

1 **The CRISPR spacer space is dominated by sequences from the species-specific**  
2 **mobilome**

3 Sergey A. Shmakov<sup>1,2</sup>, Vassilii Sitnik<sup>1</sup>, Kira S. Makarova<sup>2</sup>, Yuri I. Wolf<sup>2</sup>,  
4 Konstantin V. Severinov<sup>1,3,4</sup>, Eugene V. Koonin<sup>2,\*</sup>

5

6 <sup>1</sup>Skolkovo Institute of Science and Technology, Skolkovo, 143025, Russia

7 <sup>2</sup>National Center for Biotechnology Information, National Library of Medicine,  
8 Bethesda, MD 20894

9 <sup>3</sup>Waksman Institute for Microbiology Rutgers, The State University of New Jersey  
10 Piscataway, NJ 08854, USA

11 <sup>4</sup>Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182, Russia

12

13

14 \*For correspondence: [koonin@ncbi.nlm.nih.gov](mailto:koonin@ncbi.nlm.nih.gov)

15

16

17 **The CRISPR-Cas is the prokaryotic adaptive immunity system that stores memory of past**  
18 **encounters with foreign DNA in spacers that are inserted between direct repeats in**  
19 **CRISPR arrays**<sup>1,2</sup>. **Only for a small fraction of the spacers, homologous sequences, termed**  
20 **protospacers, are detectable in viral, plasmid or microbial genomes**<sup>3,4</sup>. **The rest of the**  
21 **spacers remain the CRISPR “dark matter”. We performed a comprehensive analysis of the**  
22 **spacers from all CRISPR-*cas* loci identified in bacterial and archaeal genomes, and found**  
23 **that, depending on the CRISPR-Cas subtype and the prokaryotic phylum, protospacers**  
24 **were detectable for 1 to about 19% of the spacers (~7% global average). Among the**  
25 **detected protospacers, the majority, typically, 80 to 90%, originate from viral genomes,**  
26 **and among the rest, the most common source are genes integrated in microbial**  
27 **chromosomes but involved in plasmid conjugation or replication. Thus, almost all spacers**  
28 **with identifiable protospacers target mobile genetic elements (MGE). The GC-content, as**  
29 **well as dinucleotide and tetranucleotide compositions, of microbial genomes, their spacer**  
30 **complements, and the cognate viral genomes show a nearly perfect correlation and are**  
31 **almost identical. Given the near absence of self-targeting spacers, these findings are best**  
32 **compatible with the possibility that the spacers, including the dark matter, are derived**  
33 **almost completely from the species-specific microbial mobilomes.**

34

35 Driven by the overwhelming success of the Cas9 and later Cpf1 endonucleases as the new  
36 generation of genome editing tools, comparative genomics, structures, biochemical activities and  
37 biological functions of CRISPR (Clustered Regularly Interspaced Palindromic Repeats)-Cas  
38 (CRISPR-associated proteins) systems have been recently explored in unprecedented detail<sup>1,2,5,6</sup>.  
39 The CRISPR-Cas are adaptive (acquired) immune systems of archaea and bacteria that store  
40 memory of past encounters with foreign DNA in unique spacer sequences that are excised from  
41 viral and plasmid genomes by the Cas adaptation machinery, or alternatively, reverse transcribed  
42 from foreign RNA and inserted into CRISPR arrays<sup>7,8</sup>. Transcripts of the spacers, together with  
43 portions of the surrounding repeats, are employed by Cas effector complexes as guide CRISPR  
44 (cr)RNAs to recognize the cognate sequences (protospacers) in the foreign genomes upon  
45 subsequent encounters, directing Cas nucleases to their cleavage sites<sup>9,10</sup> and limiting  
46 bacteriophage infection and horizontal gene transfer.<sup>REF</sup>

47 One of the burning open questions in the CRISPR area is the origin of the bulk of the spacers.  
48 For a small fraction of the spacers, protospacers have been reported, often in viral and plasmid  
49 genomes, but the overwhelming majority of the spacers remain without a match<sup>3,4,11-15</sup>. In order  
50 to get insight into the origin of this “dark matter”, we performed comprehensive searches of the  
51 current genomic and metagenomic sequence databases using all identifiable spacer sequences  
52 from complete bacterial and archaeal genomes as queries. To this end, a computational pipeline  
53 was developed that identified all CRISPR arrays from complete and partial bacterial and archaeal  
54 genomes, extracted the spacers and used them as queries to search the viral and prokaryotic  
55 subsets of the Non-redundant nucleotide database at the NCBI (NIH, Bethesda) for protospacers  
56 under stringent criteria for homology detection (Supplementary Figure 1 and Supplementary text  
57 1; see Methods for details).

58 These searches yielded 2,981 spacer matches (protospacers) in viral sequences and 23,385  
59 matches in prokaryotic sequences. We then examined the provenance of the detected  
60 protospacers across the diversity of the CRISPR-Cas systems and the prokaryotic phyla. In a  
61 general agreement with previous analyses that, however, have been performed on much smaller  
62 genomic data sets, protospacers were identified for ~7% of the spacers, with the fractions for  
63 different CRISPR-Cas subtypes ranging from 1 to 19% (Table 1). The fraction of detected  
64 protospacers was typically higher for type I and II CRISPR-Cas systems, in which it spans the  
65 entire range, compared to type III, where this fraction was uniformly low, at 1 to 2% (Table 1).

66 A similar range was detected for the fraction of spacers with matches across the bacterial and  
67 archaeal phyla (Table 2) but substantial deviations from the global average of ~7% in several  
68 phyla are notable. Thus, anomalously high fractions of spacers with matches were detected in  
69 *Spirochaetia*, *Fusobacteria* and  $\gamma$ -*Proteobacteria*. In a sharp contrast, the CRISPR arrays in  
70 archaea, especially hyperthermophiles, had low fraction of matching spacers, with none at all  
71 detected in *Thermococci* and *Thermoplasmata*; furthermore, the only phylum of  
72 hyperthermophilic bacteria, for which a large number of CRISPR arrays was identified, also had  
73 only 1% of matching spacers (Table 2). A multiple regression analysis shows that both the  
74 assignment to a CRISPR subtype and classification into an archaeal or bacterial phylum make  
75 substantial and largely independent contributions to the variation of the fraction of spacers with  
76 detectable matches; jointly, the two factors explain about 75% of the variance of that fraction

77 (see Supplementary text 1). The paucity of spacer matches in hyperthermophiles is puzzling  
78 because all these organisms possess CRISPR-*cas* loci (as opposed to only a minority among  
79 mesophiles)<sup>16</sup>, with the implication that CRISPR activity is essential for the survival of these  
80 organisms. The lack of recognizable spacers could be due to under-sampling of the respective  
81 virome and/or to preferential utilization of partially matching spacers by the CRISPR-Cas  
82 systems of thermophiles. Generally, the aspects of the biology of different groups of prokaryotes  
83 that might determine the activity of the CRISPR-Cas systems, and hence the fraction of spacers  
84 with matches, remain to be explored.

85 The CRISPR-Cas spacers have been demonstrated to insert in a polarized fashion, mostly in the  
86 beginning of arrays, adjacent to the leader sequence (although in some case, internal insertion  
87 has been observed as well), resulting in unidirectional growth of the array that, however,  
88 subsequently contracts via loss of distal spacers<sup>17,18</sup>. Indeed, a notable excess of spacers with  
89 matches was observed near the ends of the arrays, with a sharp decline downstream (Figure  
90 1A,B), indicating that a large fraction of recently acquired spacers originate from sequences  
91 available in current databases.

92 In most subtypes of CRISPR-Cas from most bacterial and archaeal phyla, 70 to 90% of the  
93 protospacers originated from virus or provirus sequences (proviruses were consistently identified  
94 with two independent approaches; see Supplementary figure 2 and Methods for details) (Tables 1  
95 and 2), in agreement with the common notion that CRISPR-Cas is primarily engaged in antiviral  
96 defense. Notably, subsets of virus-specific spacers are shared between different species and even  
97 genera of bacteria (e.g. *Staphylococcus-Streptococcus* and *Escherichia-Cronobacter*), which  
98 yields a host-virus network that includes several large connected components (Supplementary  
99 Figure 3, Supplementary data set 1). Analysis of the provenance of the non-viral protospacers  
100 showed a clear preponderance of sequences from gene families implicated in conjugal transfer  
101 and replication of plasmids, such as type IV secretion systems<sup>19</sup> (Figure 2 and Supplementary  
102 data set 2). Notably, several protospacers also originated from *cas* genes, particularly *cas3*  
103 (Figure 2 and Supplementary Table 1), recapitulating the recent finding of *cas*-matching  
104 protospacers in orphan CRISPR arrays<sup>20</sup>. Of the remaining genes containing protospacers,  
105 many are unannotated, which is typically caused by low sequence conservation, and potentially  
106 could originate from viruses or plasmids as well. A small fraction of spacer matches map to

107 genomic regions annotated as intergenic (Tables 1 and 2) but manual examination of such cases  
108 led to identification of putative protein-coding genes that apparently have been missed by  
109 genome annotation (Supplementary text 2). Complete reannotation of the available prokaryotic  
110 genomes is a demanding project outside the scope of this work but, with this caveat, only a small  
111 fraction of the detected protospacers could be traced to sequences demonstrably not originating  
112 from viruses or other mobile elements. Previous analyses of CRISPR arrays from individual  
113 bacterial and archaeal genomes have reported widely different fractions of self-matching spacers  
114 <sup>1,21</sup>. Our current, comprehensive analysis indicates that the overwhelming majority of the spacers  
115 that persist long enough to be detected are derived from viruses and other mobile elements  
116 (collectively, known as the mobilome <sup>22</sup>), apparently indicating strong selection against self-  
117 targeting spacers.

118 Where do the ~93% of the spacers that comprise the dark matter of CRISPR arrays come from?  
119 In an attempt to gain insight into the origin of these spacers, we compared the nucleotide  
120 compositions of the spacers, the respective prokaryotic genomes and the virus genomes  
121 containing the corresponding protospacers. The compositions of the three sequence sets showed  
122 near perfect correlation and were almost identical across the entire range of the GC-content;  
123 closely similar results were obtained regardless of whether all spacers or only spacers with  
124 matches were included (Figure 3A,B). Compatible results were obtained when we compared  
125 dinucleotide and tetranucleotide compositions among the same sequence sets using principal  
126 component analysis: all points formed a homogeneous cloud, without any detectable partitioning  
127 (Supplementary figures 4 and 5). Given the wide range of the GC-content covered, from ~20 to  
128 ~70% and the near indistinguishable features of the three sets of sequence, these observations  
129 strongly suggest that they all come from a single, intermixing, species-specific sequence pool.  
130 Bacteriophage genomes are generally considered to have a lower GC-content than the host  
131 genomes such that prophages form AT-rich genomic islands <sup>23</sup>, which seems to be at odds with  
132 the near perfect correlation we observed. To investigate this discrepancy, we compared the GC-  
133 content of phage and host genomes for several bacteria for which numerous phages have been  
134 characterized; all available phage genomes were included in this analysis, regardless whether or  
135 not corresponding spacers were detected. In most cases, there was indeed considerable AT-bias  
136 in phages but numerous phage genomes had the same composition as the host and spacers

137 (Figure 4). Conceivably, the spacers come from the most abundant phages that match the hosts in  
138 the GC-content.

139 We further investigated the provenance of the dark matter spacers using an alternative approach.  
140 Matches to genomes from different microbial taxa, in the range from strains within the same  
141 species to different domains (archaea and bacteria), were tallied for the CRISPR spacers and for  
142 ‘mock spacers’, i.e. 1000 randomly sampled sequence segments of the same length from each  
143 CRISPR-carrying genome. The distributions of the matches were substantially different for the  
144 two sequence sets: the spacers matched genomic sequences almost exclusively within the same  
145 species, and almost none were found outside the same genus, whereas for the mock spacers,  
146 numerous matches were detected in distantly related genomes (Figure 5A). The distributions of  
147 the number of matches per (mock) spacer are quite different also, with the spacers being largely  
148 unique or matching only a few sequences, in contrast to the distribution for the ‘mock spacers’  
149 that was dominated by a peak of abundant matches (Figure 5B). These observations indicate that  
150 the protospacers come from a sequence pool that is sharply different from the average genomic  
151 sequence in terms of evolutionary conservation. The protospacer sequences are extremely poorly  
152 conserved, which is the property of the mobilome.

153 In the present dissection of the CRISPR (proto)spacer space, we made two principal  
154 observations. First, the spacers with detectable protospacer matches that persist in CRISPR  
155 arrays originate (almost) exclusively from genomes of mobile elements, mostly viruses, but also  
156 plasmids. This is not an unexpected finding, being compatible with multiple previous  
157 observations on individual prokaryotic genomes, but the overwhelming dominance of mobilome-  
158 derived sequences is now validated quantitatively on the scale of the entire prokaryotic sequence  
159 space. Notably, the great majority of viral protospacers were actually detected in provirus  
160 sequences. In part, this could reflect bias caused by the incompleteness of the current virus  
161 sequence database but the possibility also presents that CRISPR-Cas systems play a particularly  
162 important role in the control of provirus induction. Such a mechanism is suggested by the  
163 demonstration of transcription-dependent targeting of viral genomes by some CRISPR-Cas  
164 systems<sup>24</sup>.

165 The strong selectivity of the CRISPR-Cas systems towards the mobilome is likely to stem from  
166 two sources, namely, self vs non-self discrimination at the stage of spacer incorporation and

167 selection (preferential survival) of microbial clones incorporating non-self spacers. The  
168 mechanisms of discrimination remain far from being perfectly understood but at least some  
169 preference for non-self genomes through recognition by the adaptation complex of actively  
170 replicating and repaired and/or transcribed DNA has been demonstrated<sup>24</sup>. Selection appears to  
171 be important as well because, when the nuclease activity of the effector is abolished, self-  
172 matching spacers accumulate<sup>25</sup>. The relative contributions of self vs non-self discrimination and  
173 selection to the dominance of the mobilome as the source of detectable protospacers remain to be  
174 assessed and are likely to differ across the diversity of the CRISPR-Cas systems. Regardless, the  
175 result is a (near) complete exclusion of ‘regular’ microbial sequences from the spacer space. This  
176 exclusion involves not only the host but also other microbes, suggesting that CRISPR provide  
177 protection from viruses and on many occasions prevent plasmid spread but might not create a  
178 barrier for horizontal gene transfer via other routes, such as transformation.

179 The second key finding of this work is the demonstration that CRISPR spacers, both those with  
180 matches and the dark matter, the respective microbial genomes and their viruses belong to the  
181 same genomic pool as determined by (oligo-)nucleotide composition analysis. Together with the  
182 dominance of viral and plasmid sequences among the protospacers, these observations lead to the  
183 extrapolation that the overwhelming majority, and possibly, nearly all spacers originate from the  
184 same source, namely the species-specific mobilome. Then, whence the dark matter? There seem  
185 to be two complementary explanations. First, the dramatic excess of spacers without matches  
186 over those with detectable protospacers implies that for most microbes, the ‘pan-mobilome’ that  
187 they encounter in the course of evolution is vast and still largely untapped. Second, the lack of  
188 spacer matches could be caused by progressive amelioration of the spacer sequences caused,  
189 primarily, by mutational escape of viruses, which results in the loss of information that is  
190 required to recognize protospacers, at least in a database search. In the biological setting, spacers  
191 with mismatches can still be employed for interference and/or primed adaptation<sup>26-28</sup>. Again, the  
192 relative contributions of the two factors remain to be investigated. The importance of  
193 amelioration is implied by the precipitous decline of the fraction of spacers with matches from  
194 the beginning towards the middle of arrays (Figure 1). Furthermore, in *Escherichia coli*, the only  
195 microbe, for which the virome can be considered comprehensively characterized, there are  
196 virtually no spacers with matches to the known viral genomes, suggesting that the apparently  
197 inactive CRISPR arrays in this bacterium have accumulated mismatches to the cognate

198 protospacers that render them unrecognizable<sup>29</sup>. Further characterization of the ‘pan-mobilomes’  
199 of diverse bacteria and measurement of the spacer amelioration rates should improve our  
200 understanding of the evolution of the CRISPR spacer space and the virus-host arms race.

## 201 **Methods**

202

### 203 **Prokaryotic Genome Database**

204 Archaeal and bacterial genomic sequences were downloaded in March 2016 from the NCBI FTP site  
205 (<ftp://ftp.ncbi.nlm.nih.gov/genomes/all/>). The pre-computed ORF annotation was accepted for well  
206 annotated genomes (coding density >0.6 coding sequences per kilobase), and the rest of the genomes  
207 were annotated using Meta-GeneMark<sup>30</sup> with the standard model MetaGeneMark\_v1.mod (Heuristic  
208 model for genetic code 11 and GC 30). The resulting database consisted of 4,961 completely assembled  
209 genomes and 43,599 partial, or 6,342,452 nucleotide sequences altogether (genome partitions, such as  
210 chromosomes and plasmids, and contigs).

### 211 **Detection and annotation of CRISPR arrays**

212 All contigs from the prokaryotic genome database were analyzed with CRISPRFinder<sup>31</sup> which identified  
213 61,581 CRISPR arrays and PILER-CR<sup>32</sup> which identified 49,817 arrays. Arrays were merged by coordinates  
214 (CRISPRFinder array annotation was taken in case of overlap), which produced a set of 65,194 CRISPR  
215 arrays.

216 CRISPR-Cas types and subtypes were assigned to CRISPR arrays using previously described procedures  
217<sup>16,33</sup>. All ORFs within 10 kb upstream and downstream of an array were annotated using RPS-BLAST<sup>34</sup>  
218 with 30,953 protein profiles (from the COG, pfam, and cd collections) from the NCBI CDD database<sup>35</sup>  
219 and 217 custom CRISPR-Cas protein profiles<sup>33</sup>. In cases of multiple CRISPR-Cas systems present in an  
220 examined locus, the annotation of the first detected variant was used to annotate the array.

221 Given the frequent misidentification of CRISPR arrays (Supplementary text 3), a filtering procedure for  
222 “orphan” CRISPR arrays (i.e. the arrays that are not associated with *cas* genes) was applied. A set of  
223 repeats from CRISPR arrays identified within typical CRISPR-*cas* loci was collected, and these were  
224 assumed to represent bona fide CRISPR (positive set). A BLASTN<sup>36</sup> search was performed for all repeats  
225 from orphan CRISPR arrays against the positive set, and BLAST hits were collected that showed at least



226 90% identity and 90% coverage with repeats from the positive set. All arrays that did not produce such  
227 hits against the positive set were discarded. The resulting 42,352 CRISPR arrays were used for further  
228 analysis.

## 229 Detection of Protospacers

230 A set of unique spacers was extracted from the 42,352 CRISPR arrays by comparison of the direct and  
231 reverse complement sequences. The full complement of CRISPR arrays contained 720,391 spacers in  
232 total, with 363,460 unique spacers.

233 A BLASTN search with the following command line parameters: “-max\_target\_seqs 10000000 -dust  
234 no -word\_size 8”; was performed for the unique spacer set against the virus part (NCBI taxid: 10239) of  
235 the NR/NT nucleotide collection<sup>37</sup> and against the prokaryotic database described above. The hits with  
236 at least 95% sequence identity to a spacer and at least 95% sequence coverage (i.e. allowing one or two  
237 mismatches) were accepted as protospacers. This threshold was defined from the results of a  
238 comparison of the number of spacer BLAST hits into prokaryotic and eukaryotic virus sequences  
239 (Supplementary Figure 6), where eukaryotic viruses served as a control dataset for false predictions. The  
240 threshold was set at the lowest false discovery rate of 0.06. As a result, 2,981 spacer matches were  
241 detected in viral sequences and 23,385 matches in prokaryotic sequences.

## 242 Annotation of protospacers in prokaryotic genomes

243 To identify protospacers that belong to proviruses among the 23,385 spacer matches obtained in the  
244 prokaryotic genomic sequences, the following procedure was applied:

- 245 • All ORFs within 3 kb upstream and downstream of a spacer hit were collected
- 246 • A PSI-BLAST<sup>36</sup> search for all ORFs from these loci against the virus part of the NR  
247 database<sup>37</sup>, with the following command line parameters: “-seg no -evaluate  
248 0.000001 -dbsize 20000000”, was performed
- 249 • A protospacer was classified as (pro)viral if it overlapped an ORF with a match in the  
250 viral part of NR database or if two or more ORFs with matches in the viral sequence set  
251 were identified within the neighborhood of the protospacer

252 Among the 23,385 spacer matches in prokaryotic genomes, 19,704 spacers targeted ORFs, of which  
253 16,819 of were classified as (pro)viral. Among the 3,679 spacer targeting intergenic regions, 2,799 were  
254 classified as (pro)viral.

255 The results obtained with this classification procedure were compared to those obtained with PhiSpy<sup>38</sup>,  
256 a commonly used prophage finder tool (default parameters) for the protospacer matches identified in  
257 the 4,961 completely assembled genomes. Of the 1,240 spacer matches in complete genomes, 999 hits  
258 were identified as (pro)virus-targeting by the *ad hoc* procedure described above. Using PhiSpy, 902  
259 spacers were mapped to proviruses, of which 819 overlapped with the set of 999 viral matches detected  
260 by the *ad hoc* method, indicating high consistence of the predictions by the two approaches.

261 The distribution of protospacers across CRISPR-Cas types and subtypes was obtained from the unique  
262 spacer set. In cases when a unique spacer was identified in CRISPR arrays from different subtypes, only  
263 one instance was counted. The same procedure was applied to estimate the distribution of protospacers  
264 among the bacterial and archaeal phyla.

## 265 Annotation of spacers matches in non-viral ORFs

266 The 2,885 ORFs that were targeted by spacers but not classified as viral proteins were annotated with  
267 30,953 protein profiles (COGs, pfam, cd) from the NCBI CDD database and 217 custom CRISPR-Cas  
268 protein profiles using RPS-BLAST (using *evalue* 10e-4). Profile hits were obtained for 1,616 ORFs. The  
269 1,269 ORFs with no identified profile hits were clustered using UCLUST<sup>39</sup>, with the similarity threshold of  
270 0.3. To assign ORFs to COG functional categories, the same procedure was performed against the COG  
271 proteins profiles only<sup>40</sup>. The summary statistics for the functional categories was assembled using the  
272 COG table and is available at <ftp://ftp.ncbi.nih.gov/pub/COG/COG2014/static/lists/homeCOGs.html>

273

## 274 Bipartite host-virus network analysis

275 The set of 2,981 spacer matches in the viral part of the NT/NR nucleotide collection was used to build a  
276 bipartite network with two types of nodes: CRISPR hosts and targeted viruses. All CRISPR hosts from the  
277 same genus were collapsed into a single node. Edges between network nodes were assigned when a  
278 protospacer matching a spacer in a given host was identified in in a virus. The network was visualized  
279 using the Cytoscape software<sup>41</sup>.

## 280 Nucleotide composition analysis of hosts, spacers and viruses

281 Nucleotide composition analysis was performed with the dataset of 2,104 complete genomes that  
282 contained CRISPR arrays. Frequencies of mono-, di- and tetranucleotides were calculated in genome

283 sequences. The standard “prcomp” function from the R package was used for Standard  
284 Multidimensional Scaling.

285 Species with the most extensively sampled viromes were identified from the “/host” tag in RefSeq  
286 database for double-stranded DNA viruses:

Host	Number of phages in RefSeq
Escherichia coli	144
Pseudomonas aeruginosa	103
Staphylococcus aureus	77
Propionibacterium acnes	42
Synechococcus sp.	21
Mycobacterium	21

287

288 and analyzed separately, together with the associated viruses.

289

## 290 Comparison of the distributions of spacer and random fragment 291 matches in prokaryotic genomes

292 The comparison of the matches distribution for spacers and random fragments was performed on 2,104  
293 complete genomes that contained CRISPR arrays. For each genome, 1000 random fragments, with the  
294 length equal to the median length of spacers in the given genome, were extracted. A BLASTN search  
295 against the prokaryotic database was performed for these fragments and for spacers, with following  
296 parameters: “-max\_target\_seqs 10000000 -dust no -word\_size 8”. Exact matches were selected for  
297 further analysis.

298

299 **Acknowledgements**

300 SS, KSM, YIW and EVK are funded intramural funds of the US Department of Health and Human Services

301 (to National Library of Medicine).

302

303

304

305

306

307

## References

308

- 309 1 Sorek, R., Lawrence, C. M. & Wiedenheft, B. CRISPR-mediated adaptive immune systems in  
310 bacteria and archaea. *Annu Rev Biochem* **82**, 237-266, doi:10.1146/annurev-biochem-072911-  
311 172315 (2013).
- 312 2 Mohanraju, P. *et al.* Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas  
313 systems. *Science* **353**, aad5147, doi:10.1126/science.aad5147 aad5147 [pii] 353/6299/aad5147  
314 [pii] (2016).
- 315 3 Tyson, G. W. & Banfield, J. F. Rapidly evolving CRISPRs implicated in acquired resistance of  
316 microorganisms to viruses. *Environ Microbiol* **10**, 200-207, doi:EM11444 [pii] 10.1111/j.1462-  
317 2920.2007.01444.x (2008).
- 318 4 van Houte, S., Buckling, A. & Westra, E. R. Evolutionary Ecology of Prokaryotic Immune  
319 Mechanisms. *Microbiol Mol Biol Rev* **80**, 745-763, doi:10.1128/MMBR.00011-16 80/3/745 [pii]  
320 (2016).
- 321 5 Wright, A. V., Nunez, J. K. & Doudna, J. A. Biology and Applications of CRISPR Systems:  
322 Harnessing Nature's Toolbox for Genome Engineering. *Cell* **164**, 29-44,  
323 doi:10.1016/j.cell.2015.12.035 S0092-8674(15)01699-2 [pii] (2016).
- 324 6 Komor, A. C., Badran, A. H. & Liu, D. R. CRISPR-Based Technologies for the Manipulation of  
325 Eukaryotic Genomes. *Cell* <http://dx.doi.org/10.1016/j.cell.2016.10.044> (2016).
- 326 7 Amitai, G. & Sorek, R. CRISPR-Cas adaptation: insights into the mechanism of action. *Nat Rev*  
327 *Microbiol* **14**, 67-76, doi:10.1038/nrmicro.2015.14 nrmicro.2015.14 [pii] (2016).
- 328 8 Silas, S. *et al.* Direct CRISPR spacer acquisition from RNA by a natural reverse transcriptase-Cas1  
329 fusion protein. *Science* **351**, aad4234, doi:10.1126/science.aad4234 aad4234 [pii]  
330 351/6276/aad4234 [pii] (2016).
- 331 9 Plagens, A., Richter, H., Charpentier, E. & Randau, L. DNA and RNA interference mechanisms by  
332 CRISPR-Cas surveillance complexes. *FEMS Microbiol Rev* **39**, 442-463,  
333 doi:10.1093/femsre/fuv019 fuv019 [pii] (2015).
- 334 10 Nishimasu, H. & Nureki, O. Structures and mechanisms of CRISPR RNA-guided effector  
335 nucleases. *Curr Opin Struct Biol* **43**, 68-78, doi:S0959-440X(16)30198-1 [pii]  
336 10.1016/j.sbi.2016.11.013 (2016).
- 337 11 Bolotin, A., Quinquis, B., Sorokin, A. & Ehrlich, S. D. Clustered regularly interspaced short  
338 palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**,  
339 2551-2561, doi:151/8/2551 [pii] 10.1099/mic.0.28048-0 (2005).
- 340 12 Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Soria, E. Intervening sequences of regularly  
341 spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**, 174-182,  
342 doi:10.1007/s00239-004-0046-3 (2005).

- 343 13 Pourcel, C., Salvignol, G. & Vergnaud, G. CRISPR elements in *Yersinia pestis* acquire new repeats  
344 by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary  
345 studies. *Microbiology* **151**, 653-663, doi:151/3/653 [pii] 10.1099/mic.0.27437-0 (2005).
- 346 14 England, W. E. & Whitaker, R. J. Evolutionary causes and consequences of diversified CRISPR  
347 immune profiles in natural populations. *Biochem Soc Trans* **41**, 1431-1436,  
348 doi:10.1042/BST20130243 BST20130243 [pii] (2013).
- 349 15 Childs, L. M., England, W. E., Young, M. J., Weitz, J. S. & Whitaker, R. J. CRISPR-induced  
350 distributed immunity in microbial populations. *PLoS One* **9**, e101710,  
351 doi:10.1371/journal.pone.0101710 PONE-D-14-03166 [pii] (2014).
- 352 16 Makarova, K. S. *et al.* An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev*  
353 *Microbiol* **13**, 722-736, doi:10.1038/nrmicro3569 nrmicro3569 [pii] (2015).
- 354 17 Westra, E. R. & Brouns, S. J. The rise and fall of CRISPRs--dynamics of spacer acquisition and loss.  
355 *Mol Microbiol* **85**, 1021-1025, doi:10.1111/j.1365-2958.2012.08170.x (2012).
- 356 18 Weinberger, A. D. *et al.* Persisting viral sequences shape microbial CRISPR-based immunity. *PLoS*  
357 *Comput Biol* **8**, e1002475, doi:10.1371/journal.pcbi.1002475 PCOMPBIOL-D-12-00056 [pii]  
358 (2012).
- 359 19 Smillie, C., Garcillan-Barcia, M. P., Francia, M. V., Rocha, E. P. & de la Cruz, F. Mobility of  
360 plasmids. *Microbiol Mol Biol Rev* **74**, 434-452, doi:10.1128/MMBR.00020-10 74/3/434 [pii]  
361 (2010).
- 362 20 Almendros, C., Guzman, N. M., Garcia-Martinez, J. & Mojica, F. J. Anti-cas spacers in orphan  
363 CRISPR4 arrays prevent uptake of active CRISPR-Cas I-F systems. *Nat Microbiol* **1**, 16081,  
364 doi:10.1038/nmicrobiol.2016.81 nmicrobiol201681 [pii] (2016).
- 365 21 Stern, A., Keren, L., Wurtzel, O., Amitai, G. & Sorek, R. Self-targeting by CRISPR: gene regulation  
366 or autoimmunity? *Trends Genet* **26**, 335-340, doi:10.1016/j.tig.2010.05.008 S0168-  
367 9525(10)00108-3 [pii] (2010).
- 368 22 Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. Mobile genetic elements: the agents of  
369 open source evolution. *Nat Rev Microbiol* **3**, 722-732 (2005).
- 370 23 Mortimer, J. R. & Forsdyke, D. R. Comparison of responses by bacteriophages and bacteria to  
371 pressures on the base composition of open reading frames. *Appl Bioinformatics* **2**, 47-62 (2003).
- 372 24 Goldberg, G. W., Jiang, W., Bikard, D. & Marraffini, L. A. Conditional tolerance of temperate  
373 phages via transcription-dependent CRISPR-Cas targeting. *Nature* **514**, 633-637,  
374 doi:10.1038/nature13637 nature13637 [pii] (2014).
- 375 25 Wei, Y., Terns, R. M. & Terns, M. P. Cas9 function and host genome sampling in Type II-A CRISPR-  
376 Cas adaptation. *Genes Dev* **29**, 356-361, doi:10.1101/gad.257550.114 29/4/356 [pii] (2015).
- 377 26 Semenova, E. *et al.* Interference by clustered regularly interspaced short palindromic repeat  
378 (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci U S A* **108**, 10098-10103,  
379 doi:1104144108 [pii] 10.1073/pnas.1104144108 (2011).
- 380 27 Fineran, P. C. *et al.* Degenerate target sites mediate rapid primed CRISPR adaptation. *Proc Natl*  
381 *Acad Sci U S A* **111**, E1629-1638, doi:10.1073/pnas.1400071111 1400071111 [pii] (2014).

- 382 28 Xue, C. *et al.* CRISPR interference and priming varies with individual spacer sequences. *Nucleic*  
383 *Acids Res* **43**, 10831-10847, doi:10.1093/nar/gkv1259 gkv1259 [pii] (2015).
- 384 29 Savitskaya, E. *et al.* Dynamics of Escherichia coli type I-E CRISPR spacers over 42 000 years. *Mol*  
385 *Ecol*, doi:10.1111/mec.13961 (2016).
- 386 30 Zhu, W., Lomsadze, A. & Borodovsky, M. Ab initio gene identification in metagenomic  
387 sequences. *Nucleic Acids Res* **38**, e132, doi:10.1093/nar/gkq275 gkq275 [pii] (2010).
- 388 31 Grissa, I., Vergnaud, G. & Pourcel, C. CRISPRFinder: a web tool to identify clustered regularly  
389 interspaced short palindromic repeats. *Nucleic Acids Res* **35**, W52-57, doi:gkm360 [pii]  
390 10.1093/nar/gkm360 (2007).
- 391 32 Edgar, R. C. PILER-CR: fast and accurate identification of CRISPR repeats. *BMC Bioinformatics* **8**,  
392 18, doi:1471-2105-8-18 [pii] 10.1186/1471-2105-8-18 (2007).
- 393 33 Makarova, K. S. & Koonin, E. V. Annotation and Classification of CRISPR-Cas Systems. *Methods*  
394 *Mol Biol* **1311**, 47-75, doi:10.1007/978-1-4939-2687-9\_4 (2015).
- 395 34 Marchler-Bauer, A. *et al.* CDD: a database of conserved domain alignments with links to domain  
396 three-dimensional structure. *Nucleic Acids Res* **30**, 281-283 (2002).
- 397 35 Marchler-Bauer, A. *et al.* CDD: NCBI's conserved domain database. *Nucleic Acids Res* **43**, D222-  
398 226, doi:10.1093/nar/gku1221 gku1221 [pii] (2015).
- 399 36 Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search  
400 programs. *Nucleic Acids Res* **25**, 3389-3402 (1997).
- 401 37 Database Resources of the National Center for Biotechnology Information. *Nucleic Acids Res* **45**,  
402 D12-D17, doi:10.1093/nar/gkw1071 gkw1071 [pii] (2017).
- 403 38 Akhter, S., Aziz, R. K. & Edwards, R. A. PhiSpy: a novel algorithm for finding prophages in  
404 bacterial genomes that combines similarity- and composition-based strategies. *Nucleic Acids Res*  
405 **40**, e126, doi:10.1093/nar/gks406 gks406 [pii] (2012).
- 406 39 Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**,  
407 2460-2461, doi:10.1093/bioinformatics/btq461 btq461 [pii] (2010).
- 408 40 Galperin, M. Y., Makarova, K. S., Wolf, Y. I. & Koonin, E. V. Expanded microbial genome coverage  
409 and improved protein family annotation in the COG database. *Nucleic Acids Res* **43**, D261-269,  
410 doi:10.1093/nar/gku1223 gku1223 [pii] (2015).
- 411 41 Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. L. & Ideker, T. Cytoscape 2.8: new features for  
412 data integration and network visualization. *Bioinformatics* **27**, 431-432,  
413 doi:10.1093/bioinformatics/btq675 btq675 [pii] (2011).
- 414 42 Shmakov, S. *et al.* Diversity and evolution of class 2 CRISPR-Cas systems. *Nat Rev Microbiol*,  
415 doi:10.1038/nrmicro.2016.184 nrmicro.2016.184 [pii] (2017).
- 416
- 417

418

419 Figure legends

420

421 Figure 1.

422 **Distribution of the spacers with matches along the CRISPR arrays.**

423 (A) Probability density functions for the spacers with matches (real) and for the same spacers  
424 placed randomly onto the array 100 times (random).

425 (B) Probability density function of the difference between the number of spacers with  
426 matches and randomly placed spacers along the array.

427 Given the difficulty of polarizing CRISPR arrays automatically and under the assumption that  
428 new spacers are incorporated at the leader end but not at the distal end of arrays, the results are  
429 shown from the end to the middle of the arrays.

430

431 Figure 2

432 **Breakdown of the protospacers from non-viral genes by gene family**

433 Genes implicated in conjugal transfer of plasmids and plasmid replication, a putative phage gene  
434 (not annotated as such) and *cas3* gene are color-coded. The protein family names are from the  
435 CDD database.

436

437 Figure 3

438 **Correlations between the nucleotide compositions of spacers, the genomes of the respective  
439 microbes and their viruses**

440 A. GC-content of spacers vs GC-content of microbial genomes and viruses

441 B. GC-content of spacers with matches vs GC-content of microbial genomes and viruses



442 Linear trend lines are shown for the GC-content of spacers (green) and viral genomes (red), and  
443 the  $x=y$  line is included to guide the eye.

444 Figure 4

445 **Correlations between the nucleotide compositions of spacers, genomes of bacteria with**  
446 **numerous characterized viruses and the corresponding viral genomes**

447 Figure 5

448 **Spacer sequence conservation compared to the genomic average**

449 A. Distribution of matches for the spacers and the ‘mock spacers’ across the microbial  
450 taxonomic ranks

451 B. Distributions of the number of matches to the same species per spacer for the  
452 spacers and the ‘mock spacers’

453

454

455

456

457

458

459

460

461

462

463

464

465

466 Table 1

467 **Distribution of spacers with matches among CRISPR-Cas subtypes**

CRISPR-Cas Type/subtype <sup>a</sup>	Total number of spacers	Number of spacers with hits	Fraction of spacers with hits	Spacers with matches in viral sequences, intergenic regions and non-viral ORFs (the fraction of the total number of spacers with matches is indicated)		
				Viral	Intergenic	ORFs
CAS-I	5670	513	0.09	0.79	0.08	0.13
CAS-I-A	6942	102	0.01	0.77	0.04	0.19
CAS-I-B	54781	2682	0.05	0.88	0.03	0.10
CAS-I-C	38571	2376	0.06	0.84	0.02	0.13
CAS-I-D	9096	65	0.01	0.71	0.14	0.15
CAS-I-E	59783	4475	0.07	0.84	0.03	0.13
CAS-I-F	28131	4868	0.17	0.92	0.02	0.06
CAS-I-U	7494	312	0.04	0.79	0.04	0.17
CAS-II-A	13967	2679	0.19	0.90	0.01	0.09
CAS-II-B	461	9	0.02	0.44	0.33	0.22
CAS-II-C	13022	1060	0.08	0.71	0.05	0.24
CAS-III	4662	72	0.02	0.78	0.01	0.21
CAS-III-A	9249	179	0.02	0.74	0.06	0.20
CAS-III-B	12241	260	0.02	0.86	0.05	0.10
CAS-III-C	1917	42	0.02	0.88	0.02	0.10
CAS-III-D	8345	120	0.01	0.78	0.03	0.19
CAS-IV-A	1582	147	0.09	0.72	0.03	0.24
CAS-V-A	592	5	0.01	1.00	0.00	0.00
CAS-V-B	168	8	0.05	0.88	0.00	0.13
CAS-VI-A	179	8	0.04	0.50	0.13	0.38
CAS-VI-B	682	50	0.07	0.72	0.06	0.22
CAS-VI-C	34	2	0.06	0.50	0.00	0.50
CAS-V-U	320	3	0.01	0.67	0.00	0.33
Unidentified	85462	6327	0.07	0.84	0.05	0.11
	<b>363351</b>	<b>26364</b>	<b>0.07</b>			

468

469 Identification and classification of the CRISPR-Cas systems were as previously described<sup>16,42</sup>;  
 470 CAS-I, CAS-III denote loci that could be assigned to types I and III, respectively, but not to a  
 471 specific subtype; Unidentified are orphan CRISPR arrays and incomplete CRISPR-*cas* loci.

472 Table 2

473 **Distribution of spacers with matches among bacterial and archaeal phyla**

Phylum	Total Number of spacers <sup>a</sup>	Number of spacers With matches <sup>a</sup>	Fraction of spacers with matches	Spacers with matches in viral sequences, intergenic regions and non-viral ORFs (the fraction of the total number of spacers with matches is indicated)		
				Viral	Intergenic	ORFs
Actinobacteria	54875	3614	0.07	0.76	0.05	0.19
Alphaproteobacteria	8135	120	0.01	0.69	0.07	0.24
Bacteroidetes/Chlorobi group	18611	840	0.05	0.78	0.03	0.19
Betaproteobacteria	14013	908	0.06	0.69	0.14	0.16
Chloroflexi	6523	30	0.00	0.77	0.03	0.20
Crenarchaeota	11212	119	0.01	0.90	0.02	0.08
Cyanobacteria/Melainabacteria group	20295	126	0.01	0.75	0.04	0.21
Deinococcus-Thermus	4057	85	0.02	0.75	0.04	0.21
delta/epsilon subdivisions	13588	378	0.03	0.60	0.06	0.34
Firmicutes	93332	7643	0.08	0.90	0.02	0.08
Fusobacteriia	3427	629	0.18	0.92	0.01	0.06
Gammaproteobacteria	67202	10238	0.15	0.91	0.03	0.06
Halobacteria	5121	74	0.01	0.55	0.08	0.36
Methanobacteria	2218	47	0.02	0.70	0.04	0.26
Methanococci	1639	6	0.00	0.50	0.00	0.50
Methanomicrobia	10399	141	0.01	0.91	0.02	0.06
Nitrospira	1088	13	0.01	0.85	0.00	0.15
Planctomycetes	1650	14	0.01	0.79	0.14	0.07
Spirochaetia	5114	1173	0.23	0.73	0.04	0.24
Synergistia	1702	22	0.01	0.64	0.00	0.36
Tenericutes	1050	26	0.02	0.73	0.04	0.23
Thermococci	3210	16	0.00	0.31	0.00	0.69
Thermoplasmata	1270	6	0.00	0.17	0.17	0.67
Thermotogae	3731	31	0.01	0.94	0.00	0.06
unclassified Bacteria (miscellaneous)	2814	6	0.00	0.67	0.00	0.33
	<b>356276</b>	<b>26305</b>	<b>0.07</b>			

474

475 <sup>a</sup>Only phyla with >1,000 unique spacers were included, hence slightly lower total number of  
 476 spacers than in Table 1.

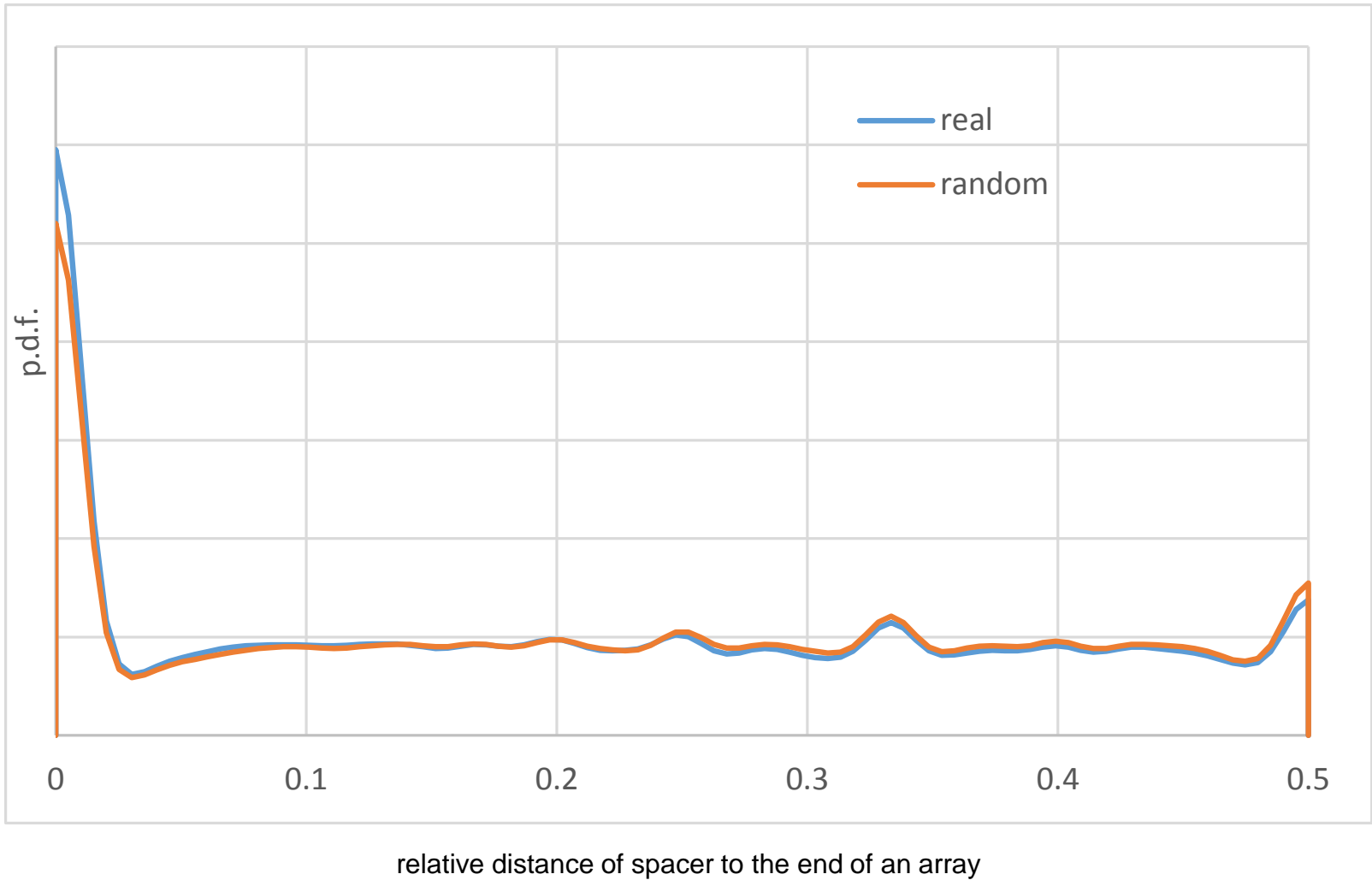


Figure 1A

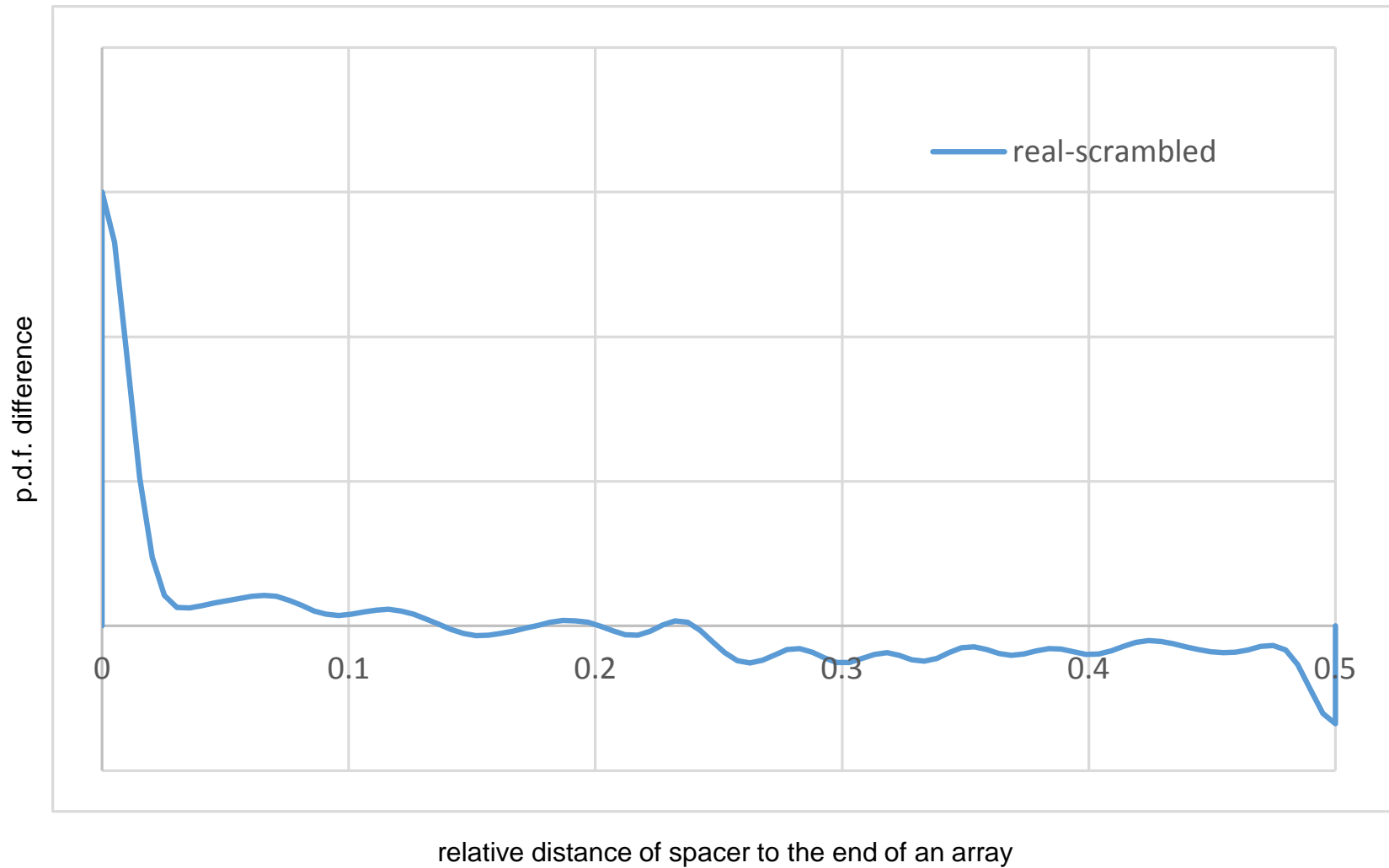


Figure 1B

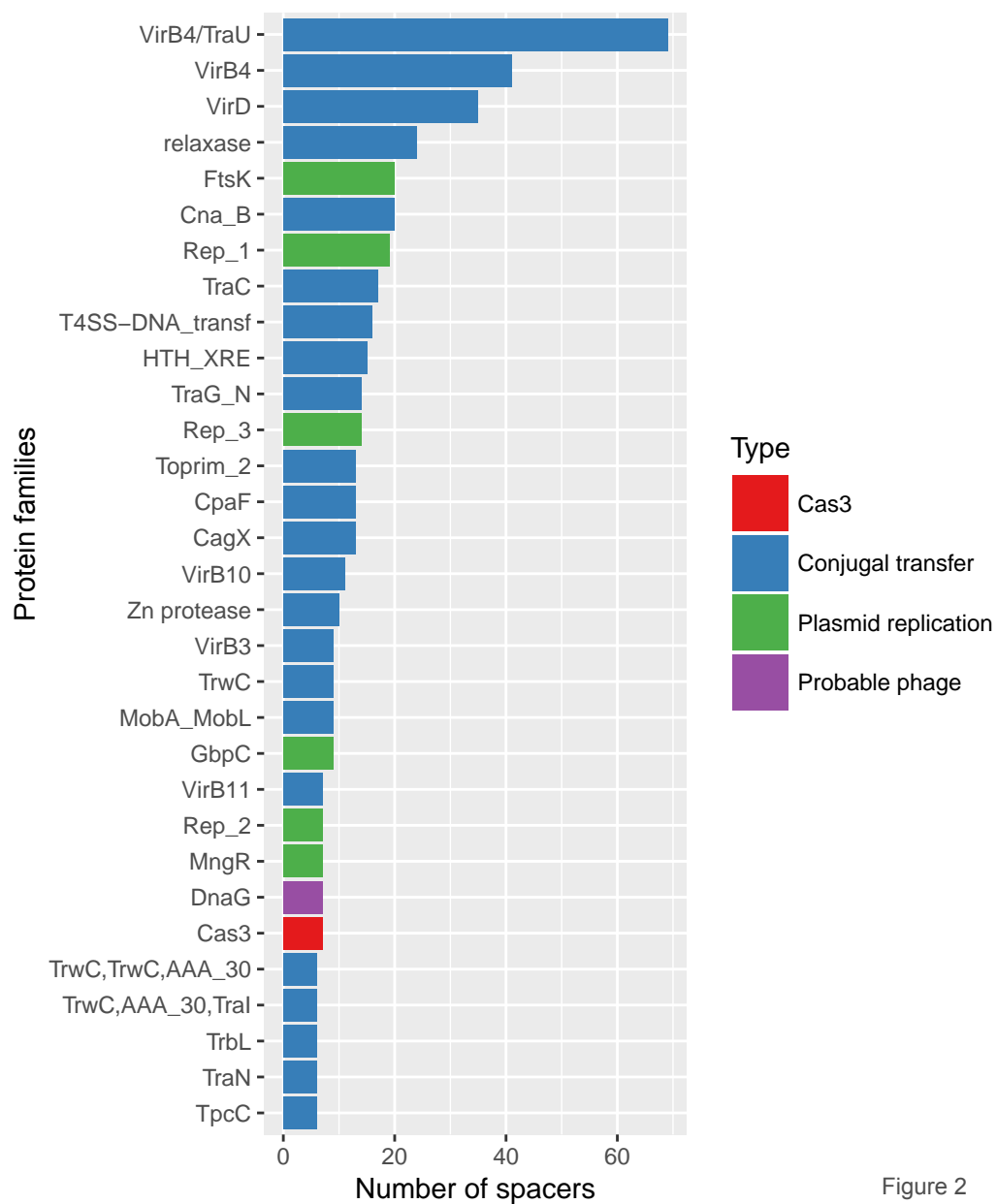
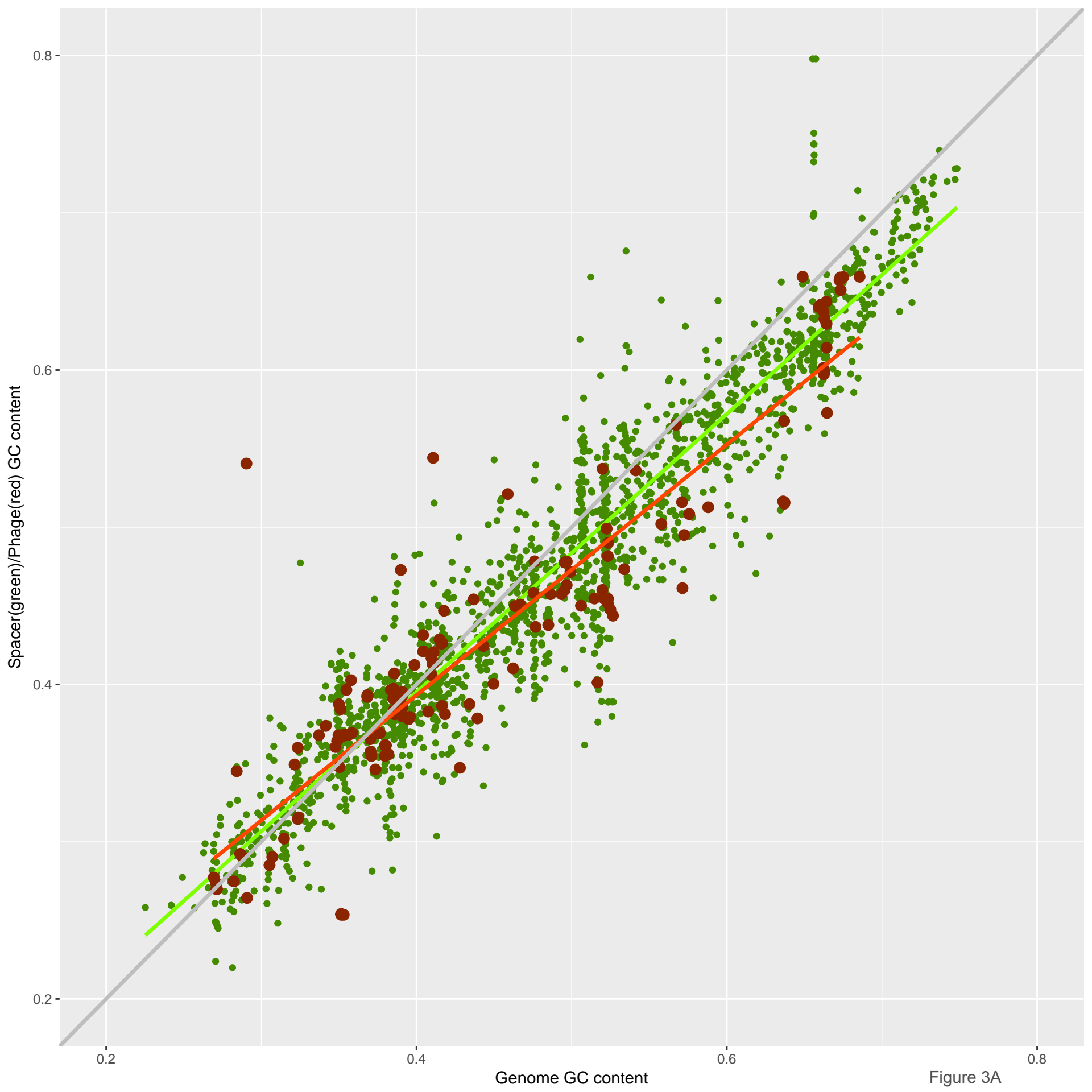


Figure 2



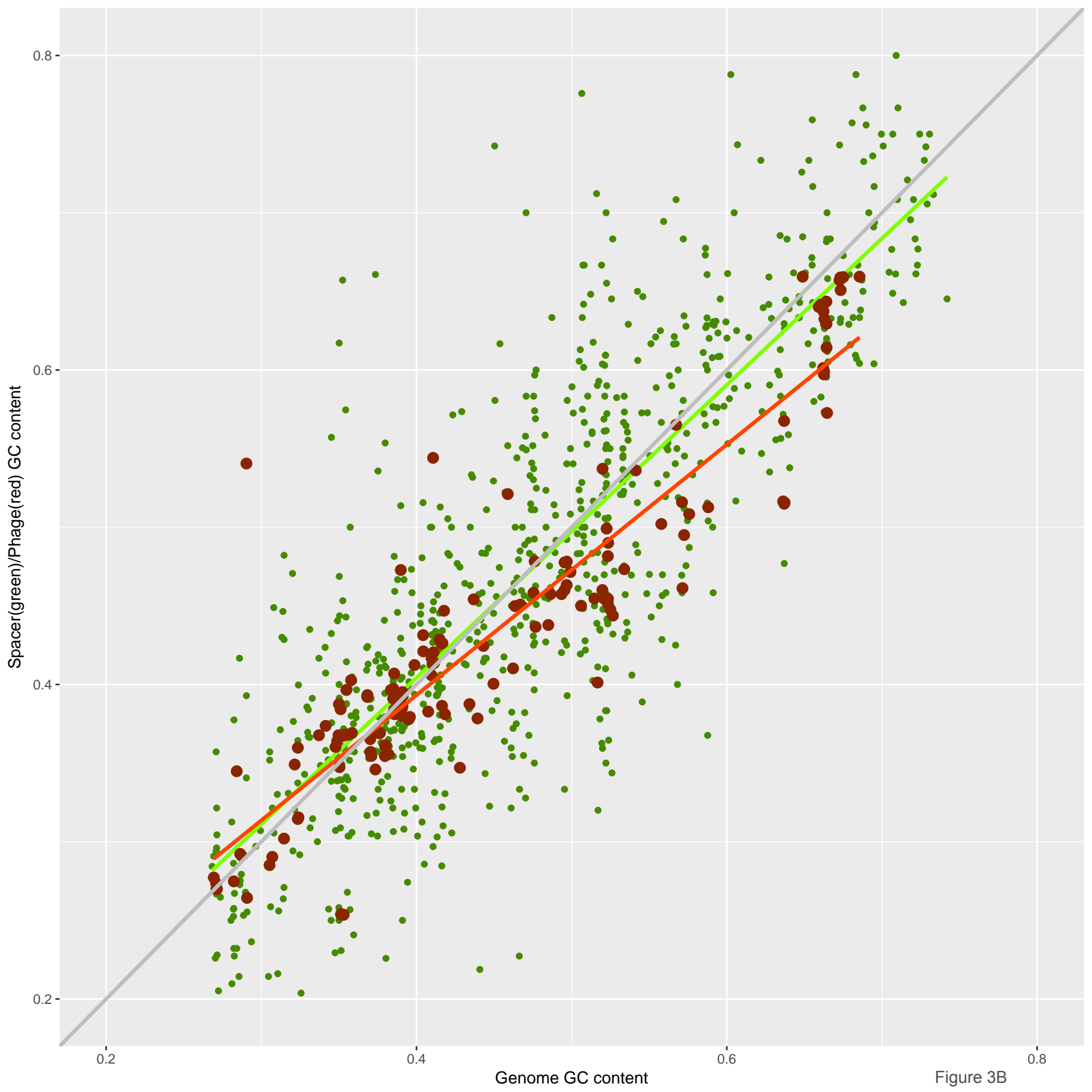


Figure 3B



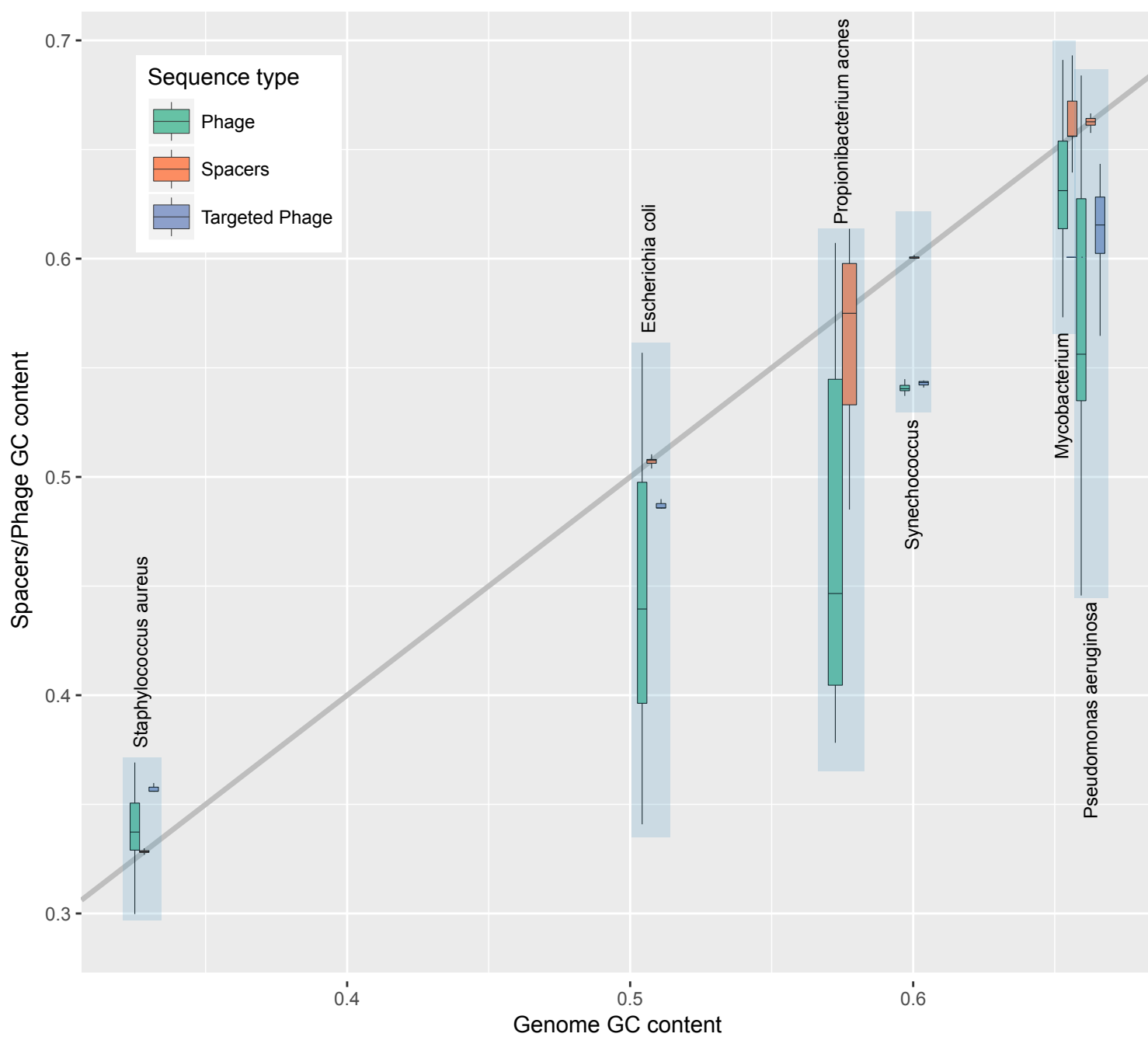


Figure 4

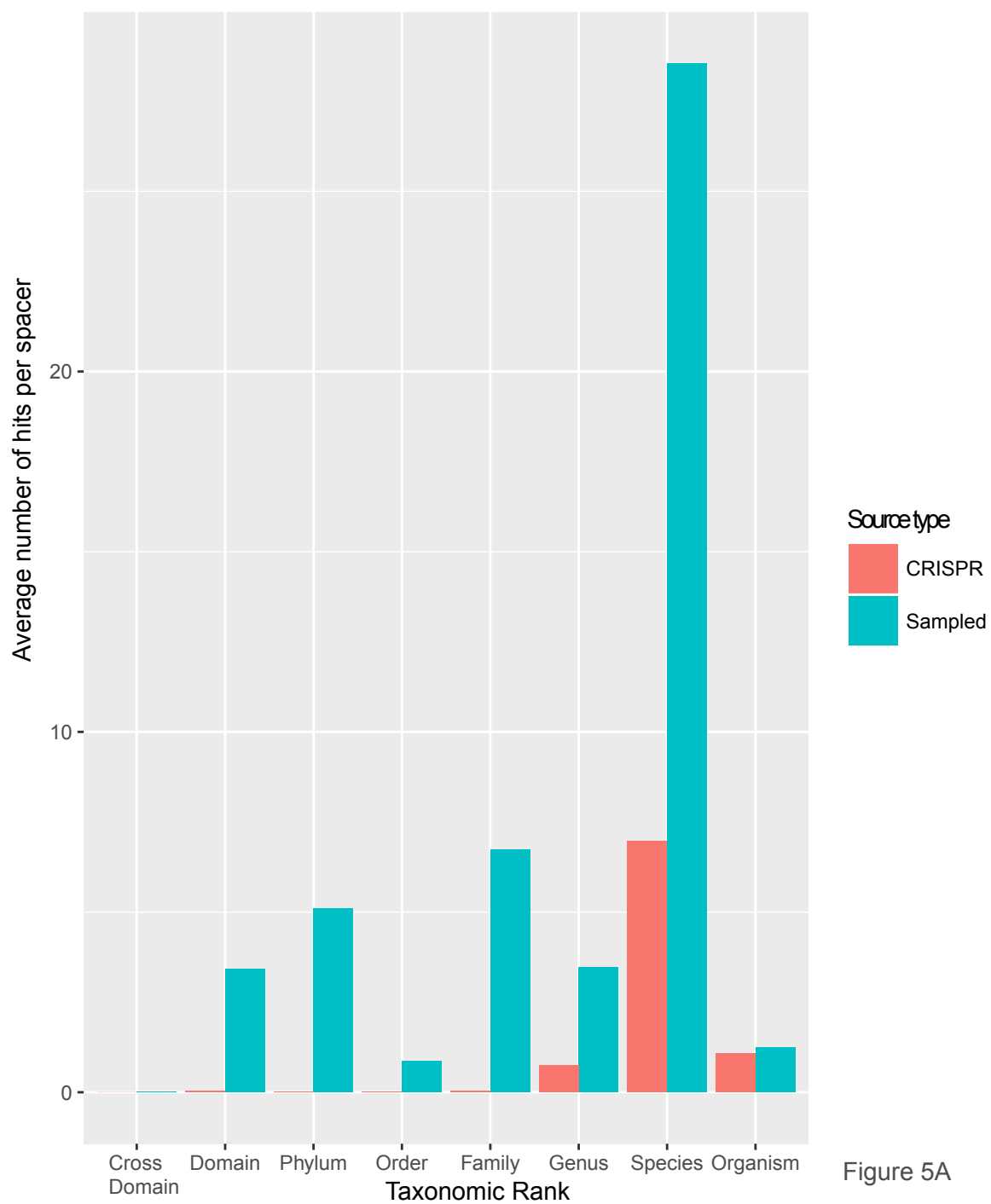


Figure 5A

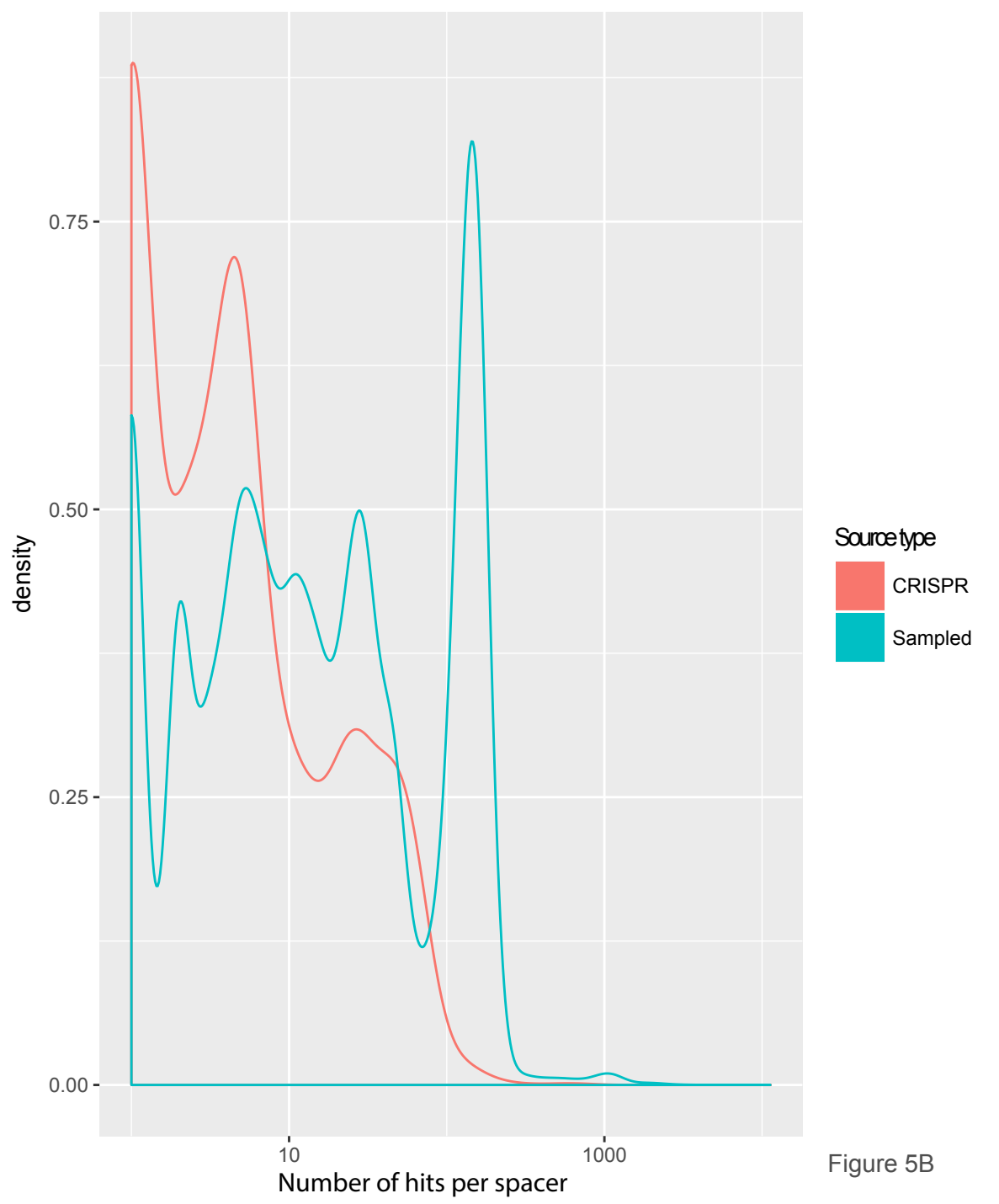


Figure 5B