

1 **BlueFeather, the singleton that wasn't: Shared gene**  
2 **content analysis supports expansion of *Arthrobacter***  
3 **phage cluster FE**

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14  
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20  
21 **Brief Running Title**

22 *Arthrobacter* phage BlueFeather

26 **Abstract**

27 Bacteriophages (phages) exhibit high genetic diversity, and the mosaic nature of  
28 the shared genetic pool makes quantifying phage relatedness a shifting target. Early  
29 parameters for clustering of related *Mycobacteria* and *Arthrobacter* phage genomes  
30 relied on nucleotide identity thresholds but, more recently, clustering of *Gordonia* and  
31 *Microbacterium* phages has been performed according to shared gene content.  
32 Singleton phages lack the nucleotide identity and/or shared gene content required for  
33 clustering newly sequenced genomes with known phages. Whole genome metrics of  
34 novel *Arthrobacter* phage BlueFeather, originally designated a putative singleton,  
35 showed low nucleotide identity but high amino acid and gene content similarity with  
36 *Arthrobacter* phages originally assigned to Clusters FE and FI. Gene content similarity  
37 revealed that BlueFeather shared genes with these phages in excess of the parameter  
38 for clustering *Gordonia* and *Microbacterium* phages. Single gene analyses revealed  
39 evidence of horizontal gene transfer between BlueFeather and phages in unique  
40 clusters that infect a variety of bacterial hosts. Our findings highlight the advantage of  
41 using shared gene content to study seemingly genetically isolated phages and have  
42 resulted in the reclustering of BlueFeather, a putative singleton, as well as former  
43 Cluster FI phages, into a newly expanded Cluster FE.

44

45 **Introduction**

46  
47 Bacteriophages are ubiquitous biological entities with an estimated  $10^{31}$  phage  
48 particles on Earth. Assuming an average length of 200 nm, they would extend 200  
49 million light years if stacked head-to-tail (Wiles 2014). Phages are found in all  
50 ecosystems in which bacteria exist and function as drivers of bacterial evolution (Keen

51 2015). They exhibit horizontal gene transfer (HGT) with each other and with bacteria,  
52 resulting in the diverse and mosaic nature of phage genomes (Pedulla et al. 2003).  
53 Despite their incredible prevalence in the environment, phages remain largely  
54 understudied (Miller-Ensminger et al. 2018).

55 Previous research on mycobacteriophages concluded that phages may exhibit a  
56 continuum of diversity, wherein genes are constantly being shuffled amongst the phage  
57 population, resulting in shared genes and sequences between different clusters (Pope et  
58 al. 2015). The immense and ever-expanding diversity of phage genomes has historically  
59 been categorized in terms of nucleotide sequence conservation, with a minimum 50%  
60 nucleotide identity and 50% span length to at least one phage in a cluster to warrant  
61 membership (Hatfull et al. 2010; Klyczek et al. 2017). A mass scale study on *Gordonia*  
62 phages also identified a spectrum of genetic diversity, as clusters did not have clear  
63 boundaries (Pope et al. 2017). Numerous phages lacked the requirement of 50%  
64 nucleotide identity but shared many genes, suggesting a relatedness not captured by  
65 nucleotide comparisons alone. This relatedness was confirmed with a gene content  
66 network phylogeny and subsequently, the cluster assignment parameter for *Gordonia*  
67 phages (Pope et al. 2017), and later for *Microbacterium* phages (Jacobs-Sera et al. 2020),  
68 was adjusted to 35% shared gene content with at least one phage in a cluster.  
69 Mycobacteriophages, as well as *Gordonia* and *Microbacterium* phages, exhibited this  
70 spectrum; however, the extent of diversity varies depending on the current known  
71 phage population, which in turn affects how clustering is carried out. *Arthrobacter*  
72 phages were previously found to exchange genes more slowly than *Gordonia* phages,  
73 and the 50% nucleotide clustering parameter was considered sufficient at the time (Pope

## *Arthrobacter* phage BlueFeather

74 et al. 2017). Further studies on *Arthrobacter* phages found these phages to be  
75 genetically isolated with highly variable gene content for phages that can infect a range  
76 of host species. With this great diversity, nucleotide identity was used to separate  
77 *Arthrobacter* phages into 10 distinct clusters and 2 singletons (Klyczek et al. 2017), and  
78 this parameter has been considered sufficient to categorize the limited number of  
79 *Arthrobacter* phages until recently.

80 Singleton phages can serve as the seeds to start new clusters or be extremely  
81 distinct, as they lack the nucleotide identity and/or shared genes required for clustering  
82 with known phages. In this study, the genome of novel *Arthrobacter* phage BlueFeather  
83 was examined for nucleotide and amino acid identity with other known phages.  
84 BlueFeather lacked sufficient nucleotide conservation for clustering according to  
85 nucleotide-based parameters, and was thus designated a putative singleton. Phage  
86 BlueFeather did, however, have notable amino acid conservation and shared gene  
87 content with other *Arthrobacter* phages previously assigned to Clusters FE and FI,  
88 suggesting it may not be as isolated as its putative singleton status implied. The  
89 outcomes of this research on phage BlueFeather provided evidence for the reclustering  
90 of phage BlueFeather, as well as phages formerly assigned to Cluster FI, into a newly  
91 expanded Cluster FE.

92

## 93 **Materials and methods**

94

## 95 **Sample collection and direct isolation**

96 Soil was collected from Los Angeles, CA in a residential area located at  
97 34.05638889° N, 118.445010000° W. Direct isolation of phages was performed by  
98 shaking a soil sample and 2X PYCa broth (Yeast Extract 1 g/L, Peptone 15 g/L, 4.5mM  
99 CaCl<sub>2</sub>, Dextrose 0.1%) in conical tubes at 250 RPM at 25°C for 1.5 hours. After  
100 incubation, the solution was filtered through a 0.22 µm syringe and spotted onto  
101 *Arthrobacter globiformis* B-2979 (*A. globiformis*). Plaque purifications were performed  
102 and a high titer lysate was filter-sterilized to be used in subsequent characterization  
103 experiments.

## 104 **Transmission electron microscopy**

105 Transmission electron microscopy (TEM) was performed on BlueFeather lysate.  
106 The sample was placed onto a carbon-coated electron microscope grid and stained with  
107 1% uranyl acetate. Phage particles were visualized using the CM120 Instrument  
108 (Philips, Amsterdam, Netherlands) and micrographs were captured. Phage head and  
109 tail lengths were measured using ImageJ (Schneider, Rasband, and Eliceiri 2012).

## 110 **Genome sequencing and assembly**

111 Viral DNA was isolated with the Wizard® DNA Clean-Up System (cat # A7280,  
112 Promega, WI, USA). Sequencing libraries were constructed with the NEBNext® Ultra™  
113 II DNA Library Prep kit (New England Biolabs, MA, USA), and sequenced by Illumina-  
114 MiSeq at the Pittsburgh Bacteriophage Institute to an approximate shotgun coverage of  
115 3538x. Genome assembly and finishing were performed as previously described  
116 (Russell 2018).

117 **Gene annotation**

118 Genomes were annotated as described previously (Pope and Jacobs-Sera 2018)  
119 using DNA Master (<http://cobamide2.bio.pitt.edu/>) and PECAAN  
120 (<https://pecaan.kbrinsgd.org/>) for auto-annotation. GLIMMER (Delcher et al. 1999) and  
121 GeneMark (Besemer and Borodovsky 2005) were used to predict protein-coding regions  
122 along with their start and stop sites. Manual annotation was performed using  
123 Phamerator (Cresawn et al. 2011), Starterator (*SEA-PHAGES/Starterator* [2016] 2020), and  
124 host-trained and self-trained GeneMark coding potential maps to support or refute auto-  
125 annotation predictions (Besemer and Borodovsky 2005). Gene functions were determined  
126 using PhagesDB BLAST (<https://phagesdb.org/blastp/>), NCBI BLAST (Altschul et al.  
127 1990), HHpred (Soding, Biegert, and Lupas 2005) and CDD (Marchler-Bauer et al. 2014). The  
128 presence of transmembrane proteins was determined using TMHMM (Krogh et al. 2001)  
129 and TOPCONS (Tsirigos et al. 2015). The annotated complete genome was deposited to  
130 GenBank under the accession number MT024867.

131 **Gene content comparisons**

132 Phage genomes used in this study are available from [phagesdb.org](http://phagesdb.org) (Russell and  
133 Hatfull 2017). Gepard was used to perform sequence analysis to identify regions of  
134 homology between nucleotide sequences or amino acid sequences of different phages  
135 (Krumsiek, Arnold, and Rattei 2007). Concatenated whole genome nucleotide and whole  
136 amino acid sequences were used to create dot plots with word sizes of 15 and 5,  
137 respectively.

138 SplitsTree was used to generate a network phylogeny in order to reveal the  
139 genetic distance between *Arthrobacter* phages (Huson 1998). BlueFeather and up to 10

## *Arthrobacter* phage BlueFeather

140 representative phages from each *Arthrobacter* cluster were selected from the  
141 Actino\_Draft database (version 366) for comparison.

142 The gene content calculator on PhagesDB (<https://phagesdb.org/genecontent/>)  
143 was used to calculate Gene Content Similarity (GCS), the percentage of shared genes  
144 in phams (groups of genes with related sequences), between BlueFeather, Cluster FE,  
145 and former Cluster FI phages (Cresawn et al. 2011). Gene Content Dissimilarity (GCD)  
146 and maximum GCD gap (MaxGCDGap) were calculated using scripts described  
147 previously (Pope et al. 2017). Heatmaps and scatter plots were created using Prism 8.0.0  
148 (GraphPad Software, San Diego, California, USA) and were used for quantitative  
149 analysis and visualization of GCS and GCD values.

150 PhagesDB Pham View was used to gather information about phages with genes  
151 in the same phams as BlueFeather's (Russell and Hatfull 2017). PECAAN was used to  
152 obtain the nucleotide sequences for each BlueFeather gene  
153 (<https://discover.kbrinsgd.org>). The BiologicsCorp online GC content calculator was  
154 used for each gene in the genome (<https://www.biologicscorp.com/tools/GCContent>).

## 155 **Results**

### 156 **BlueFeather is a siphovirus with a short genome**

157 Phage BlueFeather was isolated from a soil sample via direct isolation on *A.*  
158 *globiformis* B-2979 at 25°C and had a bullseye plaque morphology 5 mm in diameter  
159 (Fig 1a). Transmission electron microscopy (TEM) at 67,000X magnification showed an  
160 average phage capsid diameter and tail length of 48 ± 8 nm and 156 ± 53 nm,  
161 respectively (Fig 1b). The long, flexible, non-contractile tail suggested BlueFeather's  
162 classification as a *Siphoviridae* (Yuan and Gao 2017).

163        BlueFeather's genome had a length of 16,302 bp, 64.30% GC content, and  
164    genome ends with 15 base 3' sticky overhangs (CCACGGTTCCCGTCC). Phages that  
165    infect *Arthrobacter* hosts have genome lengths that range from 15,319 bp (Toulouse) to  
166    70,265 bp (PrincessTrina) (Klyczek et al. 2017). The average *Arthrobacter* phage genome  
167    length (as of May 2020) was 46,968 bp with a standard deviation of 20,619 bp and a  
168    median length of 53,859 bp, suggesting that most *Arthrobacter* phages have genomes  
169    notably larger than that of BlueFeather. BlueFeather's genome contained 25 manually  
170    annotated genes; 18 were of known function, 6 were orphans—meaning they have not  
171    been identified in any other known phage—and 1 was a reverse gene (Fig 2). The left  
172    arm of the genome had highly conserved genes amongst siphoviral *Arthrobacter*  
173    phages, such as those encoding terminase, portal protein, head-to-tail adapter, and tail  
174    proteins (Klyczek et al. 2017). Tail tube and sheath genes were absent, confirming the  
175    classification of BlueFeather as a siphovirus. Genes characteristic of the lytic life cycle,  
176    such as lysin A and holin, were identified; however, there were no genes that would  
177    indicate BlueFeather's ability to undergo a lysogenic life cycle, suggesting that  
178    BlueFeather is not a temperate phage (Shi et al. 2012).

179    **Dot plot comparisons revealed synonymous substitutions in  
180    BlueFeather's genome**

181        Phage BlueFeather was originally classified as a singleton on PhagesDB due to  
182    low nucleotide identity with other known phages. Nucleotide and amino acid dot plots  
183    were created to qualitatively compare BlueFeather to the most similar *Arthrobacter*  
184    phages, including those in Cluster FE (Corgi, Idaho, Noely) and former Cluster FI  
185    (Whytu, Yavru), as identified by BLASTn. Due to the limited number of sequenced

186 *Arthrobacter* phages, many of the clusters have few members. Of the 28 *Arthrobacter*  
187 clusters on PhagesDB (as of May 2020), 17 clusters have between 2-4 phages  
188 (including the former Cluster FI). As expected, phages originally assigned to the same  
189 cluster had alignments indicating large regions of nucleotide similarity (Grose and  
190 Casjens 2014), while comparison of BlueFeather's genome to phages originally assigned  
191 to Clusters FE and FI revealed no homologous sequences (Fig 3a). Unexpectedly, dot  
192 plot analysis of concatenated amino acid sequences with a word size of 5 revealed  
193 numerous regions of amino acid sequence similarity between these phages (Fig 3b).  
194 This reflects, at present, perhaps one of the clearest examples in which a group of  
195 phages lack nucleotide identity while sharing considerable amino acid identity.

196 **Gene similarity demonstrates a close relationship between**  
197 **BlueFeather and phages of Cluster FE and former Cluster FI**

198 Gene Content Similarity (GCS) is a key metric in quantifying phage genetic  
199 relationships and is calculated by averaging the number of shared genes between two  
200 phages (Mavrich and Hatfull 2017). GCS was calculated for BlueFeather, Cluster FE  
201 phages, and phages originally assigned to Cluster FI. BlueFeather shared over 35% of  
202 genes with most Cluster FE phages, and over 55% of genes with former Cluster FI  
203 phages. Over 35% of genes were shared in each pairwise comparison performed, with  
204 the exception of phage Idaho (34.18% shared gene content with BlueFeather) (Fig 4a).  
205 Given that BlueFeather was originally determined to be a singleton, it was surprising to  
206 find GCS greater than the recently adopted threshold of 35% for clustering other phage  
207 populations (Jacobs-Sera et al. 2020; Pope et al. 2017). Gene Content Dissimilarity (GCD)  
208 is the opposite of GCS and was used to calculate the maximum GCD gap

## Arthrobacter phage BlueFeather

209 (MaxGCDGap), a metric that represents the degree of isolation between a phage and a  
210 selected phage population (Pope et al. 2017). GCD was calculated for BlueFeather and  
211 all *Arthrobacter* phages. There was a MaxGCDGap of 44.45% between BlueFeather and  
212 and Whytu, indicating a relatively high degree of separation between BlueFeather and  
213 the rest of the *Arthrobacter* phage population (Fig 4b). *Arthrobacter* phages which  
214 exhibited pairwise GCD values with BlueFeather of less than 1 were found in Clusters  
215 AN, AU, AM, AZ, AV, AL, FE, AO, FH, FF, and former Cluster FI, indicating shared  
216 gene content. GCD was then calculated for BlueFeather and all known phages in the  
217 PhagesDB Actino\_draft database (Fig 4c). Similar to the *Arthrobacter* GCD plot, phages  
218 assigned to Cluster FE and former Cluster FI were the least dissimilar to BlueFeather. It  
219 is notable that in this comparison, there were 63 additional phages ranging from 0.959  
220 to 0.975 GCD, meaning BlueFeather shares a low number of genes with many non-  
221 *Arthrobacter* phages. Non-*Arthrobacter* phages which exhibited pairwise GCD values  
222 with BlueFeather of less than 1 were found in *Microbacterium* phage Cluster EE,  
223 *Mycobacterium* phage Clusters N, I, P, and the singleton IdentityCrisis, as well as  
224 *Gordonia* phage Clusters DT, CW and the singleton GMA4.

225 To compare the relationships between the *Arthrobacter* phage population as  
226 whole and the phages assigned to Cluster FE, former Cluster FI, and BlueFeather, a  
227 SplitsTree network phylogeny of the phams from each *Arthrobacter* phage cluster was  
228 generated to examine the genetic distance between the phages. As expected,  
229 BlueFeather was shown to be more genetically similar to phages originally assigned to  
230 Clusters FE and FI than to any other *Arthrobacter* phage clusters (Fig 5). BlueFeather  
231 demonstrated a closer pham similarity to former Cluster FI phages Whytu and Yavru

232 than to Cluster FE phages Idaho, Noely and Corgi; however, these phages altogether  
233 formed a distinct branch from the rest of the phages sampled and together comprise the  
234 newly expanded Cluster FE.

235 **BlueFeather genome exhibits evidence of horizontal gene  
236 transfer**

237 Given that BlueFeather shares genes with phages infecting distinct hosts, we  
238 investigated its genome for potential evidence of horizontal gene transfer (HGT). A  
239 whole genome heatmap was created using common metrics for evidence of HGT for  
240 each gene in the genome. As of March 2020, 4 genes in BlueFeather were considered  
241 to have the most convincing evidence for HGT based on GC content and prevalence in  
242 phages that infect unique bacterial hosts: genes 2, 15, 19, and 24 (Fig 6).

243 Typically, viral genes have about the same (Bohlin and Pettersson 2019) or slightly  
244 lower GC content (de Melo et al. 2019) compared to their bacterial hosts, suggesting that  
245 genes with higher GC content may have been horizontally transferred. BlueFeather had  
246 an overall average GC content of 64.30% and *Arthrobacter globiformis* mrc11 was  
247 found to have an overall GC content of 65.9% (Sahoo et al. 2019). BlueFeather gene-  
248 specific average GC contents ranged from 59.30% to 70.30%, and genes with  
249 maximum average GC contents were considered for HGT. This included genes 15 and  
250 24 with GC contents of 70.3% and 70.1%, respectively.

251 It is increasingly understood that phages infecting different hosts may share  
252 considerable gene content through processes such as HGT (Pope et al. 2017). For each  
253 gene in the BlueFeather genome, we calculated the number of unique isolation hosts for  
254 phages possessing a pham found in BlueFeather. Gene 2 belongs to a pham with

255 member genes found in phages that infect *Gordonia malaquae* BEN700 and  
256 *Arthrobacter* sp. ATCC 21022. Gene 15 belongs to a pham with member genes found in  
257 phages that infect *A. globiformis* B-2979, *A. sp.* ATCC 21022, *Mycobacterium*  
258 *smegmatis* mc<sup>2</sup>155, *G. malaquae* BEN700, and *Gordonia rubripertincta* NRRL B-16540.  
259 Gene 19 was the only reverse gene in the BlueFeather genome, and this gene was only  
260 found in BlueFeather and in phages infecting *Microbacterium foliorum* NRRL B-24224  
261 SEA and *Microbacterium paraoxydans* NWU1.

262

## 263 **Discussion**

264 Our research was focused on the genomic and evolutionary relationships  
265 between the novel *Arthrobacter* phage BlueFeather and other known phages,  
266 particularly those originally assigned to Clusters FE and FI. Previous studies have  
267 shown that new clusters can be formed when novel phages are found to be similar to  
268 former singletons, as demonstrated by the formation of Cluster AS from *Arthrobacter*  
269 singleton Galaxy (Klyczek et al. 2017). BlueFeather, originally designated as a putative  
270 singleton phage, exhibits over 35% GCS with nearly all phages originally in Cluster FE  
271 and over 55% GCS with those formerly assigned to Cluster FI. The conservation of  
272 amino acids, rather than nucleotides, suggests a history of purifying selection via many  
273 synonymous mutations in which deleterious mutations were filtered out (Ngandu et al.  
274 2008). Moreover, this presents some of the most clear evidence to date of highly  
275 conserved amino acid sequences despite the absence of significant nucleotide  
276 conservation amongst related phages.

## *Arthrobacter* phage BlueFeather

277 There is a high degree of synteny between these phages as well. While  
278 BlueFeather's original designation as a singleton would imply low genomic relatedness  
279 to other phages (Pope et al. 2015), gene similarity between BlueFeather and phages  
280 from Cluster FE and former Cluster FI—in excess of 35%—indicates conservation of gene  
281 functions and genome architecture despite extensive divergence of nucleotide identity.  
282 While *Arthrobacter* phages have been clustered according to nucleotide identity in the  
283 past (Klyczek et al. 2017), this study on BlueFeather, Cluster FE, and the former Cluster  
284 FI highlights the importance of continually reevaluating clustering parameters,  
285 particularly when different parameters may result in different cluster assignments.  
286 Moreover, BlueFeather has the smallest genome of all *Arthrobacter* singletons, and it is  
287 possible that clustering parameters may also need to take genome size into account.  
288 Given that GCS reflects the number of genes shared as a proportion of the total number  
289 of genes for each phage, the same number of shared genes would yield higher GCS in  
290 comparisons between smaller genomes.

291 Gene content dissimilarity demonstrated that BlueFeather has a MaxGCDGap of  
292 44.45% with phage Whytu, which was originally assigned to Cluster FI. BlueFeather  
293 was found to be least dissimilar with Cluster FE and former Cluster FI phages; this was  
294 supported by a network phylogeny of representative *Arthrobacter* phages that indicated  
295 great diversity between clusters, but revealed that phage BlueFeather forms a distinct  
296 branch with Cluster FE and former Cluster FI phages. Additionally, many phages were  
297 found to share between 0-10% GCS with BlueFeather. While this is too low to warrant a  
298 significant phylogenetic relationship, it reinforced the observed continuum of diversity in  
299 phage populations. Previous research found *Arthrobacter* phage clusters to be very

## *Arthrobacter* phage BlueFeather

300 discrete (Klyczek et al. 2017). Even so, this low yet seemingly widespread display of  
301 shared genes, as well as BlueFeather's unexpected relationships with Cluster FE and  
302 former Cluster FI phages, provides new insight into the genetic landscape of  
303 *Arthrobacter* phages. Few phages were previously assigned to Cluster FE and the  
304 former Cluster FI, representing only 5 of the 306 sequenced and manually annotated  
305 *Arthrobacter* phages (as of May 2020). On the other hand, there are 1,906 sequenced  
306 *Mycobacterium* phages (as of May 2020), which has allowed for a more thorough  
307 investigation of the mycobacteriophage continuum of diversity. As more *Arthrobacter*  
308 phages are sequenced, we expect to observe similar trends in these host-dependent  
309 genetic landscapes.

310 Unlike singleton phages that are replete with orphans (Klyczek et al. 2017; Pope et  
311 al. 2015), the BlueFeather genome, originally designated as a putative singleton, is  
312 composed predominantly of genes with known functions that have been assigned to  
313 phams. BlueFeather has less than half as many genes as current *Arthrobacter*  
314 singletons and contains highly conserved genes required for viral mechanisms. These  
315 vital functional genes have been more thoroughly studied and as a result, are more  
316 likely to be found in phams with predicted functions (Cresawn et al. 2011). Additionally,  
317 given that pham assignments are performed on the basis of amino acid identity, it is  
318 unsurprising that many of the phams containing these vital functional genes are shared  
319 amongst BlueFeather, Cluster FE, and former Cluster FI phages, despite the lack of  
320 significant nucleotide identity in gene encoding sequences.

321 Markers of horizontal gene transfer (HGT) included unexpectedly high GC  
322 content, as well as multiple bacterial hosts on which phages sharing genes with

## *Arthrobacter* phage BlueFeather

323 BlueFeather were isolated (Pope et al. 2015). BlueFeather shared phams with a  
324 multitude of non-*Arthrobacter* phages from various clusters, which allowed us to identify  
325 multiple regions as having evidence for HGT. These potential HGT events serve to  
326 magnify phage diversity and promote the phenomenon of genetic mosaicism.  
327 BlueFeather serves as yet another example of the highly intricate mosaic relationships  
328 which exist among phages, a feature of the genetic landscape which makes phage  
329 taxonomy an increasingly difficult task.

330 In sum, this research has led to the reclustering of BlueFeather and phages  
331 formerly assigned to Cluster FI into a newly expanded Cluster FE. Recent observations  
332 in which there appear to be limited nucleotide conservation but high shared gene  
333 content, as observed in this newly expanded cluster, support the notion that clustering  
334 methods should be continually reevaluated and optimized as more phages are  
335 sequenced (Pope et al. 2017). This study thus provides valuable insight into the  
336 continuum of diversity amongst *Arthrobacter* phages, while also supporting a 35%  
337 shared gene content clustering parameter as was previously adopted for *Gordonia* and  
338 *Microbacterium* phages (Pope et al. 2017; Jacobs-Sera et al. 2020). Further investigation  
339 into novel phages is essential to understand the complex phage landscape. As more  
340 *Arthrobacter* phages are discovered, it is likely that we will discover many more phages  
341 like BlueFeather which belong to clusters whose close relationships become apparent  
342 only through the lens of shared gene content.

343

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352

## 353 **Author contributions**

354 A.B., B.H., E.L., I.V., K.G., K.H., and S.D. performed experiments and drafted the  
355 paper; S.D., A.K., J.M.P. and A.F. revised the paper; and J.M.P. and A.F. supervised  
356 the research. This research was funded in part by the Microbiology, Immunology and  
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358

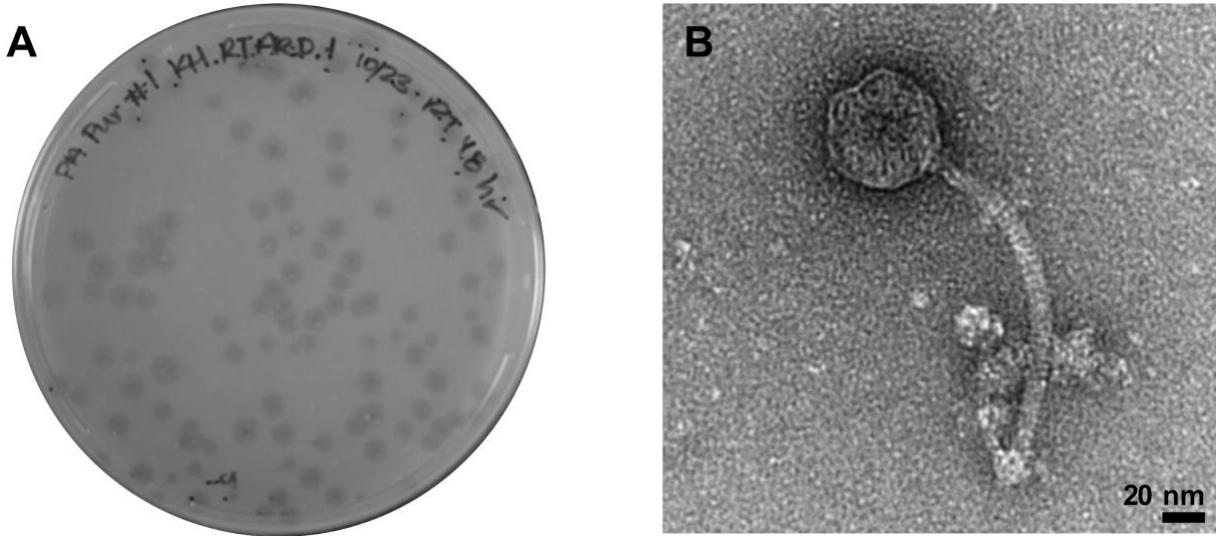
## 359 **Author disclosure statement**

360 The authors declare that there is no conflict of interest regarding the publication  
361 of this article.

362

363 **Figures**

364



365

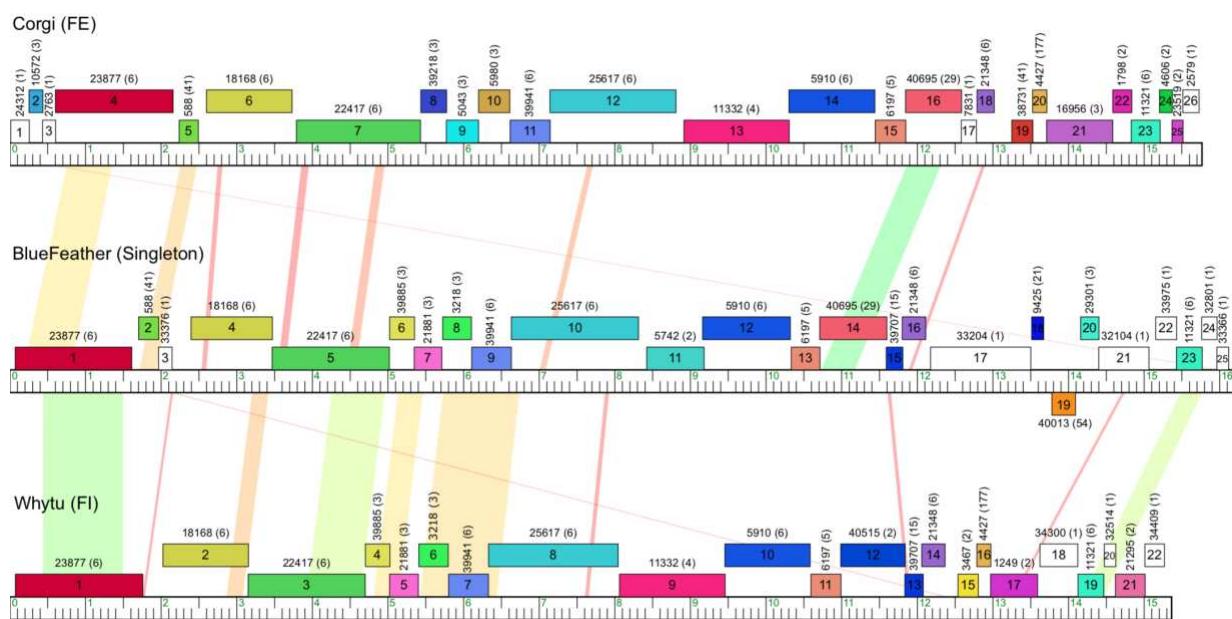
**Fig 1. BlueFeather is a Siphovirus. (A)** A picked plaque was suspended in phage buffer, then tested in a plaque assay for purification. Plaque morphology was consistent with bullseye plaques around 5 mm in diameter. **(B)** TEM image of BlueFeather at 366 67,000X magnification. The capsid was estimated to be  $48 \pm 8$  nm and the tail  $156 \pm 53$  367 nm.

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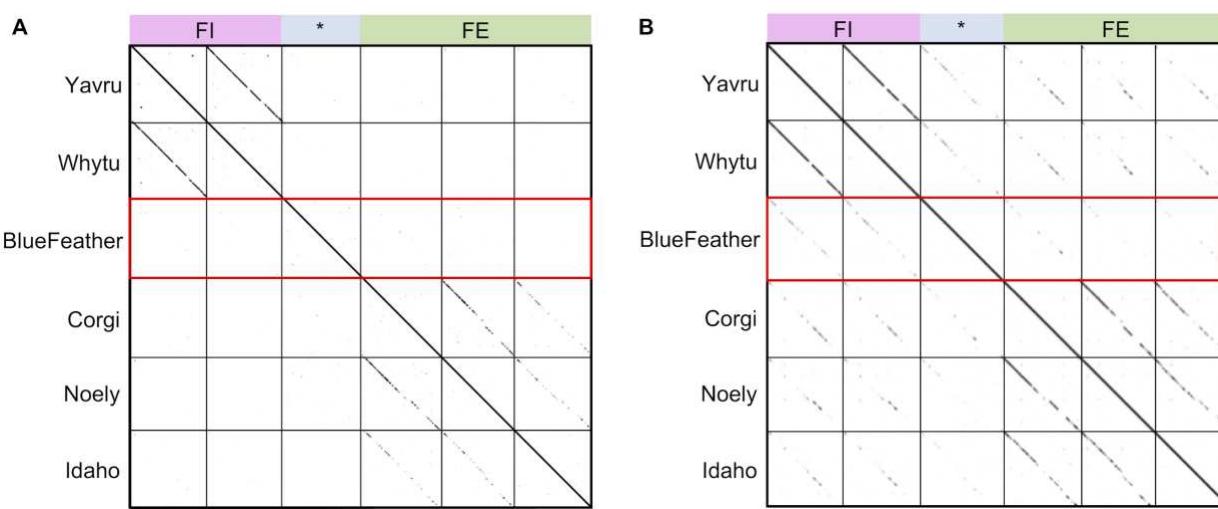
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372

373 **Fig 2. BlueFeather genome shares little nucleotide similarity but many phams**  
374 **with Cluster FE and former Cluster FI.** The BlueFeather genome is linear with a  
375 relatively small length of 16 kbp. Of the 25 identified ORFs, 18 were of known function,  
376 6 were orphans and 1 was a reverse gene. BlueFeather had little BLASTn homology to  
377 its most similar phages, as indicated by the limited orange and yellow shading.

378

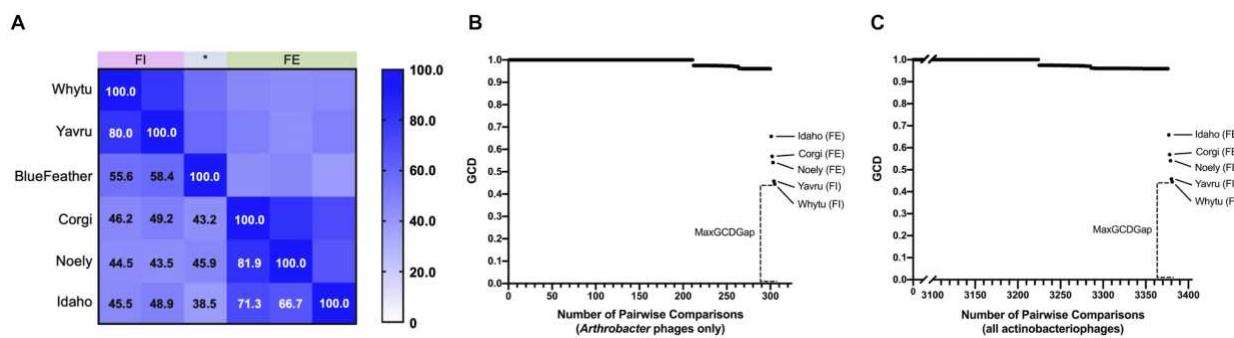


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380  
381 **Fig 3. Dot plots suggest shared amino acids but not nucleotides.** Whole genomes  
382 and proteomes for each phage were concatenated and dot plots were created using  
383 Gepard. Original cluster information is denoted along the top of each figure, with phage  
384 BlueFeather indicated by \*. **(A)** A whole genome dot plot with word size of 15 indicates  
385 strong intracluster nucleotide similarities with both FE and former FI phages. No

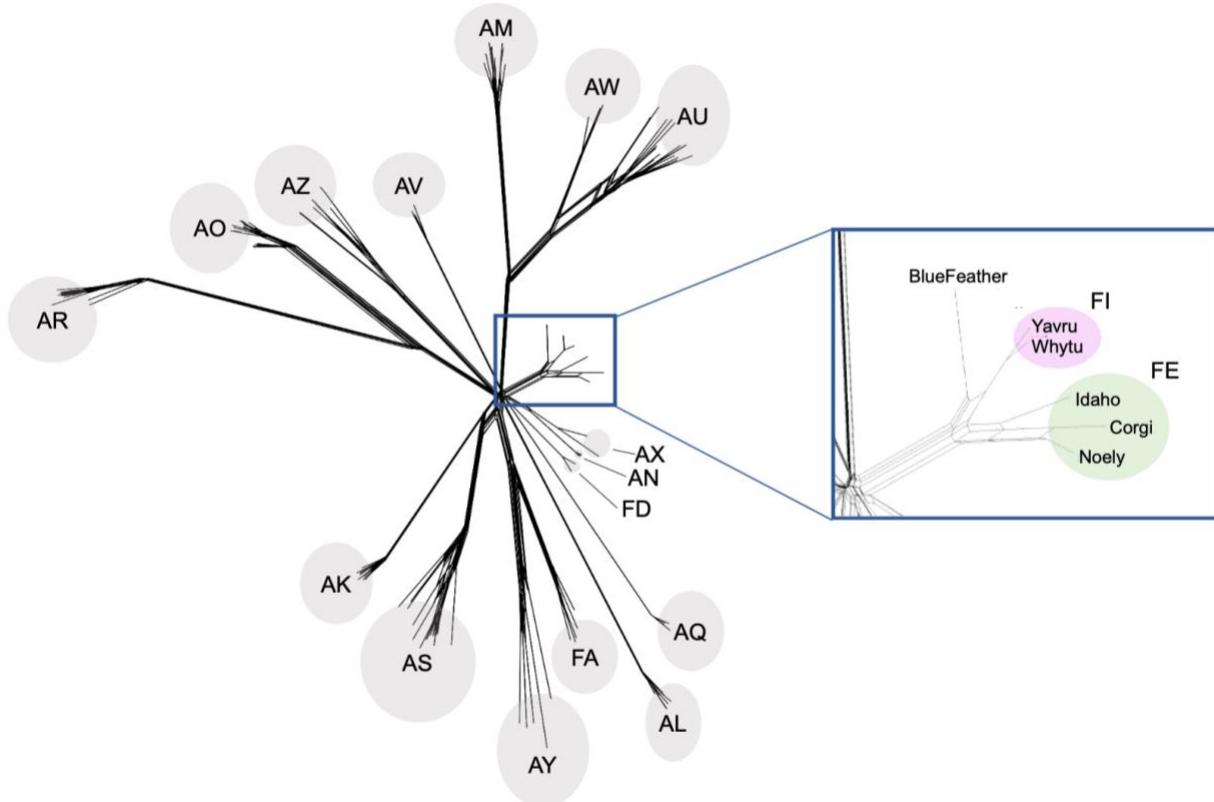
387 intercluster nucleotide similarities were observed, indicating BlueFeather does not share  
388 significant nucleotide sequences with any of these phages. **(B)** A whole proteome dot  
389 plot with a word size of 5 indicated the same introcluster amino acid similarities seen in  
390 the genome dot plot, but there were also amino acid similarities observed between  
391 BlueFeather, Cluster FE, and former Cluster FI phages. BlueFeather appeared to have  
392 greater amino acid similarity with phages originally assigned to Cluster FI.  
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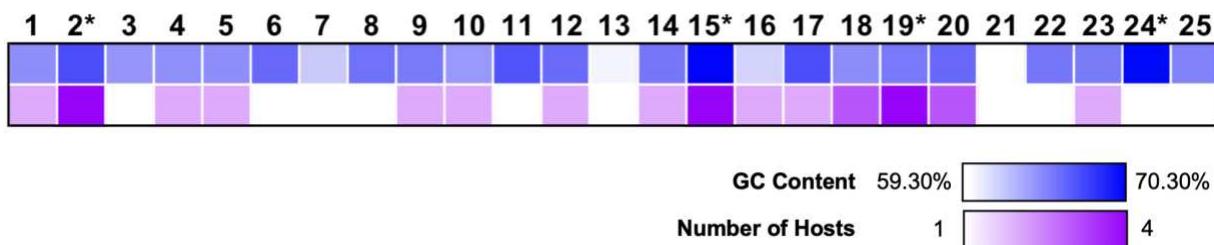


397 **Fig 4. BlueFeather shares the most phams with phages originally assigned to**  
398 **Cluster FE and former Cluster FI (A)** Gene Content Similarity (GCS) between  
399 BlueFeather, Cluster FE and the former Cluster FI was calculated with the PhagesDB  
400 GCS calculator using the number of shared phams. There was high introcluster GCS,  
401 and BlueFeather showed higher GCS values with former Cluster FI. **(B)** Gene Content  
402 Dissimilarity (GCD) output values of all pairwise comparisons of BlueFeather and all  
403 *Arthrobacter* phages (305), ordered by magnitude. Cluster FE and former Cluster FI  
404 were found to be least dissimilar to BlueFeather, with a MaxGCDGap of 44.45%,  
405 between BlueFeather and Whytu. **(C)** GCD output values of all pairwise comparisons of  
406 BlueFeather and all phages in PhagesDB (3381). MaxGCDGap remained at 44.45%.  
407 There are no non-*Arthrobacter* phages that are less dissimilar to BlueFeather than  
408 Whytu. BlueFeather shares up to 10% of genes with at least 63 non-*Arthrobacter*  
409 phages.

## Arthrobacter phage BlueFeather



410  
411  
412 **Fig 5. Expanded Cluster FE includes BlueFeather and former FI phages.** A  
413 SplitsTree was generated in order to group *Arthrobacter* phages based on pham  
414 similarity. Ten representative phages from each cluster were selected to measure  
415 evolutionary relatedness. While there is great diversity of *Arthrobacter* phages,  
416 BlueFeather forms a relatively small branch with phages originally assigned to Cluster  
417 FE and the former Cluster FI. These phages, boxed in blue, comprise the expanded FE  
418 Cluster.  
419  
420



421  
422 **Fig 6. Evidence of horizontal gene transfer in the BlueFeather genome.** The GC  
423 content for each gene in BlueFeather's genome ranged from 59.30%-70.30%, with an  
424 average of 64.30%. The number of unique isolation hosts that were represented in each  
425 pham ranged from 1-4. Genes with unexpectedly high values were considered to be the  
426 result of horizontal gene transfer. There were four genes with the most convincing  
427 evidence, indicated by \*.

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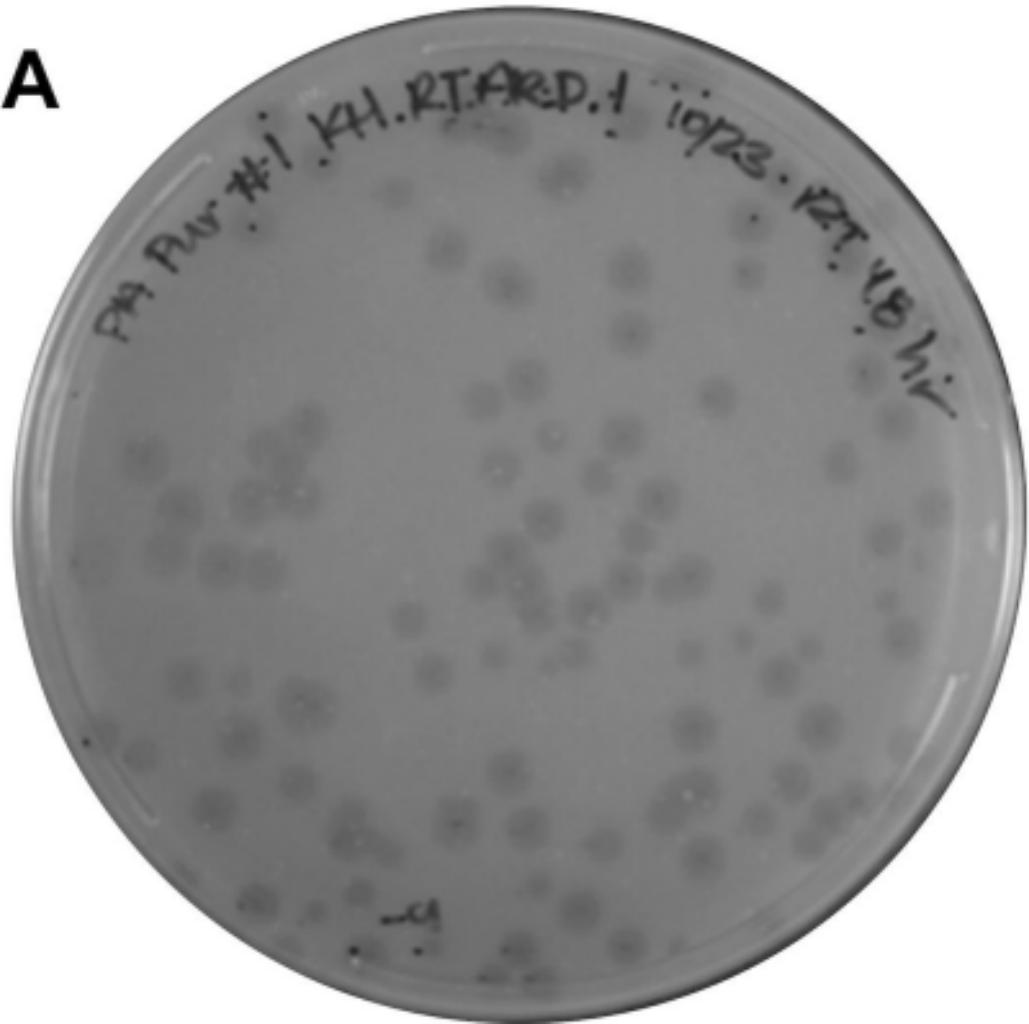
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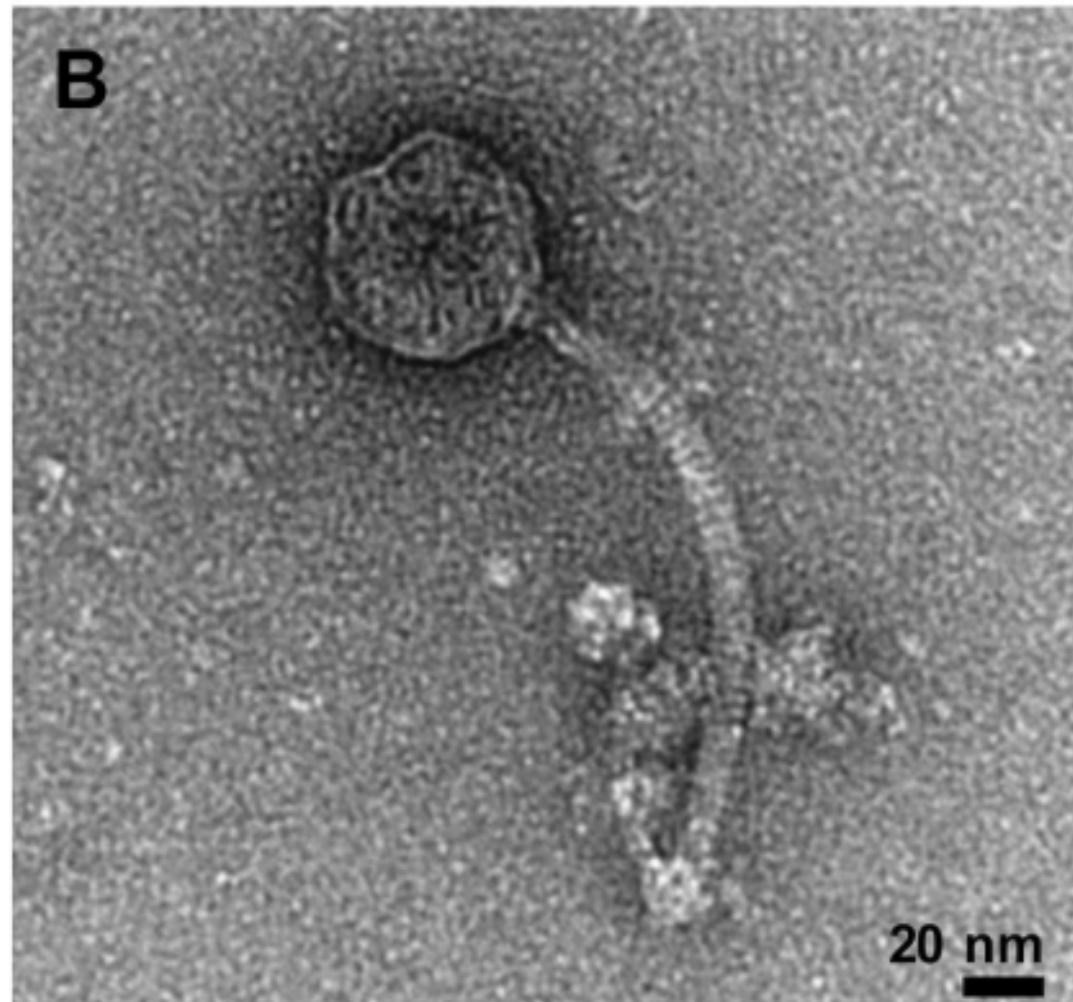
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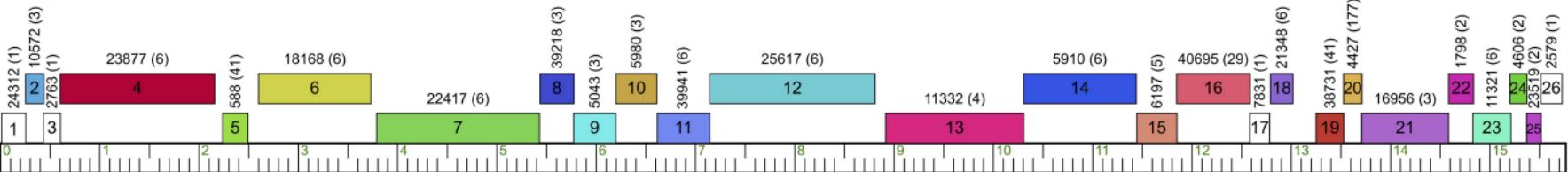
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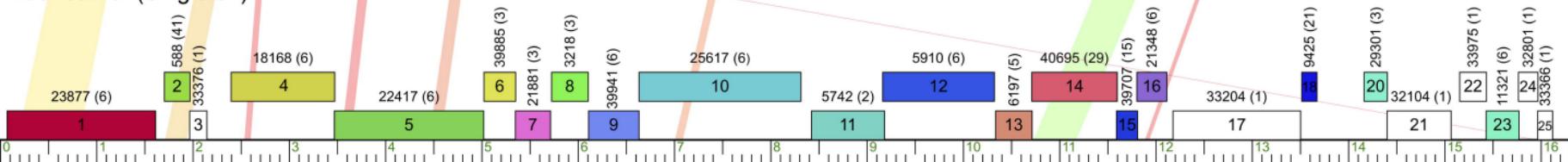
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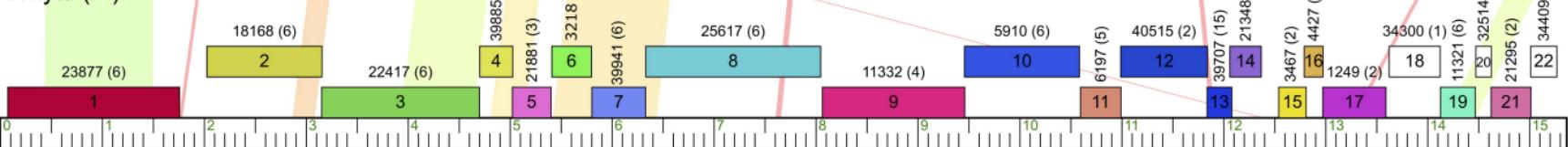
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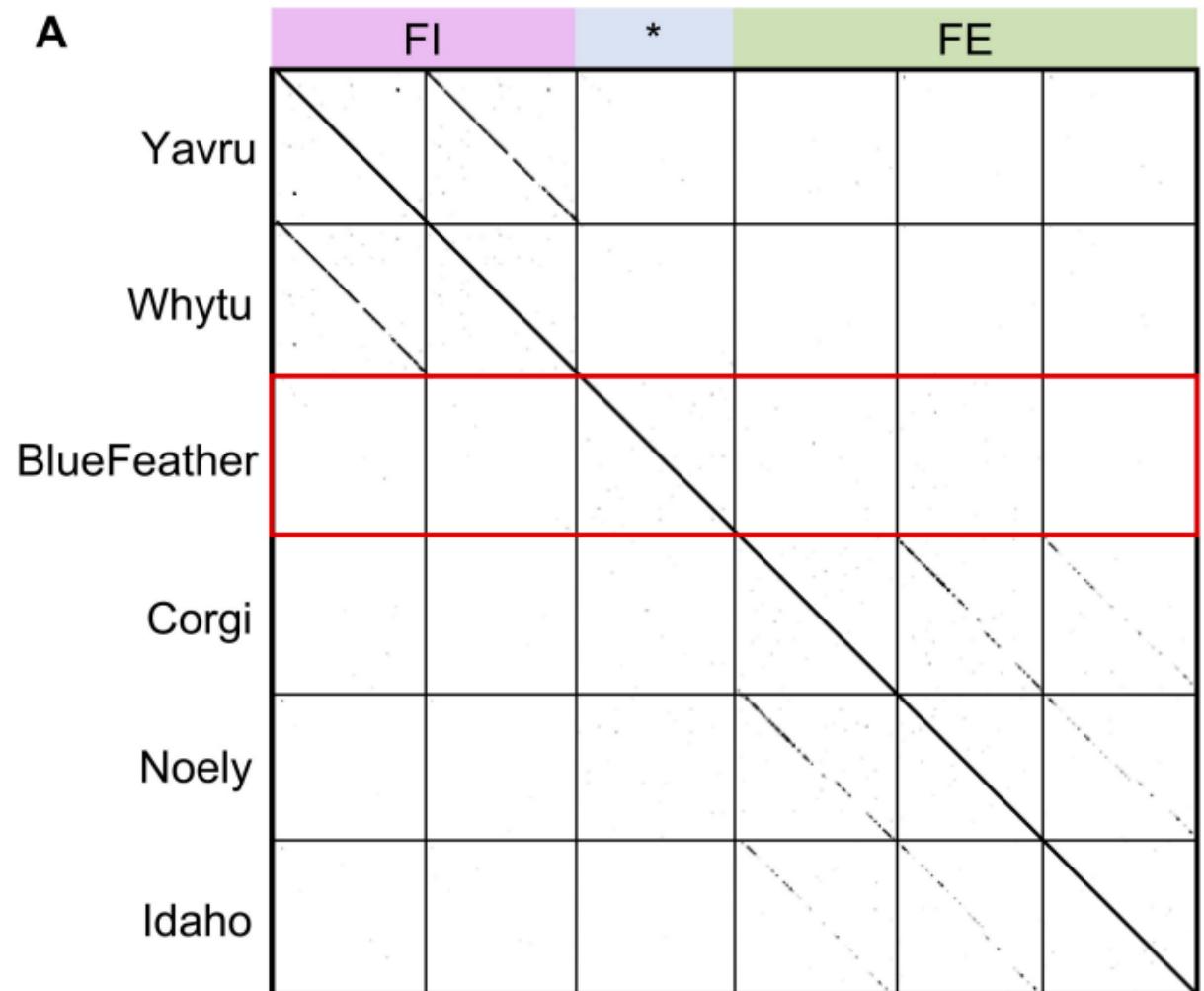
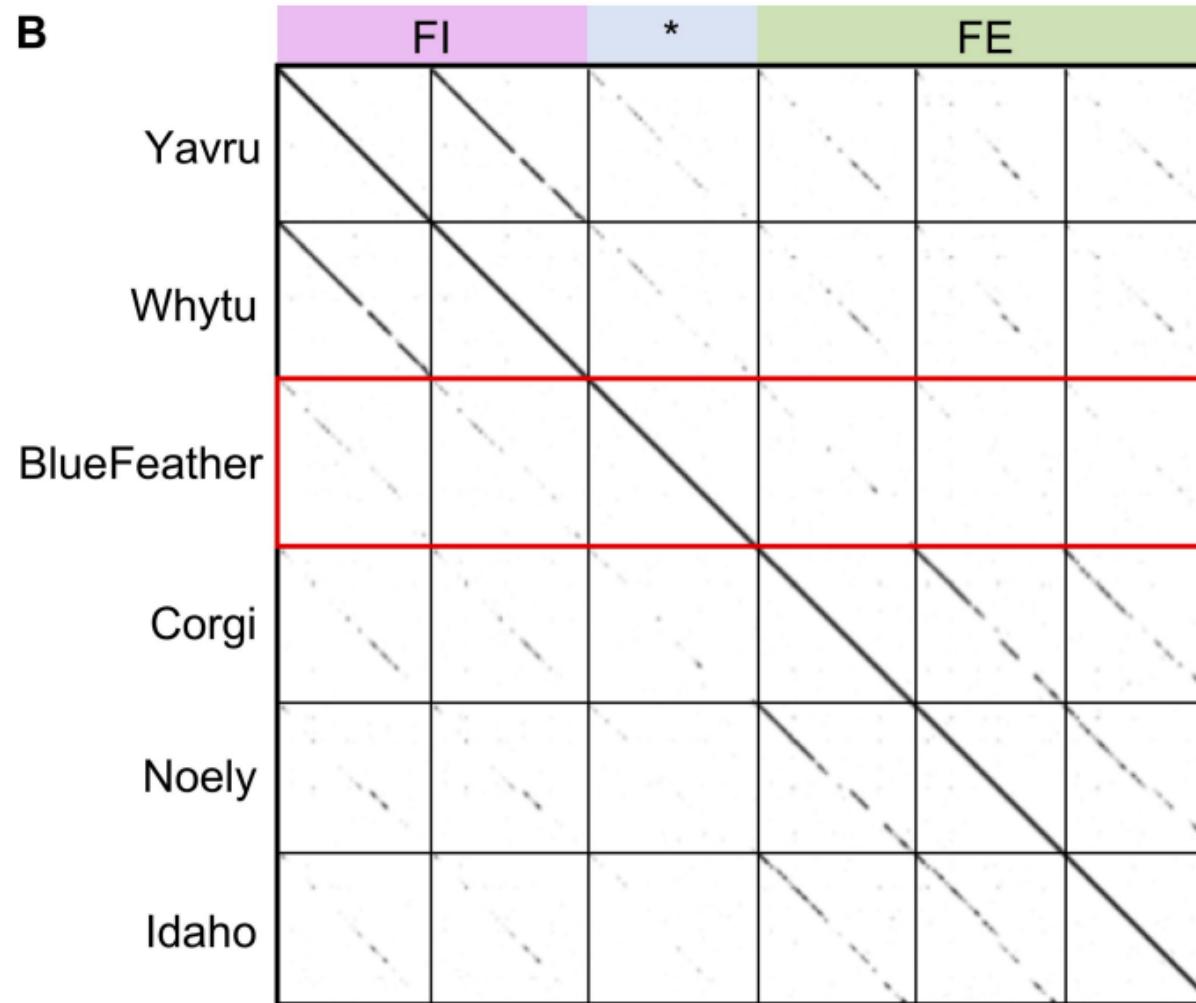


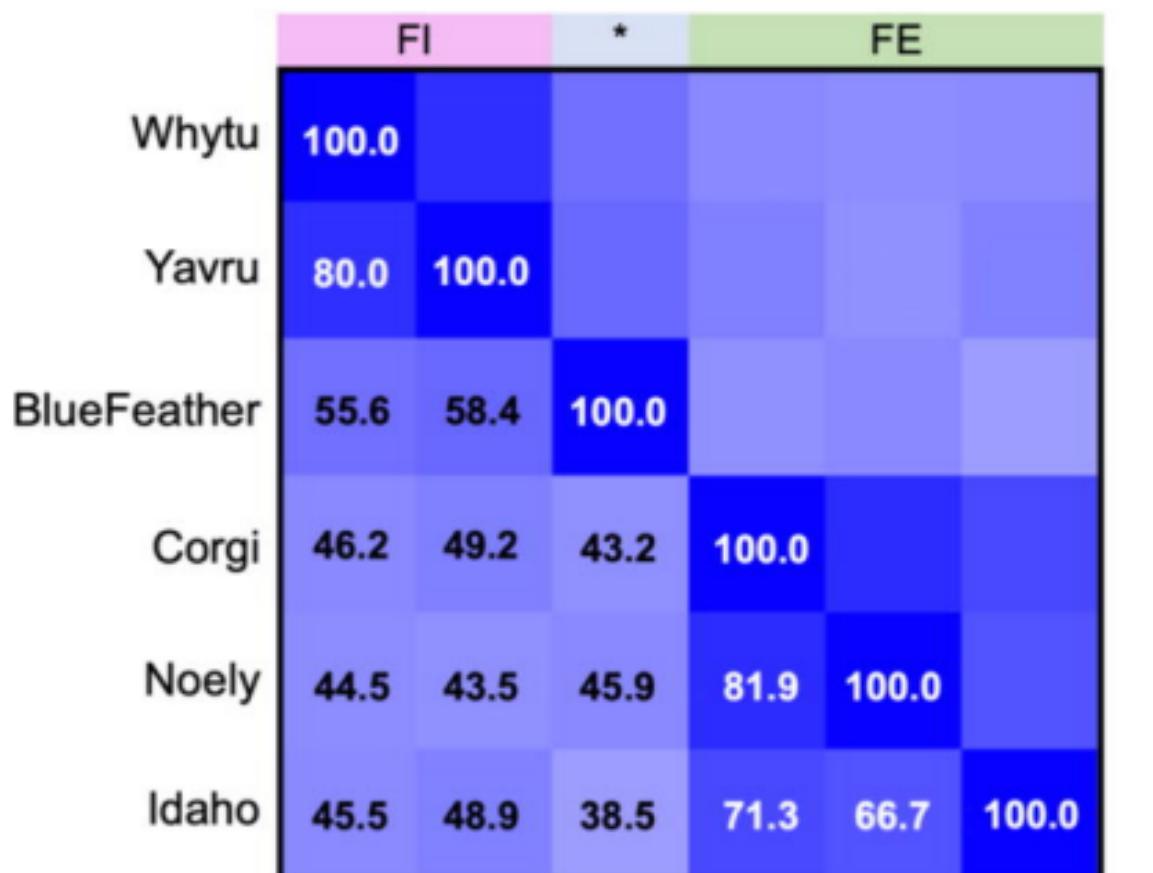
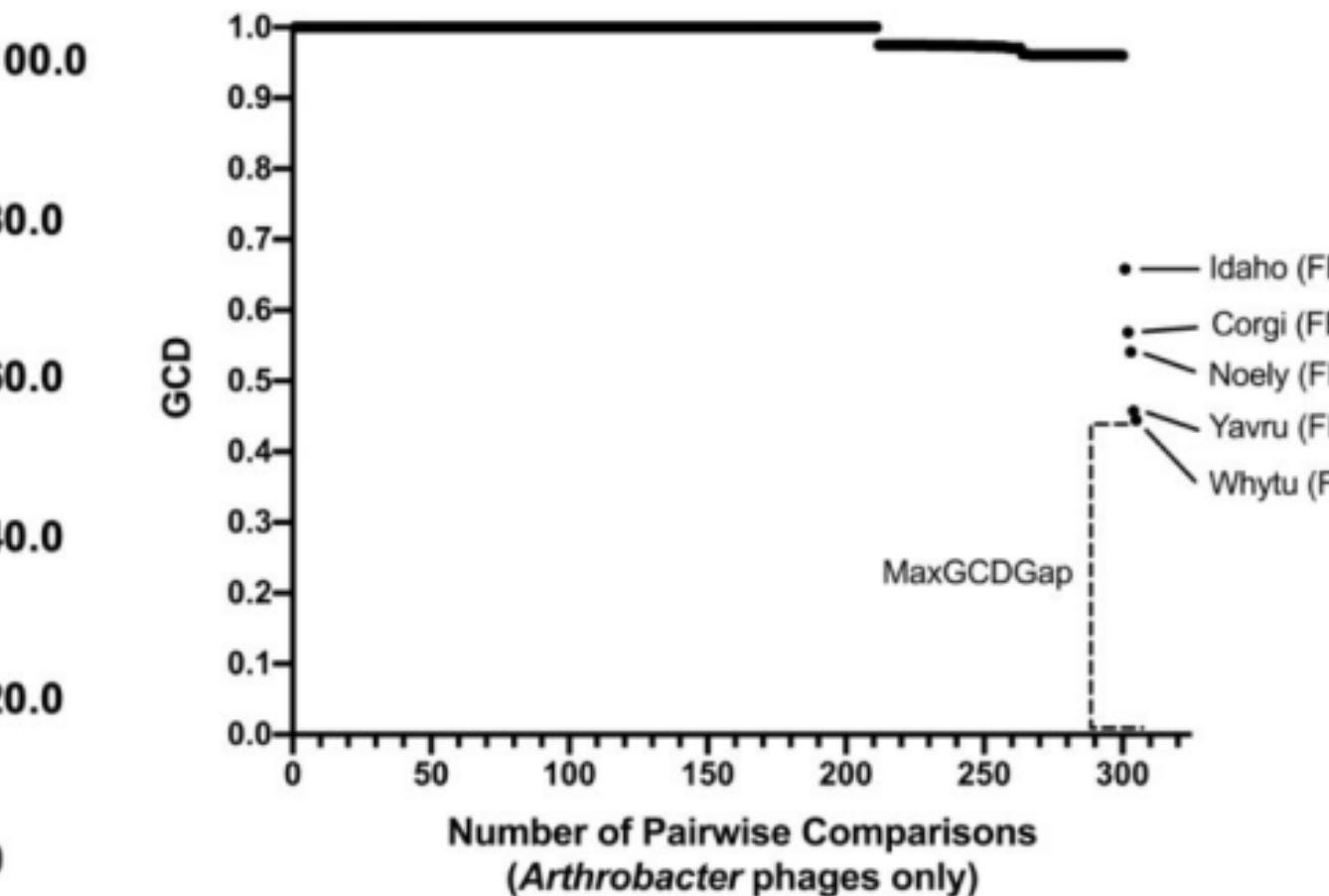
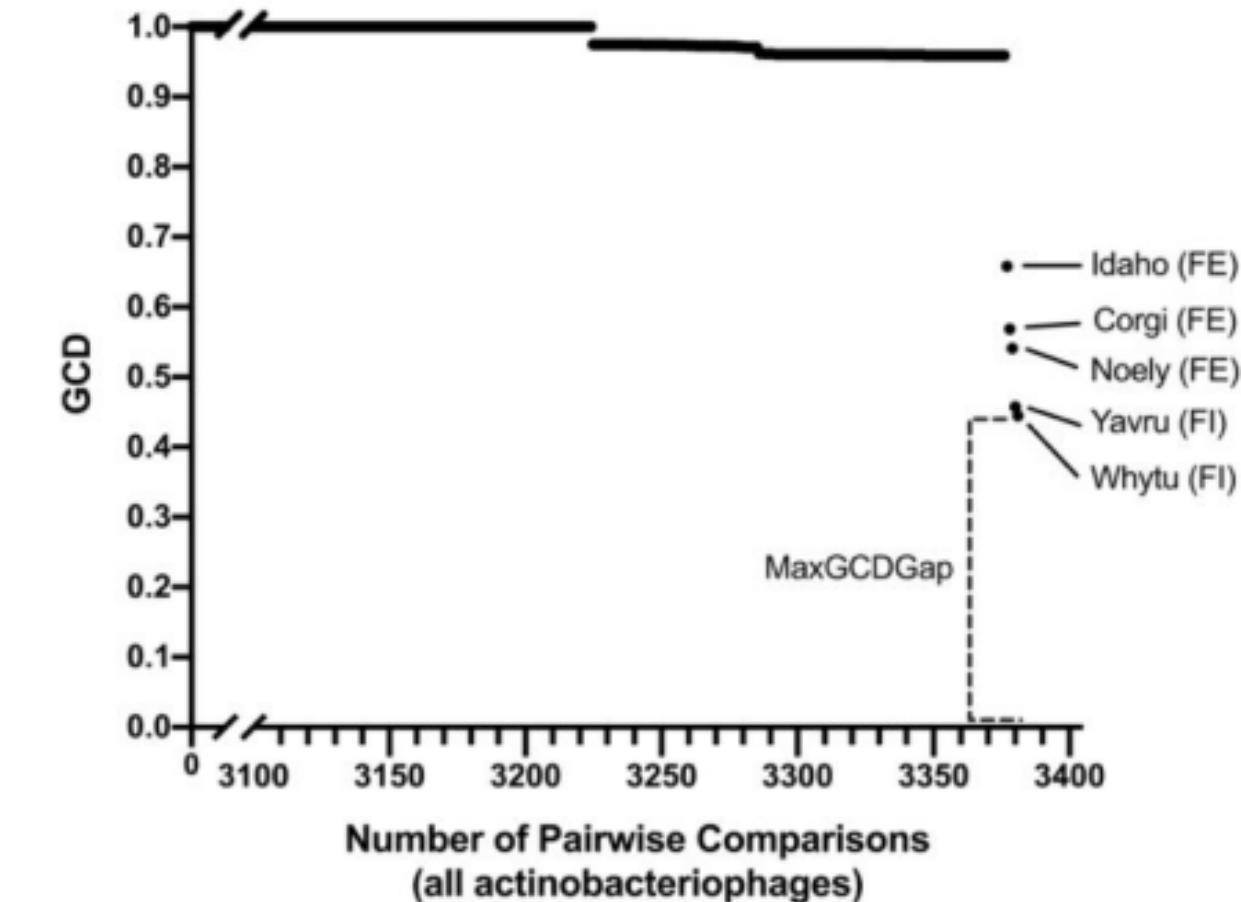
## BlueFeather (Singleton)

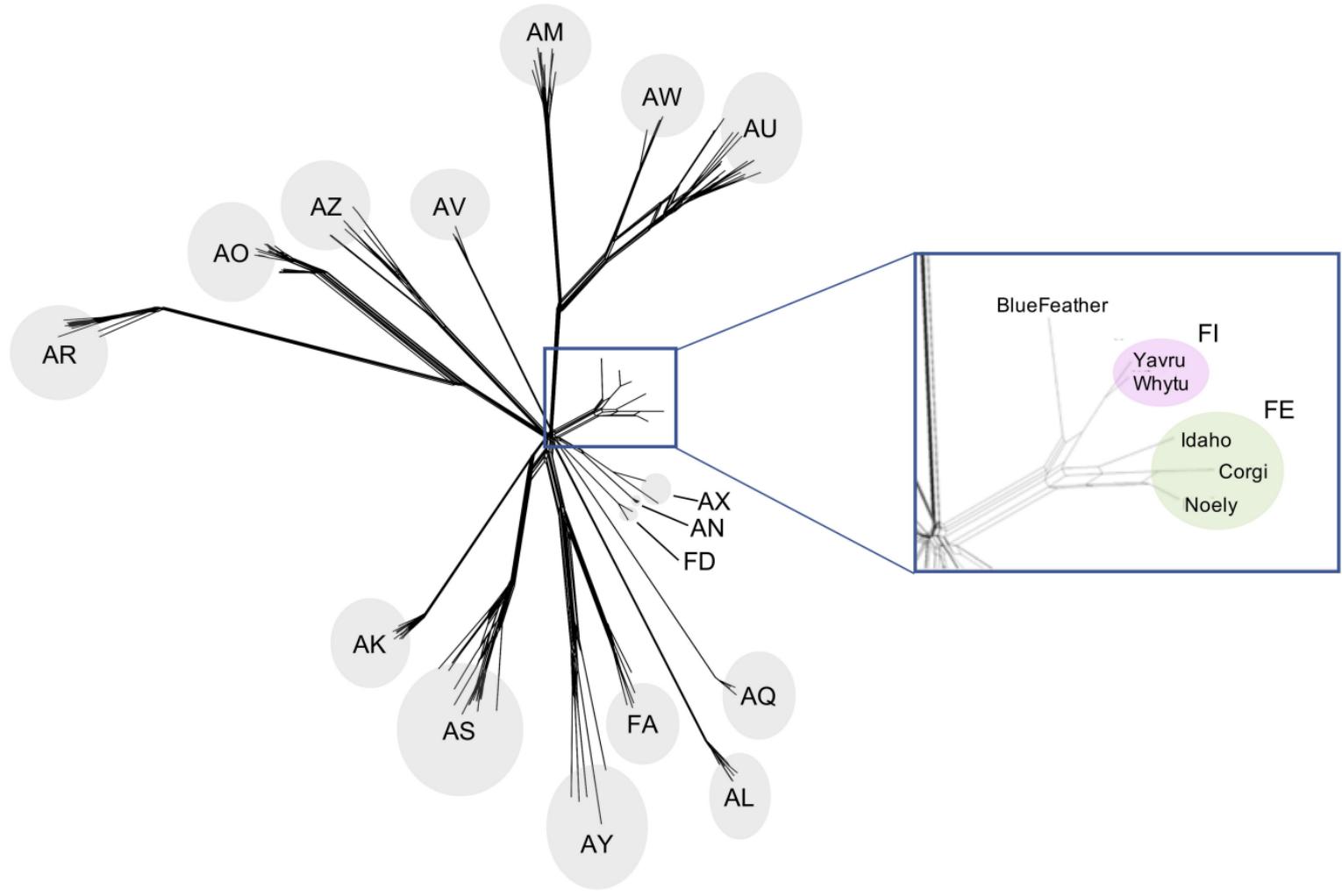


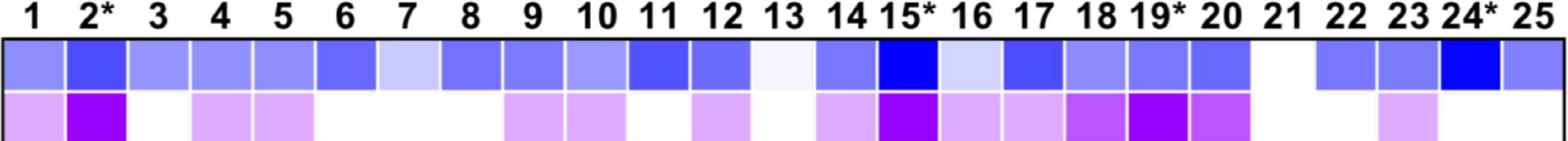
## Whytu (FI)



**A****B**

**A****B****C**





**GC Content** 59.30% 70.30%

**Number of Hosts** 1 4