Identification of *Klebsiella* capsule synthesis loci from whole genome data

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Abstract

Background: *Klebsiella pneumoniae* and close relatives are a growing cause of healthcare-associated infections for which increasing rates of multi-drug resistance are a major concern. The *Klebsiella* polysaccharide capsule is a major virulence determinant and epidemiological marker. However, little is known about capsule epidemiology since serological typing is not widely accessible, and many isolates are serologically non-typeable. Molecular methods for capsular typing are needed, but existing methods lack sensitivity and specificity and fail to take advantage of the information available in whole-genome sequence data, which is increasingly being generated for surveillance and investigation of *Klebsiella*.

Methods: We investigated the diversity of capsule synthesis loci (K loci) among a large, diverse collection of 2503 genome sequences of *K. pneumoniae* and closely related species. We incorporated analyses of both full-length K locus DNA sequences and clustered protein coding sequences to identify, annotate and compare K locus structures, and we propose a novel method for identifying K loci based on full locus information extracted from whole genome sequences.

Results: A total of 134 distinct K loci were identified, including 31 novel types. Comparative analysis of K locus gene content detected 508 unique protein coding gene clusters that appear to reassort via homologous recombination, generating novel K locus types. Extensive nucleotide diversity was detected among the *wzi* and *wzc* genes, both within and between K loci, indicating that current typing schemes

based on these genes are inadequate. As a solution, we introduce *Kaptive*, a novel software tool that automates the process of identifying K loci from large sets of *Klebsiella* genomes based on full locus information.

Conclusions: This work highlights the extensive diversity of *Klebsiella* K loci and the proteins that they encode. We propose a standardised K locus nomenclature for *Klebsiella*, present a curated reference database of all known K loci, and introduce a tool for identifying K loci from genome data (https://github.com/katholt/Kaptive). These developments constitute important new resources for the *Klebsiella* community for use in genomic surveillance and epidemiology.

Key Words:

Klebsiella, capsule, K locus, genomic epidemiology, polysaccharide variation

55 **Background**

56 *Klebsiella pneumoniae*, *Klebsiella variicola* and *Klebsiella quasipneumoniae* are
57 ubiquitous, encapsulated Gram-negative bacteria. They can be carried
58 asymptomatically in the human gut or nasopharynx [1] but are also opportunistic
59 pathogens, frequently associated with human disease and recognised as a significant
60 threat to global health. Antimicrobial resistance, particularly multi-drug resistance
61 and resistance to the carbapenems, is a major concern. Notably, there are a number
62 of multi-drug resistant clones, which are distributed world-wide and are reported to
63 cause outbreaks of healthcare-associated infections [2,3]. There are also increasing
64 reports of invasive, community-acquired *K. pneumoniae* disease in many Asian
65 countries [4]. While this phenomenon is not yet well understood, it is associated
66 with 'hypervirulent' *K. pneumoniae* strains expressing specific capsular serotypes
67 known as K1, K2 and K5 [5,6].
68
69 In order to control the emerging threat of *K. pneumoniae sensu stricto, K. variicola*
70 and *K. quasipneumoniae* (hereafter collectively referred to as *K. pneumoniae* unless
71 otherwise stated), there is an urgent requirement for genome-based surveillance.
72 Recent advances in understanding *K. pneumoniae* population structure [7,8]
73 highlight immense genomic diversity and provide a framework for tracking this
74 pathogen. Useful strategies involve analyses of lineages or multi-locus sequence
75 types in combination with resistance and virulence gene characterisation, e.g. using
76 tools such as SRST2 [9] and BIGSdb [8]. Additionally, there have been successful *K.*
77 *pneumoniae* outbreak investigations using genomic analysis [3,10]. However,
78 methods for tracking *K. pneumoniae* capsular variation are currently lacking.

The polysaccharide capsule is the outer most layer of the *K. pneumoniae* cell, which protects the bacterium from desiccation, phage and protist predation [11]. The capsule is also a key virulence determinant due to its antiphagocytic properties [12–14]. There are 77 immunologically distinct *K. pneumoniae* capsule types (K-types) defined by serology, mostly based on work done in the 1950s-70s [15–17]. However, serological typing requires specialist techniques and reagents not available to most microbiology laboratories, so it is very rarely applied. Furthermore, between 10% and 70% of *K. pneumoniae* isolates are serologically non-typeable, either because they express a novel capsule (most commonly for clinical isolates) or are non-capsulated [6,18,19].

K. pneumoniae employ a Wzy-dependent capsule synthesis process [11,20] and the genes required for capsule synthesis and assembly are located at the capsule polysaccharide synthesis locus (K locus). The K locus varies in length from 10 to 30 kbp [21–26] and includes a set of 'common' genes in the terminal regions, which encode the core capsule biosynthesis machinery (e.g. *galF, wzi, wza, wzb, wzc*, *gnd* and *ugd*). The central region of the K locus is highly variable, encoding the capsule-specific sugar synthesis, processing and export proteins plus the core assembly components Wzx (flippase) and Wzy (capsule repeat unit polymerase). The *wzx* and *wzy* genes are more diverse than those of the other core assembly components and do not have a fixed position within the K locus [22].

102 K locus nucleotide sequences and annotations are now available for a large number
103 of *K. pneumoniae* isolates, including the 77 K-type reference strains [3,21–23,25,27–
104 29]. Serological K-types are generally defined by distinct sets of genes in the variable
105 central region of the K locus. This is usually due to the presence of entirely different
106 sets of protein coding sequences; however two types (K22 and K37) are
107 distinguished by a single point mutation resulting in a premature stop codon that
108 affects acetyltransferase function [22].
109
110 A number of molecular K-typing schemes have been developed that take advantage
111 of the conserved K locus structure: restriction fragment length polymorphism ('C-
112 typing') [30], *wzi* and *wzc* typing [31,32] and capsule-specific *wzy* PCR-based typing
113 [25,33]. C-typing comprises PCR amplification of a large region of the K locus (from
114 upstream of *wzi* to within *gnd*), followed by *Hinc*II restriction. In contrast, *wzi* and
115 *wzc* typing each comprise PCR amplification and nucleotide sequencing of regions of
116 a single gene, *wzi* and *wzc* respectively. Within the *wzi* scheme, unique alleles are
117 associated to specific K-types [32]. Within the *wzc* scheme, K-types are assigned
118 based on the level of *wzc* nucleotide similarity to a reference sequence, with a
119 threshold of 94% [31]. These molecular typing methods are less technically
120 challenging than serological techniques and are more discriminatory [30–32].
121 However, none of the methods have been widely adopted and regardless of the
122 method, a substantial proportion of isolates remain non-typeable. As a
123 consequence, the true extent of *K. pneumoniae* capsule diversity remains unknown.
124

125 Here we report the K loci from a collection of 2503 *K. pneumoniae*. We identify 31
126 novel K loci, and provide evidence that limited additional diversity remains to be
127 discovered in *K. pneumoniae*. We define a standardised nomenclature for *Klebsiella*
128 K loci, provide a curated reference database and introduce *Kaptive*, a tool for rapid
129 identification of reference K loci from genome data, which will greatly facilitate
130 genomic surveillance efforts and evolutionary investigations of this important
131 pathogen.
132
133 **Methods**
134 We obtained a total of 2600 *K. pneumoniae* genomes (2021 publicly available
135 genomes and 579 novel genomes from a diverse set of isolates collected in
136 Australia). Sequence reads were generated locally or obtained from the European
137 Nucleotide Archive (accessions listed in **Table S1, Additional File 1**); 916 genomes
138 that were publicly available as assembled contigs only were downloaded from
139 PATRIC [34] and the NCTC3000 project [35]. For isolates sequenced in this study (n =
140 579) DNA was extracted and libraries prepared using the Nextera® XT 96 barcode
141 DNA kit and 125 bp paired-end sequence reads were generated on the Illumina
142 HiSeq 2500 platform.
143
144 All paired-end read sets were filtered to remove reads with mean Phred quality
145 score <30, then assembled *de novo* using SPAdes v3 [36]. Genomes were excluded
146 from the study if they were duplicate samples, if there was evidence of
147 contamination or mixed culture measured by: (i) <50% reads mapped to the NTUH-
148 K2044 reference chromosome (accession: AP006725.1); (ii) the ratio of

149 heterozygous/homozygous SNP calls compared to the reference chromosome
150 exceeded 20%; (iii) the total assembly length was >6.5Mb, or >6.0Mb with evidence
151 of >1% non-*Klebsiella* read contamination as determined by MetaPhlAn [37]; or (v)
152 the assembly was low quality i.e total length <5Mb.
153
154 ***Existing high quality K locus reference sequences***
155 K locus nucleotide sequences for each of the 77 K-type references and two
156 serologically non-typeable strains published previously [21–26] were obtained from
157 GenBank or directly from the authors (accessions in **Table S2, Additional File 2**). A
158 total of 12 additional K locus sequences had been published prior to the K-type
159 references [3,27,29,38]; we have compared these loci to those of the 77 K-type
160 references [21–26] and identified seven that were novel. These seven novel loci plus
161 17 distinct loci described in our recent survey [28] were added to the non redundant
162 list of K locus reference sequences, resulting in a total of 103 loci (see **Table S2,**
163 **Additional File 2**).
164
165 ***Identification of novel K loci***
166 In order to identify novel K loci we first classified each genome by similarity to
167 previously known loci. BLASTn [39] was used to search each genome assembly for
168 sequences with similarity to those of annotated K locus coding sequences (CDS)
169 usually located between *galF* and *ugd* inclusive (minimum coverage 80%, minimum
170 identity 50%). Transposase CDS present in the published K locus reference
171 sequences were excluded from this analysis since they are not K locus specific. Up to
172 three missing CDS were tolerated for K locus assignment, to allow for assembly

173 problems and insertion sequence (IS) insertions (see **Figure S1, Additional File 3**).
174 This approach can successfully distinguish the 77 K-type reference loci (with the
175 exception of K22 and K37).
176
177 Genomes that could not be assigned a K locus were investigated further: BLASTn was
178 used to identify the *galF* and *ugd* genes within the assembly, and single contig loci
179 were extracted. The assembly graph viewer Bandage [40] was used to identify K loci
180 that did not assemble on a single contig or where *galF* and/or *ugd* where missing.
181 Loci were clustered, with identity and coverage thresholds of 90%, using CD-HIT-EST
182 [41,42]. A representative sequence from each cluster was annotated with Prokka
183 [43], using all proteins in the 77 reference K-type loci as the primary annotation
184 database. Novel K locus sequences were deposited in Genbank (accessions:
185 LT603702 - LT603735; also included in the Kaptive database at
186 https://github.com/katholt/Kaptive/).
187
188 Recombination in K loci was investigated by aligning nucleotide sequences for the
189 eight common genes (extracted from the reference annotations) using MUSCLE [44].
190 This generated a 9944 bp concatenated gene alignment which was used as input for
191 maximum likelihood (ML) phylogenetic inference with RAxML [45] (best scoring tree
192 from five runs each of 1000 bootstrap replicates with gamma model of rate
193 heterogeneity), and recombination analysis using ClonalFrameML [46] (run for 1000
194 simulations and using the ML phylogeny as the starting tree).
195
196 ***Amino acid clustering***

197 Predicted amino acid sequences of all annotated K locus coding regions were
198 translated from the DNA sequences using BioPython and clustered with CD-HIT
199 [41,42] (90%, 80%, 70%, 60%, 50%, 40% identity). We explored the co-occurrence of
200 predicted protein clusters present in three or more K loci each (excluding the
201 common proteins and the initiating glycosyltransferases, WbaP and WcaJ, n = 115
202 clusters for analysis): Pairwise Jaccard similarity scores were calculated as $J\ (A, B) =$
203 $A\ \cap B\ /\ A\ \cup B$ and were used to draw a weighted edge graph with the igraph R
204 package v 1.0.1 [47]. A weight threshold was determined empirically as 0.61 and all
205 edges for which $J < 0.61$ were removed.
206
207 ***Wzc and wzi nucleotide sequence determination***
208 We characterised *wzi* and *wzc* sequence diversity and explored the association with
209 K loci. We used SRST2 [9] to determine *wzi* alleles defined in the *K. pneumoniae*
210 BIGSdb [48]. BLASTn was used to determine alleles in genomes available only as
211 assemblies. Novel alleles were submitted to the *K. pneumoniae* BIGSdb for official
212 designation. *Wzc* sequences were extracted from genome assemblies by BLASTn
213 search against a database of previously published alleles [31]. Sequences were
214 aligned with MUSCLE [44] and pairwise nucleotide divergences calculated.
215
216 ***Kaptive, a tool for identification of K loci in genome data***
217 We developed an extended procedure for identification and assessment of full-
218 length K loci among bacterial genomes based on BLAST analysis of assemblies. The
219 procedure has been automated and is implemented in a freely available open source

220 software tool, *Kaptive* (https://github.com/katholt/Kaptive). For full details see
221 **Additional File 3, Figures S2 and S3.**
222
223 ***Comparison of Kaptive, wzi and wzc typing results***
224 *Kaptive, wzi* and *wzc* typing were applied to the 86 genomes that had matched
225 serological typing information available (**Table S3, Additional File 4)**. *Wzi* alleles
226 were determined as described above, and used to predict serotypes by comparison
227 to the *K. pneumoniae* BIGSdb. *Wzc* sequences were extracted as above and genomes
228 were assigned to serotypes if the sequence was <6% divergent from the
229 corresponding reference [31].
230
231 **Results**
232 We analysed K loci in a final dataset comprising 2503 high quality genomes, including
233 2298 *K. pneumoniae sensu stricto*, 144 *K. variicola*, 57 *K. quasipneumoniae* and four
234 unclassified *Klebsiella* spp. (**Table 1** and **Table S1, Additional File 1**). Also included
235 were 10 publicly available genomes representing the more distantly related
236 *Klebsiella oxytoca* (**Table S4, Additional File 3**), as we hypothesised that these
237 organisms may share capsule synthesis loci with *K. pneumoniae*. Isolates were
238 collected between 1932 and 2014 (see **Figure S4, Additional File 3**) and from eight
239 different geographic regions spanning six continents (see **Figure S5, Additional File**
240 **3**).
241
242
243

244 **Table 1: *K. pneumoniae* genomes investigated in this study**

Dataset	Count	Reference	Notes
Bialek-Davenet *et al.*	33	[8]	Investigation of multi-drug resistant and hypervirulent clones
Bowers *et al.*	160	[49]	Isolates mostly of clonal group 258
Davis *et al.*	77	[50]	Isolates from human UTI and animal meats in Arizona, USA
Deleo *et al.*	69	[29]	Isolates of clonal group 258
Ellington	185	[51]	Multidrug resistant isolates from a hospital in Cambridge, UK
Holt *et al.*	274	[7]	Global diversity study
Lee *et al.*	27	[52]	Isolates from pyogenic liver abscess disease, Singapore
NCTC3000	81	[35]	Isolates from the Public Health England NCTC reference collection
PATRIC	811	[34]	Genome assemblies submitted to GenBank
Stoesser *et al.* 2013	69	[53]	Isolates from health-care associated infections, Oxford, UK
Stoesser *et al.* 2014	55	[54]	Isolates collected during an outbreak in Nepal
Struve *et al.*	67	[55]	Predominantly isolates of clonal group 23
The *et al.*	76	[3]	Isolates from two outbreaks in Nepal
Wand *et al.*	35	[56]	Historical isolate collection
Novel isolates	484	This study	Diverse collection of Australian hospital surveillance isolates

245

246 A total of 1371 *K. pneumoniae* genomes could be putatively assigned to 63 of the 77
247 K-type reference loci, and a further 918 to one of the other 25 previously published K
248 loci. Among the remaining 213 genomes, 106 were assigned to 29 novel K loci,
249 bringing the total number of known K loci to 132. A further six genomes harboured
250 deletion mutants of known K loci, two had IS variants of known loci and one had an
251 IS variant of a novel locus (see below). For 93 genomes (3.7%), no K locus could be
252 determined; however we found evidence of the presence of three or more K locus-
253 associated genes in all such genomes and consider the lack of assignment was likely
254 attributable to low quality sequence data (low read depth and/or fragmented

255 assembly) rather than complete K locus deletion. Complete details of K locus
256 assignments to all genomes are given in **Table S1** in **Additional File 1**.
257
258 A locus previously associated with K66 was identified in one *K. oxytoca* genome and
259 the K74 locus in four *K. oxytoca* genomes; these matches were very close to the *K.*
260 *pneumoniae* reference sequences (100% coverage and >93% nucleotide identity in
261 all cases). Two novel K loci were identified in three *K. oxytoca* genomes, increasing
262 the total known *Klebsiella* K loci to 134 (**Table S4, Additional File 3**).
263
264 We estimated the extent to which we had captured the repertoire of K locus
265 diversity in the *K. pneumoniae* population (**Figure 1**). The rarefaction curves were
266 estimated from (i) the full genome set for which K loci were assigned (n = 2410; grey
267 lines in **Figure 1**); (ii) a 'non-redundant' genome set from which highly biased sub-
268 samples such as outbreaks were removed (n = 1081; blue lines in **Figure 1**); and (iii)
269 genomes from the non-redundant set representing each of the distinct species, *K.*
270 *pneumoniae sensu stricto, K. variicola* and *K. quasipneumoniae* (black lines in **Figure**
271 **1**). In comparison to that for the full genome set (grey), the non-redundant (blue)
272 curve better represents the true diversity of the *K. pneumoniae sensu lato*
273 population. Note that neither reached the total number of known K loci, since 13 of
274 the serologically defined K loci [22] were not represented at all in our 2503 genomes.
275 The rarefaction curves for each of the three *Klebsiella* species within the non-
276 redundant dataset were highly similar to one another, indicating similar levels of
277 capsule diversity within each species (**Figure 1**). There was no strong evidence of
278 species specificity: across our entire genome collection 46 distinct K loci were

279 identified only among *K. pneumoniae sensu stricto*, while three and two were
280 identified only among *K. variicola* and *K. quasipneumoniae*, respectively, however
281 these differences are likely an artefact of the much larger sample size currently
282 available for *K. pneumoniae sensu stricto*.
283
284 ***K locus nomenclature***
285 We used a standardised K locus nomenclature based on that proposed for
286 *Acinetobacter baumannii* [57]. Each distinct *Klebsiella* K locus was designated as KL
287 (K locus) and a unique numeric identifier. The K-type reference K loci were assigned
288 the same numeric identifier as the corresponding K-type, for example K1 is encoded
289 by the KL1 locus. K loci for which capsule types have not yet been phenotypically
290 defined were assigned identifiers starting from 101 (note KL101 and KL102
291 correspond to those previously named as KN1 and KN2).
292
293 K loci with IS insertions disrupting the region were distinguished from orthologous
294 IS-free variants by using -1, -2. This nomenclature was consistently applied to the 10
295 K-type reference K loci published previously that include one or more ISs
296 [21,22,25,26]. Deletion variants derived from a known K locus were given the suffix -
297 D1, -D2, etc.
298
299 ***K locus reference database***
300 We curated a K locus reference database of complete annotated K locus sequences
301 for all of the 134 loci (**Table S2, Additional File 2**). Where possible sequences were
302 included at their full length, from the start of *galF* to the end of *ugd*. Where a

303 previously published sequence did not span the full length of the locus or contained
304 an IS, we substituted the complete, IS-free K locus sequence from a genome in our
305 collection if available (39 of 51). Where no naturally occurring IS-free variants were
306 available, we manually generated an IS-free synthetic sequence (**Table S2,**
307 **Additional File 2**). IS-free sequences are included in a primary K locus reference
308 database, while all available IS or deletion variant K locus references are included in
309 an accompanying variant database, both available at
310 https://github.com/katholt/Kaptive.
311
312 ***K locus structures***
313 All of the novel K loci identified in this study conformed to the common structure
314 described previously (**Figure S6**) [20–22]. We also identified six deletion variants
315 within our genome collection (KL5-D1, KL20-D1, KL30-D1, KL62-D1, KL106-D1 and
316 KL107-D1). Each putative deletion variant was missing several common genes but
317 the remaining regions showed a high degree of similarity to other apparently
318 complete K loci, which we suggest represent the ancestral forms. Isolate NCTC10004
319 (recorded as serotype K11 in the UK National Culture Type Collection) and four other
320 genomes carried a K locus that was nearly identical to the previously published K11 K
321 locus reference sequence [22]. However, the latter lacked the essential *wzx* gene
322 plus two other neighbouring genes, and was not identified among any other
323 genomes. We assume the NCTC10004 locus represents the full length KL11 locus and
324 designate the original K11 reference as KL11-D1 (note it is unclear whether the
325 original sequenced reference isolate had retained the ability to produce a capsule,
326 since the serological typing was performed decades earlier [22]). In four of the

327 deletion variants the deleted region was replaced by an IS, which may have
328 mediated the deletion.
329
330 Of the other IS related variants, KL157-1 contained an IS*903* family IS without an
331 obvious deletion. In addition, we identified two novel IS variants of K-type reference
332 loci (KL15-1 and KL22-1), and five IS-free variants of K-type reference loci (KL3, KL6,
333 KL38, KL57, KL81), plus one other previously published K locus (KL103). In total,
334 seven IS insertions were associated with neither a deletion nor a rearrangement
335 event. In contrast, the KL22-1 locus included a translocation of part of the
336 lipopolysaccharide (LPS) locus to the centre of the K locus, plus an inversion of the 3'
337 portion of the K locus (**Figure 2**). The translocated and inverted regions were bound
338 at each end by a copy of IS*Kpn26*.
339
340 ClonalFrameML analysis of the nucleotide sequences of common K locus genes
341 identified a high number of putative recombination events (n=382) between the
342 reference K loci. These events were not distributed equally across the nucleotide
343 alignment (**Figure 3A**); rather the genes closest to the central variable region of the K
344 locus were affected by a greater number of recombination events compared to
345 those at the ends of the locus.
346
347 ***Variation in K locus gene content***
348 A total of 2675 predicted proteins from 134 complete K loci were clustered at
349 various identity levels (see **Methods**), resulting in 1496 to 508 clusters. As the
350 identity threshold was reduced the number of clusters continued to fall and showed

351 no signs of stabilising, even between 50% and 40% identity (**Figure S7**), and we
352 believe the latter is a lower bound for sensible comparison. At 40% identity, the core
353 capsule assembly proteins GalF, Wzi, Wza, Wzb, Gnd and Ugd each formed a single
354 cluster, and were present in nearly all loci (**Figure 3**). The Wzc sequences clustered
355 into two groups, and each locus encoded one Wzc protein (except KL50). In contrast,
356 Wzx (flippase) clustered into 42 groups and Wzy (capsule repeat unit polymerase)
357 clustered into 83 groups, highlighting the extreme diversity of these proteins
358 compared to the other core capsule assembly machinery proteins (**Figure 3**).
359
360 There were 374 clusters among the remaining proteins, almost all of which were
361 associated with sugar synthesis and processing (**Figure 3**). The initiating sugar
362 transferase proteins WbaP (undecaprenyl-phosphate galactosephosphotransferase)
363 and WcaJ (undecaprenyl-phosphate glucose phosphotransferase) were grouped into
364 two clusters. These proteins are considered essential for capsule synthesis.
365 Concordantly each locus encoded a single protein from one of these two clusters.
366 RmlB, RmlA, RmlD and RmlC, which are associated with synthesis and processing of
367 rhamnose and typically encoded together in a single operon, were each represented
368 by a single cluster. Similarly, the mannose synthesis and processing proteins, ManC
369 and ManB, were grouped into a single cluster each. The associated operons *rmlBADC*
370 and *manCB* were present in 55 and 73 K loci, respectively (14 loci contained both
371 operons, **Figure 3**). In contrast, 360 of the remaining 366 protein clusters were
372 present in fewer than ten K loci each (**Figure 3E**).
373

374 Co-occurrence analysis identified 18 correlated groups of K locus proteins, ranging in
375 size from two to five protein clusters (pairwise Jaccard similarity ≥0.61 for all pairs in
376 the group, **Figure 4** and **Table S5, Additional File 5**). One group included the four Rml
377 protein clusters; interestingly this group also included a WcaA glycosyltransferase,
378 which was present in 67.3% of *rmlBADC*-containing K loci and no *rmlBADC*-negative
379 loci (X-squared = 70.09, p-value < 2.2e-16 by two-sided proportion test). Similarly
380 another group included the ManCB proteins and the putative mannosyl transferase,
381 WbaZ, which was present in 65.8% of *manCB*-containing K loci and one *manCB*-
382 negative locus (X-squared = 56.159, p-value = 6.683e-14 by two-sided proportion
383 test). In addition, several groups included proteins for which the associated genes
384 were located sequentially in their K loci (e.g. *wckG*, *wckH* and *wzx* in KL12, KL29 and
385 KL42) consistent with linked gene transfer.
386
387 ***Diversity of wzc and wzi gene sequences***
388 We confidently assigned *wzi* alleles to 2461 *K. pneumoniae* genomes, including 390
389 distinct alleles, 218 of which were novel. Median pairwise nucleotide divergence was
390 7%. Among the non-redundant genome set there were 54 *wzi* alleles represented by
391 at least five genomes and of these, 15 (28%) were associated with more than one K
392 locus type (**Table S1, Additional File 1**). Much of the *wzi* allelic variation appeared to
393 result from accumulation of mutations within K loci. Among the 67 K loci for which
394 we had ≥5 representative sequences, 64 (95.5%) were associated with two or more
395 *wzi* alleles, and there was a general trend towards increasing *wzi* allelic diversity with
396 increasing K locus representation (**Figure 5**).
397

398 We extracted *wzc* sequences from 1041 of 1082 genomes in the non-redundant
399 genomes set (**Figure 6**). In general, genomes sharing the same K locus (n=6262
400 pairwise observations) showed lower *wzc* nucleotide divergence than those with
401 different K loci (n=491,775 pairwise observations), but the distributions overlapped
402 substantially (**Figure 6**). Notably, there were five distinct combinations of K loci for
403 which one or more pairs harboured *wzc* sequences that were <6% divergent (the
404 cut-off for K-type assignment as described in [31]; KL1 and KL112, KL9 and KL45,
405 KL15 and KL52, KL30 and KL104, KL40 and KL135). Conversely, some K loci (KL45,
406 KL112) had more than 25% *wzc* nucleotide diversity between representatives of the
407 same K locus.
408
409 ***Kaptive – capsule locus (K locus) typing and variant evaluation from genome data***
410 To facilitate easy identification of K loci from genome assemblies, we developed the
411 command-line software tool *Kaptive,* which is an extension of the analysis procedure
412 described above, as shown in **Figure 7** (also see **Additional File 3**). We used *Kaptive*
413 with our primary *Klebsiella* K locus reference database to rapidly type the K loci in
414 our collection of 2503 *Klebsiella* genomes, and obtained confident K locus calls for
415 2412 genomes (96.4%, see **Additional File 3** for further details).
416
417 We compared the K locus calls from *Kaptive*, *wzc* and *wzi* typing to serological typing
418 results for 86 isolates for which both genome and serology data were available
419 ([7,18,35], see **Table S3, Additional File 4**). Five of six isolates that were non-
420 typeable by serological techniques were identified by *Kaptive* as carrying KL16, KL54,
421 KL81, KL111 and KL149. The KL16, KL54 and KL81 calls were in agreement with *wzc*

422 and *wzi* typing results; the other two K loci were not present in the *wzi* or *wzc*
423 schemes and so were not typeable by those methods. Among the 80 serologically
424 typeable isolates, the three molecular methods were generally in agreement with
425 one another, although concordance with recorded phenotypes was quite low (65-
426 74%, **Table S3**). Call rates were highest for *Kaptive* (95%), followed by *wzc* (89%) and
427 *wzi* typing (75%).
428
429 **Discussion**
430 The number of distinct *Klebsiella* K loci (now 134) is striking and exceeds that
431 described for capsule synthesis loci in other bacterial species such as *A. baumannii,*
432 *Streptococcus pneumoniae* and *Neisseria meningitidis.* Furthermore, the diversity is
433 an order of magnitude greater than that recently described for *Klebsiella* LPS, the
434 other major *Klebsiella* surface antigen [28]. This suggests that the K locus is subject
435 to strong diversifying selection. Given that these bacteria are not obligate pathogens
436 and are ubiquitous in non-host-associated environments [58,59], it seems likely that
437 the factors driving selection are not immune pressures from humans or other hosts,
438 but may include phage and/or protist predation.
439
440 Two novel K loci were identified from *K. oxytoca*, a close relative of *K. pneumoniae*.
441 The KL66 and KL74 K-type reference loci were also identified among *K. oxytoca*
442 genomes. Little is known about *K. oxytoca* capsules, though a report from Japan in
443 2012 identified several other *K. pneumoniae*-associated capsules among *K. oxytoca*
444 isolates from blood and bile infections [60]. Together these findings indicate that *K.*
445 *oxytoca* is able to exchange genetic material with *K. pneumoniae.* Therefore, *K.*

446 *oxytoca* represents a potential reservoir of virulence, drug resistance and other
447 genes for *K. pneumoniae,* and warrants greater research attention.
448
449 Our analysis confirms there are strong constraints on the structure of K loci, which
450 generally include *galF, cpsACP, wzi, wza, wzb* and *wzc* at the 5' end, *gnd* and *ugd* at
451 the 3' end, and a highly variable set of genes in the centre (**Figure 3**). Our data also
452 reveal the extensive diversity of proteins encoded in the variable central region, with
453 499 unique proteins identified across the 134 K loci. These genes ranged in
454 frequency from 0.7% to 54.7% of the K loci. Among those represented in at least
455 three K loci, approximately half co-occurred in groups ranging from two to five
456 genes.
457
458 The molecular evolutionary events driving K locus diversification are not yet well
459 understood, but likely include a combination of point mutation, IS-mediated
460 rearrangements, and homologous recombination within the locus, resulting in the
461 mosaic structure summarised in **Figure 3**. It has been shown, in both *A. baumannii*
462 [61,62] and *S. pneumoniae* [63], that recombination within the capsule synthesis
463 locus can drive capsule exchange between distinct clones. We recently speculated
464 that this may also be true for *K. pneumoniae* [27] and the recombination analysis
465 presented here supports this theory. The genes closest to the central variable region
466 of the K locus (i.e. *wzb, wzc* and *gnd*), showed evidence of the greatest number of
467 recombination events, consistent with the hypothesis that they act as regions of
468 homology for recombination events that shuffle the central region of the locus.
469

470 The prediction of capsule phenotypes from genome data is complex, as capsule
471 expression is a highly regulated process that involves loci outside the K locus region
472 [64], and so presence of an identical K locus sequence does not guarantee an
473 identical phenotype. However it is likely that K loci encoding distinct sets of proteins
474 are associated with distinct capsule phenotypes, as is the case for the vast majority
475 of K-type reference strains [22]. Therefore, our data suggest that there are at least
476 134 distinct *Klebsiella* capsule types. Note that this is a lower bound estimate since
477 there are likely additional K loci in the wider population that were not in the current
478 genome collection, and our analysis did not capture differences that may arise from
479 point mutations and small-scale insertions or deletions (e.g. in the case of K22 and
480 K37 described previously [22]). Furthermore, while we did not attempt to thoroughly
481 characterise IS variants, several such variants were apparent. The potential
482 functional impacts of IS insertions likely vary depending on their location in the
483 locus, but may include up-regulation, loss of capsule production and/or more subtle
484 changes in sugar structures [65–67]. However, functional studies are required to
485 understand these effects and to improve the prediction of phenotypes.
486
487 Serological typing of *Klebsiella* isolates is notoriously difficult and rarely performed.
488 We were able to compare genotypes (whole-locus typing using *Kaptive*, as well as
489 *wzi* and *wzc* typing schemes) with phenotypes on just 86 isolates for which both
490 sequences and serotypes were available. Of the 19 discordant genotype vs
491 phenotype results, two were due to deletion variants and were resolved by running
492 *Kaptive* with the K locus variants database. Interestingly, one of these isolates was
493 non-typeable by serology, *wzi* or *wzc* typing, but recognized as a specific K locus

494 deletion variant by *Kaptive*. This highlights a benefit of our whole-locus typing
495 approach; it provides epidemiologically relevant information even when the K locus
496 is interrupted. Another isolate was serologically typed as K54 but genotyped by
497 *Kaptive* as KL113, which has sequence homology with KL54 (>84% nucleotide
498 identity over 76% of the locus) and may encode a serologically similar or cross-
499 reacting capsule. The other cases of discordance had no obvious explanation,
500 however it is likely that some result from serological typing errors or from mutations
501 arising during subculture (as identified for the K11 reference isolate above), neither
502 of which we were able to check. Some discordance may also be due to unpredictable
503 serological cross-reactions.
504
505 Given the problems with serotyping and the comparative robustness and
506 widespread access to genome sequencing, we anticipate that genotyping will remain
507 the preferred method for tracking capsular diversity in *Klebsiella*. Due to the
508 extensive diversity and potential for ongoing evolution, we strongly advocate for
509 classification based on complete, or near complete K locus sequences, rather than
510 single genes such as *wzi* or *wzc,* which can be misled by substitutions and horizontal
511 gene transfer. *Kaptive* analyses the full-length K locus nucleotide sequences and
512 assesses the presence of all K locus associated genes by protein BLAST search, thus
513 the approach is resilient to spurious results that may arise due to sequence
514 divergence. Furthermore, the information provided allows users to determine
515 confidence in the results and to identify putative novel K loci or variants of known
516 loci if desired (see **Additional File 2**).
517

518 **Conclusions**

519 We report an investigation of K loci among a large collection of 2513 *Klebsiella*
520 genomes. We identified 31 novel K loci, increasing the total number of known loci to
521 134, almost twice the number of serologically defined K-types. We defined a
522 standardised *Klebsiella* K locus nomenclature and developed a curated reference
523 database, which captures the majority of the extensive diversity in the *K.*
524 *pneumoniae* population. Lastly, we developed a simple program, *Kaptive,* for the
525 detection of reference K loci from genome assemblies. These new resources will
526 greatly facilitate evolutionary investigations and genomic surveillance efforts for this
527 and other important bacterial pathogens.

528

529 **List of Abbreviations**

530 K-type: capsule type; K locus: capsule synthesis locus; IS: insertion sequence; CDS:
531 coding sequence; LPS: lipopolysaccharide

532

533 **Additional Files**

534 **Additional file 1 (Excel spreadsheet, xlsx)**

535 **Table S1.** *K. pneumoniae* genome data analysed in this study. Accession numbers, K
536 locus designations and summarised *Kaptive* typing results are provided.

537

538 **Additional file 2 (Excel spreadsheet, xlsx)**

539 **Table S2.** *Klebsiella* K locus references, accession numbers and isolate names.

540

541 **Additional file 3 (Word document, docx)**

542 **Supplementary Methods and Results**

543 **Table S4.** *K. oxytoca* genomes analysed in this study and K locus designations.

544 **Figures S1 – S7.**

545

546 **Additional file 4 (Excel spreadsheet, xlsx)**

547 **Table S3.** *Kaptive*, *wzi, wzc* and serological typing results for 86 *K. pneumoniae*

548 genomes for which serological typing information were available.

549

550 **Additional file 5 (Excel spreadsheet, xlsx)**

551 **Table S5.** Jaccard similarity scores for 115 K locus protein clusters for which the

552 associated genes were present in at least three K loci.

553

554 **Declarations**

555 ***Availability of data and material***

556 The datasets generated and/or analysed during the current study are available in the

557 European Nucleotide Archive and/or PATRIC genome database, accession numbers

558 are listed in **Table S1**. Novel K locus nucleotide sequences have been deposited in

559 GenBank (accessions listed in **Table S2**) and are also distributed together with our re-

560 annotated and curated set of all known K loci at https://github.com/katholt/Kaptive.

561

562 ***Competing interests***

563 None declared.

564

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568

569 ***Authors' Contributions***

570 KLW and KEH designed the study, collected and analysed data, designed the *Kaptive*

571 tool and wrote the manuscript. RRW designed and implemented the *Kaptive* tool

572 and wrote the manuscript. CG and AJ generated serological and sequence data for

573 Australian isolates. RF and NT contributed to data analysis and interpretation. All

574 authors read and approved the final manuscript.

575

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762
763 **Figure Legends**
764
765 **Figure 1: Rate of discovery of distinct K loci with increasing genome sample size**.
766 Curves indicate the accumulation of distinct K loci (mean ± SE) in different genome
767 sets. Grey; all genomes (n=2410, excluding *K. oxytoca*). Blue; non-redundant
768 genomes (n=1081, excludes genomes from investigations of disease outbreaks and
769 specific clonal groups). Black; species specific genome sets (*K. pneumoniae* refers to
770 *K. pneumoniae sensu stricto*, SE not shown). Inset box shows a zoomed view of the
771 bottom-left section of the plot, as indicated by the dashed box.
772
773 **Figure 2: Example K locus structures and comparisons**
774 Coding sequences are represented as arrows coloured by predicted function of the
775 protein product and labelled with gene names where known. Grey bars indicate
776 regions of similarity identified by BLAST comparison, darker shading indicates higher
777 sequence identity. **(A)** Comparison of deletion variant KL15-D1 and insertion
778 sequence variant KL15-1 with the synthetic KL15 locus. **(B)** Comparison of the
779 insertion sequence variant KL22-1 with the K-type reference KL22 locus. The

780 downstream LPS (lipopolysaccharide synthesis) operon (pink arrows) has been

781 translocated into the K locus.

782

783 **Figure 3: Composition and diversity of *Klebsiella* K loci**

784 **(A)** Putative recombination events among the common K locus genes. Values plotted
785 are relative number of events per 100 bp window, inferred using ClonalFrameML. **(B)**
786 Representation of a generalised K locus structure. Arrows represent K locus coding
787 regions coloured by predicted protein product as in **Figure 2**. Percentage values
788 indicate the number of reference K loci containing each gene (total 134 references).
789 Note that 13 of the K locus references partially or completely exclude *ugd*, although
790 it is known to be present in 11 of these loci [22]. Thus we counted these 11 as
791 containing *ugd*. The locations within this structure at which *wzx* (C), *wzy* (D) and
792 sugar processing genes (E) have been found to occur are indicated. **(C, D, E)** Diversity
793 of proteins encoded by *wzx* (C), *wzy* (D) and sugar processing genes (E) annotated
794 amongst the 134 K locus reference sequences. Bar charts indicate the frequency of
795 each predicted protein cluster.

796

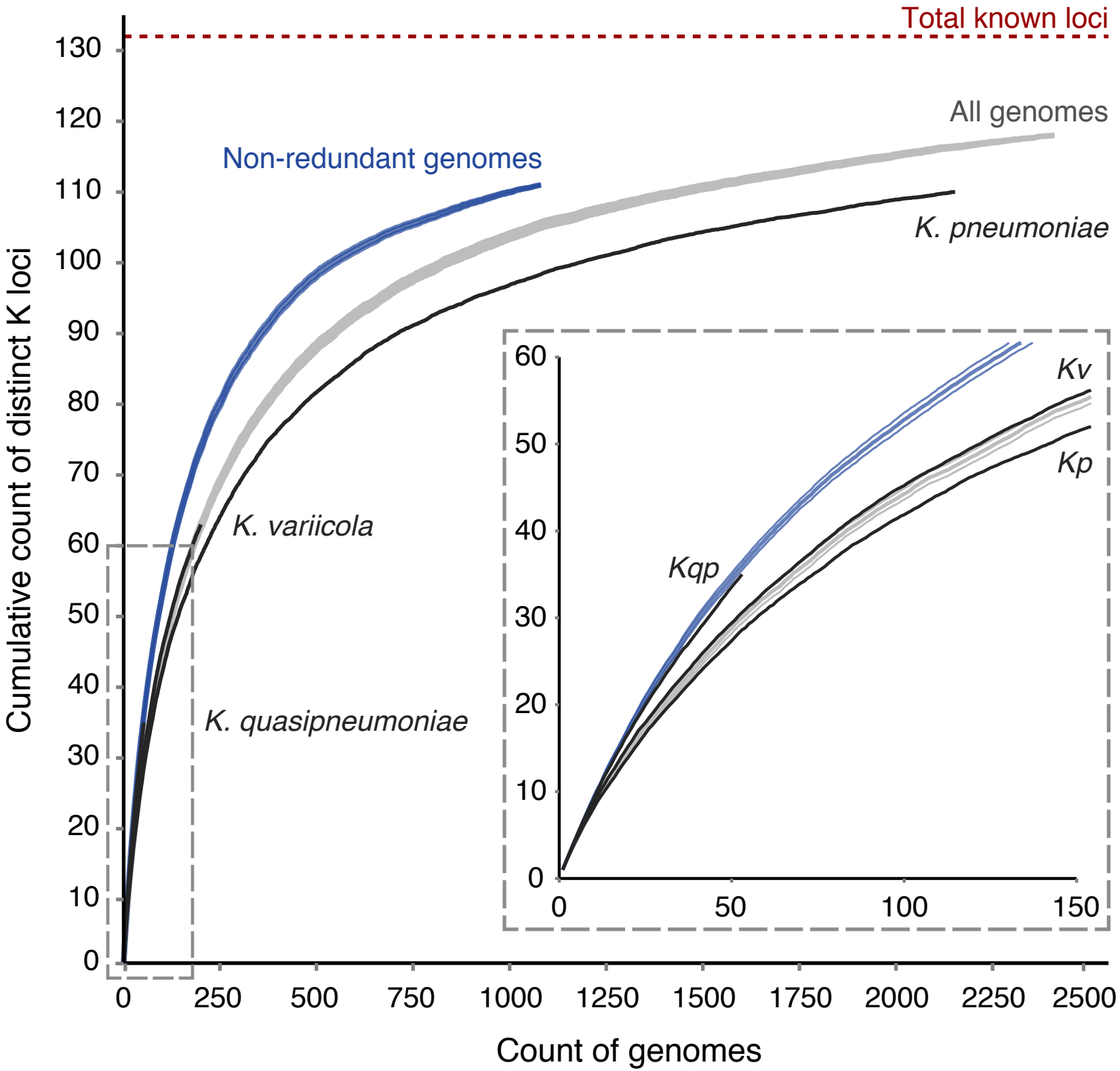
797 **Figure 4: Co-occurrence of *wzx, wzy* and sugar processing genes across reference K**
798 **loci**

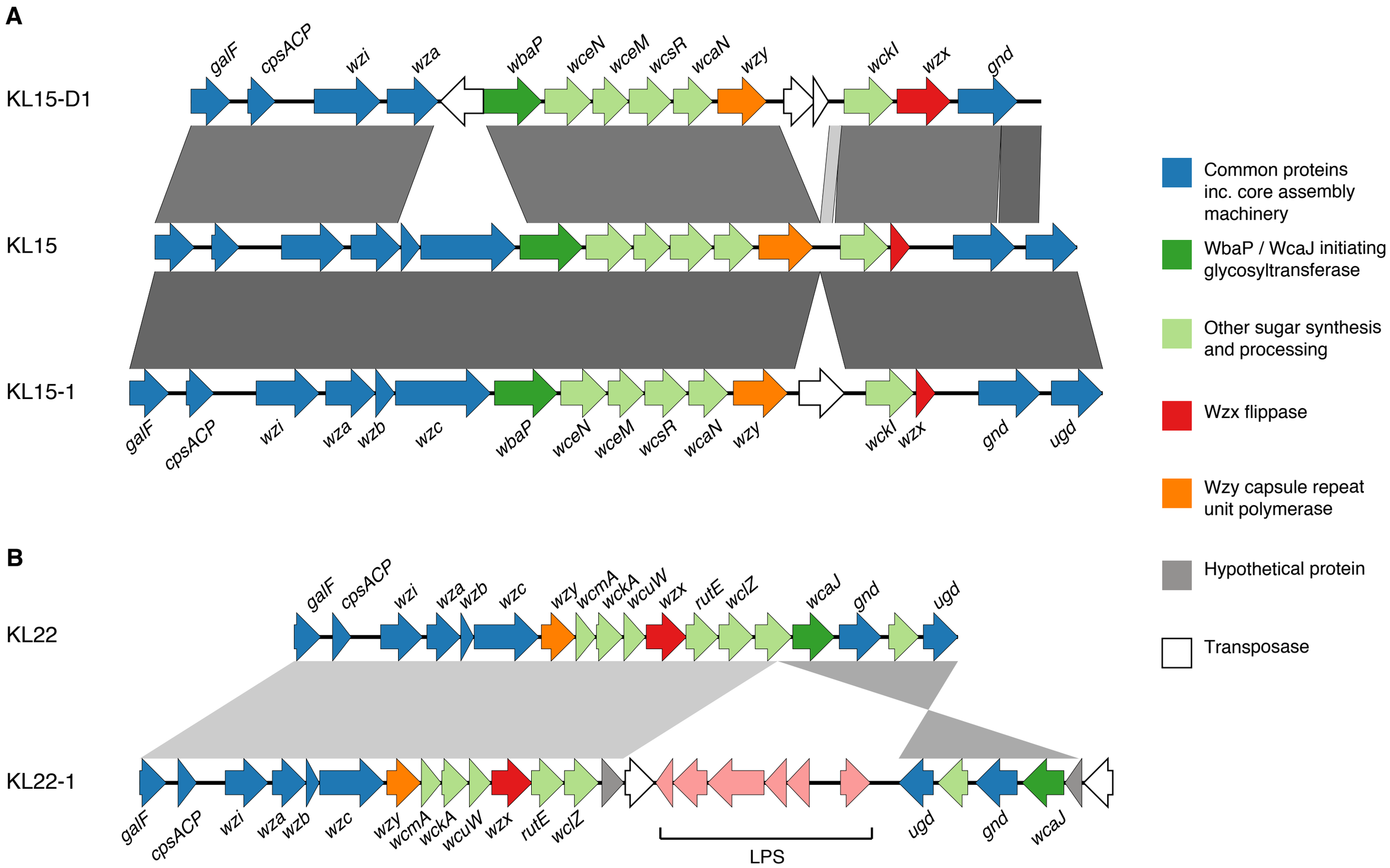
799 Nodes represent genes, labelled by name (HYP: hypothetical protein) and coloured
800 by protein product as in Figures 2 and 3: Wzx (red), Wzy (Orange), mannose
801 synthesis/processing proteins (dark purple), rhamnose synthesis/processing proteins
802 (light purple), other proteins (green). Edge widths are proportional to Jaccard index
803 (J) and are shown for all pairs where $J \geq 0.61$. Numbers represent co-occurrence
804 group assignments as defined in **Table S5, Additional File 5**.

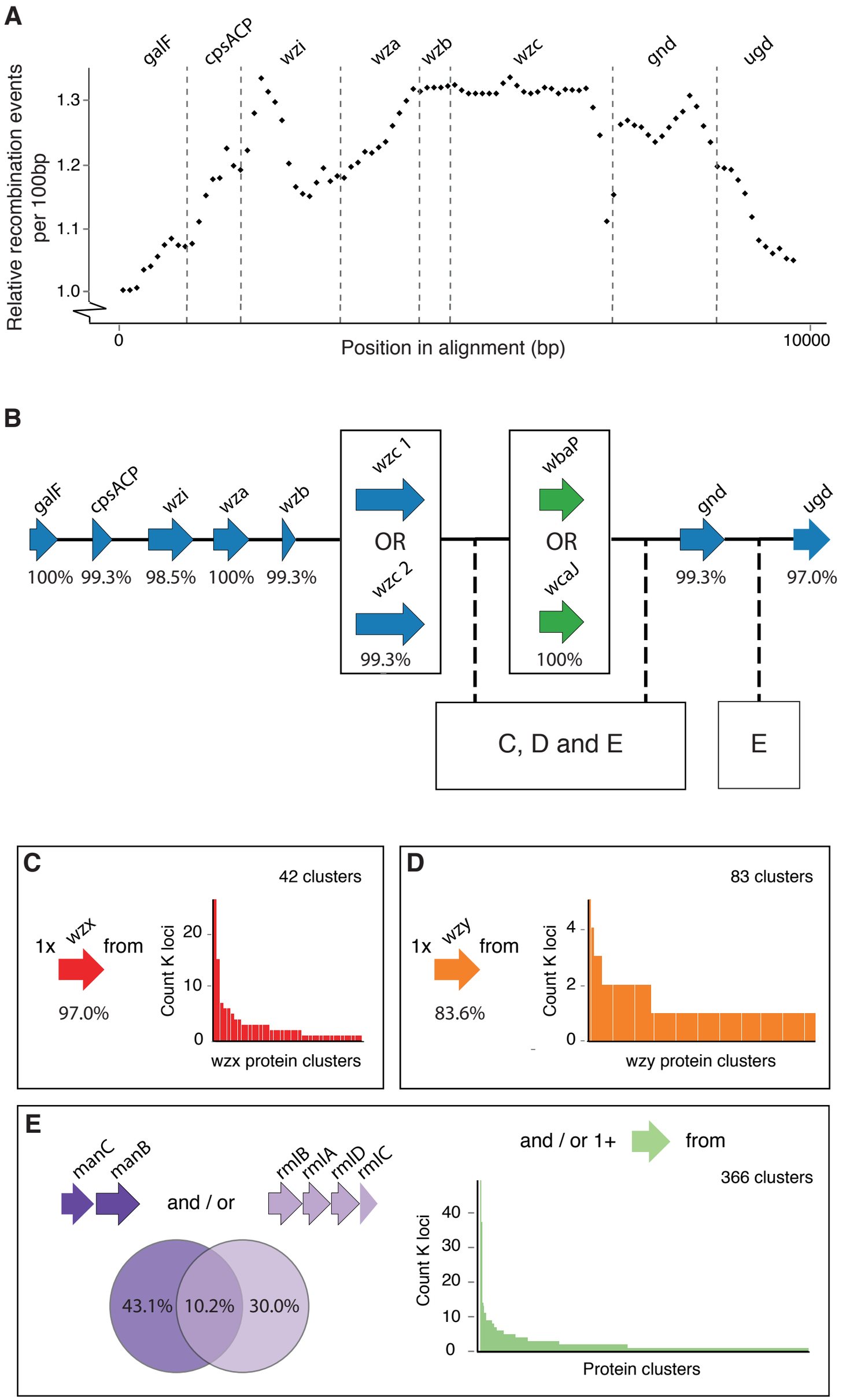
805

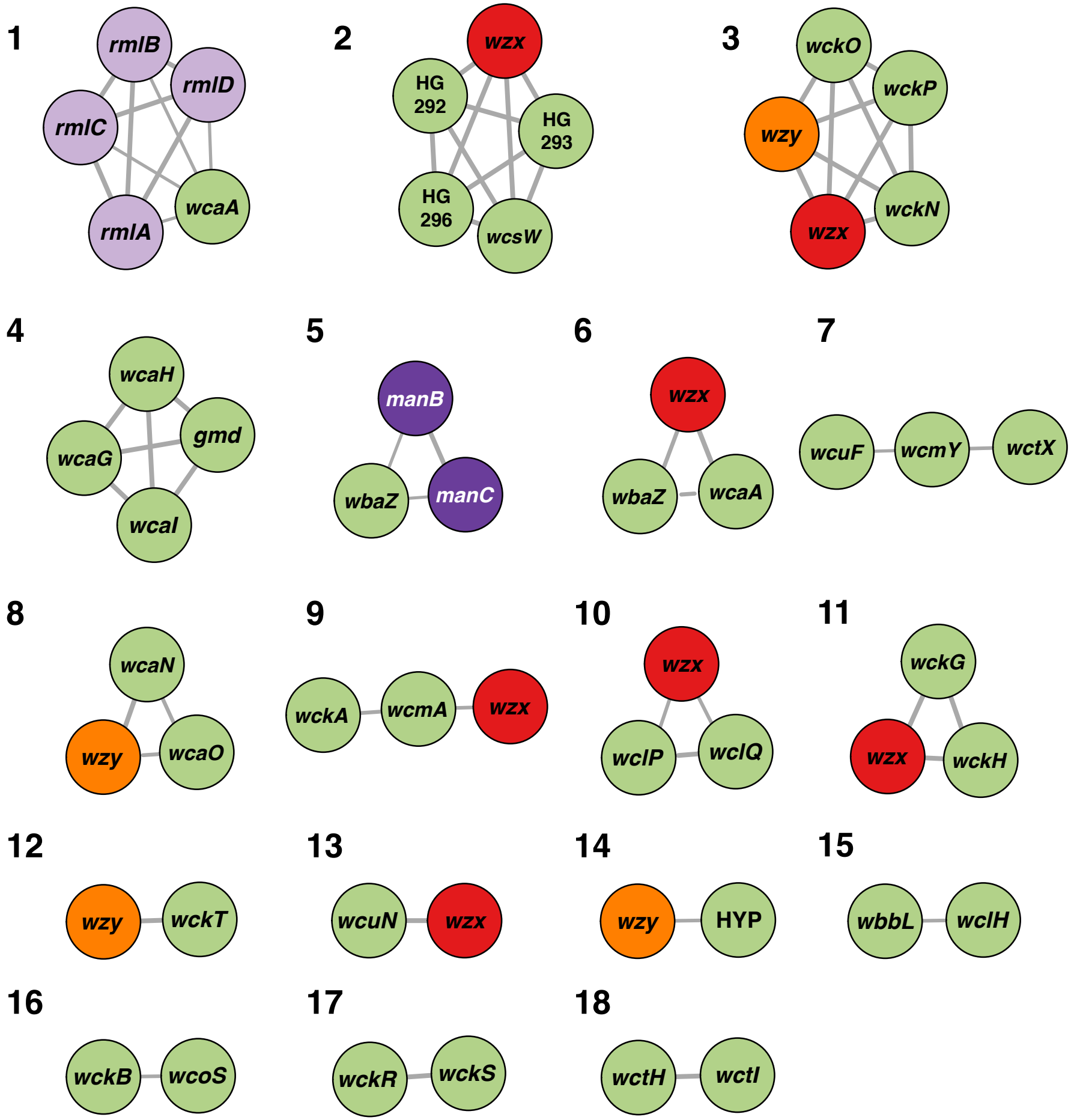
806 **Figure 5: Allelic diversity of *wzi***

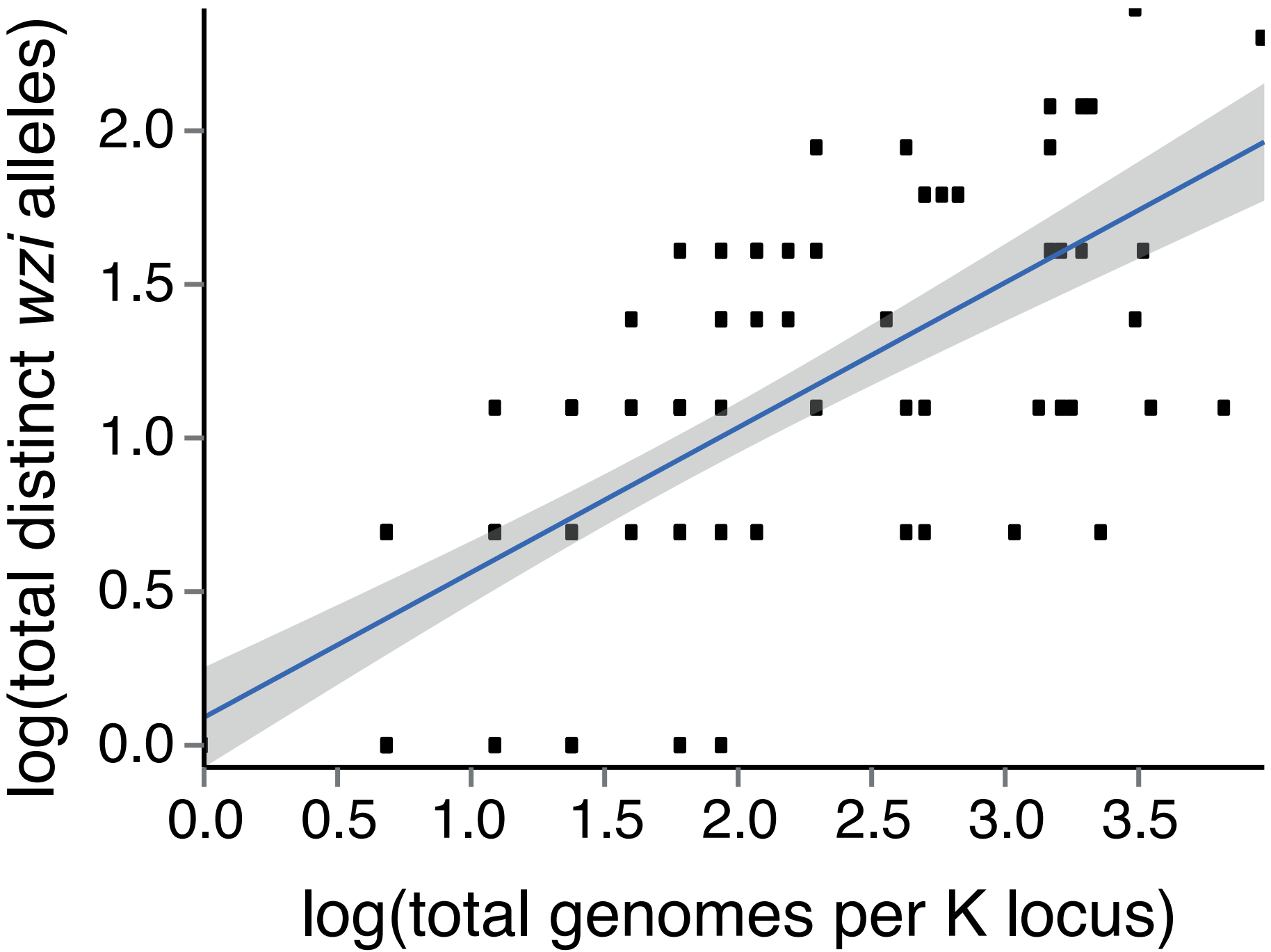
807 Within K locus *wzi* allelic diversity increases with total K locus representation. The
808 blue line represents the least-squares regression and grey shading indicates the 95%
809 confidence interval.
810
811 **Figure 6: *wzc* nucleotide diversity**
812 Barplots showing distribution of pairwise *wzc* nucleotide divergence for pairs of
813 genomes with the same (light blue) or different (dark blue) K loci. The inset box
814 shows a zoomed view of the lower end of the distribution.
815
816 **Figure 7. Summary of the *Kaptive* analysis procedure**
817 *Kaptive* takes as input a set of annotated reference K loci in GenBank format and one
818 or more genome assemblies, each as a single FASTA file of contigs. *Kaptive* performs
819 a series of BLAST searches to identify the best-match K locus in the query genome
820 and assess the presence of genes annotated in the best-match locus (expected
821 genes) and those annotated in other loci (unexpected genes) both within and
822 outside the putative K locus region of the query assembly. The output is a FASTA file
823 containing the nucleotide sequence(s) of the K locus region(s) for each query
824 assembly and a table summarising the best-match locus, gene content and potential
825 problems with the match (e.g. the assembly K locus region is fragmented, expected
826 genes are missing from the K locus region or at low identity, or unexpected genes
827 are present) for each query assembly. CDS = coding sequence.

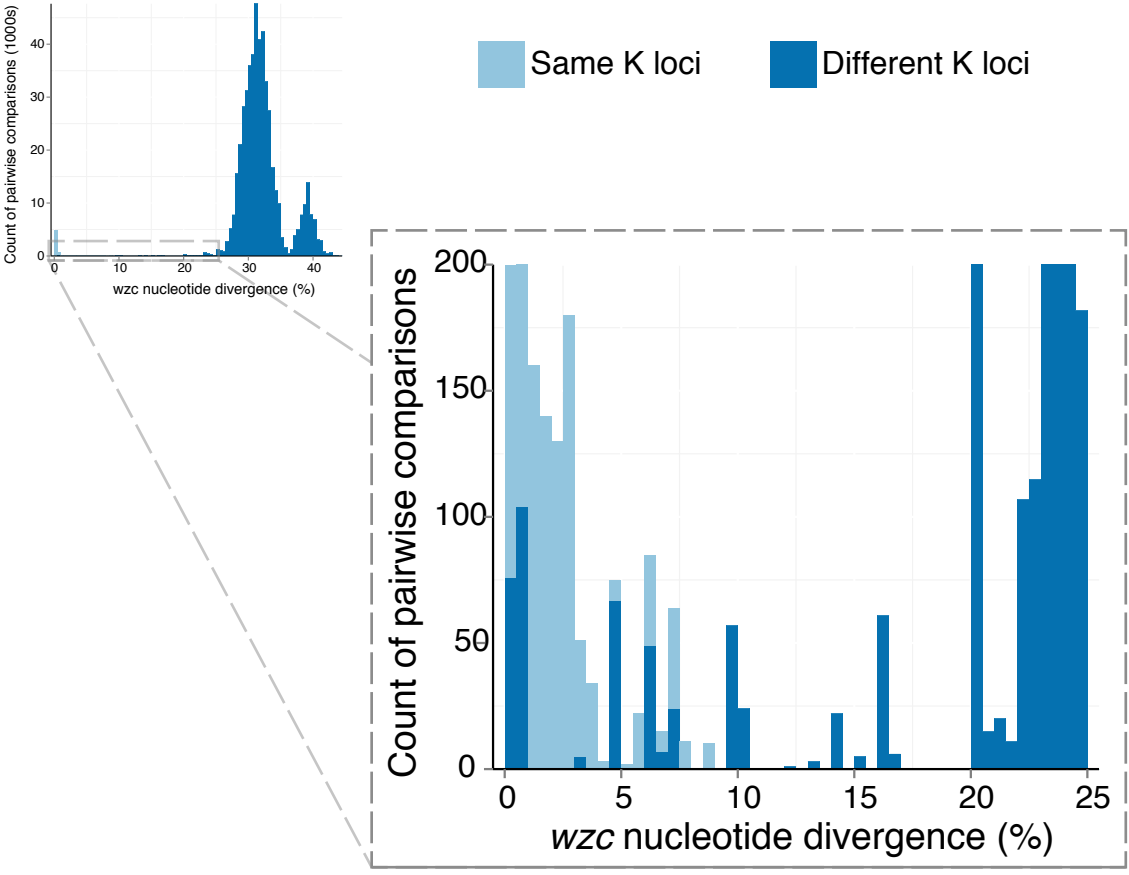

Total known loci
All genomes
Non-redundant genomes
K. pneumoniae
K. variicola
K. quasipneumoniae
Kv
Kp
Kqp
Cumulative count of distinct K loci
Count of genomes
0
10
20
30
40
50
60
70
80
90
100
110
120
130
0
250
500
750
1000
1250
1500
1750
2000
2250
2500
0
10
20
30
40
50
60
0
50
100
150

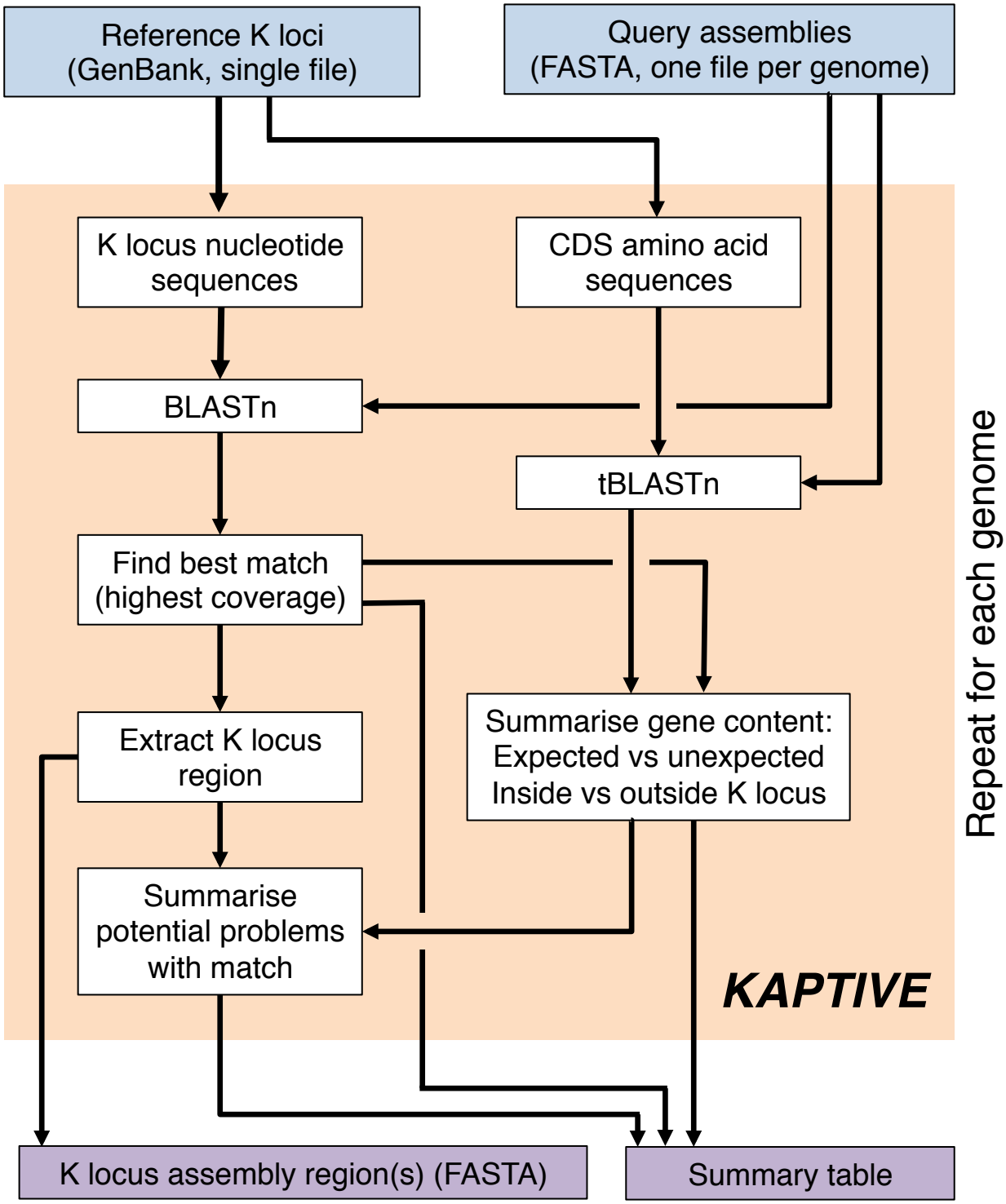

A
KL15-D1
galF
cpsACP
wzi
wza
wbaP
wceN
wceM
wcsR
wcaN
wzy
wckI
wzx
gnd
KL15
KL15-1
galF
cpsACP
wzi
wza
wzb
wzc
wbaP
wceN
wceM
wcsR
wcaN
wzy
wckI
wzx
gnd
ugd
B
KL22
galF
cpsACP
wzi
wza
wzb
wzc
wzy
wcmA
wckA
wcuW
wzx
rutE
wclZ
wcaJ
gnd
ugd
KL22-1
galF
cpsACP
wzi
wza
wzb
wzc
wzy
wcmA
wckA
wcuW
wzx
rutE
wclZ
ugd
gnd
wcaJ
LPS
Common proteins inc. core assembly machinery
WbaP / WcaJ initiating glycosyltransferase
Other sugar synthesis and processing
Wzx flippase
Wzy capsule repeat unit polymerase
Hypothetical protein
Transposase


A
galF
cpsACP
wzi
wza
wzb
wzc
gnd
ugd
Relative recombination events per 100bp
1.3
1.2
1.1
1.0
0
10000
Position in alignment (bp)
B
galF
cpsACP
wzi
wza
wzb
100%
99.3%
98.5%
100%
99.3%
wzc 1
OR
wzc 2
99.3%
wbaP
OR
wcaJ
100%
gnd
ugd
99.3%
97.0%
C, D and E
E
C
42 clusters
1x
wzx
from
97.0%
Count K loci
20
10
0
wzx protein clusters
D
83 clusters
1x
wzy
from
83.6%
Count K loci
4
2
0
wzy protein clusters
E
manC
manB
and / or
rmlB
rmlA
rmlD
rmlC
43.1%
10.2%
30.0%
and / or 1+
from
366 clusters
Count K loci
40
30
20
10
0
Protein clusters


1
rmlB
rmlD
rmlC
wcaA
rmlA
2
wzx
HG 292
HG 293
HG 296
wcsW
3
wckO
wckP
wzy
wckN
wzx
4
wcaH
gmd
wcaG
wcaI
5
manB
wbaZ
manC
6
wzx
wbaZ
wcaA
7
wcuF
wcmY
wctX
8
wcaN
wzy
wcaO
9
wckA
wcmA
wzx
10
wzx
wclP
wclQ
11
wckG
wzx
wckH
12
wzy
wckT
13
wcuN
wzx
14
wzy
HYP
15
wbbL
wclH
16
wckB
wcoS
17
wckR
wckS
18
wctH
wctI


log(total distinct wzi alleles)
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
log(total genomes per K locus)


Count of pairwise comparisons (1000s)
0
10
20
30
40
0
10
20
30
40
wzc nucleotide divergence (%)
Same K loci
Different K loci
Count of pairwise comparisons
0
50
100
150
200
0
5
10
15
20
25
wzc nucleotide divergence (%)


Reference K loci
(GenBank, single file)
Query assemblies
(FASTA, one file per genome)
K locus nucleotide sequences
CDS amino acid sequences
BLASTn
tBLASTn
Find best match
(highest coverage)
Extract K locus region
Summarise gene content:
Expected vs unexpected
Inside vs outside K locus
Summarise potential problems with match
KAPTIVE
Repeat for each genome
K locus assembly region(s) (FASTA)
Summary table