

1 **Generation of lineage-resolved complete metagenome-assembled genomes by precision**
2 **phasing**

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20 **Abstract**

21 Microbial communities in many environments include distinct lineages of closely related
22 organisms which have proved challenging to separate in metagenomic assembly, preventing
23 generation of complete metagenome-assembled genomes (MAGs). The advent of long and
24 accurate HiFi reads presents a possible means to address this challenge by generating complete
25 MAGs for nearly all sufficiently abundant bacterial genomes in a microbial community. We
26 present a metagenomic HiFi assembly of a complex microbial community from sheep fecal
27 material that resulted in 428 high-quality MAGs from a single sample, the highest resolution
28 achieved with metagenomic deconvolution to date. We applied a computational approach to
29 separate distinct haplotype lineages and identified haplotypes of hundreds of variants across
30 hundreds of kilobases of genomic sequence. Analysis of these haplotypes revealed 220 lineage-
31 resolved complete MAGs, including 44 in single circular contigs, and demonstrated improvement
32 in overall assembly compared to error-prone long reads. We report the characterization of multiple,
33 closely-related microbes within a sample with potential to improve precision in assigning mobile
34 genetic elements to host genomes within complex microbial communities.

35 **Introduction**

36 The creation of reference-quality, species-level assemblies from metagenome communities
37 is exceedingly difficult. In particular, generating a complete genome assembly of a microbe closely
38 related to a more abundant member of the community has been an elusive goal. Previous short-
39 read studies have resulted in high-quality metagenome-assembled genomes (MAG)¹ only after
40 extensive polishing and manual curation of initial contigs². However, if a community contains
41 thousands of organisms at different levels of abundance, manual curation of each MAG to achieve
42 reference quality is extremely laborious. Generally, the MAGs assembled from short reads are
43 represented by hundreds or even thousands of contigs, many of which have fragmented open
44 reading frames (ORFs) at their ends. A major source of discontinuity in metagenome assembly
45 appears to be from the prevalence of high sequence identity orthologous genes and operons³. These
46 genomic features tend to be repetitive in the community and can preclude complete assembly
47 unless the data include sequencing reads that span the entire shared region. Furthermore, nearly all
48 short-read and long-read assembly algorithms typically collapse the variant features into a single
49 representation that does not reflect the true strain- or species-level diversity of a subpopulation

50 within the community^{4,5}, moreover, consensus assemblies might include various artifacts arising
51 from the variation collapsing procedure, e.g. frame shifts, complicating downstream analysis⁶.
52 Ambiguity resulting from the metagenomic assembly of short-reads or error-prone long-reads⁷ has
53 therefore left the possibility of first-pass characterization of microbial strains out of reach.

54 Generation of high-quality assemblies of individual microbial lineages within
55 metagenomes remains a substantial challenge. Binning methods were developed to address issues
56 with assembly fragmentation and organize contigs into candidate MAGs based on assumptions of
57 shared sequence composition⁸ or orthologous linkage data⁹. The presence of single copy genes
58 (SCG) expected to be in all bacterial and archaeal lineages has been proposed as a measure of the
59 completeness and redundancy within these bins². High-quality draft MAGs are defined in the
60 literature as having over 90% of the expected count of SCG with less than 5% redundancy of their
61 prevalence¹. However, bacterial and archaeal lineages may contain significant accessory gene
62 content¹⁰ that is not assessed using these metrics. Even though bins are often generalized to
63 represent distinct microbial taxonomic units in a sample, they are rarely assumed to accurately
64 represent true, genetically distinct microbial populations in a sample. This problem has been
65 addressed by multiple studies^{11,12}, and precise definitions for individual, highly resolved MAGs
66 remain contextual to each study. Similar to one of these studies¹¹, we focus on generating separate
67 representative reference genomes for distinct microbial lineages within an individual metagenome,
68 which we define as “lineage-resolved MAGs”^{1,13}. Combined with prior definitions of SCG quality
69 metrics, we further extend the term to “lineage-resolved complete MAGs” for all such assemblies
70 which have high degrees of SCG completeness (> 90%), low degrees of SCG redundancy (< 10%),
71 and the separation of all observable variant lineages of microbial taxa into individual MAGs. Tools
72 have been developed to identify or separate lineage-resolved complete MAGs from metagenomic
73 bins post-hoc, but these tools often rely on co-assembly data, assembly graphs or various statistical
74 methods to overcome biases in read-alignments to estimate strains from observed genetic variant
75 data and therefore require more curation to properly disentangle lineages from MAGs^{11,14,15}.
76 Furthermore, these workflows are designed primarily to identify strain lineages from alignments
77 of short-read data and do not capture variant linkage data from longer read datasets. A recent
78 attempt to adapt uncorrected long reads to this purpose requires the use of manual curation and *a*
79 *priori* estimates of strain numbers in order to achieve optimal results¹². An intuitive and automated

80 method to generate lineage-resolved complete MAGs is needed for analysis of more complex
81 metagenome communities in order to reduce the time required to validate results.

82 Recent improvements in long-read sequencing technologies (such as Oxford Nanopore or
83 Pacific Biosciences) have dramatically improved the quality of de novo genome assemblies of
84 large eukaryotic genomes^{16,17}. However, due to the high error rate of long error-prone reads,
85 assembly algorithms still fail to disambiguate between highly similar sequences, such as segmental
86 duplications in the human genome¹⁸. The recent development of highly accurate HiFi reads from
87 circular consensus sequencing (CCS) on the Pacific Biosciences platform resulted in long accurate
88 reads with error rates below 1% across the length of the read¹⁹ providing opportunity to improve
89 assembly quality²⁰ and even resolve both haplotypes of diploid genomes^{21,22}. Recent attempts to
90 sequence and assemble metagenomes with long error-prone reads²³⁻²⁵ or linked reads³ have
91 resulted in few successes. While it has been demonstrated that long error-prone reads result in
92 longer contigs than short reads^{23,24}, the assembly of nearly all members of the community in
93 singular circular contigs has still been elusive. Much of this may be due to the imperfect “length
94 versus error-rate” trade-off²⁶. Although the recently introduced metaFlye assembler improved
95 reconstruction of complex environmental metagenomes using long reads⁵, it subsequently
96 produced collapsed representations of similar bacterial strains.

97 Long and accurate HiFi reads have recently resulted in the first complete human genome
98 assembly by the Telomere-to-Telomere Consortium and opened the era of “complete (T2T)
99 genomics¹⁷”. Thus, this new technology could be suitable for traversing and assembling the highly
100 repetitive orthologous genomic features present in metagenomes into lineage-resolved complete
101 MAGs, enabling a new era of “complete metagenomics”. Furthermore, variant calling using HiFi
102 reads has the added benefit of providing single molecule, physical evidence of sequence variant
103 linkage that can be used to define discrete haplotypes in MAGs. In this paper, we leverage the use
104 of HiFi reads in a metagenome assembly and demonstrate that metaFlye assembly with HiFi reads
105 produce more lineage-resolved complete MAGs compared to assemblies generated with
106 uncorrected long-reads without the need for manual curation. Additionally, we present a
107 computational approach to phase alternative SNP haplotypes in these MAGs to provide finer
108 resolution of descendant lineage variation in the sample.

109

110 **Results**

111 **Assembly of the sheep gut microbiome**

112 We extracted high molecular weight DNA from a fecal sample of an adult sheep collected
113 during necropsy to determine cause of death. The resultant DNA prep was sequenced using a short-
114 read (Illumina, San Diego, CA) and a long-read (PacBio, Menlo Park, CA) sequencing technology,
115 with the latter using the CCS method to generate HiFi reads from the error-prone subreads (for a
116 technical definition, see Methods). The short and HiFi reads comprised 154 and 255 total
117 Gigabases (Gbp) in 1,024,375,790 and 22,118,393 reads, respectively, with the latter representing
118 higher depth of coverage compared to most previous reports of long-read metagenome assembly.
119 metaFlye assembly of HiFi reads resulted in a total of 57,259 contigs with a contig N50 of 279 kb,
120 including 127 contigs that fit the criteria of a high-quality draft¹ (or by our terms, “complete”)
121 MAG. Among the MAG-quality contigs, 44 (35%) represented closed circles in the metagenome
122 assembly graph (see Table 1).

123 **Long accurate reads result in significantly improved metagenome assemblies**

124 We hypothesized that substantial improvements in assembled contig completeness
125 statistics were primarily due to the lower error rates of reads providing less ambiguity in resolving
126 structural complexity in microbial genomes, so we sought to create an experimental design that
127 would quantify the benefits of using an equivalent amount of long error-prone reads. Comparisons
128 of metagenome assemblies based on generation of separate library types from the same sample
129 may suffer from differences in microbial composition, the temporal nature of samples and the
130 likelihood of sampling particular microbes in the community. As such, comparisons of a separate
131 library of long error-prone continuous long reads (CLR) taken from the same DNA sample are
132 unlikely to control for all of the confounding variables that impact the quality of the downstream
133 assembly.

134 We devised an approach for an apples-to-apples comparison of HiFi and CLR reads by
135 extracting subreads from the original HiFi reads to generate a series of “pseudo-CLR” (pCLR)
136 datasets. We generated three separate assemblies corresponding to the first (pCLR1), second
137 (pCLR2) and third (pCLR3) full-length sub-read, respectively (Figure 1a). These subreads share
138 the same error profile as PacBio CLR sequencing (8-15% error rate²⁷) but were equivalent in length

139 to their parental HiFi reads. We assembled these datasets using metaFlye and compared them to
140 our HiFi assembly to quantify the benefits of using long and accurate reads instead of long error-
141 prone reads. The average pCLR contig was longer than the average HiFi contig in all
142 Superkingdoms except the Eukaryotes (Figure 1b). However, the total assembly length of pCLR
143 contigs was lower than the HiFi assembly in all categories except the unassigned, “no-hit” lineage
144 (Figure 1c). In the Archaea and Bacteria annotated contigs, the pCLR assemblies had an average
145 of 61 high-quality draft genomes with an average of 22 predicted circular complete genomes,
146 representing a 48% and 50% reduction, respectively, compared to the HiFi assembly (Table 1;
147 Figure 1d).

148 Binning the HiFi contigs with Hi-C linkage data (see Methods) resulted in 428 complete
149 MAGs (> 90% SCG completeness and < 10% SCG contamination), which is the largest number
150 of reference-quality MAGs reported from a single sample, to our knowledge (Supplementary Table
151 1). The pCLR assemblies also resulted in a substantial quantity of complete MAGs, with an
152 average of 335 MAGs in each assembly (78% of the HiFi total). We hypothesized that one factor
153 contributing to the lower number of MAGs in the pCLR assemblies could be a smaller number of
154 contigs from distinct, related lineages that were more correctly represented in the HiFi assembly.
155 Consistent with this hypothesis, a cumulative assembly length plot suggested that a larger
156 proportion of complete MAGs in the HiFi dataset were of low relative abundance (with coverage
157 below 10x) compared to MAGs in the pCLR assemblies (Figure 2a). Comparisons of bin SCG
158 completeness and average depth of coverage also indicated that the HiFi assembly had more low-
159 coverage complete MAGs than the pCLR assemblies (Figure 2b). The contrast between HiFi and
160 pCLR assemblies was more pronounced in bins that had > 90% SCG completeness (Figure 2c),
161 where the pCLR assemblies contained mainly bins with more than 10X coverage and as much as
162 1000X coverage compared to the HiFi complete MAGs. The distribution of coverage for complete
163 MAGs is consistent with the hypothesis that HiFi assembly resolved pCLR bins into higher
164 resolution, lower coverage bins that had been compressed into single bins in the pCLR assembly.

165

166 **Lineage-resolved MAGs enabled by assembly with HiFi reads**

167 Our experimental design allowed us to test the hypothesis that assembly with HiFi reads
168 had separated distinct lineages into individual assemblies within metagenomes compared to

169 assemblies with pCLR reads. We first classified HiFi and pCLR complete MAGs into predicted
170 phylogeny using GTDB-TK²⁸, resulting in 197 and 187 distinct Genera, and 15 and 14 distinct
171 Phyla classifications, respectively (Supplementary Figure 3). There were 22 genera unique to the
172 HiFi dataset, compared to 8 among all three pCLR datasets, and one phylum unique to HiFi bins
173 (Supplementary Figures 4,5 and 6). Several cases where the HiFi assembly had more assembled
174 bins for a taxon than the pCLR assemblies were also identified (Supplementary Table 2). A clear
175 example of this was for a lineage assigned to the Clostridia class, which had three assembled bins
176 in the HiFi assembly. These three bins had estimated MASH²⁹ distance scores between 0.05-0.07,
177 suggesting that they are separate assemblies of related organisms within this class and possibly
178 represent different species within genus or strains within species (Supplementary Tables 3 and 4).
179 Comparisons of alignments of contigs to the assembly graphs for these bins show clear separation
180 of MAGs within the HiFi dataset and comparatively heterogeneous regions of alignment in
181 equivalent, collapsed pCLR MAGs (Figure 3a). Separation of these HiFi complete MAGs was
182 further compared to the three pCLR datasets through MASH kmer profile comparisons, which
183 revealed that only one bin per pCLR assembly fell within a predicted MASH distance of 0.10 from
184 any of the three HiFi MAGs. This suggested that the pCLR assemblies had collapsed the distinct
185 components of the separate HiFi MAGs into single bins. Indeed, the pCLR contig bins
186 corresponding to the Clostridia class had uneven depths of coverage averaging approximately 45-
187 fold, suggesting they represent composites of distinct lineages, compared to consistent coverage
188 across the contigs for the HiFi bins (Figure 3b). Moreover, this consistent read depth in the HiFi
189 bins varied with the three bins having approximately 10x, 20x, and 33x coverage, demonstrating
190 the potential to accurately deconstruct subtypes across a range of relative abundance. This
191 outcome has significant implications in the use of read coverage in resolving strain lineages from
192 metagenomes.

193 The Clostridia class was instructional but was not the only example of collapsed
194 assemblies present in the pCLR MAGs. A total of 15, 10 and 11 pCLR MAGs were found to be
195 condensed orthologs of 31, 23 and 25 HiFi bins in the pCLR1-3 assemblies, respectively
196 (Supplementary Figures 7, 8 and 9). We also identified other MAGs within the HiFi assembly that
197 are likely species- or strain-resolved assemblies using a nearest neighbor distance analysis with a
198 low MASH pairwise distance cutoff (≤ 0.07 distance). These MAGs likely represent “lineage-
199 resolved” assemblies of individual subpopulations within the same sample as they are separate

200 assemblies of organisms from the same genus or species given this distance cutoff. We identified
201 18 such MAGs within the HiFi assembly, which was triple the amount in the pCLR assemblies (an
202 average of six lineage-resolved MAGs; Supplementary Table 5). These HiFi MAGs had solitary
203 representatives in the pCLR assemblies, suggesting that such fine-scale differences in sequence
204 content and structural variation are likely to be lost in assemblies of long error-prone reads.

205 **Improving resolution within lineage-resolved complete MAGs using HiFi reads**

206 Comparison of pCLR and HiFi bins demonstrated that HiFi assembly resolves sub-lineages
207 even at the stage of initial contig output from the metaFlye assembler. This result motivated us to
208 investigate whether we could further resolve HiFi bins into lineage-resolved complete MAGs
209 using SNP variant data as attempted previously¹⁴. We identified several MAGs that still had single
210 nucleotide polymorphism (SNP) variation above that expected from read error rates within SCG
211 regions. Alignments of short-reads were unable to distinguish true polymorphic sites, particularly
212 in highly repetitive or frequently orthologous gene regions (Figure 3c), so we developed a
213 computational approach to resolve lineages in metagenomes. This approach required an ability to
214 distinguish between polymorphisms within a lineage and structurally variant subtypes within a
215 MAG, which in turn required an ability to simultaneously consider depth of coverage and
216 haplotype information.

217 Since this problem has similarities to phasing isoforms of transcripts in the context of
218 variable expression from parental alleles in gene expression studies, we adapted the phasing
219 algorithm of the IsoPhase workflow^{30,31} into a new tool called MAGPhase to identify SNPs on
220 individual HiFi reads and to phase them across identified single copy gene regions in each MAG.
221 To avoid potential false positive SNP haplotypes due to errors in reads, we only call variants in
222 SCG regions that have at least 10 spanning HiFi reads, and are prevalent at significant proportions
223 of read depth as assessed by a Fisher exact test with Benjamini-Hochberg³² correction (see
224 Methods). Phased SNP haplotypes were identified in each target region and the maximum number
225 of haplotype alleles was counted for each MAG to assess the upper boundary for SCG variation in
226 each MAG. A majority of HiFi MAGs (220; 52% of the total) had zero identified alternate
227 haplotype alleles, suggesting that many lineages were well resolved by the HiFi assembly or did
228 not have detectable polymorphic subpopulations in the sample (Table 2). This is in contrast to the
229 pCLR assemblies, of which an average of 118 MAGs (35% of the total) were found to have zero

230 haplotype alleles (Supplementary Table 6). Polymorphic HiFi MAGs were found to exhibit as
231 many as 25 unique haplotype alleles within SCG regions, suggesting localized regions of genetic
232 drift. This is further supported by the fact that, among 48 HiFi haplotype loci with more than 10
233 unique alleles, we found that 40% (122/305 haplotypes) differed from the original reference
234 sequence by three or fewer bases, suggesting fixation of neutral mutations in subpopulations³³.
235 Median coverage of the alternative alleles in these hotspot regions was an average of five HiFi
236 reads across the length of the haplotype, suggesting that most of these alternative haplotypes were
237 likely not caused by read errors.

238 Comparisons of aligned short-reads to polymorphic HiFi MAGs revealed limitations in the
239 use of short-reads for strain heterogeneity assessments. Using the previously identified example
240 of the lineage-resolved Clostridia MAGs, we identified 7, 1 and 0 alternative haplotype loci on
241 HiFi bins 451, 452, and 471 respectively (Figure 3b). Closer examination of these regions revealed
242 clear variant patterns in individual, aligned HiFi reads, demonstrating the power of using these
243 data for phasing haplotypes from metagenome bins (Figure 3b). These signatures were not readily
244 apparent or were heavily fragmented in the short-read alignments to the HiFi bins (Figure 3c).
245 Furthermore, read pileups in lineage-resolved complete HiFi MAGs and orthologous pCLR
246 collapsed MAGs were instructional in determining how read mapping could be used in
247 downstream variant calling workflows. Comparing orthologous regions between the HiFi MAG
248 451 and pCLR1 MAG 451 (the similarity in number was a coincidence), the visual determination
249 of haplotypes within the selected HiFi MAG is trivial (Figure 3c). One haplotype lineage
250 containing a large insertion of sequence is clearly visible from read pileups and is identified by
251 MAGPhase. By contrast, the pCLR1 MAG has four distinguishable haplotype alleles, consistent
252 with the properties of a collapsed assembly. HiFi MAG 451 can consequently be separated into
253 two separate lineage-resolved complete MAGs using these identified haplotypes, whereas the
254 pCLR MAG is more difficult to resolve. In addition to this example, we identified 35 and 32
255 complete HiFi MAGs that had only 1 or 2 identified alternative SNP haplotypes that could be
256 separated into an additional 70 and 96 lineage-resolved complete MAGs, respectively. However,
257 we note that 220 of our complete MAGs had zero identified haplotypes without any need for
258 manual curation, and therefore fit the criteria of lineage-resolved complete MAGs by default. We
259 adopt this tally of 220 lineage-resolved complete MAGs as a final, conservative estimate of
260 metaFlye assembly of our HiFi read dataset to demonstrate the lack of need for extensive post-hoc

261 editing. In both assemblies, short-read alignments also failed to consistently identify variants
262 within identified haplotype alleles, regardless of the quality of the underlying MAG.

263 The paucity of consistent signal and the smaller power to link variants into haplotypes
264 appears to limit the use of short-reads for variant phasing in complex metagenome communities.
265 Furthermore, the prevalence of many ambiguous short-read alignments with a mapping quality
266 score of 0 (MapQ0) in haplotype regions suggests that these regions are highly repetitive in the
267 overall assembly and do not provide sufficient unique sequence for short-read alignment. The
268 percentage of short-read MapQ0 alignments out of the total were 7%, 9% and 17% for bins 451,
269 452 and 471, respectively, suggesting that large portions of these bins would be otherwise
270 intractable to variant profiling using short-read data. Indeed, a 5 kb window analysis across the
271 entirety of the HiFi assembly identified 18% of the assembly is covered by windows that have
272 ratios of MapQ0 alignments to total alignments greater than 0.50. Naturally occurring variation is
273 unlikely to be detected in these windows via short-read alignments due to mapping ambiguity. By
274 contrast, the proportion of high MapQ0 HiFi alignment windows were found to constitute only ~
275 2% of the length of the assembly, suggesting that 98% of the assembly contains sufficient unique
276 sequence for HiFi read alignment (Supplementary Figure 10).

277 **Improvements in functional genetics analysis**

278 We illustrate the advantages of HiFi reads in functional annotation of a metagenome by
279 predicting biosynthetic gene clusters (BGCs) that are notoriously difficult to identify in fragmented
280 assemblies³⁴. We identified 1,400 complete and 350 partial BGCs (the latter being defined as lying
281 on a contig edge) in the HiFi assembly using antiSMASH³⁵. To the best of our knowledge, this
282 represented the largest number of complete BGCs ever reported in metagenomic assemblies and a
283 40% increase over discovery rates in the three pCLR assemblies (showing 1,245 BGCs on
284 average), with appreciable increases in the detection of important nonribosomal peptide synthetase
285 (NRPS, 25 %) and ribosomally synthesized and post-translationally modified peptide (RiPP, 40%)
286 BGC classes (Fig. 4a). This substantial increase in detected BGCs is not commensurate with the
287 increase in assembly size (15% more assembled HiFi sequence), suggesting that the BGC
288 prediction was significantly improved in the HiFi assembly. Interestingly, nearly all identified
289 BGCs were classified as novel, in the sense that no reference gene clusters of known function were
290 found with >50% of their genes showing homology, illustrating the capabilities of long reads for

291 exploration of novel natural products. We identified 40% more novel BGCs in the HiFi assembly
292 than in the pCLR assemblies (Fig. 4b). Finally, more partial BGCs were identified in the HiFi
293 assembly (Fig. 4c).

294

295 **Improved resolution of mobile DNA association analysis**

296 Candidate viral contigs were identified in each assembly using an alignment-based
297 approach (see Methods). Candidate viral contigs ranging from 5-250 kb in length were identified
298 in each assembly using an alignment-based approach (see Methods). The higher count of
299 assembled viral contigs in the HiFi assembly ($n = 383$) compared to the pCLR assemblies (average:
300 276; stdev: 20) suggested that the breadth of viral diversity in the sample was best represented in
301 that assembly. We conducted an association analysis of viral contigs to candidate microbial hosts
302 using Hi-C links and partial long-read alignments by application of a previously published
303 workflow²³. A resulting network analysis showed that the majority of the viral associations were
304 found between the viral *Siphonoviridae* family and bacterial hosts (Fig 5a), regardless of the use
305 of the HiFi assembly (211 associations), or the pCLR assemblies (185.7 average associations;
306 Supplementary Figures 11, 12 and 13). More associations due to long-read overlaps were identified
307 in the HiFi viral network than the pCLR network (Fig 5b), likely due to improved alignment
308 mapping rates in that assembly. Interestingly, the HiFi assembly provided more evidence of Virus-
309 Archaea associations (60 archaeal contigs) than the pCLR datasets (8.5 mean contigs), primarily
310 via partial long-read alignment metrics which are evidence of genomic integration via a lysogenic
311 life-cycle phase²³(Fig 5c). This increase in Archaea-viral associations is likely due to the increased
312 assembly of Archaea-origin sequence in the HiFi assembly (Figure 1c), which enabled the
313 detection of integrated archaeal virus sequence.

314 Our HiFi assembly also contained many predicted short circular contigs (< 1 Mbp) that
315 likely represented complete plasmid sequence. Using the SCAPP³⁶ plasmid assembly tool, we
316 identified 5,528 candidate plasmid contigs within the HiFi assembly. We identified 298 plasmid-
317 contig associations in the HiFi dataset using Hi-C linkage data (Fig 5d). The largest subgraph
318 (degree = 18) consisted of an interesting association between six plasmid contigs and 25 candidate
319 bacterial hosts (Supplementary Figure 14), in which one plasmid was predicted to inhabit members
320 of 13 different bacterial genera, suggesting inter-genera mobility of this plasmid. We also predicted

321 associations between identified plasmid contigs and three genera of Archaea, including
322 *Methanobrevibacter* and *Methanosphaera*, which were previously not known to carry naturally-
323 occurring plasmids³⁰.

324

325 **Discussion**

326 The goal of metagenome assembly is to create representative reference genomes for the
327 majority of organisms that comprise the sample. However, our data suggests that both short and
328 long error-prone reads produce collapsed assemblies that would otherwise require extensive
329 manual curation to resolve into reference-quality resources. Here, we show that metaFlye
330 assemblies using HiFi reads generate lineage-resolved complete MAGs for single samples without
331 the need for curation (Figure 3). Furthermore, we found good representation of organisms that tend
332 to be prevalent at lower relative abundance in the community in assembled MAGs (Figure 2a), but
333 we nevertheless assembled them to meet the criteria for high-quality draft genomes¹ (Figure 2c).
334 These complete MAGs appear to be resolved with respect to structural variation and orthologous
335 gene sequence compared to closely related (< 10% MASH distance) lineages as evidenced by the
336 assembly graph comparisons. Our data suggests that lineage-resolved complete MAGs are difficult
337 to generate using long error-prone reads, and our experimental design shows that the accuracy of
338 HiFi reads is a necessary element of this result. Sketch-based comparisons revealed that several of
339 the HiFi MAGs (23 - 31 MAGs; 6 - 7% of total) were condensed into collapsed assemblies in the
340 pCLR datasets. The collapsed pCLR bins present in the pCLR dataset were found to be poor
341 representatives of the actual genomic sequence of the organisms based on read alignment metrics
342 (Figure 3b) and variant phasing analysis (Figure 3c). This may present a future challenge for other
343 long-read metagenome assemblies, as lineage-resolved MAGs are most likely to be collapsed in
344 such surveys, particularly if several closely-related species are present in the sample.

345 Our variant phasing method with HiFi reads greatly simplifies variant lineage detection
346 within a sample through the detection of discrete haplotypes. Existing short-read-based strain-
347 resolution algorithms rely on multiple sample observations and statistical variant linkage analysis
348 in order to determine potential microbial lineages^{11,37}. By contrast, HiFi reads provide suitable
349 accuracy and length to enable easy identification of linked variants within a single sample. We
350 identified phased haplotypes of up to 309 SNPs and phase variants across segments as large as 300

351 kbp in our HiFi MAGs (Table 2). Rather than limiting analysis of microbial lineages to average
352 nucleotide ID (ANI) thresholds that may be biased due to short-read alignment inaccuracy, HiFi
353 reads allow for detection of haplotypes segregating in a sample that have as low as 2% (5 reads
354 out of 300) relative abundance of the reference MAG haplotype. To enable this degree of
355 classification, we provide a pipeline and workflow called MAGPhase, based on the
356 cDNA_Cupcake API that is the first to use HiFi reads for haplotype analysis on metagenome
357 assemblies (https://github.com/Magdoll/cDNA_Cupcake). Our IGV alignment diagrams show
358 that evidence supporting the prevalence of these SNP haplotypes is visually verifiable due to the
359 accuracy of HiFi reads. We provide tools to reproduce these diagrams within the MAGPhase
360 workflow to assist future surveys using this data. Even when using MAGs produced by long error-
361 prone reads (pCLR assemblies) as a reference, MAGPhase can still produce discernable SNP
362 haplotypes that could be used to identify descendant lineages (Figure 3c). This means that existing
363 references from isolates or other long-read metagenome surveys could be used in tandem with
364 HiFi reads for strain-typing. However, we note that HiFi alignments to lower quality MAGs are
365 likely to contain far more noise than when using lineage-resolved complete MAGs as references,
366 so *de novo* HiFi-based assemblies are still preferred in this context.

367 We acquired several biological insights from our data that were provided almost
368 exclusively by the HiFi assembly and HiFi reads. Use of the antiSMASH³⁵ detection tool identified
369 40% more BGCs in the HiFi assembly than the highest count in the next best pCLR assembly. The
370 antiSMASH results also provided insights into the functional potential of secondary metabolic
371 pathways in the sheep gastrointestinal tract; for example, 19 BGCs were found in the HiFi data
372 that show high similarity to a recently identified class of gene clusters encoding the production of
373 proteasome inhibitors from the human gut microbiota³⁸, indicating that these functions may be of
374 similar importance for host colonisation in ruminants as they are in humans. More of these BGCs
375 were predicted to be novel in the HiFi assembly, and were furthermore not found to be resolved
376 replicates of compressed consensus sequences in the pCLR assemblies. Additionally, we identified
377 several novel associations of mobile genetic elements in our sample using a combination of Hi-C
378 linkage data and HiFi read alignment overlaps. Both the pCLR assemblies and HiFi assembly had
379 similar profiles of viral-host association links, with a notable exception in the case of links between
380 Archaea and viral contigs. The HiFi assembly detected a higher quantity (n= 60) and greater
381 complexity (diameter = 7) of archaeal-viral associations primarily through HiFi read overlaps.

382 Host-plasmid analysis using Hi-C links also identified broad host-specificity for several
383 assembled, circular plasmids in our HiFi dataset. In total, we identified 424 and 298 potential host-
384 viral and host-plasmid links in our HiFi dataset, which represents one of the most substantial
385 associations of mobile element activity in a single sample to date. Most of these associations were
386 exclusive to the HiFi assembly and were not identified in replicate pCLR assemblies.

387 The improvements in lineage resolution and haplotype phasing offered by HiFi reads
388 present new opportunities but also a major dilemma. HiFi reads are currently more expensive to
389 generate than equivalent amounts of short-reads and long error-prone reads. Additionally, HiFi
390 reads tend to be shorter than reads generated by PacBio CLR mode or Oxford Nanopore platforms
391 due to shorter molecule fragment size requirements for circular consensus sequencing (CCS) to
392 obtain enough passes across the insert (minimum of 3) for CCS error correction. This could limit
393 their application in large-scale metagenomic surveys; however, we note that DNA fragment size
394 distributions from recent long-read metagenome assembly surveys often do not exceed 10 kbp in
395 size^{23,24}. In the absence of a reliable protocol to generate metagenome WGS datasets with read
396 N50 values above 100 kbp as per typical “ultra-long” library preparations¹⁷, the choice between
397 longer CLR datasets and higher quality HiFi datasets could be a false dilemma. We previously
398 reported that the use of long error-prone reads resulted in a four-fold increase in contig N100K
399 statistics over a comparable short-read assembly²³. In this study, we found that the use of the same
400 amount of near-equivalent length, HiFi long-reads resulted in a 2.5-fold increase in SCG complete
401 contigs over assemblies constructed from their constitutive subreads. Another HiFi-specific
402 advantage is the assembly of lineage-resolved MAGs that were otherwise condensed in pCLR
403 assemblies, and the phasing of variant haplotypes to distinguish finer resolution differences in
404 populations in the sample.

405 To our knowledge, this is the first time that it has been possible to examine the population
406 structure of metagenomes using whole assemblies and read-phased haplotype alleles, and it creates
407 more exciting possibilities for future study. Our analysis suggests that such insights can only be
408 gained through the use of long (> 5 kb) reads with suitably low (~ 1%) error rates, as the former
409 criteria enables the spanning of orthologous genomic regions and the latter enables the separation
410 of species/strain-level haplotypes into separate assemblies. These results were obtained with
411 relatively minimal efforts, requiring only assembly and binning of HiFi reads with Hi-C data,

412 thereby obviating the need for extensive manual curation. Resulting lineage-resolved complete
413 MAGs and phased SNP haplotypes are the first realization of “complete metagenomics” - isolate-
414 quality genome assemblies for microbial organisms from complex metagenome samples.

415

416

417 **Online Methods**

418 **Long-read DNA sequencing and subread extraction**

419 A fecal sample was taken from a young (<1 year old) wether lamb of the Katahdin breed.
420 The animal died while on pasture and postmortem was diagnosed with combined *Strongyloides*
421 and coccidial infection. The sample was acquired postmortem following the USDA ARS IACUC
422 protocol #137.0 during routine necropsy to determine cause of death. The sample had a watery
423 texture consistent with diarrhea and apparent parasite eggs were observed within the sample, which
424 was transferred to a 50ml tube, mixed to make as homogenous as possible, and aliquoted into 1.5
425 ml microfuge tubes. DNA was extracted in small batches from approximately 0.5g/batch using the
426 QIAamp PowerFecal DNA kit as suggested by the manufacturer (QIAGEN) with moderate bead
427 beating and sheared using a Digilab Genomic Solutions Hydroshear instrument (Digilab). The
428 sheared DNA was size-selected to approximately 9-18 kb on a SAGE ELF instrument to final
429 target size which varied from 9 kbp up to 16 kbp followed by library preparations using the
430 SMRTbell Template Prep kit v1.0 as described (20). Sequence data was collected over time and
431 included 46 SMRT cells on a Sequel instrument using 10 library preparations, with 24 cells of v2
432 chemistry and average inserts of 9-10 kbp and 22 cells of v3 chemistry and average inserts of 14
433 kbp. An additional 8 cells representing individual library preparations were sequenced on a Sequel
434 II instrument using v1.0 chemistry and average inserts of 14 kbp. Subreads and CCS reads were
435 generated using SMRTLink software v6.0 CCS protocol and default settings. An average of 35%
436 of subreads per cell were converted to CCS corrected reads (range 1-63%). This resulted in 255
437 Gbp of total CCS reads from both the Sequel I (45 Gbp of the total) and Sequel II (210 Gbp)
438 sequencing runs. A subset of this data (46 Sequel I SMRTcells) representing 18% (45 Gbp) of the
439 total dataset was previously assembled as validation data in the metaFlye assembler publication⁵.
440 The Sequel II dataset was filtered after CCS correction to retain only reads that fit HiFi quality

441 standards (3+ full length passes and average read quality scores > Q20). We note that a small
442 proportion of our Sequel I dataset (4,350 reads; 0.02% of the total number of CCS reads) consisted
443 of reads that did not meet HiFi read quality standards (average Q scores above 20) as this dataset
444 had been filtered with a prior version of the SMRTLink software. These reads were retained as
445 they comprised a very small proportion of the total dataset.

446 Subreads were extracted from the converted CCS reads to provide a suitable comparison
447 between uncorrected and corrected long read datasets. First, all of the constitutive subreads of the
448 CCS reads were identified from subread BAM files. Using a custom script
449 ([https://github.com/njdbickhart/python_toolchain/blob/master/assembly/extractPacbioCLRFrom](https://github.com/njdbickhart/python_toolchain/blob/master/assembly/extractPacbioCLRFromCCSData.py)
450 [CCSData.py](https://github.com/njdbickhart/python_toolchain/blob/master/assembly/extractPacbioCLRFromCCSData.py)), the second, third and fourth subreads were separately extracted into FASTQ files
451 designated pCLR1, pCLR2, and pCLR3, respectively (the first subread does not typically
452 encompass the complete DNA fragment, so was discarded). Statistics on subread lengths from the
453 Sequel I and Sequel II datasets are shown in Supplementary Figures 1 and 2, respectively. Due to
454 sequence read falloff in later subreads, the pCLR3 dataset was truncated relative to the pCLR1 and
455 pCLR2 datasets. In the Sequel I dataset, a small proportion of reads (51 reads; ~0.001%) did not
456 have a fourth (pCLR3) subread, making the third replicate dataset smaller than the others. This
457 resulted in a reduction of 104 Mbp of sequence in this dataset (48.763 Gbp) compared to the
458 pCLR1 or pCLR2 extracted subreads (48.830 Gbp). The original CCS reads were organized into
459 a dataset hereafter referred to as the “HiFi” reads and the three subread replicates were labelled
460 pCLR1-3 in the chronological order in which they were sequenced in the subread BAM files.

461 **Short-read sequencing and Hi-C library preparation**

462 An approximately 2g subsample of frozen homogenized fecal material was provided to
463 Phase Genomics (Seattle, WA) for Hi-C contact map construction using their Proximeta service.
464 The restriction endonucleases Sau3AI and MluCI were used to generate separate Hi-C sequencing
465 libraries as previously described³⁹. Using a total of 107 million paired-end reads from both Hi-C
466 libraries were generated for analysis. A separate portion of the extracted DNA from the fecal
467 sample was saved for short-read “whole genome shotgun” (WGS) DNA sequencing. Truseq PCR-
468 free libraries were created from this sample as previously described⁴⁰ and were sequenced on an
469 Illumina NextSeq 500. A total of 149 Gbp of WGS short reads were generated from this sample.

470 **Genome assembly, read alignment and binning**

471 Reads from the HiFi and pCLR datasets were assembled into contigs using the metaFlye⁵
472 genome assembler, version 2.7-b1646 for HiFi reads and version 2.7.1-b1590 for pCLR reads. The
473 assembler was run in metagenome mode (“—meta”) flag and the “—pacbio-hifi” and “—pacbio-
474 raw” data prefix flags were used for input HiFi reads and the pCLR reads, respectively. We note
475 that the “—pacbio-hifi” input designation only uses reads that have average error rates below 1%
476 for the disjointig and contig phases of the workflow. This means that only HiFi quality reads
477 (Q20+) were used to generate the initial graphs and final contigs of the HiFi assembly. However,
478 all input reads were used in the consensus polishing step of metaFlye. All assemblies were polished
479 with two rounds of Pilon⁴¹ correction using the previously generated, WGS, short-read datasets.
480 Contigs shorter than 1000 bp in all assemblies were removed from further analysis. Closed circular
481 contigs were identified from metaFlye assembly reports. WGS short reads were aligned to the
482 assemblies using BWA MEM⁴² using default settings. HiFi reads were aligned using Minimap2⁴³
483 with the “-x asm 20” preset setting as recommended by the developers. Window-based alignment
484 analysis was conducted by using custom python scripts
485 (https://github.com/njdbickhart/python_toolchain/blob/master/sequenceData/getBAMMapQ0Rat
486 [ios.py](#)).

487 Hi-C read-pairs were aligned to each assembly using BWA MEM with the “-5SP” flag to
488 disable attempts to pair reads according to normal Illumina paired-read settings. Resulting BAM
489 files from Hi-C reads were sorted by read name. Hi-C alignments were used in the Bin3c⁴⁴ binning
490 pipeline to generate a set of bins for each assembly. Bin quality was assessed by CheckM² and
491 DASTool⁴⁵ single copy gene metrics. MAGs were identified from bins that had > 90% SCG
492 completeness and less than 10% SCG contamination estimates from the DASTool quality
493 assessment data.

494 **Taxonomic assignment**

495 We distinguish between contig-level and bin-level taxonomic classification to demonstrate
496 differences in pCLR/HiFi assembly quality and assign representative taxonomy of the final
497 polished bins, respectively. Contigs were assigned to candidate taxa using the Blobtools v1.0⁴⁵
498 taxify pipeline, using models from the Uniprot (release: 2017_07) database as described
499 previously²³. Contigs that did not meet the Blobtools threshold for taxonomic assignment, or were
500 identified as belonging to faulty database entries (e.g. the “Cetacean” lineage) were labeled as “no-

501 hit” taxa. Viral contigs identified from this analysis were used in subsequent virus association
502 analysis (see methods section below). Predicted viral contigs were separately verified using the
503 CheckV⁴⁶ pipeline using the “end-to-end” workflow, multithreaded, and with normal settings.

504 The GTDB-TK v1.0²⁸ ‘classify_wf’ workflow was used to assign candidate taxonomic
505 affiliation to all assembled bins. Default GTDB-TK settings were used with the only exception
506 being the setting of the ‘—pplacer_cpus’ argument to ‘1’ as recommended by the authors. In cases
507 where GTDB-TK was unable to assign a taxonomic lineage, a consensus of contig-level
508 assignments from the Blobtools taxify pipeline were used to assign candidate taxonomic affiliation
509 for the bin. The prevalence of three or more contigs in the MAG indicating the same species-level
510 taxonomy were used when possible. In the case of “ties” between contig-level taxonomic
511 consensus, the final taxonomic consensus was resolved to the lowest possible level (ie. genus or
512 family).

513 **MagPhase lineage-resolution and orthologous MAG identification**

514 We first sought to identify orthologous bins among each of the pCLR assemblies and the
515 HiFi assembly in order to provide direct comparisons among similar assembled taxonomic groups.
516 To identify orthologous bins, we used MASH v2.2²⁹ sketches of all HiFi bins as a reference against
517 queries of all pCLR bins. MASH sketch settings were -s 100000 and -k 21, with all other settings
518 left at the default. The MASH “dist” command was used with a cutoff of 0.10 distance to identify
519 orthologous MAGs which is approximately equivalent to an average nucleotide identity of 90%
520 between hits. Multiple reference and query hits were allowed and retained for future comparisons.

521 HiFi reads were realigned to the HiFi and pCLR assembly bins using minimap2⁴³ as
522 previously described and alignment files were converted to BAM file format using Samtools⁴⁷. To
523 reduce the possibility of supplementary or split-read alignments impacting downstream variant
524 calling, we filtered these alignments from the HiFi read BAM files. We then used these filtered
525 alignment files for variant calling and haplotype identification using MAGPhase. The MAGPhase
526 algorithm attempts to identify full-length SNP variant haplotypes in a greedy fashion within a
527 given set of genomic coordinates. By default, only genomic coordinates that have at least 10 full
528 length reads (10X coverage) are considered for variant calling. Initial variants used in read phasing
529 are identified from HiFi read alignment pileups. To distinguish between potential errors in reads
530 and SNPs, we model expected errors at a rate of 0.5% and test observed variant coverages against

531 an expected error variant coverage using the Fisher exact test. To correct for further multiple
532 hypothesis testing across an entire region, we employ a Benjamini-Hochberg³² procedure to
533 estimate modified p values. The default p value cutoff for a variant site to be included is less than
534 0.10 for the modified p value. Once a set of candidate variants is called, the program then attempts
535 to phase them into haplotypes based on their observed presence in HiFi reads. The entire length of
536 an alternate haplotype is imputed using the physical linkage of previously identified SNP variants
537 on individual HiFi reads that span the region. Missing variant information from imputation (due
538 primarily from chimeric read alignments or the presence of read errors) is denoted with question
539 marks (?) in the final haplotype dataset.

540 In order to reduce the potential expansion of haplotype counts due to recombination, we
541 phased HiFi reads within identified SCG regions of each HiFi and pCLR bin. CCS reads that
542 extended over the edges of SCG regions were included in haplotype phasing, so if two SCG regions
543 were within short distances from each other, phased variant haplotypes could extend further.
544 Partially imputed haplotypes (haplotypes that contained question marks (“?”)) were excluded from
545 analysis as these could have resulted from chimeric read alignments or base call errors on selected
546 SNP variant sites within the haplotype. Haplotypes were considered alternative alleles based on
547 read depth, with lower depth haplotypes considered to be alternatives to the highest read depth
548 allele at that loci. Haplotypes that included fewer than 3 SNPs were filtered as these tended to have
549 lower counts of read alignments and higher alternate allele haplotype counts. If a MAG was found
550 to have no SNP variants that fit the read depth statistical requirements, it was considered to be a
551 “lineage-resolved” MAG. MAGs that had unfiltered SNP variants that were otherwise unable to
552 be assigned to haplotypes with 3 or more SNPs were not considered to be lineage-resolved and
553 were labeled as “polymorphic.” Read depth and read clustering were assessed through custom
554 Python scripts
555 ([https://github.com/njdbickhart/python_toolchain/blob/master/metagenomics/plotMagPhaseOutp
556 ut.py](https://github.com/njdbickhart/python_toolchain/blob/master/metagenomics/plotMagPhaseOutput.py)) and IGV⁴⁸ plots.

557 **Gene cluster prediction and functional annotation**

558 The four assembled metagenomes in FASTA format were used as input for antiSMASH
559 version 5³⁵, which predicted the genes using Prodigal⁴⁹. The generated output was used to group
560 BGCs into six different BGC classes: RiPP, NRPS, Terpene, PKS, Saccharide and Others. Also,

561 from the annotated Genbank files, BGCs could be classified into either the “Partial” category
562 (when they were found on a contig edge) or into the “Complete” category (when this was not the
563 case). Finally, predicted BGCs with fewer than 50% of the genes having hits to the best
564 KnownClusterBlast hit, which is obtained from searching all BGCs in the MiBIG database v 2.0⁵⁰
565 were considered “Novel”. When this condition was not satisfied, the BGCs were classified into
566 the “Known” group.

567 **Virus and plasmid association analysis**

568 Viral contigs were identified from Blobtools taxonomic assignment for use in the
569 association analysis. Genome completeness of these viral contigs was estimated by the CheckV
570 1.0 ‘end_to_end’ workflow⁴⁰ (See supplementary table 7). Given the potential novelty of
571 assembled viral genomes in this dataset, the “Not-Determined” and “Medium” completeness viral
572 contigs were not filtered prior to the association analysis. CCS read overlaps and Hi-C link data
573 were used to identify potential host-viral associations as previously described²³. Briefly, read
574 overlap data consisted of CCS reads that partially mapped to both viral and non-viral contigs.
575 Associative Hi-C links consisted of cases where the number of inter-contig Hi-C pair alignments
576 between viral and non-viral contigs were three standard deviations above the average count for all
577 contigs. Both datasets were compared for overlap, and network plots were generated using the
578 Python NetworkX version 2.5 module. The analysis workflow and network plotting were
579 automated using the following script:
580 <https://github.com/njbickhart/RumenLongReadASM/blob/master/viralAssociationPipeline.py>

581 Plasmids were identified using the SCAPP workflow with the metaFlye HiFi assembly
582 graph (“gfa” file) and aligned short-read BAM files to the final, polished assembly fasta file³⁶. The
583 default settings were used apart from the setting of the “-k/--max_kmer” value to “0” in order to
584 disable kmer-based tokenization of sequence reads. SCAPP plasmid nodes were filtered if they
585 were shorter than 5 kb or longer than 1 megabase in length prior to alignment. Plasmid node
586 orthologs in each main assembly were identified through minimap2⁴³ alignments and were
587 removed prior to alignment. Hi-C reads were aligned to this modified reference using bwa MEM⁴²
588 and alignment files were converted to BAM format using samtools⁴⁷. The alignment file was used
589 in the aforementioned viral-association workflow script to identify substantial links between
590 candidate plasmids and host contigs. Contig level annotation via the Blobtools⁴⁵ taxify pipeline

591 was used to classify each candidate host by Kingdom. Networks were visualized using the Python
592 NetworkX version 2.5 module.

593 **Data Availability**

594 The HiFi Sheep dataset, Hi-C reads and WGS short-reads are available on NCBI Bioproject
595 PRJNA595610 at accession ids SRX7628648, SRX10704191, and SRX7649993, respectively.
596 Whole metagenome assemblies and MAG bins for the pCLR and HiFi datasets are available at the
597 following DOI: <https://doi.org/10.5281/zenodo.4729049>

598 **Code Availability**

599 The MAGPhase script and codebase are part of the https://github.com/Magdoll/cDNA_Cupcake
600 github repository. Custom scripts used to analyze MAGs and visualize the data are part of the
601 following github repository: https://github.com/njdbickhart/python_toolchain.

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609

610 **Author Contributions**

611 TPLS and DMB conceived the project with extensive modifications introduced on the advice of
612 IL and PAP. SBS and TPLS were responsible for collecting the sample and generating the
613 sequence data. DB and MK produced the assemblies and conducted a large proportion of reported
614 analysis. VPA and MHM identified biosynthetic gene clusters in the dataset. DMB, AZ and IM
615 identified mobile genetic elements in the sample. ET developed the MagPhase algorithm with
616 inputs from DMB. DMB, TPLS, MK and PAP wrote the manuscript. All authors read and
617 contributed to the final manuscript.

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726

727 **Figure Legends**

728 **Figure 1.** Contig-level comparison of pCLR and HiFi assemblies. a. Strategy for generating the
729 read sets for the three pCLR and the HiFi assemblies. b. Comparison of contig length distributions
730 in the four assemblies demonstrating a tendency for pCLR assembly to create longer contigs. c.
731 Comparison of the total length of each assembly after separation of contigs into predicted
732 Superkingdoms demonstrating an increased length from HiFi assembly among assigned
733 Superkingdom and reduced length in unassigned bin. d. Comparison of the completeness of pCLR
734 and HiFi assemblies based on the presence of >90% expected single-copy genes with <5%
735 redundancy.

736

737

738 **Figure 2.** The cumulative length of assembled HiFi bins (a) peaks at lower depths of coverage at
739 a faster rate than the cumulative lengths of pCLR bins, suggesting that lower abundance taxa were
740 more likely to be assembled by HiFi reads. Comparisons of average short-read coverage against
741 single copy gene completeness estimates (b) for high-quality bins revealed a substantial number
742 of HiFi bins below the 10X coverage threshold compared to the pCLR datasets. This is particularly
743 enhanced in the >90% completion category, where the average coverage of the HiFi bins is lower
744 than that of each pCLR assembly, and several HiFi bins have less than 1X average short-read
745 coverage as opposed to no equivalent coverage-profile pCLR bins.

746

747 **Figure 3.** Lineage resolved MAGs (a) in the HiFi assembly often corresponded to two or more
748 compressed bins in the pCLR assemblies. In this example, we show comparative alignments of
749 HiFi bins (colored according to the legend) on superset graphs of three HiFi bins (left-most graph)
750 and a single pCLR1 bin (right-most graph). pCLR graph alignments show bifurcation and
751 trifurcation of sequence into bubbles that were otherwise condensed in the final assembly. Dark
752 red tinted boxes correspond to IGV plots in (c). Using our newly developed MAGPhase algorithm
753 (b), we identified several locations where multiple SNP-derived haplotype alleles are present in
754 each bin (alternating colors) and estimated their relative depth compared to all HiFi read
755 alignments. IGV plots of specific loci within these bins (c) show the power of this method to easily
756 distinguish between haplotypes without the need for extensive statistical post-hoc analysis.
757 Comparative alignments of HiFi reads to HiFi bin 451 show only one alternate allele, whereas the
758 equivalent region in the pCLR1 bin 451 shows as many as four alternate alleles (labeled on the
759 figures). Furthermore, comparisons with short-read alignments revealed the inadequacy of short-
760 reads to identify phased haplotypes within these highly resolved MAGs.

761

762 **Figure 4.** The HiFi assembly revealed approximately 25% more complete Biosynthetic Gene
763 Clusters (BGCs) than the average pCLR assembly (a). This increase was manifested in all
764 identified BGC classes (colors in legend) and was not exclusive to one particular class. As found
765 in other metagenome assembly datasets, the majority of identified BGCs were novel in all

766 assemblies (b), but the HiFi assembly had a higher proportion of novel BGCs than the other
767 assemblies. Additionally, the HiFi assembly contained more partial BGCs (c) of any assembly.

768

769 **Figure 5.** A network plot of predicted host-virus associations (a) identified through HiFi read
770 overlaps (blue), Hi-C links (green) and both data types (red) revealed new viral genomes that have
771 broad host specificity. In addition, the HiFi assembly was better able to identify candidate viral-
772 Archaeal associations than those detected in the pCLR datasets. Viral-host associations were
773 predominantly identified through HiFi read alignments (b) and the HiFi assembly had a higher
774 proportion of this evidence compared to the average pCLR assembly. Highlighting the difference
775 in domain detection between the assemblies, more Viral-Archaeal links (c) were identified in the
776 HiFi assembly compared to the pCLR assemblies. Using Hi-C link data, we were also able to
777 identify candidate hosts for assembled plasmid sequence (d) in the HiFi assembly.

778

779

780 **Tables:**

781 **1. Assembly quality statistics**

782

Assembly	Contigs	Assembly Length (Mbp)	Contig N50 (Kbp)	HQ Draft Contigs ¹	Circular Contigs ²	Circular + HQ Draft Contigs
HiFi	57,259	3,424	280	123	49	44
pCLR1	48,338	2,985	185	54	21	18
pCLR2	48,790	3,008	187	65	28	26
pCLR3	56,456	2,978	181	64	26	22

783

784 ¹ Contigs that were determined to have greater than 90% single copy gene (SCG) completeness
785 and less than 5% SCG redundancy.

786 ² Contigs larger than 1 Mbp in size that were predicted to be circular by the metaFlye assembler.

787

788

789

790 **2. MagPhase Haplotyping results**

791

	HiFi	pCLR1	pCLR2	pCLR3
Total complete MAGs	428	345	345	315
Average Contig count per MAGs	8.3	10.8	11.4	11.9
Zero Haplotype MAGs ¹	220	130	136	89
Percent Polymorphic MAGs ²	48.5%	62.3%	60.6%	71.7%

792

Average Haplotype Variant Length (bp) ³	20.1	27.1	24.1	34.0
Average Haplotype Genomic Length (bp) ⁴	1151.3	1106.1	1057.8	961.2

Maximum Haplotype Genomic Length (bp)	336,899	463,082	480,257	493,333
Average Haplotype Alleles per Locus ⁵	4.18	4.72	4.43	5.06
Maximum Haplotype Alleles per Locus	25	59	54	60

793

794

795 ¹ Metagenome assembled genomes (MAG) that did not have detectable SNP haplotypes that could be
796 linked with HiFi reads.

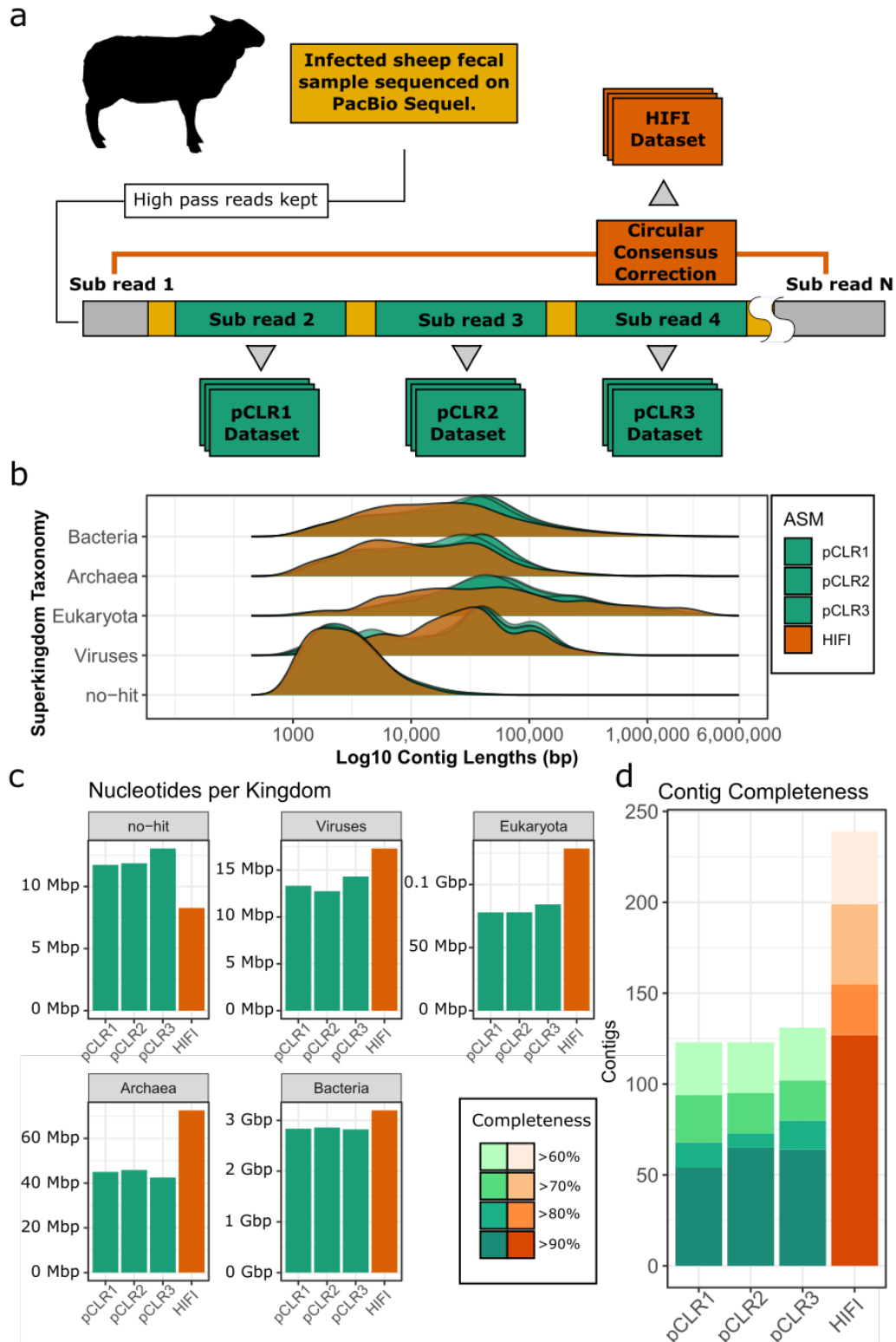
797 ² The number of MAGs that had at least one detectable alternative SNP haplotype allele.

798 ³ Haplotype variant length represents the count of polymorphic SNP loci within an identified haplotype.

799 ⁴ Genomic length was defined as the distance in bases on the assembled contig from the first
800 polymorphic SNP site to the final site.

801 ⁵ A haplotype allele was defined as a unique permutation of polymorphic SNP variants that were
802 identified in one consistent region in the genome that met thresholds for detection.

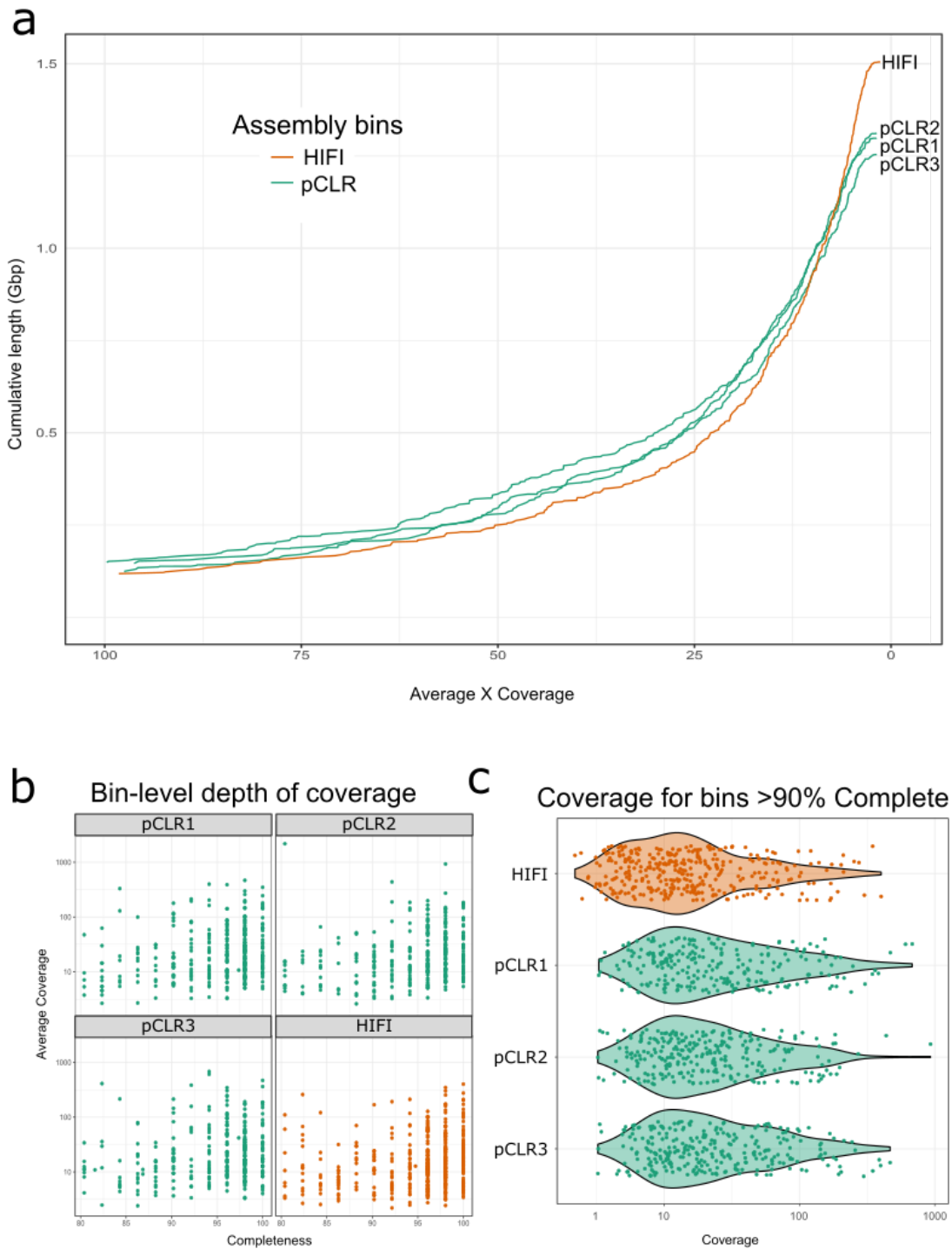
803 **Figure 1. Contig-level statistics of assembled datasets**



804

805

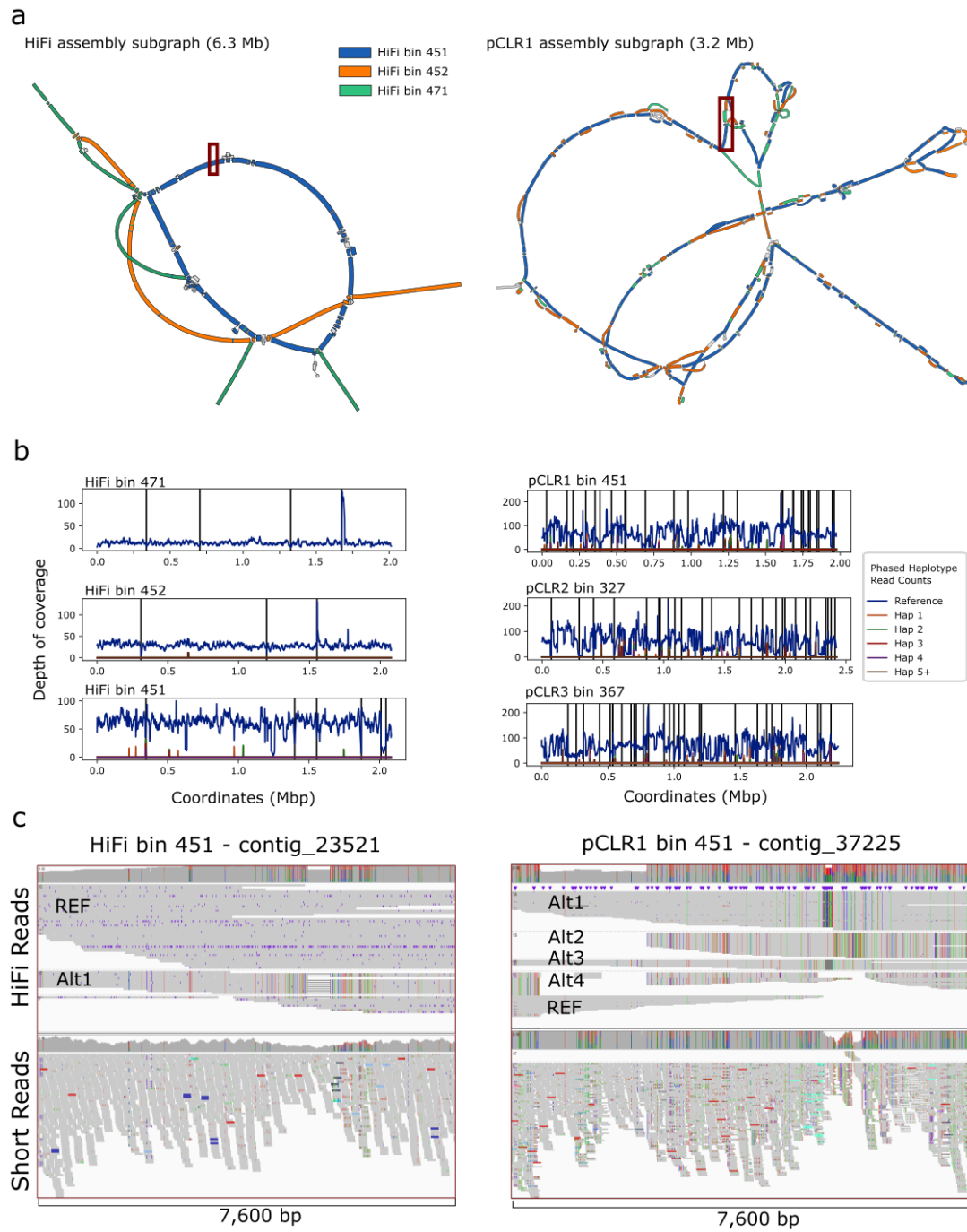
806 **Figure 2. HiFi metagenomic bins better represent lower-abundance taxa**



807

808

809 **Figure 3. Lineage-resolved metagenome assembled genomes**



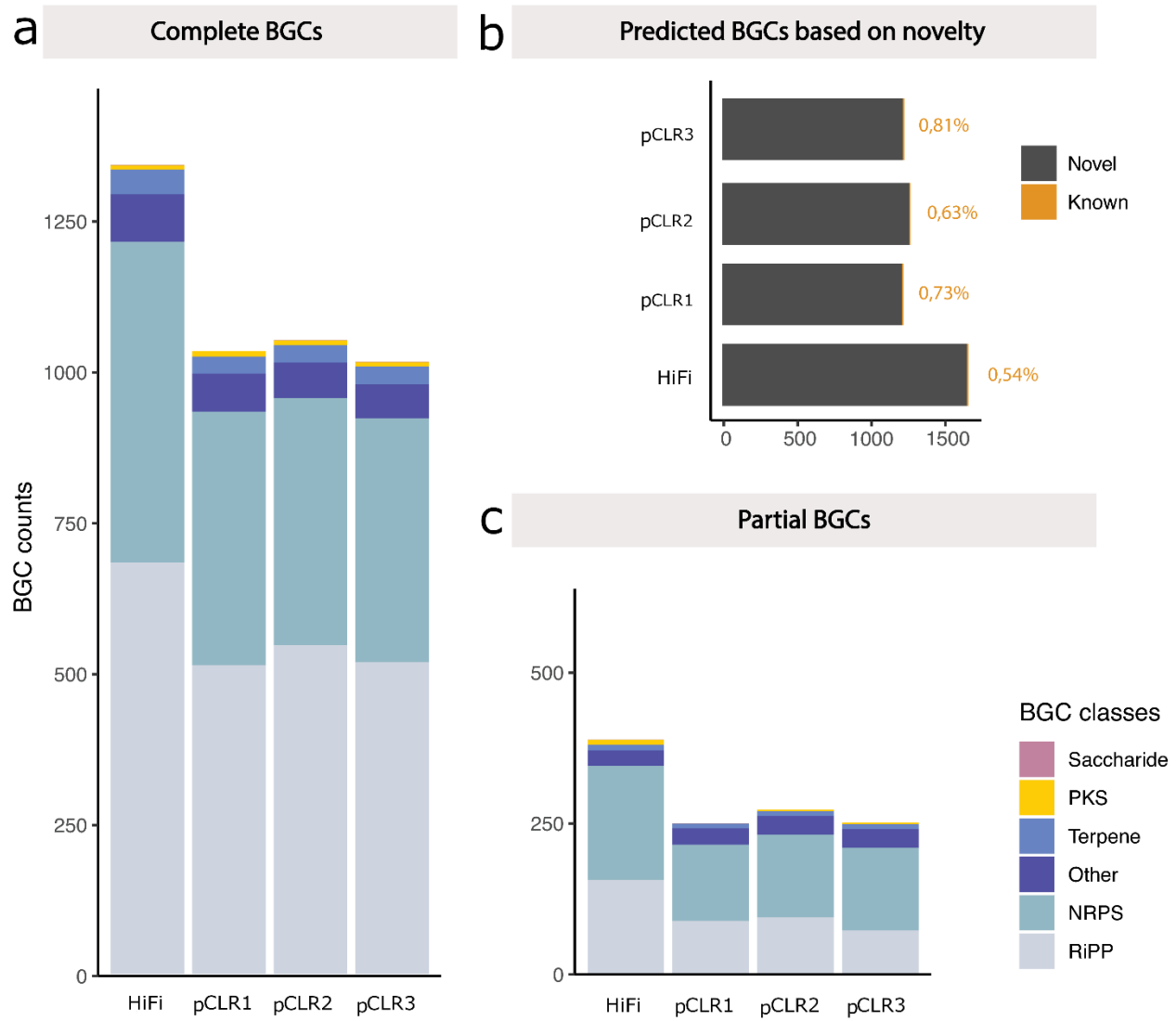
810

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812

813 **Figure 4. Improvements in functional genetic annotation**

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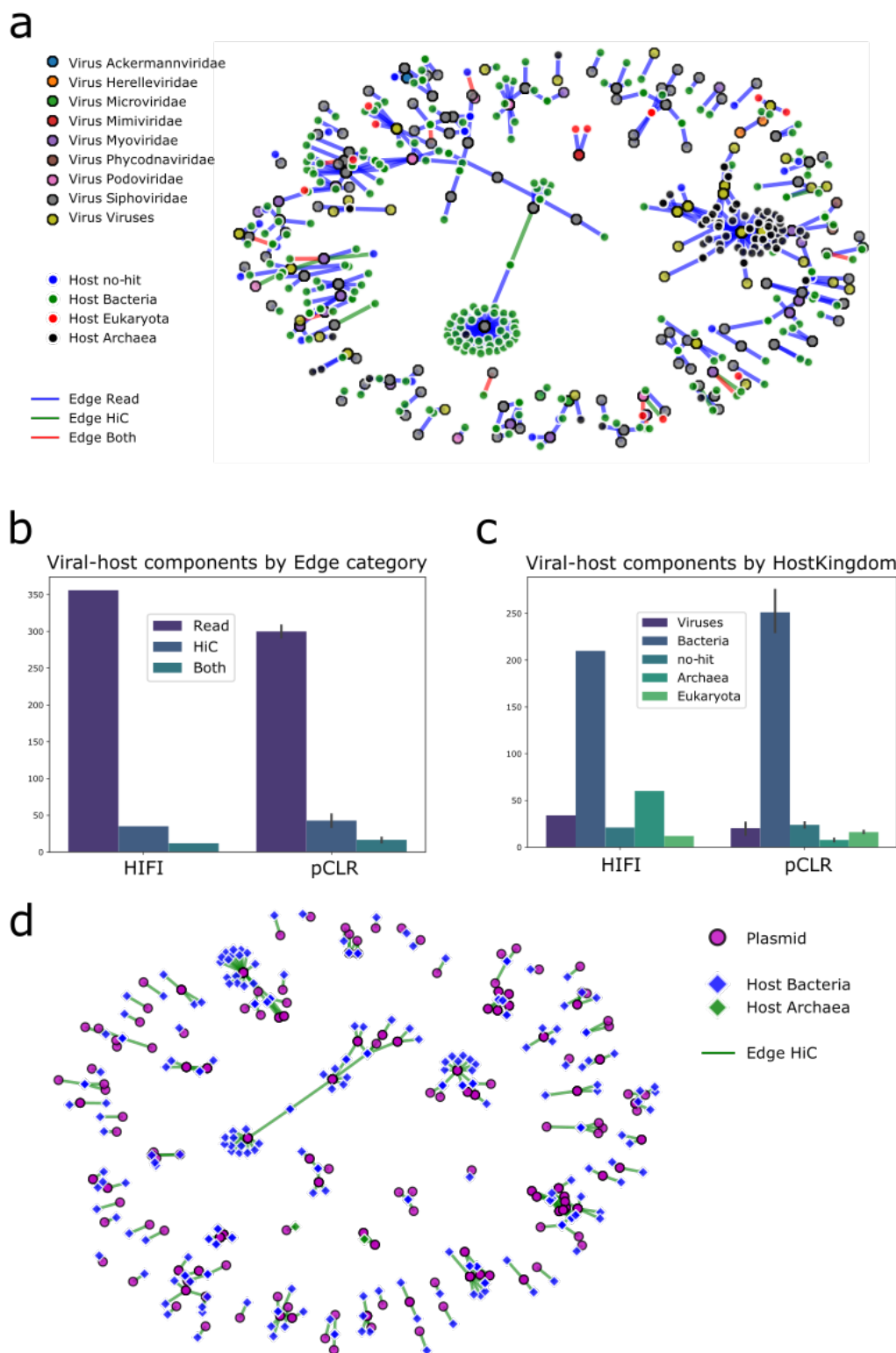


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818 **Figure 5. Improved detection of mobile genetic elements**



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820