Phylogenomic analysis of the understudied *Neisseriaceae* species reveals a poly- and paraphyletic *Kingella* genus

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Abbreviations: ANI: Average Nucleotide identity; dDDH: digital DNA-DNA hybridization, OGRI: overall genome relatedness index, COGs: clusters of orthologous gene groups; MuLDi: Multicellular, longitudinally-dividing; DUS: DNA-uptake sequences, MALDI-TOF MS: Matrix-assisted laser desorption-ionization time of flight mass spectrometry, CA: common ancestor

Abstract

Taxonomic classification and phylogenetic analysis of the *Neisseriaceae* family has focused on the pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Less is known about the relationships of commensal *Neisseria* species and other *Neisseriaceae* genera, raising the possibility that the phylogeny of this family may not agree with taxonomy. In this study we used available nucleotide sequences and a phylogenetic approach to assess the *Kingella* genus and its relatives. We found that this genus is both paraphyletic and polyphyletic. *Kingella potus* is more closely related to *Neisseria bacilliformis* than other *Kingella* species. The *Alysiella* and *Simonsiella* genera form a distinct clade within the *Kingella* genus that is closely related to the pathogens *K. kingae* and *K. negevensis*. We find a phylogenetic relationship between *Conchiformibius, Alysiella, Simonsiella,* and *Kingella,* which we name the CASK clade. Finally, we define the gene sets that differentiate each genus of the CASK clade from one another and from the rest of the *Neisseriaceae* family.

Importance

Understanding the evolutionary relationships between the species in the *Neisseriaceae* has been a persistent challenge in bacterial systematics due to high recombination rates in these species. Previous studies of this family have focused on *N. meningitidis* and *N. gonorrhoeae*. However, previously understudied *Neisseriaceae* species are gaining new attention, with *K. kingae* now recognized as a common human pathogen and with *Alysiella* and *Simonsiella* being unique in the bacterial world as multicellular organisms. A better understanding of the genomic evolution of the *Neisseriaceae* can lead to identification of the specific genes and traits that characterize the remarkable diversity of this family.

24 **Introduction**

25 Defining taxonomic relationships is a cornerstone of microbial systematics, but in species
26 that reproduce asexually and trade genetic information through active horizontal gene transfer
27 across the species boundary, defining the relationships between species can be challenging. As
28 in many taxonomic disciplines, the relationships amongst and within species have traditionally
29 been established using bacterial cell morphology and other phenotypic properties. With the
30 advent of molecular techniques such as DNA-DNA hybridization, 16S rRNA gene sequencing,
31 multilocus sequence typing (MLST), and matrix-assisted laser desorption/ionization time of flight
32 (MALDI-TOF), this system gave way to the so called polyphasic classification strategy that seeks
33 to consider both phenotypic and molecular data (1, 2). As whole genome sequences became
34 readily available, a newer version of this system, taxonomogenomics, seeks to incorporate this
35 rich genomic data into taxonomical descriptions (3–6). This approach also has the benefit of being
36 explicitly phylogenetic, providing a non-arbitrary approach to classification (5, 7, 8). While there
37 is still controversy about how best to integrate phenotypic and phylogenomic data in bacterial
38 taxonomy, there is a general consensus that taxonomy should be reflective of the evolutionary
39 relationships and that monophyly is a desirable characteristic of taxonomic groups.

40 The *Neisseriaceae* family is a group of Gram-negative, β-proteobacteria that currently
41 includes 20 genera: *Neisseria*, *Wielerella*, *Vitreoscilla*, *Uruburuella*, *Stenoxybacter*, *Snodgrassela*,
42 *Simonsiella*, *Rivicola*, *Prolinoborus*, *Paralysiella*, *Morococcus*, *Kingella*, *Eikenella*, *Crenobacter*,
43 *Craterilacuibacter*, *Conchiformibius*, *Bergeriella*, *Aquella*, *Amantichitinum*, and *Alysiella* (9–11).
44 The majority of prior work devoted to understanding the taxonomic relationships between these
45 genera has focused primarily on the pathogenic *Neisseria* species, *N. meningitidis* and *N.*

46 *gonorrhoeae* (2, 8, 12–17). Early work using 16S rRNA failed to properly resolve species
47 boundaries, resulting in several polyphyletic genera (18). In addition, pervasive horizontal gene
48 transfer (HGT) and recombination in the *Neisseriaceae* further increases taxonomic ambiguity,
49 with clonal lineages punctuated by large recombination events between distant relatives (14, 19).
50 HGT results in the formation of ill-defined species with ambiguous species boundaries (20). To
51 help resolve this ambiguity, recent studies of the commensal *Neisseria* species employed core
52 genome MLST (cgMLST) and proposed genus specific cut-offs for defining species using genome
53 relatedness indices (8, 19, 20). However, these studies did not comprehensively address other
54 polyphyletic genera within the *Neisseriaceae*.

55 The *Kingella* genus includes five taxa: *K. potus, K. oralis, K. denitrificans, K. negevensis,*
56 and *K. kingae* (21–27). Recently, two novel species have been proposed, namely *K. bonacorsii*
57 *and K. pumchi* (28, 29). All of the *Kingella* species are typically associated with the oral or
58 oropharyngeal microbiome, a common niche for other *Neisseriaceae*. Isolates of *K. kingae*, *K.*
59 *negevensis*, and *K. potus* have all been associated with invasive disease in immunocompetent
60 individuals (23, 25, 30). In contrast, *K. oralis* and *K. denitrificans* are present in dental plaque and
61 in gingival and periodontal samples and only rarely cause invasive disease, typically in
62 immunocompromised individuals (26, 31, 32). *Kingella* species are oxidase positive, catalase
63 negative, rods and typically form short chains (33).

64 A review of published phylogenetic relationships among the commensal *Neisseria* species
65 reveals a high level of variability in the proposed taxonomic relationships, primarily owing to the
66 genes selected and the method used to perform the analysis (15, 34–36). In many of these studies
67 the *Kingella* genus is polyphyletic, and the genera that interrupt a monophyletic clade with

68 *Kingella* species differ in different published analyses (for examples see 24, 33, 34, 37, 38). Most
69 isolates used for defining taxonomic nomenclature in this genus were collected prior to pervasive
70 whole genome sequencing and were assigned to *Kingella* as a result of phenotypic and/or
71 minimal sequence-based analyses. Additionally, as the frequency of culture-dependent and
72 culture-independent microbiome studies have become more prevalent, the number and
73 diversity of formally accepted species in the *Neisseriaceae* have increased (13). Here, we seek to
74 reassess the relatedness of the five species in the *Kingella* genus using whole genomes and a
75 phylogenomic approach.
76
77 **Results**
78 ***Kingella potus* is distinct from the other species in the *Kingella* genus**
79 To examine the *Kingella* species, 16S rRNA gene sequences for isolates from the
80 *Neisseriaceae* were collected for phylogenetic reconstruction (Fig. 1). By 16S rRNA relatedness
81 alone, it is clear that a major driver of the polyphyletic structure of *Kingella* is *K. potus.* This
82 species was recovered from an infected wound caused by an animal bite and is more closely
83 related to *Neisseria bacilliformis* than it is to any currently described *Kingella* species (25).
84 Phenotypically, *K. potus* and *N. bacilliformis* are characterized for a limited number of
85 characteristics, though both species are non-hemolytic, indole-negative coccobacilli that produce
86 acid from glucose, maltose, and fructose (Table S3).
87 To better understand the relationship between *K. potus* and *N. bacilliformis*, we
88 calculated average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) between
89 the type strains of these species. The ANI between *K. potus* and *N. bacilliformis* was calculated at

90 86.75%, below the established species cut-off of 95% (39). However, the ANI between *K. potus*
91 and *N. bacilliformis* is appreciably greater than the ANI calculated between *K. potus* and other
92 *Kingella* species, which ranges between <75% (*K. kingae*) and 78.8% (*K. denitrificans*) (Table 1,
93 Fig. 2). The calculated dDDH between these species falls below species thresholds at 30% (95%
94 confidence interval 27.6-32.5%) (Table 2).
95
96 **The *Kingella* genus is paraphyletic**
97 To further define the relationships among *Kingella* and closely related species, whole
98 genome sequences for select species in the *Neisseriaceae* were downloaded from NCBI. As 16S
99 rRNA gene sequences have been historically used to define species in this group, all following
100 analyses were limited to species most closely related to *Kingella* by 16S rRNA gene phylogeny
101 (Fig. 1). This group includes *Neisseria elongata, Neisseria sheyganii, N. bacilliformis, Eikenella*
102 *corrodens, Alysiella filiformis, Alysiella crassa, Simonsiella muelleri, Conchiformibius steedae, K.*
103 *oralis, K. bonacorsii, K. potus, K. denitrificans, K. kingae,* and *K. negevensis. N. elongata*, *N.*
104 *sheyganii,* and *E. corrodens* were included as outgroups for these analyses. In total, 134 whole
105 genome sequences were downloaded from NCBI and annotated with Prokka.
106 To minimize the impact of HGT and variability in the pangenome, Roary was used to
107 identify the core genome (40). Core genes were clustered at the 75%, 80%, 85%, 90%, and 95%
108 sequence similarity level and were limited to only those clusters of orthologous groups (COGs)
109 found in all genomes. Core gene alignments were generated and used to reconstruct maximum-
110 likelihood phylogenies (Fig. 3). Both the Robinson-Foulds distance and normalized Nye Similarity
111 metrics were used to quantify tree similarity. Each phylogeny was highly congruent with the

112 others based on these metrics, and each included a polyphyletic clade for the genus *Kingella* (Fig.
113 3B and C). We therefore proceeded with the lowest similarity cutoff of 75% for further analyses
114 (Fig. 3A). In each of these analyses, *Conchiformibius* spp., *Alysiella* spp., *Simonsiella* spp, and
115 *Kingella* spp. (with the exception of *K. potus*) form a closely related group that we will refer to as
116 the CASK clade. Phenotypic data on the species in the CASK clade are extremely limited, limiting
117 the utility of phenotypic properties in understanding relationships within the group (Table S3).

118 Based on a whole genome approach, *Alysiella* and *Simonsiella* form a clade within the
119 *Kingella* genus (Fig. 3A), dividing *K. oralis* and *K. denitrificans* from *K. negevensis* and *K. kingae.*
120 ANI, dDDH, and G+C% were calculated for type strains of *Kingella*, *Alysiella*, and *Simonsiella* and
121 are shown in Tables 1 and 3. All of these overall genome relatedness indices (OGRI) support a
122 close relationship between the species of these three genera.

123 Additionally, we calculated pairwise ANI scores for each of the genomes listed above. The
124 ANI scores confirm the majority of the proposed species classifications except for *K. bonacorsii*,
125 an unclassified *Kingella* isolate that was recently proposed to be a new species (28). This isolate
126 was recovered from a gingival sample, and the authors report a 16S rRNA similarity of 98.7%, ANI
127 of 95.83%, and dDDH of 63.6% relative to *K. oralis* UB-38, as well as non-identification by MALDI-
128 TOF mass-spectrometry (28). We replicated each of the genomic calculations reported as well as
129 additional dDDH calculations using alternative formulae and found that choice of formula is
130 essential, as only *d4* yielded a dDDH below the accepted values for species cutoffs (Tables 1 and
131 4, Fig. 2) (39, 41).

132 ***Kingella* species are unified by gene presence/absence in the pangenome**

133 Finally, another recent evolutionary study of these taxa has used less stringent similarity
134 and gene presence cutoffs to define the core genome for species in the *Neisseriaceae* for
135 subsequent phylogenetic analyses (34). We replicated this analysis by reanalyzing the data using
136 a sequence similarity cut off of 50% for genes present in 80% of analyzed genomes. By lowering
137 the threshold for a gene to be considered part of the core genome, we increase the number of
138 genes considered from 109 at the 75% similarity level up to 620 genes. Notably, as we increased
139 the number of genes considered part of the core genome by decreasing the stringency of our
140 Roary analysis, the presence of a polyphyletic *Kingella* clade remains constant (Fig. 4A),
141 demonstrating the robust relationships between these genera that are obtained when we
142 consider the core genome of species in the *Neisseriaceae*.

143 To better understand how the pangenome impacts the relationships described in the
144 above analyses, we reconstructed the phylogeny considering only gene presence and absence in
145 the pangenome. This analysis was performed with a binary COG presence/absence matrix as
146 defined by Roary with a 75% similarity cut-off with phylogenetic inference in IQ-Tree. For the first
147 time in any of our analyses, all named *Kingella* species except for *K. potus* are monophyletic,
148 excluding the *Simonsiella* and *Alysiella* genomes. To identify genes that drive the unification of
149 *Kingella sensu stricto* (*Kingella s.s.*), IQ-Tree was used to perform ancestral reconstructions of the
150 species in this tree. These ancestral reconstructions facilitate the identification of the genes that
151 drive the unification of *Kingella* into a monophyletic clade. Discounting statistically ambiguous
152 sites, monophyly is driven by the loss of eight genes in *Kingella s.s.* (Table 4), all of which fall in
153 the accessory genome. There are no gene gains that support this clade, and therefore this
154 monophyly is not well supported.

155 These data further suggest that horizonal gene transfer (HGT) can confound our ability to
156 define species relationships and that limits on HGT may be important to consider when defining
157 species in the *Neisseriaceae.* HGT is driven by DNA uptake sequences (DUS) in the *Neisseriaceae*
158 (42–44), which are largely thought to be species specific. The DUS in the CASK clade have been
159 previously characterized (42). Type genomes for each species were used to define DUS
160 preference by word search. *Alysiella filliformis*, *Alysiella crassa*, *Simonsiella muelleri*, and *Kingella*
161 *oralis* genomes are enriched for the AG-SimDUS (AGGCTGCCTGAA) and the AG-KingDUS
162 (AGGCAGCCTGAA). *Kingella kingae, Kingella negevensis,* and *Kingella denitrificans* genomes are
163 enriched for the AA-King3DUS (AAGCAGCCTGCA). *Conchiformibius steedae* and *Conchiformibius*
164 *kuhniae* do not appear to use any of these previously defined DUS.
165
166 **The CASK species are unique based on cellular morphology and lifestyle.**
167 To identify the genes that differentiate the species in the CASK clade and to define clusters of
168 orthologous groups (COGs) unique to groups of interest, Scoary was used to analyze the pangenome
169 analysis generated by Roary at the 75% sequence similarity level. The presence of genes is defined as
170 diagnostic for the in-group if they are 100% specific, are 100% sensitive, and have a $p > 0.001$. These
171 cutoffs reduce the impact of shared mobile genetic elements (MGEs) such as prophages or genomic
172 islands.
173 First, the CASK clade was compared against the rest of the species included in the phylogenetic
174 analyses (Fig. 5A, table S5). This clade is enriched for 1360 COGs that are absent in the rest of the analyzed
175 species, providing strong evidence supporting the evolutionary relatedness of this group. The average
176 genome size for the species in this group is approximately 2,000 coding sequences (CDS). To understand
177 putative roles of these COGs in the CASK species, COG groups and Gene Ontology (GO) functional

178 annotations were defined with eggnog mapper (Fig. 5B). Approximately half of the COGs were assigned
179 to the "function unknown" group (n = 656). Of the remaining COGs, the majority play putative roles in cell
180 wall, membrane, and envelope biogenesis (n = 276); DNA replication, recombination, and repair (n = 224);
181 or amino acid transport and metabolism (n = 205).

182 Next, *Kingella sensu stricto (Kingella s.s.)*, comprised of *K. oralis, K. denitrificans, K. negevensis,*
183 and *K. kingae*, was compared to the rest of the CASK clade. Only 67 COGs meet the criteria to be diagnostic
184 for *Kingella s.s* (Table S6). When *Alysiella* and *Simonsiella* or the pathogenic *Kingella* spp. were compared
185 to the rest of CASK clade, we identified only 41 or 79 diagnostic COGs, respectively (Fig. 5A). Again, we
186 assigned putative functional roles for the COGs in each of these groups (Fig. 5C). The COGs diagnostic for
187 *Kingella s.s*. and the pathogenic *Kingella* spp (*K. negevensis* and *K. kingae*) are primarily involved in
188 carbohydrate transport and metabolism or nucleotide transport and metabolism. By contrast, *Alysiella*
189 and *Simonsiella* diagnostic COGs are involved in DNA replication, recombination, and repair or cell wall,
190 membrane, and envelope biogenesis (Fig. 5C).

191 Finally, we compared the genomes of the type-strain for each species in the CASK clade using
192 BLASTn against *K. kingae* ATCC 23330 (Fig. 5D). We found high levels of nucleotide sequence similarity
193 across the *K. kingae* genome relative to the related species. There are few notable regions of dissimilarity,
194 which appear to be MGEs in the *K. kingae* genome.

195

196 **Discussion**

197 Classification of the species in the *Neisseriaceae* has been difficult using traditional metrics of
198 relatedness, both phenotypically and phylogenetically. As a result, the family has been restructured
199 several times since its inception to include or exclude genera (15, 24, 36, 45–47). Species in this family
200 resist robust phylogenetic classification due to pervasive horizontal gene transfer and recombination,
201 including in the 16S rRNA sequence (18–20, 42). Due to the large diversity of species and variability,

202 phenotypic classification using strict phenotypic classification is either difficult or impossible without
203 additional sampling. For example, presence of catalase is conserved among *Neisseria* isolates, and yet
204 isolates of *N. elongata* have been recovered that lack the catalase enzyme(33). Furthermore, as more
205 species are proposed using culture-independent methods or based on a single isolate, it will be essential
206 to establish phylogenomic species definitions for the *Neisseriaceae* (1, 18, 20).

207 In this study, we employed a phylogenetic approach to define the evolutionary relationships
208 within a major subgroup of the *Neisseriaceae*, defined by the genera *Eikenella, Conchiformibius, Alysiella,*
209 *Simonsiella, Kingella*, and select *Neisseria* species. Examination by 16S rRNA sequence unveiled a very
210 recent common ancestor between *K. potus* and *N. bacilliformis*, which was further supported by several
211 other genome relatedness indices (Fig. 1, Tables 1,3). *K. potus* is a catalase-negative species collected from
212 an infected wound that resulted from a bite by a captive South American Kinkajou (*Potus flavus*) (25). *N.*
213 *bacilliformis* is an opportunistic pathogen that has been recovered from human infections, varies in the
214 production of catalase (48), and is phenotypically identical to *N. elongata* subsp. *elongata.* These species
215 were reported nearly simultaneously, preventing their direct comparison. Our reanalysis suggests that *K.*
216 *potus* is much more closely related to *N. bacilliformis* than to any other *Kingella* species, though it is likely
217 a distinct species from *N. bacilliformis*. Inclusion in the genus *Kingella* was likely due to a negative catalase
218 test, underscoring the importance of a genomic approach to classification of these isolates.

219 The phylogenomic analyses also uncovered close evolutionary relationships between
220 *Conchiformibius* sp., *Alysiella* sp., *Simonsiella* sp., and *Kingella* sp., which we have named the
221 "CASK" clade (Fig. 3). The CASK clade is a major branch of the *Neisseriaceae* that includes both
222 invasive pathogens (*K. negevensis* and *K. kingae*) and oral-associated commensals. *Alysiella* and
223 *Simonsiella* are genera associated with the oral microbiome of non-human mammals (15, 47, 49).
224 *Conchiformibius* spp., *Alysiella* spp., and *Simonsiella* spp. are remarkable for their multicellularity,

225 which is the result of incomplete septation by the forming peptidoglycan during bacterial division
226 (34).

227 The reproducibility of the core genome phylogenetic structure very strongly supports the
228 close relationship between the species of the CASK clade and the exclusion of *K. potus* (Fig. 3-4).
229 We hypothesized that prior misclassification could be due to high levels of horizontal gene
230 transfer of accessory genes between *Kingella* (*sensu stricto*) genomes. To test this hypothesis, we
231 generated a presence/absence matrix generated by Roary at 75% sequence similarity level, a
232 technique that emphasizes accessory genes over core genes, and we reconstructed a phylogeny
233 using the RAxML binary model with gamma model of rate heterogeneity and bootstrapped 100
234 times (Fig. 3B). This was the only analysis in which we observed a monophyletic *Kingella* genus,
235 though *Kingella potus* was still excluded. We favor the analyses that are based on nucleotide
236 sequence of the core genome because they contain much more genomic information (~76,500
237 variable sites compared to ~29,000 variable genes) and likely lessen confounding signal from
238 recombined areas. A separate whole gene-based analysis also identified more diagnostic genes
239 uniting *Alysiella*, *Simonsiella,* and the pathogenic *Kingella* species (n=79) than genes uniting the
240 *Kingella* genus *sensu strictu* (n=69). Additionally, as *Simonsiella*, *K. oralis*, and *K. kingae* utilize
241 specific DNA-uptake sequences (DUS), suggesting they may be able to share DNA more easily
242 among themselves as compared to the rest of the *Neisseriaceae*. Given that members of the
243 *Neisseriaceae* are highly recombinogenic and undergo significant HGT of accessory genes, we
244 believe that the core genome is a higher fidelity evolutionary record for this family (20).

245 The CASK clade has a large set of diagnostic genes that differentiate these species from
246 the remainder of the *Neisseriaceae*. Interestingly, this group is enriched for processes that impact

247 cell wall and envelope biogenesis (Fig. 5B), likely driven by the unusual cellular morphologies of
248 *Conchiformibius* spp., *Alysiella* spp., and *Simonsiella* spp. These genera are distinctive for being
249 multicellular, longitudinally-dividing (MuLDi) species that comprise the only known animal
250 multicellular bacterial symbionts (34). Nyongesa and co-workers hypothesize that the MuLDi
251 phenotype evolved twice in the *Neisseriaceae,* first in *Conchiformibius* species and then in the
252 common ancestor of *Alysiella* and *Simonsiella*, a hypothesis that is strongly supported by our
253 phylogenetic analyses. This hypothesis is further supported by consideration of the diagnostic
254 genes that differentiate *Alysiella* and *Simonsiella* from the rest of this group (Fig. 5C). We again
255 find an enrichment for COGs implicated in cellular morphology, as we would expect if these
256 genera evolved the MuLDi phenotype independently of *Conchiformibius* spp. By contrast,
257 *Kingella s.s.* is enriched for COGs associated with nutrient transport and metabolism (Fig. 5C).
258 Our study suggests that the current taxonomic classifications of species in the *Kingella* genus
259 may not reflect evolutionary history. Based on our results, *K. potus* is more related to *N.*
260 *bacilliformis* than to other *Kingella* species by 16S rRNA, OGRI, and whole genome analyses and
261 should probably be assigned to the genus *Neisseria* or another genus entirely. Additionally,
262 phylogenomic analysis of the core genomes of *Alysiella* and *Simonsiella* indicates their close
263 relationship to the *Kingella* genus and supports their reassignment to *Kingella*, resulting in a
264 monophyletic genus.
265
266 **Methods**
267 *Phylogenetic Analysis*

840 of the available 16S rRNA gene sequences for isolates from the *Neisseriaceae* and in the RefNR database were downloaded from the SILVA r138.1 database on September 08, 2022 (50, 51). 16S sequence accession numbers are listed in table S1. 16S rRNA sequences were aligned with MAFFT, and a phylogeny was reconstructed using RAxML v. 8.4.2 with the general time reversible model of nucleotide substitution and gamma model of rate heterogeneity, and bootstrapped 100 times (52, 53).

Whole genome sequences for *Neisseria elongata, Neisseria sheyganii, N. bacilliformis, Eikenella corrodens, Alysiella filiformis, Alysiella crassa, Simonsiella muelleri, Conchiformibius steedae, K. oralis, K. bonacorsii, K. potus, K. denitrificans, K. kingae,* and *K. negevensis* were downloaded from the NCBI Genome database on September 09, 2022. Sequence accession numbers are listed in table S2. In total, 134 genomes were reassembled with Prokka v1.14.6, using default settings for bacterial sequences (54). Phenotypic characteristics for each of these species were collected by literature review and are listed in table S3. Assemblies were analyzed with Roary v3.13.0. to identify the core genome (40). Core genes were clustered at the 75%, 80%, 85%, 90%, and 95% sequence similarity level and were limited to only those clusters of orthologous groups (COGs) found in all genomes (-cd 100), unless otherwise noted. Additionally, other recent evolutionary study of these taxa has used less stringent similarity and gene presence cutoff to define the core genome for species in the *Neisseriaceae* for subsequent phylogenetic analyses (8, 34). We replicated this analysis by reanalyzing the data using a sequence similarity cut off of 50% for genes present in 99% or 80% of analyzed genomes. Nucleotide sequences of core genes were compared in a codon-aware alignment by PRANK within Roary, and core gene

289 alignments were used to reconstruct phylogenies using RAxML as above. Phylogenetic trees were
290 annotated using Figtree v.1.4.4 and ITOL (55). Bootstrap values >70 are shown.

291 To calculate phylogenetic tree similarity, final trees were analyzed in R using the Ape v5.6-
292 2 (56), ggtree v3.1.2. (57), and TreeDist v.2.5.0 (58, 59) packages . The Robinson-Fould distance
293 and normalized Nye Simillarity Score were determined for each pair of trees (58–61).

294 Ancestral reconstructions were performed using the Roary gene presence/absence (PA)
295 matrix generated at the 75% sequence similarity cut-off. The matrix was converted into a FASTA
296 formatted file of binary data using R. This file was used for phylogenetic reconstruction in IQ-Tree
297 v 1.6.12 with automatic model calling (62, 63). Branch support was determined via ultrafast
298 bootstrapping (-bb 1000) (64), and ancestral states (-asr) were calculated with default cut-offs.
299 Full inferred ancestral state matrices are available in Supplementary File 2. To determine the
300 COGs that unite *Kingella s.s.*, the inferred PA matrix of common ancestor of *Alysiella* spp.,
301 *Simonsiella* spp., and *Kingella s.s.* (Node 7), was compared to the inferred PA matrix of common
302 ancestor of *Kingella* spp. Statistically ambiguous sites present in either PA matrix were removed
303 from this analysis.

304 *Gene Set Enrichment*

305 Scoary v 1.6.16 was used to further analyze the gene presence absence matrix generated
306 by Roary at the 75% sequence similarity cutoff (65). The -r flag was used to limit the set of strains
307 considered in this analysis. COGs were eliminated from this analysis if the Bonferroni-corrected
308 p-value >0.0001. Diagnostic genes are only those genes which 1) meet this statistical threshold,
309 2) are enriched in the clade of interest, and 3) have specificity and sensitivity of >80%.

310 To identify pathways that are enriched in each phylogenetic group, the representative
311 COG sequence was collected from the Roary output files and compiled into a gene set. Gene sets
312 were analyzed with the COG database using eggNOG-mapper v 2.1.9 (66, 67), with a DIAMOND
313 alignment (68) and default settings for bacterial species (Table S4-9). Results were plotted using
314 Graphpad Prism.

315 *Genome Relatedness Indices*

316 Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) between the
317 type strains of these species using FastANI v.1.33 and the Type Strain Genome Server (TYGS),
318 respectively (69–71).

319

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516

517 **Tables.**

518 **Supplemental Tables are attached.**

519 **Table 1. Average ANI scores calculated among taxa of interest.** ANI scores were calculated using
520 FastANI and are shown only if the ANI >75%. Empty cells denote data excluded from the table.

	N. bacilliformis	*K. denitrificans*	*K. oralis*	*K. negevensis*	*K. kingae*	*N. elongata*	*A. crassa*	*A. filiformis*	*S. muelleri*
K. potus	86.75	78.88	78.74	<75	<75	80.7	-	-	-
K. bonacorsii	-	79.84	96.41	78.06	77.9	78.76	77.98	77.87	77.14
A. crassa	-	77.81	77.96	79.37	79.74	<75	100	85.43	79.79
A. filiformis	-	77.81	77.96	79.37	79.74	<75	85.43	100	78.75
S. muelleri	-	<75	76.9	79.16	79.96	<75	79.79	78.75	100

521

522

523 **Table 2. dDDH values of *K. potus* calculated against closely related taxa.** dDDH was calculated
524 using TYGS using each of the possible algorithms, as well as the difference in G+C content
525 between *K. potus* and each of the subject type strains.

Query strain	Subject strain	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4 , in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
K. potus NCTC13336	*N. bacilliformis* ATCC BAA-1200	45.6	[42.2 - 49.0]	30	[27.6 - 32.5]	41	[38.0 - 44.0]	1.86
K. potus NCTC13336	*N. elongata* subsp. *glycolytica* ATCC 29315	20.3	[17.1 - 23.9]	22.5	[20.2 - 25.0]	19.7	[17.0 - 22.8]	3.71
K. potus NCTC13336	*K. negevensis* Sch538	12.9	[10.2 - 16.2]	23.5	[21.2 - 25.9]	13.3	[10.9 - 16.0]	12.24

526

527

528 **Table 3. OGRI of *Kingella*, *Alysiella*, and *Simonsiella*.** dDDH was calculated using TYGS using each
529 of the possible algorithms, as well as the difference in G+C content between type strains of
530 *Alysiella* or *Simonsiella* species and each of the closest related subject type strains.

Query strain	Subject strain	dDDH (d0, in %)	C.I. (d0, in %)	dDDH (d4, in %)	C.I. (d4, in %)	dDDH (d6, in %)	C.I. (d6, in %)	G+C content difference (in %)
A. crassa NCTC10283	*A. filiformis* DSM 16848	21.7	[18.5 - 25.3]	34.4	[31.9 - 36.9]	21.9	[19.1 - 25.0]	1.29
A. crassa NCTC10283	*W. bovis* CCUG 44465T	18.4	[15.3 - 22.0]	33.4	[31.0 - 35.9]	18.8	[16.1 - 21.8]	2.67
A. crassa NCTC10283	*S. muelleri* ATCC 29453	16.9	[13.8 - 20.4]	23.2	[20.9 - 25.6]	16.9	[14.3 - 19.8]	3.83
A. crassa NCTC10283	*K. kingae* ATCC 23330	16.3	[13.3 - 19.8]	23.8	[21.5 - 26.3]	16.4	[13.8 - 19.3]	1.45
A. crassa NCTC10283	*K. negevensis* Sch538	15.6	[12.7 - 19.0]	23.3	[21.0 - 25.8]	15.7	[13.2 - 18.6]	0.17
A. crassa NCTC10283	*K. bonacorsii* Marseille-Q4569	14.2	[11.4 - 17.6]	22.7	[20.5 - 25.2]	14.5	[12.0 - 17.3]	8.71
A. crassa NCTC10283	*K. oralis* ATCC 51147	14.2	[11.4 - 17.6]	22	[19.7 - 24.4]	14.5	[12.1 - 17.3]	9.01
A. filiformis DSM 16848	*A. crassa* NCTC10283	21.8	[18.6 - 25.5]	34.1	[31.7 - 36.6]	22	[19.2 - 25.1]	1.25
A. filiformis DSM 16848	*W. bovis* CCUG 44465T	17.2	[14.2 - 20.8]	30.5	[28.1 - 33.0]	17.5	[14.9 - 20.5]	3.95
A. filiformis DSM 16848	*K. kingae* ATCC 23330	16.5	[13.5 - 20.0]	24	[21.7 - 26.5]	16.5	[14.0 - 19.5]	0.17
A. filiformis DSM 16848	*K. negevensis* Sch538	15.1	[12.2 - 18.6]	24.1	[21.8 - 26.6]	15.3	[12.8 - 18.2]	1.11
A. filiformis DSM 16848	*S. muelleri* ATCC 29453	15.1	[12.2 - 18.6]	23.7	[21.4 - 26.1]	15.3	[12.8 - 18.2]	5.12
A. filiformis DSM 16848	*C. steedae* DSM 2580	15.1	[12.2 - 18.5]	22.6	[20.3 - 25.0]	15.3	[12.8 - 18.2]	4.17
A. filiformis DSM 16848	*K. bonacorsii* Marseille-Q4569	14.6	[11.7 - 18.0]	22.8	[20.5 - 25.2]	14.8	[12.3 - 17.6]	7.43
A. filiformis DSM 16848	*K. oralis* ATCC 51147	14.6	[11.8 - 18.0]	22.2	[19.9 - 24.7]	14.8	[12.4 - 17.7]	7.72

531

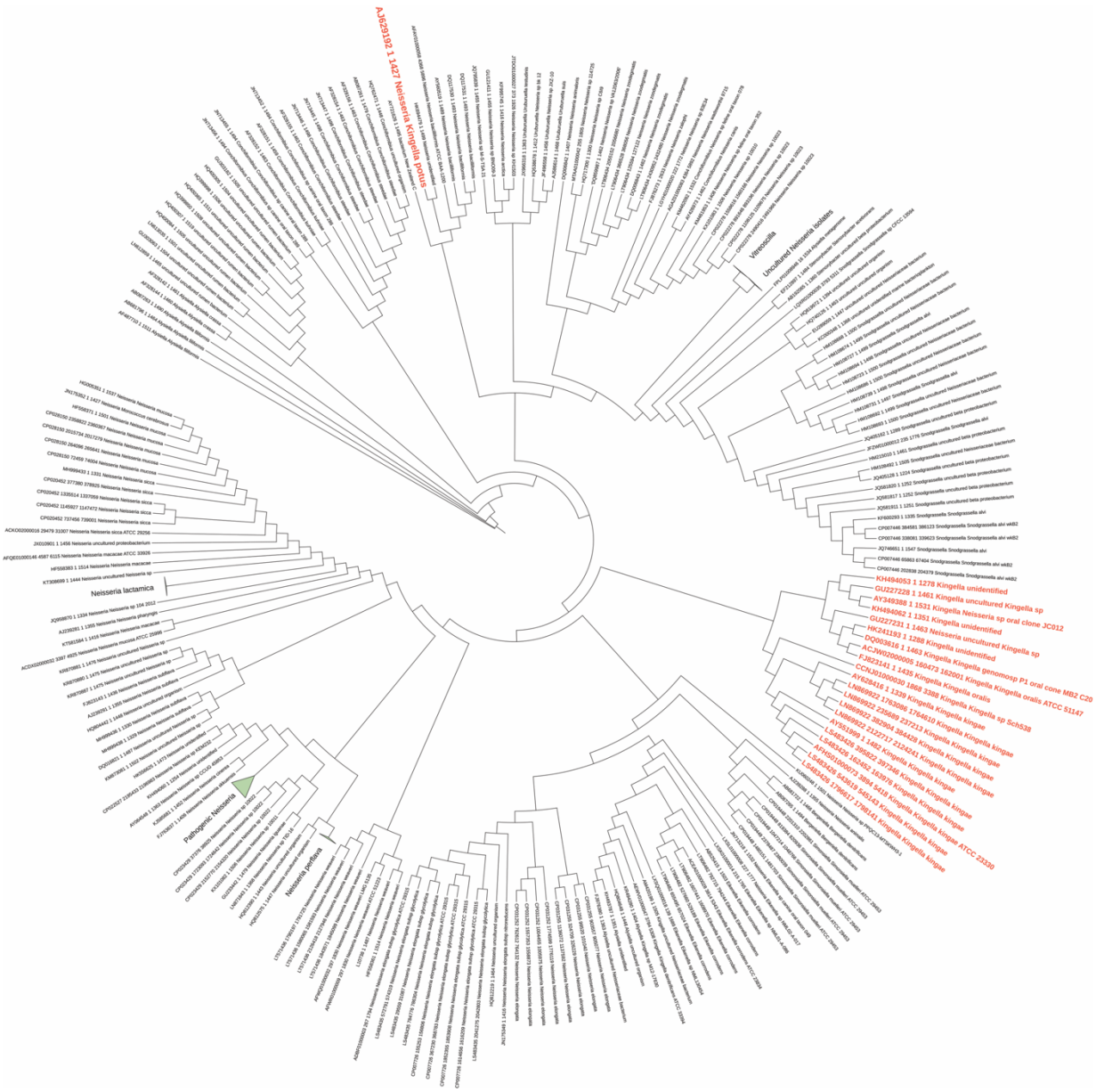
532

533 **Table 4. COGs responsible for the unification of *Kingella s.s.* in the accessory genome.**

	COG	State	p_0[1]	p_1[2]		State	p_0[1]	p_1[2]	COG name[3]	Predicted Function[3]
Last Common Ancestor *Alysiella, Simonsiella,* and *Kingella s.s.*	742	1	0.15414	0.84586	Last Common Ancestor of *Kingella s.s.*	0	0.98197	0.01803	group_2532	Protein IscX
	766	1	0.02993	0.97007		0	0.97924	0.02076	group_2994	hypothetical protein
	771	1	0.02993	0.97007		0	0.97924	0.02076	*purU*	hypothetical protein
	923	1	0.24673	0.75327		0	0.97821	0.02179	group_3650	Sulfur carrier protein TusA
	3159	1	0.06887	0.93113		0	0.96883	0.03117	group_6852	Isocitrate lyase
	3277	1	0.35969	0.64031		0	0.96819	0.03181	*thiG*	Thiazole synthase
	3284	1	0.35969	0.64031		0	0.96819	0.03181	group_5996	Imidazole glycerol phosphate synthase subunit HisH
	3586	1	0.13975	0.86025		0	0.95545	0.04455	group_5655	Isocitrate dehydrogenase [NADP]

534
535 1 – Maximum likelihood posterior probability that this COG was absent at this site in the inferred
536 ancestral reconstruction. Genes were called as absent if p_0 >= 0.95.
537 2 - Maximum likelihood posterior probability that this COG was present at this site in the inferred
538 ancestral reconstruction. Genes were called as present if p_0 >= 0.95.
539 3 – Predicted names and functions of COGs of interest were recovered from the associated Roary
540 matrix. Sequential COGs are not necessarily syntenic in any given genome.
541
542

543 **Figures and Legends.**



544

545 **Figure 1.** High quality 16S rRNA sequences were downloaded from the SILVA database for the

546 family Neisseriaceae (taxid: 408). Sequences were aligned with MAFFT, and a maximum

547 likelihood phylogeny was constructed using RAxML. Species that belong a species in the genus

548 *Kingella* are colored red. By 16S rRNA relatedness, *Kingella* forms a polyphyletic clade with *K.*

549 *potus* falling outside of the rest of the genus. *K. potus* forms a clade with the oral-associated

550 *Neisseria bacilliformis*. Bootstrap values greater than 70% are shown.

551

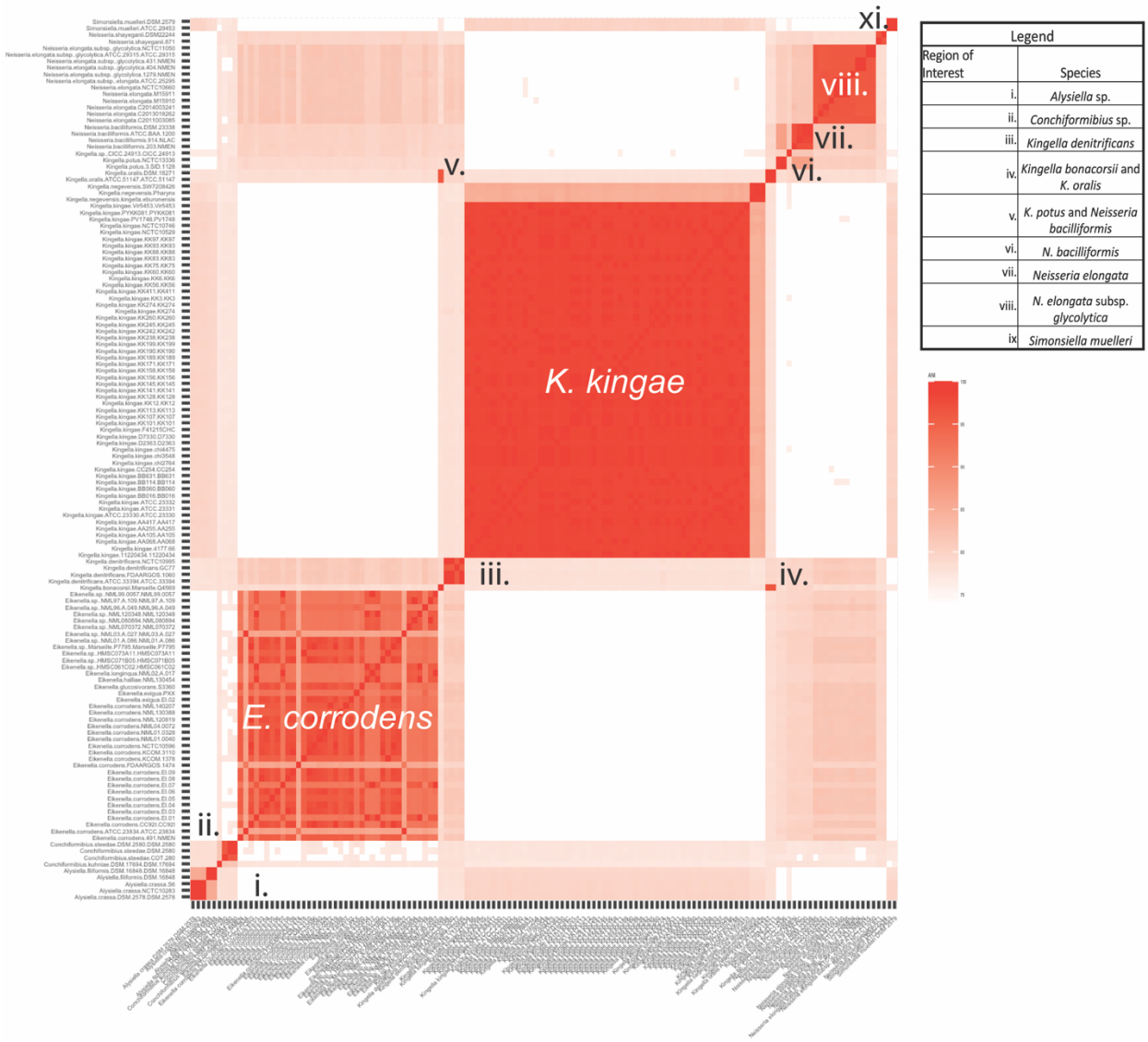
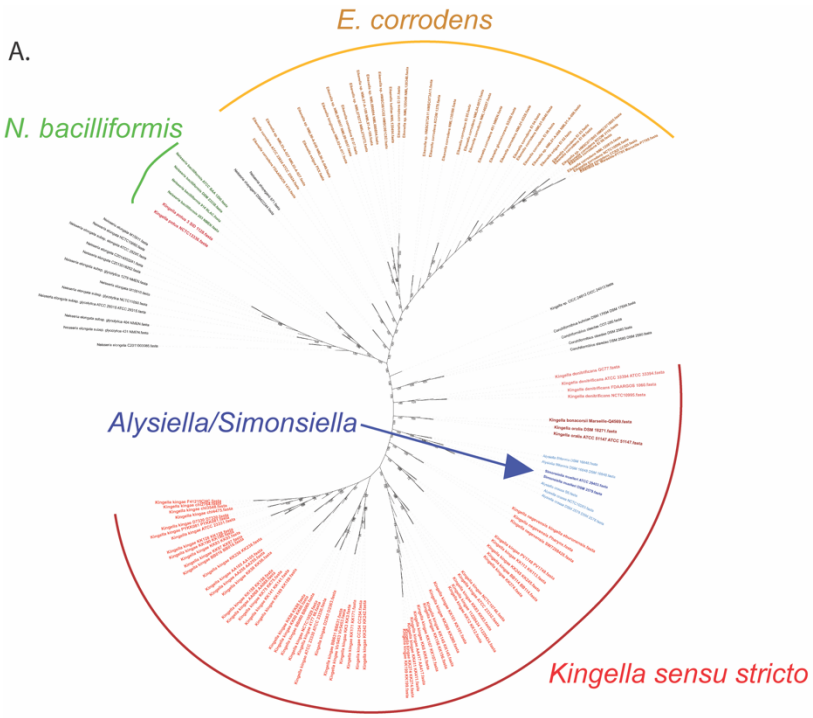


Figure 2. ANI scores comparing sequenced isolates from species in the *Neisseriaceae* most closely related to the genus *Kingella*. ANI scores were calculated between publicly available genomes downloaded from NCBI using FastANI. Several regions of interest are indicated on the heatmap, including the species delimiters, and regions of interest comparing *K. bonacorsii* and *K. oralis* (iv) and *K. potus* and *N. bacilliformis* (v). The resulting ANI matrix was rendered as a heatmap using R.



B.

Robinson-Foulds distance

Roary Cutoff Scores	95	90	85	80	75
95	0	0.133	0.183	0.166	0.270
90	0.133	0	0.128	0.151	0.263
85	0.183	0.128	0	0.174	0.271
80	0.166	0.151	0.174	0	0.232
75	0.270	0.263	0.271	0.232	0

C.

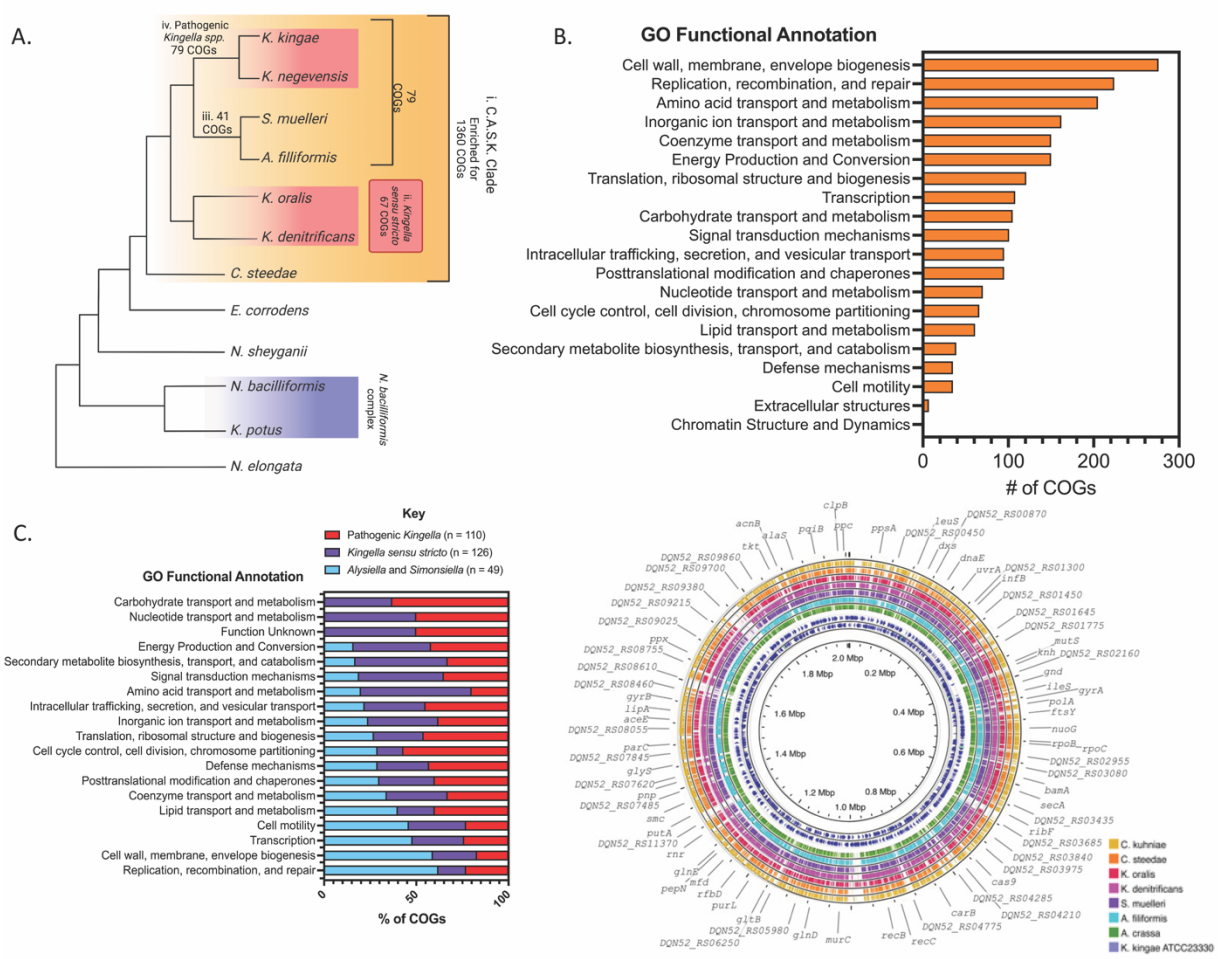
Normalized Nye Similarity Score

Roary Cutoff Scores	95	90	85	80	75
95	1	0.8685	0.8182	0.8271	0.7307
90	0.8685	1	0.8714	0.8282	0.7407
85	0.8182	0.8714	1	0.8216	0.7384
80	0.8271	0.8282	0.8216	1	0.7685
75	0.7307	0.7407	0.7384	0.7685	1

Figure 3. A. The core genome of the strains from the 13 species most closely related to *Kingella kingae* was determined using Roary with a minimum homology cutoff of 75%. The core genomes, which encompass approximately two million sites, were aligned with MAFFT and used to reconstruct a maximum likelihood phylogeny. As above, *K. potus* is only distantly related to the rest of the species in the genus *Kingella.* Additionally, the genera *Alysiella* and *Simonsiella* fall within the *Kingella* clade, suggesting the core genome of the species in each of these genera is very closely related to that of *Kingella.* Bootstraps greater than 70% are shown. **B-C.** The Robinson-Foulds and Normalized Nye similarity score congruency metrics were used to quantify changes in tree architecture between phylogenies reconstructed using core genome alignments. The Nye similarity score was normalized against the number of possible rearrangements given the number of leaves in the tree.

Figure 4. A. The core genome of the strains from the 13 species most closely related to *Kingella kingae* was determined using Roary with a minimum homology cutoff of 50% with COGs present in at least 80% of the species. The core genomes, which encompass approximately two million

577 sites, were aligned with MAFFT and used to reconstruct a maximum likelihood phylogeny. As
578 above, *K. potus* is only distantly related to the rest of the species in the genus *Kingella.*
579 Additionally, the genera *Alysiella* and *Simonsiella* fall within the *Kingella* clade, suggesting the
580 core genome of the species in each of these genera is very closely related to that of *Kingella.*
581 Bootstraps greater than 70% are shown. The phylogeny is rooted by the *Neisseria elongata* clade.
582 **B.** A binary gene presence/absence matrix was generated using Roary at the 75% similarity cut-
583 off, and includes both the core and accessory genes of all species. A phylogenetic tree based on
584 this matrix was constructed using RAxML via the GAMMA model of rate heterogeneity. This
585 analysis, based solely on gene content unites *Kingella sensu stricto* into a monophyletic group.
586 Bootstraps greater than 70% are shown. The phylogeny is rooted by the *Neisseria elongata* clade.
587



588

589 **Figure 5. A**. Scoary was used to generate lists of genes differentially encoded genes comparing

590 (i) CASK species against the *Neisseriaceae*, (ii) *Kingella sensu stricto* (iii) *Alysiella* and *Simonsiella*

591 or (iii) pathogenic *Kingella* against the CASK clade. Finally, *Alysiella* and *Simonsiella* COGs were

592 compared against the pathogenic *Kingella.* Diagnostic COGs are defined as COGs with 100%

593 sensitivity and specificity to the in-group, and filtered to $p < 0.001$. Please see Supplemental

594 tables 5-9 list for full COGs sets. **B-C.** Diagnostic gene sets for each group were annotated using

595 eggNOG mapper, and classified by Gene Ontology functional groups using the NCBI COG

596 database. B. The CASK clade is enriched for unique COGs with putative roles in cell wall,

597 membrane and envelope biogenesis, as well as DNA replication, recombination and repair. COGs

598 with no functional annotations were omitted from this graphic (n = 656 COGs). **C.** Similarly, the
599 majority of the unique COGs present in *Alysiella* and *Simonsiella* may function as part of DNA
600 replication, recombination and repair pathways and cell wall, membrane and envelope
601 biogenesis. *Kingella sensu stricto* and the pathogenic *Kingella* species encode more unique COGs
602 implicated in carbohydrate, amino acid, or nucleotide transport and metabolism. **D.** Type strains
603 of each species in the CASK clade was compared to the *K. kingae* ATCC 23330 genome (inner
604 circle) using BLASTn. Bars in each ring represent regions of sequence similarity between
605 genomes. Each of the CASK species shows significant sequence similarity to nearly all of the *K.*
606 *kingae* reference, further demonstrating how closely related each of these species are. Regions
607 of dissimilarly map to regions that encode putative MGEs, particularly prophages.
608