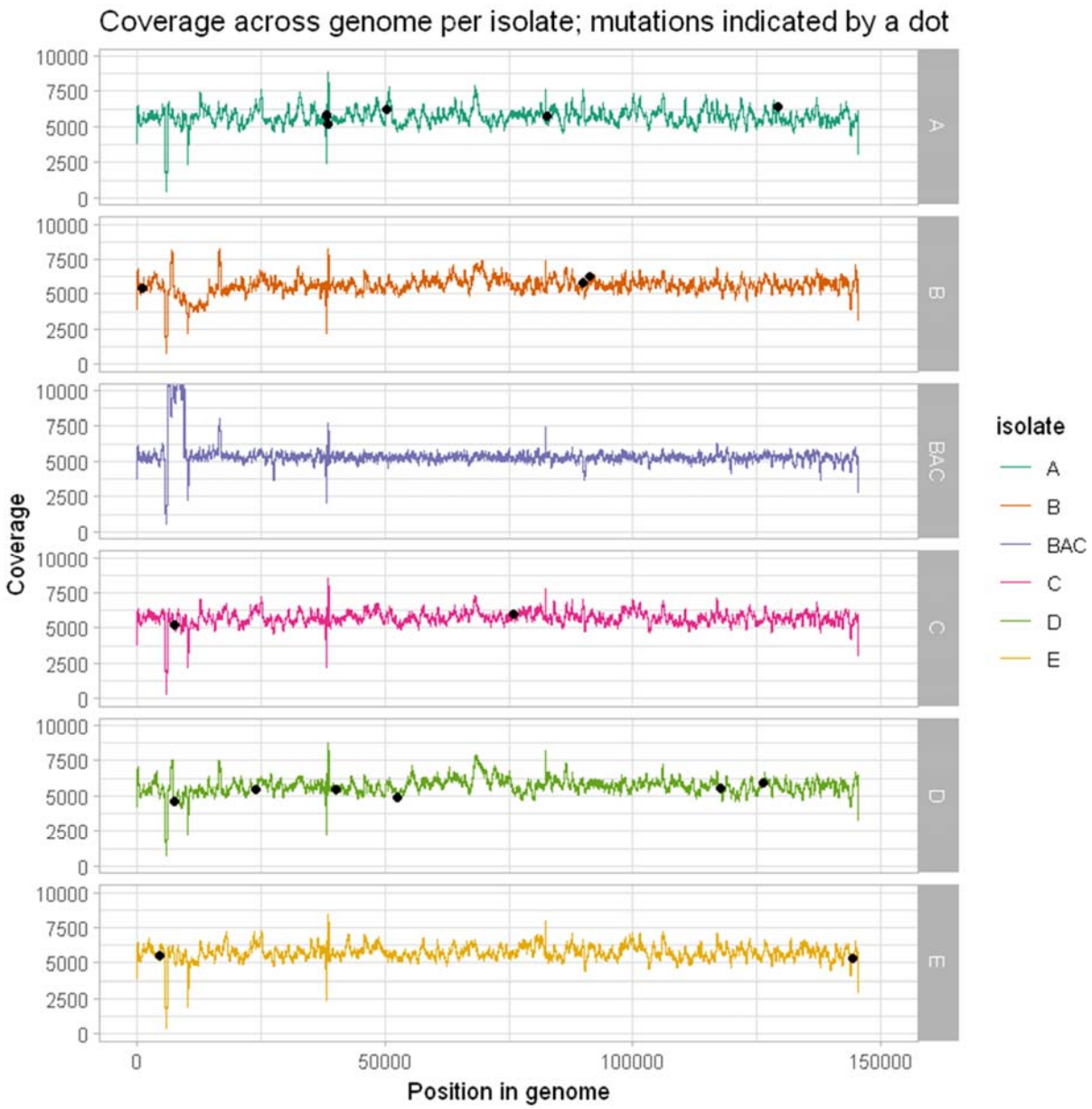

Figure S2: We show coverage along the genome for each evolved line (A, B, C, D and E) as well as ancestral strain BAC. Position of mutations observed at mutation frequency threshold value (*τ*) = 1 are shown as black dots. Coverage patterns are similar between the different isolates. The peak observed at around 10000 bp for the BAC isolate is due to the presence of empty bacmid vectors in sequencing data and is omitted from mutation calling.

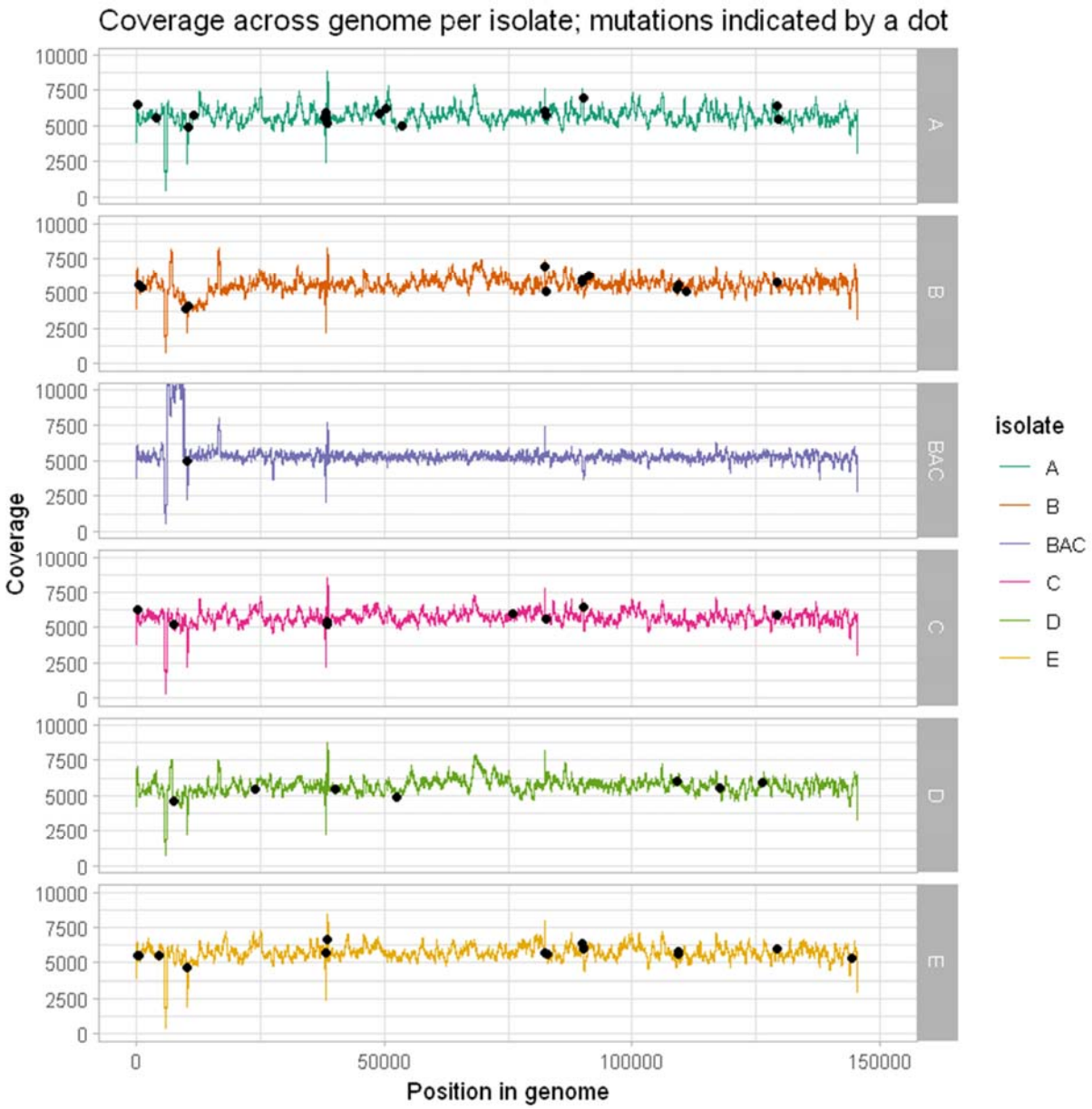

Figure S3: We show coverage along the genome for each evolved line (A, B, C, D and E) as well as ancestral strain BAC. Position of mutations observed at mutation frequency threshold value (τ) = 0.5 are shown as black dots. Coverage patterns are similar between the different isolates. The peak observed at around 10000 bp for the BAC isolate is due to the presence of empty bacmid vectors in sequencing data and is omitted from mutation calling.

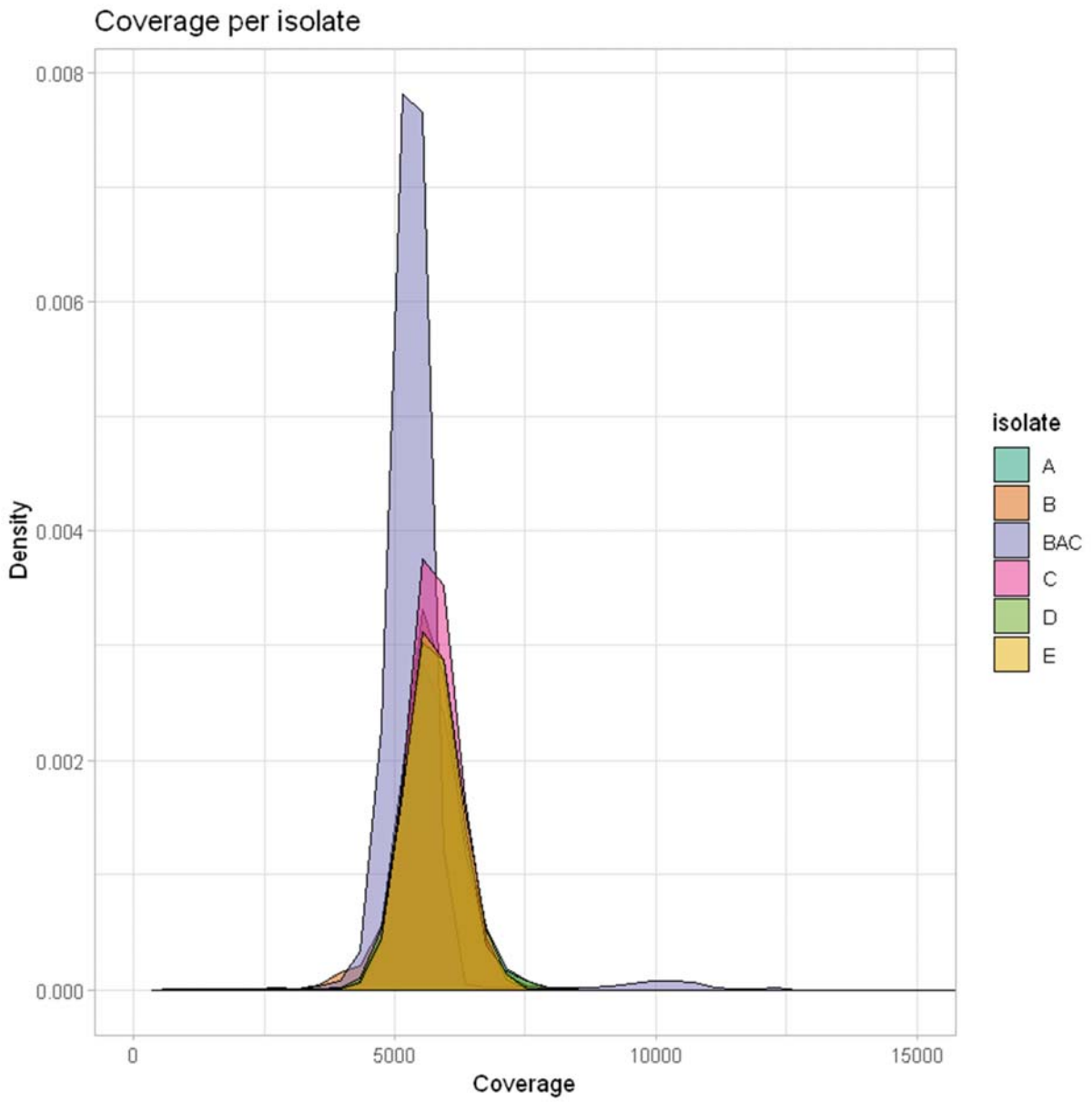

Figure S4: Coverage distribution per isolate after subsampling to approximately equal mean coverage. Isolates have a mean coverage of around 5500. The BAC isolate is showing an additional peak at a coverage of around 10000, which is explained by the presence of empty bacmid vectors in sequencing data.

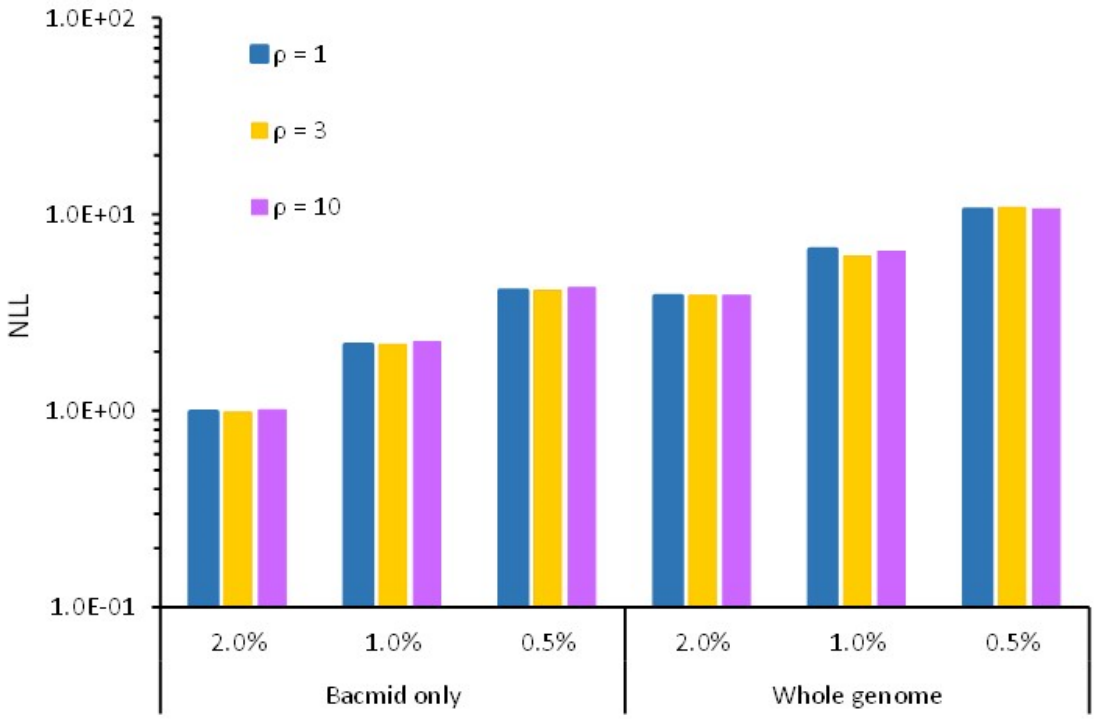

Figure S5: We show the negative log likelihood (NLL) for models fitted with different mutation frequency threshold values (τ), given as a percentage. Mutation frequency threshold values clearly effect model fit, as they have an effect on the number of mutations that will be detected. By contrast, assumptions on the value for the parameter that determines the mode of virus replication (ρ) had little effect on model fit. This result is not surprising however, given that our model does not consider the frequency of mutations, but simply the number of bases with a mutation frequency greater than τ.

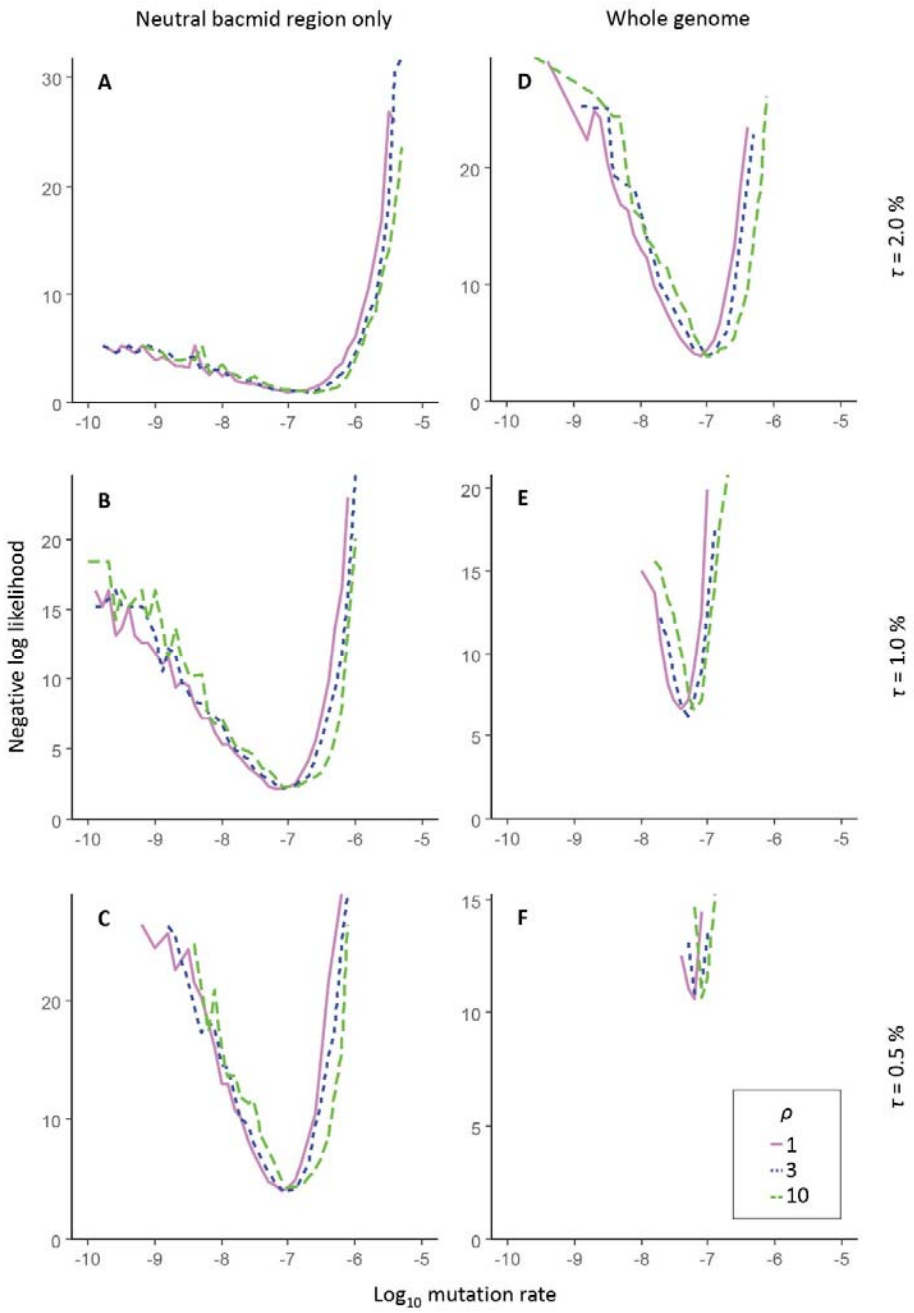

Figure S6: Results of the fitting of the simulation model to the experimental data, for making mutation rate estimates. For all panels, the abscissa is the $\log_{10}$ mutation rate and the originate the negative log likelihood. Panels on the left correspond to the neutral bacmid region data, and panels on the right to the whole genome data. Different thresholds for the detection of mutations (τ) were also employed, ranging from 0.5% (bottom row) to 1% (middle row) to 2% (top row). The different colors and line types indicate model fit for different values of ρ, the parameter that describes the viral mode of replication. Whereas higher values of ρ lead to lower mutation rate estimates, this model parameter does not impact the minimum for the NLL.

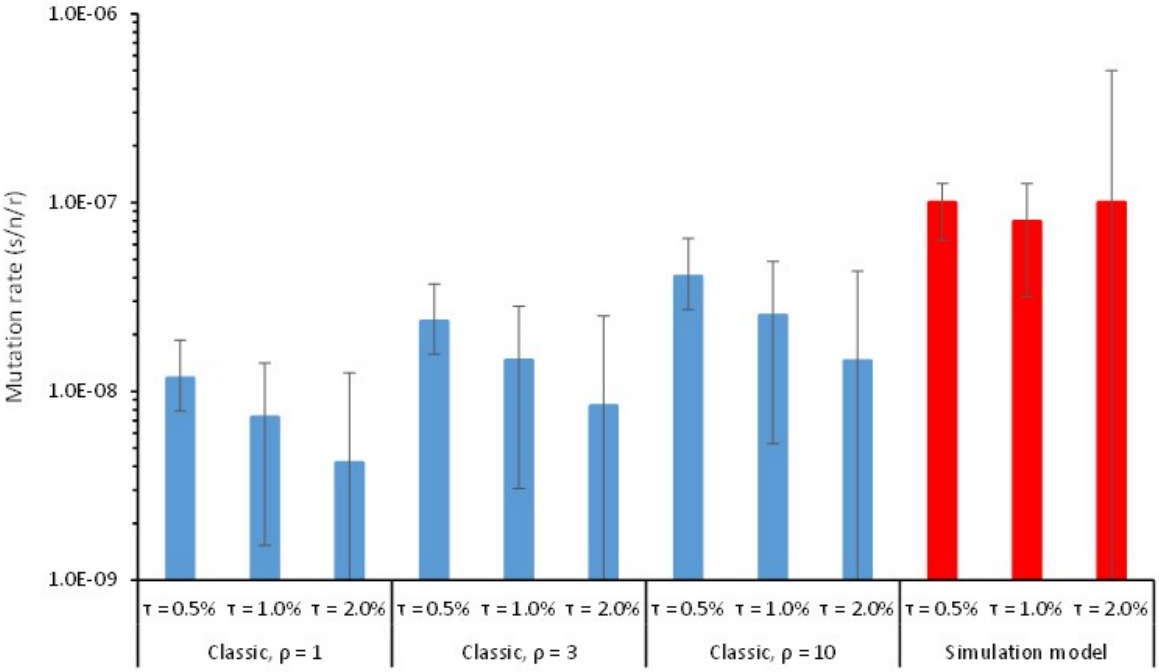

Figure S7: Estimate of mutation rate (s/n/r) using established methods applied to deep sequencing data (blue bars, samples categorized as "Classic"), as an alternative to our approach using a simulation-based model (red bars, samples categorized as "Simulation model"). Mutation rates were estimated for different values of the viral mode of replication (ρ) and with different values for the threshold of mutation detection (τ). Error bars represent the 95% fiducial limits, as determined by bootstrapping. When the lower fiducial limit extends beyond the lower limit of the axis, this indicates a lower fiducial limit of zero. Overall, these estimates were lower than those obtained with the approach employing a simulation model. As baculoviruses most likely employ rolling circle amplification, replication is likely to have a high value of ρ. Furthermore, this method is clearly sensitive to differences in the mutation detection threshold, as the cumulative frequency of mutations above this value is used required to estimate mutations and no correction is made for this threshold. Therefore, the best estimates with this approach assume the highest value of ρ and the lowest mutation detection thresholds (τ), provided all mutations are assumed to be bona fide. The estimate for these conditions (τ = 0.5% and ρ = 10), renders an estimate of $\mu = 4 \times 10^{-8}$ which is lower but roughly similar than for our simulation-based approach ($\mu \sim 10^{-7}$).