

# <sup>1</sup> Predicting stop codon reassignment improves functional <sup>2</sup> annotation of bacteriophages

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24 **Abstract**

25 The majority of bacteriophage diversity remains uncharacterised, and new intriguing  
26 mechanisms of their biology are being continually described. Members of some phage  
27 lineages, such as the *Crassvirales*, repurpose stop codons to encode an amino acid by using  
28 alternate genetic codes. Here, we investigated the prevalence of stop codon reassignment in  
29 phage genomes and subsequent impacts on functional annotation. We predicted 76  
30 genomes within INPHARED and 712 vOTUs from the Unified Human Gut Virome catalogue  
31 (UHGV) that repurpose a stop codon to encode an amino acid. We re-annotated these  
32 sequences with modified versions of Pharokka and Prokka, called Pharokka-gv and Prokka-  
33 gv, to automatically predict stop codon reassignment prior to annotation. Both tools  
34 significantly improved the quality of annotations, with Pharokka-gv performing best. For  
35 sequences predicted to repurpose TAG to glutamine (translation table 15), Pharokka-gv  
36 increased the median gene length (median of per genome medians) from 287 to 481 bp for  
37 UHGV sequences (67.8% increase) and from 318 to 550 bp for INPHARED sequences (72.9%  
38 increase). The re-annotation increased mean coding density from 66.8% to 90.0%, and from  
39 69.0% to 89.8% for UHGV and INPHARED sequences. Furthermore, the proportion of genes  
40 that could be assigned functional annotation increased, including an increase in the number  
41 of major capsid proteins that could be identified. We propose that automatic prediction of  
42 stop codon reassignment before annotation is beneficial to downstream viral genomic and  
43 metagenomic analyses.

44 **Main Body**

45 Bacteriophages, hereafter phages, are increasingly recognised as a vital component of  
46 microbial communities in all environments where they have been studied in detail. Phages  
47 are known to drive bacterial evolution and community composition through predator-prey  
48 dynamics and their potential as agents of horizontal gene transfer. The use of viral  
49 metagenomics, or viromics, has massively expanded our understanding of global viral  
50 diversity and shed light on the ecological roles that phages play.

51

52 Much of the study into viral communities has been conducted on the human gut. Here,  
53 viromics has uncovered ecologically important viruses that are difficult to bring into culture  
54 using standard laboratory techniques<sup>1</sup>, shown potential roles of viruses in disease states<sup>2</sup>,  
55 and allowed for the recovery of enormous phage genomes larger than any brought into  
56 culture<sup>3</sup>. As the majority of phage diversity remains uncharacterised, new and enigmatic  
57 diversification mechanisms are being described continually, including the potential use of  
58 alternative translation tables.

59

60 Lineage-specific stop codon reassignment has been described previously in  
61 bacteriophages<sup>4,5</sup>, whereby a stop codon is repurposed to encode an amino acid. Notably,  
62 annotations of Lak “megaphages” assembled from metagenomes were observed to exhibit  
63 unusually low coding density (~70%) when genes are predicted using the standard bacterial,  
64 archaeal and plant plastid genetic code (translation table 11)<sup>3</sup>, much lower than the value  
65 observed for most cultured phages of ~90%<sup>6</sup>. The Lak megaphages were predicted to  
66 repurpose the TAG stop codon into an as-of-yet unknown amino acid<sup>3</sup>. More recently,  
67 uncultured members of *Crassvirales* have been predicted to repurpose TAG to glutamine  
68 (translation table 15), and TGA to tryptophan (translation table 4)<sup>5</sup>, and since then the use of  
69 translation table 15 has been experimentally validated in two phages belonging to  
70 *Crassvirales*<sup>7</sup>. As this feature may be widespread in human gut viruses, we trained a fork of  
71 Prodigal<sup>8</sup>, named prodigal-gv, to predict stop codon reassignment in phages<sup>9</sup> and  
72 implemented in the pyrodigal-gv library to provide efficient Cython bindings to Prodigal-gv  
73 with pyrodigal<sup>10</sup>. Additionally, the virus discovery tool geNomad incorporates pyrodigal-gv to  
74 predict stop codon reassignment for viral sequences identified in metagenomes and

75 viromes<sup>9</sup>. However, the detection of translation table 15 still has limited support in many  
76 tools, and the impacts of stop codon reassignment are rarely considered in viral genomics  
77 and metagenomics.

78

79 To assess the extent of stop codon reassignment in studied phage genomes and the impacts  
80 on functional annotation, we extracted phage genomes from INPHARED<sup>6</sup> and predicted  
81 those using alternative stop codons. We also added high-quality and complete vOTUs from  
82 the Unified Human Gut Virome Catalog (UHGV; <https://github.com/snayfach/UHGV>)  
83 predicted to use alternative codons. The viral genomes were re-annotated using modified  
84 versions of the commonly used annotation pipelines Prokka<sup>11</sup>, and Pharokka<sup>12</sup> implementing  
85 prodigal-gv/pyrodigal-gv for gene prediction (Supplementary Methods). Hereafter, the  
86 modified versions are referred to Prokka-gv and Pharokka-gv.

87

88 From INPHARED, 49 genomes (0.24%) were predicted to use translation table 15, and 27  
89 (0.13%) were predicted to use translation table 4. From the UHGV, 666 vOTUs (1.2%) were  
90 predicted to use translation table 15 and 46 (0.08%) were predicted to use translation table  
91 4. These genomes and vOTUs were not constrained to one particular clade of viruses, being  
92 predicted to occur on both dsDNA viruses of the realm *Duplodnaviria* and ssDNA viruses of  
93 the realm *Monodnaviria*, suggesting it is a phenomenon that has arisen on at least two  
94 occasions (Supplementary Table 1). The lower frequency of these genomes in cultured  
95 isolates (INPHARED) versus human viromes (UHGV) may be due to culturing and sequencing  
96 biases, perhaps including modifications to DNA that are known to be recalcitrant to  
97 sequencing.

98

99 Although the mechanism for stop codon reassignment in phages is not fully understood,  
100 suppressor tRNAs are suggested to play a role<sup>4,13</sup>. Consistent with previous findings, we  
101 found 375/715 (52.4%) phages predicted to use translation table 15 encoded at least one  
102 suppressor tRNA corresponding to the *amber* stop codon (Sup-CTA tRNA), and 11/73 (15.1%)  
103 of those predicted to use translation table 4 encoded at least one suppressor tRNA  
104 corresponding to the *opal* stop codon (Sup-TCA tRNA)<sup>4,13,14</sup>. Although fewer of those  
105 predicted to use translation table 4 encoded the relevant suppressor tRNA, 22/27 (81%) of  
106 the INPHARED phages predicted to use translation table 4 were viruses of *Mycoplasma* or

107 *Spiroplasma*. As *Mycoplasma* and *Spiroplasma* are known to use translation table 4, many of  
108 the viruses predicted to use translation table 4 may be simply using the same translation  
109 table as their host.

110

111 Prediction of stop codon reassignment led to improved annotations for both Prokka and  
112 Pharokka, although the extent of this varied with the two datasets, translation tables, and  
113 annotation pipelines tested. As Pharokka-gv outperformed Prokka-gv on all metrics tested,  
114 only Pharokka-gv is discussed further, and the equivalent results for Prokka-gv can be found  
115 in Supplementary Results.

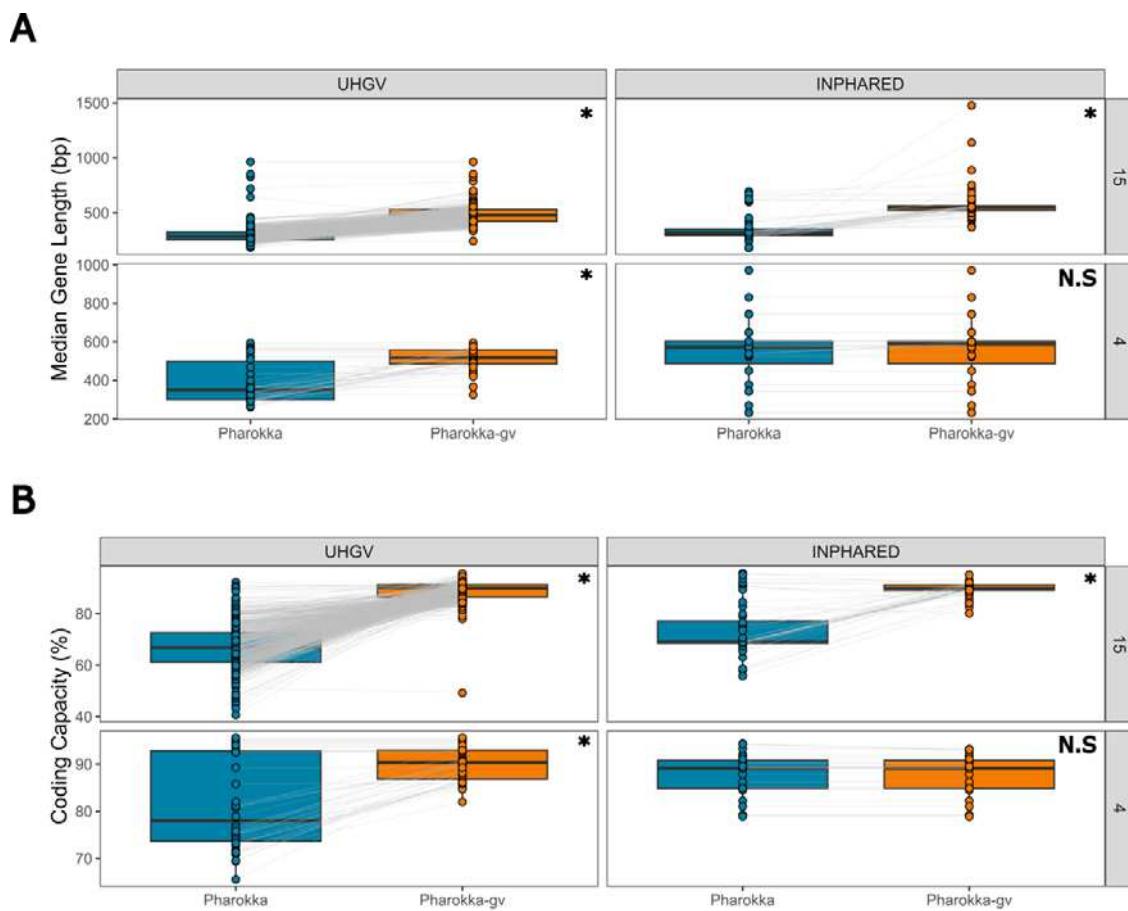
116

117 The largest differences were observed for sequences predicted to use translation table 15,  
118 for which Pharokka-gv increased the median gene length (median of per genome medians)  
119 from 287 to 481 bp for UHGV sequences (67.8% increase) and from 318 to 550 bp for  
120 INPHARED sequences (72.9% increase; Figure 1A). This was also reflected in an increase of  
121 median coding capacity from 66.8% to 90.0% for UHGV, and 69.0% to 89.8% for INPHARED  
122 (Figure 1B). Overall, these improved gene calls led to an increased gene length, and a  
123 reduction in the number of predicted genes per kb and the number of genes that could not  
124 be assigned functional annotations (Supplementary Figure 2; Supplementary Table 2). As it is  
125 commonly used as a phylogenetic marker for bacteriophages, we investigated how  
126 commonly the major capsid protein (MCP) could be identified with and without predicted  
127 stop codon reassignment<sup>15</sup>. For those viruses we predicted to use translation table 15,  
128 annotation using the default translation table 11 only resulted in the MCP being identified in  
129 407/715 (56.9%) of the genomes. In contrast, using translation table 15 with Pharokka-gv,  
130 we could identify the MCP in 475/715 (66.4%).

131

132 When investigating the sequences for which translation table 4 was predicted to be optimal,  
133 a substantial increase was also observed for UHGV sequences, with Pharokka-gv increasing  
134 median gene length (median of per genome medians) from 350 to 518 bp (a 48.0% increase  
135 in length; Figure 1A), resulting in an increase of coding capacity from 78.0% to 90.4% (Figure  
136 1B). However, the same was not observed for the 27 INPHARED genomes predicted to use  
137 translation table 4. Reannotation resulted in a modest increase in median gene length  
138 (median of per genome medians) from 573 to 588 bp (a 2.6% increase in length; Figure 1A).

139 Median coding capacity was not increased, with both Pharokka and Pharokka-gv obtaining  
140 89.1% (Figure 1B). As the median gene length and coding capacity for INPHARED sequences  
141 predicted to use translation table 4 are in line with expected values, their prediction may be  
142 a false positive. Reassuringly, the prediction of translation table 4 has not hindered the  
143 quality of annotations where it may be a false positive.



144

145

146 **Figure 1.** Re-annotating with predicted stop codon reassignment increases the quality of  
147 annotations. Comparison of **(A)** median predicted gene length (bp) and **(B)** coding capacity  
148 (%) for INPHARED genomes and UHGV vOTUs annotated with Pharokka (translation table 11  
149 only) and Pharokka-gv (prediction of stop codon reassignment), grouped by dataset and  
150 predicted stop codon reassignment. Asterisk indicates significance at  $P \leq 10e-10$  with  $P$   
151 determined by a simple T test and adjusted with the Benjamini-Hochberg procedure.

152 The analysis of viral (meta)genomes relies on accurate protein predictions, with predicted  
153 ORFs being used in common analyses, including (pro)phage prediction, functional  
154 annotation, and phylogenetic analyses. The clear differences in protein predictions  
155 with/without predicted stop codon reassignment will likely have downstream impacts upon  
156 these analyses. However, this phenomenon is not yet widely considered in viral  
157 (meta)genomics. We have demonstrated the impacts of stop codon reassignment in the  
158 functional annotation of phages, and provide tools for the automatic prediction and  
159 annotation of viral genomes that repurpose stop codons. Our analysis highlights the need for  
160 accurate viral ORF prediction, and further experimental validation to elucidate the  
161 mechanisms of stop codon reassignment.

162 **Data Availability**

163 The genomes used in this analysis are from two publicly available datasets; INPHARED  
164 (<https://github.com/RyanCook94/inphared>) and the Unified Human Gut Virome (UHGV;  
165 <https://github.com/snayfach/UHGV>). The details of included sequences are shown in  
166 Supplementary Table 1. The code for Prokka-gv is available on GitHub  
167 (<https://github.com/telatin/metaprokka>). The code for Pharokka is available on GitHub  
168 (<https://github.com/gbouras13/pharokka>). The code for Prodigal-gv is available on GitHub  
169 (<https://github.com/apcamargo/prodigal-gv>). The code for Pyrodigal-gv is available on  
170 GitHub (<https://github.com/althonos/pyrodigal-gv>).  
171

172 **Competing Interests**

173 The authors have nothing to declare.  
174

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227 **Supplementary Methods**

228

229 **Datasets**

230 A multifasta file of phage genomes was downloaded from INPHARED  
231 (<https://github.com/RyanCook94/inphared>; September 2023)<sup>6</sup>. Stop codon reassignment of  
232 INPHARED genomes was predicted using Prodigal-gv v2.11.0  
233 (<https://github.com/apcamargo/prodigal-gv>), a fork of Prodigal written to improve viral gene  
234 calling<sup>8</sup>. Those predicted to use translation table 4 or 15 were retained for downstream  
235 analysis.

236

237 The Unified Human Gut Virome Catalog (UHGV) was filtered for high quality and complete  
238 vOTUs deemed to be a “high confidence” virus and predicted to use either translation table  
239 4 or 15 (<https://github.com/snayfach/UHGV>). Stop codon reassignment had already been  
240 predicted for UHGV vOTUs using Prodigal-gv and is available in the UHGV metadata.

241

242 **Prokka**

243 A fork of Prokka v1.14.5<sup>11</sup> was written that incorporates an initial stage of ORF prediction  
244 using Prodigal-gv v2.11.0 (<https://github.com/apcamargo/prodigal-gv>)<sup>8</sup>. A first gene calling  
245 step is used to infer the genetic code most likely adopted by the genome, then the predicted  
246 genetic code is used to perform the translation FASTX::Seq, which we updated to accept  
247 code 15 ([metacpan.org/pod/FASTX::Seq](https://metacpan.org/pod/FASTX::Seq))<sup>16</sup>. The code for this is available at  
248 ([github.com/telatin/metaprokka](https://github.com/telatin/metaprokka)). We included publicly available HMMs of the PHROGs  
249 database in our Prokka-gv annotations  
250 ([http://s3.climb.ac.uk/ADM\\_share/all\\_phrogs.hmm.gz](https://s3.climb.ac.uk/ADM_share/all_phrogs.hmm.gz))<sup>17</sup>. The fork is installable from  
251 Bioconda as ‘metaprokka’.

252

253 **Pharokka**

254 Pharokka v1.5.0<sup>12</sup> was updated to include support for pyrodigal-gv implementing pyrodigal-  
255 gv as a gene predictor. This is specified by using ‘-g prodigal-gv’ when running Pharokka. The  
256 updated code is available on GitHub (<https://github.com/gbouras13/pharokka>). Pharokka  
257 uses tRNAscan-SE for predicting tRNAs<sup>14</sup>.

258

259 **Statistical Analyses and Data Visualisation**

260 To test for significance in differences of results, a simple paired T test was performed in R

261 v4.2.2<sup>18</sup> and P-values were adjusted using the Benjamini-Hochberg procedure<sup>19</sup>. Figure 1 was

262 produced using ggplot2 v3.4.2<sup>20</sup>.

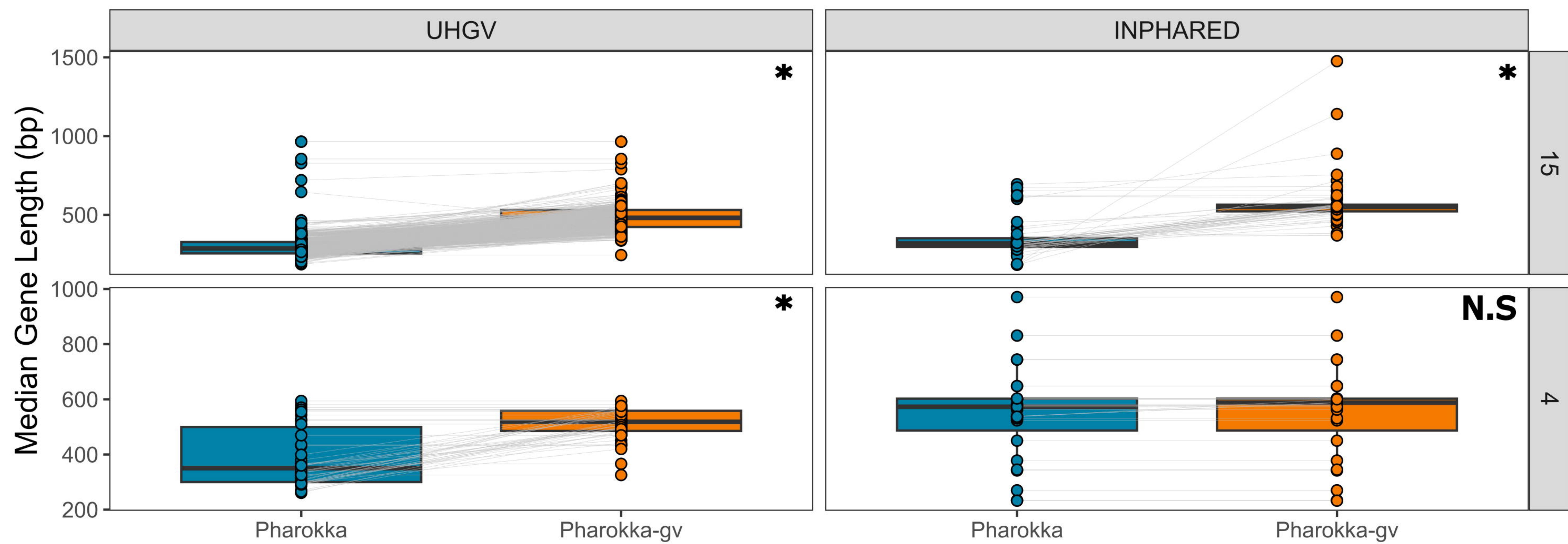
263 **Supplementary Results**

264 **Prokka-gv Annotations**

265 For Prokka-gv, the largest differences were observed for sequences predicted to use  
266 translation table 15, for which Prokka-gv increased the median gene length (median of per  
267 genome medians) from 276 to 396 bp for UHGV sequences (43.5% increase), and from 309  
268 to 483 bp for INPHARED sequences (56.3% increase). This was also reflected in an increase  
269 of median coding capacity from 66.6% to 86.7% for UHGV, and from 69.2% to 87.3% for  
270 INPHARED. As it is commonly used as a phylogenetic marker for bacteriophages, we  
271 investigated how commonly the major capsid protein (MCP) could be identified with and  
272 without predicted stop codon reassignment<sup>15</sup>. For sequences predicted to use translation  
273 table 15, the MCP could be identified on 382/715 (53.4%) sequences with Prokka and this  
274 was marginally increased to 386/715 (53.9%) with Prokka-gv.

275

276 When investigating the sequences for which translation table 4 was predicted, a substantial  
277 increase was also observed for UHGV sequences, with Prokka-gv increasing median median  
278 gene length from 319 to 460 bp (44.2%), resulting in an increase of coding capacity from  
279 78.4% to 91.4%. However, the same was not observed for INPHARED sequences predicted to  
280 use translation table 4. These sequences observed a modest increase in median median  
281 gene length from 573 to 584 bp (1.8%) for Prokka-gv. Median coding capacity was not  
282 increased with Prokka and Prokka-gv both obtaining 86.2%.

**A****B**