Comparative genomics and phylogenomics of *Campylobacter* unveil potential novel species and provide insights into niche segregation

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Abstract

Campylobacter is a bacterial genus associated with community outbreaks and gastrointestinal symptoms. Studies on *Campylobacter* generally focus on specific pathogenic species such as *C. coli* and *C. jejuni*. Currently, there are thousands of publicly available *Campylobacter* genomes, allowing a more complete assessment of the genus diversity. In this work, we report a network-based analysis of all available *Campylobacter* genomes to explore the genus structure and diversity, revealing potentially new species and elucidating genus features. We also hypothesize that the previously established clade III of *C. coli* is in fact novel species (referred here as *Campylobacter spp12*). Finally, we found a negative correlation between pangenome fluidity and saturation coefficient, with potential implications to the lifestyles of distinct *Campylobacter* species. Since pangenome analysis depend on the number of available genomes, this correlation could help estimate pangenome metrics of *Campylobacter* species with less sequenced genomes, helping understand their lifestyle and niche adaptation. Together, our results indicate that the *Campylobacter* genus should be re-evaluated, with particular attention to the interplay between genome structure and niche segregation.

Keywords: Network analysis; *Campylobacter* diversity; Pangenome.

INTRODUCTION

Campylobacter is a Gram-negative Epsilonproteobacteria genus of motile bacteria that can infect humans and other animals. *Campylobacter* species are the leading cause of bacterial foodborne infections, especially transmitted via the consumption of undercooked chicken meat, contaminated water, or dairy products [1]. The genus comprises 34 species and 7 subspecies according to LPSN (List of Prokaryotic names with Standing in Nomenclature) [2] (on March 2021).

Among all known *Campylobacter* species, *C. coli* and *C. jejuni* are responsible for over 90% of human campylobacteriosis [1]. The ease of adaptation of these two species to multiple hosts led the World Health Organization to define *Campylobacter* as a pathogen of critical priority for the study and development of new antibiotic treatments [3]. Because of their clinical relevance, *C. coli* and *C. jejuni* have the largest number of publicly available genomes among *Campylobacter* species, creating an unintended bias for genus-wide population structure and ecological studies.

Campylobacter species occupy a wide range of niches [4]. For example, *C. coli* is differentiated into three clades (I, II, and III) [5]. While isolates from clade I are usually associated with acute diarrhea in humans, those belonging to clades II and III are mainly found in environmental sources (e.g., lakes) [5,6]. In contrast, species such as *C. jejuni* present a more clonal population structure with host-specific clonal complexes (e.g., CC-42 and CC-61), which are associated with cattle and sheep [7]. Importantly, these niche preferences can be related to genome sequences, with bacteria with similar lifestyles sharing a greater number of genes.

Next-generation sequencing technologies have been revolutionizing our understanding of bacterial diversity [8,9]. However, a systematic assessment of *Campylobacter* genomes is yet to be conducted. Here, we employ a network approach to explore *Campylobacter* diversity and taxonomic classification.

Results

Dataset collection and type strains

We retrieved 3,319 *Campylobacter* genomes from the RefSeq database [10] in August 2020. After removing genomes with completeness below 90% and duplication levels (a proxy for contamination) greater than 5%, we created a dataset with 3261 *Campylobacter* genomes (Table S1). According to the NCBI classification, 46.24% and 29.9% of the genomes belong to *C. jejuni* and *C. coli*, respectively (Table S1), highlighting an expected overrepresentation of these species. These results also uncovered possible misclassification problems, since overrepresented species might overshadow less studied ones. The LPSN comprises 41 validly named and published *Campylobacter* representative genomes (Table S2), encompassing 34 species and 7 subspecies. We conducted an Average Nucleotide Identity (ANI) analysis with all type strain genomes and confirmed that all pairs of type strains from different species had ANI values lower than 95% (the threshold to define bacterial species [11]). We also identified

three subspecies with ANI lower than 95% with their species' type strains (*C. fetus* subsp. *testudinum*, *C. lari* subsp. *concheus* and *C. pinnipediorum* subsp. *caledonicus*), suggesting that these strains are likely from different species.

Network analysis identifies potentially new *Campylobacter* species

We conducted a network analysis based on Mash distances (see methods for details). In this network, nodes and edges represent genomes and the estimated identities (1 – Mash; minimum threshold of 95%), respectively. Each community in the resulting network is considered a different species, as described elsewhere [9]. Species networks inferred with genomic distance or identity are highly structured, with few overlapping communities [9,12,13]. In our *Campylobacter* network, we found 65 genome communities (Figure 1), which is above the expected number considering the 34 species available in LPSN. Out of those, only 36 contained type strain genomes and were named after their species names, propagating this classification to all the genomes of the community. Two of these 36 communities were represented by genomes from the subspecies *C. lari* subsp. *concheus* and *C. fetus* subsp. *testudinum*.

We named the 29 communities without a type genome as *Campylobacter "sppX",* where X represents an arbitrary community number (see methods). We found three large unnamed communities: *Campylobacter spp5* (134 genomes), corresponding to the *C. concisus* GSII; *Campylobacter spp12* (78 genomes), representing the well-known *C. coli Clade III*; and *Campylobacter spp33* (37 genomes), comprising genomes from isolates obtained exclusively from swine feces [14] that form a sister group with *C. lanienae* (Figure 2). These results support that a significant part of the *Campylobacter* diversity remains largely unexplored.

Besides investigating potentially new species, we also constructed a distance tree to infer the proximity of their respective communities to type species (Figure 2). We observed that *C. coli* is the sister group of *C. jejuni*, as previously described [5]. The *C. coli* population is structured in three main clades [15]. We observed that the *C. coli* clade III is an independent community in the distance network and is paraphyletic in relation to the other *C. coli* genomes (Figure 2). Although *C. coli* clade II is a monophyletic sister group of clade I in the distance tree, the high number of connections in the network with other *C. coli* genomes does not support its separation as an independent community.

We also corroborated the split of *C. concisus* into two distinct genomospecies (GSI and GSII), as previously proposed [16]. Within the 216 *C. concisus* genomes available in RefSeq, 82 belong to *C. concisus* GSI, while 134 genomes formed a distinct community named as *Campylobacter spp5* (*C. concisus* GSII). The two communities also differ in GC content (37.22% in *C. concisus* and 39.19% *in Campylobacter spp5*; Wilcoxon test, p-value < 0.001) and genome sizes (1.9 Mbp in *C. concisus* and 2.0 Mbp in *Campylobacter spp5*; Wilcoxon test, p-value < 0.001), supporting their split into two different species. Accordingly, it has been proposed that the GC content within *Campylobacter* species should not vary by more than 1% [17].

The distance tree also provides insights into novel species and niche segregation. For example, *C. fetus* subsp. *testudinum* is probably a new species, typically found in mammals.

121 Furthermore, we also observed a clear division of *C. lari* into two clades, corresponding to the
122 two subspecies previously proposed based on 16S rRNA: *C. lari* subsp. *lari* and *C. lari* subsp.
123 *concheus* [18]. These results imply that the *C. lari* phylogeny also warrants revisions.
124
125 **Campylobacter phylogeny of representative genomes and species reclassification**
126 We used all core single-copy genes from 65 representative genomes (Table 2) to reconstruct
127 the *Campylobacter* phylogenetic tree. This phylogenetic reconstruction uncovered a
128 remarkable difference in GC content across clades. (Figure 3). For example, the clade
129 comprising *C. showae* and *C. rectus* have GC content greater than 40%, far above the 30.40%
130 found in the *C. jejuni* clade.
131 We also used the representative genomes to estimate a misclassification rate of 7.57%
132 in *Campylobacter spp* (247 misclassified genomes; Table S3). We considered as misclassified
133 only those genomes that were re-assigned either to a different validly published species or to
134 a new community (*Campylobacter sppX*). Therefore, genomes identified as "*Campylobacter*
135 *sp.*" were not considered misclassified if assigned to a community.
136 We found extreme misclassification levels in eight known species (Table 1),
137 particularly *C. showae* (81.82%, n=11). Species such as *C. concisus* and *C. coli* showed
138 significant reclassification levels due to their known heterogeneity (61.86, n=215 and 7.96%,
139 n=980, respectively). Further, genomes submitted to the RefSeq database as *C. aviculae*, *C.*
140 *estrildidarum*, *C. taeniopygiae,* and *C. troglodytis* were marked as reclassified because of the
141 absence of these species in LPSN at the time of the analysis. Finally, 92 genomes previously
142 identified as *Campylobacter sp.* (54.76%). were assigned to communities composed by known
143 species, particularly *C. jejuni*.
144 We also compared the results obtained by the genome analysis with the
145 *Campylobacter* classification provided by GTDB. GTDB has a standardized framework to
146 classify genomes in higher taxonomic ranks (e.g., genus) [19] by following a phylogenomic
147 approach based on a set of conserved single-copy proteins. This divides *Campylobacter* in five
148 different genera (Table S1): *Campylobacter*, *Campylobacter A*, *Campylobacter B*,
149 *Campylobacter D*, and *Campylobacter E*. Importantly, this distribution is coherent with the
150 phylogenetic reconstruction and community analysis described here. The largest genus,
151 *Campylobacter E*, comprised the *C. coli* and *C. jejuni* communities, whereas the second largest
152 genus, *Campylobacter A*, harbored the monophyletic group of *C. concisus*, *Campylobacter*
153 *spp5*, *C. curvus* and *C. pinnipediorum*.
154
155 **Pangenome analysis**
156 The pangenome analysis of a bacterial species provides insights into its lifestyle [21]. A
157 pangenome is the total set of non-redundant genes in a species. It comprises the core
158 genome, the set genes present in all isolates; the accessory genome, with genes present in
159 more than one but not in all isolates; and the isolate-exclusive genes [22]. We computed the
160 genomic fluidity and pangenome openness to investigate the overall architecture of the
161 *Campylobacter* pangenome (Table 3, Figure 4). The genomic fluidity measures how dissimilar

genomes are at the gene level [23], while pangenome openness reflects the propensity to detect new genes, as new genomes are included in the analysis. Since sample size can bias pangenome estimation, we analyzed only those communities with more than 20 genomes.

Previous analysis on the connection between pangenome openness and genomic fluidity have shown that these two metrics are not necessarily correlated, with both pangenome fluidity and saturation being related to its openness [24]. We found that genomic fluidity of *Campylobacter* is negatively correlated with saturation coefficient (α) (ρ = -0.8733; p-value = 0.0004), with higher values of α representing a closer pangenome. We found a significant regression equation ($F_{1;9}$ = 28.927; p-value < 0.0001), with an R^2 of 0.73 (Figure 4b) and predicted genomic fluidity of $0.8187 - 0.8467\alpha$, meaning that the genomic fluidity decreased 0.8467 units for each unit of the parameter α. This result provides a model for estimating the genomic fluidity of species without enough genomes to be included in the pangenome analysis.

We observed that *C. hepaticus* is genetically conserved and homogeneous. This species has the lowest diversity and the highest saturation coefficient (closed pangenome) (Figure 4a) across *Campylobacter* communities, supporting a low genomic diversity and reduced HGT in this species. However, it is important to mention that many *C. hepaticus* genomes are from the same bioproject, which might result in unrealistic diversity estimates for this species. On the other hand, species such as *C. jejuni* and *C. coli* have a high genomic fluidity, which is in line with the generalist ecological character of both species, with isolates from various host sources.

DISCUSSION

The *Campylobacter* genus is often associated with gastrointestinal diseases and antibiotic resistance, having a strong study bias towards clinically relevant species such as *C. jejuni* and *C. coli*. Here, we analyzed 3,261 *Campylobacter* genomes from several species and sources. We identified 65 different communities within *Campylobacter*, way over than the 34 species originally listed on the LPSN. We found a portion of these novel communities (here considered as potential new species) to be closely related to some *Campylobacter* groups such as the *C. lari* and *C. fetus* groups. Our ANI-based network analysis also showed that 7.57% of the *Campylobacter* genomes warrants phylogenetic reclassification.

C. concisus is an oral bacterium that consists of two genomospecies (GSI and GSII) that are undistinguishable at the phenotypic level [16]. Our results support this division, as we identified two *C. concisus* communities, the first containing the species' type strain with 82 genomes, and *Campylobacter spp5* with 134 genomes. Similarly, we also found support to separate *C. coli* in two communities: one comprising the original type strain and other 902 genomes, and a second community with 78 genomes (referred as *Campylobacter spp12*).

We also reported strong evidence for potentially novel genomospecies. For example, *Campylobacter spp33* comprises 37 genomes of isolates obtained only from swine feces [14], supporting a specific host niche occupation in *C. fetus*-related species [26]. The *C. fetus* clade belongs to a separate genus in GTDB (Table S1) [20]. We can further divide this clade using

203 characteristics like the low capacity to cause diseases in humans (*Campylobacter lanienae*-
204 related species) and selenium metabolism deficiency (*Campylobacter spp33* clade [14]). The
205 identification of *Campylobacter spp33* uncovered a novel species in the *C. fetus* group, which
206 should be further investigated for a more thorough and formal description.
207 We found that genomic fluidity and pangenome saturation coefficient are negatively
208 correlated in the *Campylobacter* genus. Although these two parameters are usually
209 conceptually associated, previous findings have shown that there was not a direct correlation
210 between their values, but that fluidity was just one of the determinants of genome openness
211 [24]. *C. hepaticus* showed a close and homogeneous pangenome, although one must consider
212 that a high fraction of the *C. hepaticus* genomes were from the same study, which could result
213 in a potential sample bias that resulted in the underestimation of the *C. hepaticus* diversity.
214 Finally, we it is worth mentioning that more in depth investigations of the large unnamed
215 communities described here should be conducted to validate them as novel species.
216
217
218
219 **METHODS**
220 **Dataset collection**
221 We downloaded 3,319 *Campylobacter* genomes available on RefSeq using Datasets v4.2.0
222 (https://www.ncbi.nlm.nih.gov/datasets/) in August 2020. We used BUSCO v4.1.0 [27] and
223 removed the genomes with completeness lower than 90%, and duplication greater than 5%.
224 Genomic metadata were gathered from RefSeq using the Reutils package v. 0.2.3
225 (http://mirrors.ucr.ac.cr/CRAN/web/packages/R.utils/R.utils.pdf).
226 All the genomes were analyzed using Mash v1.1 [12]. We compared different Mash
227 sketch values to find the parameter with the lowest error rate [9]. We used the mean distances
228 generated by sketch sizes of 1000, 2000, and 5000 bp, removing genome distances greater
229 than 0.8. We used the reciprocal of Mash distance to estimate the identity (1 – Mash). For
230 type strain analyses, we computed ANI using pyani v0.2.10 [28].
231 The network analyses of the distances between the genomes were conducted using
232 igraph v1.2.7 [29]. We generated a weighted graph with nodes and edges representing genomes
233 and genetic identities between them, respectively. Only edges representing identities greater
234 than 95% were retained.
235
236 **Type strains validation**
237 The pre-set *Campylobacter* type strains were retrieved from LPSN [2] in March 2021. The valid
238 accession numbers of each species and subspecies were manually gathered, and we used the
239 information in LPSN to identify validly reported and documented type genomes (34
240 representing species, 6 representing subspecies) (Table S2). Next, we conducted an ANI
241 analysis with Pyani v0.2.10 [28] to verify that all the genome pairs are at least 95% identical to
242 each other.
243

Network analysis

We employed the label propagation algorithm to detect communities in the genome distance network, as previously reported [9]. Communities with type genomes were named after them, while communities without a type genome were arbitrarily identified as *Campylobacter sppX*, where X represents the number of the community. These communities also received a random representative genome. The named communities were contrasted with RefSeq to estimate the level of misclassification.

Phylogeny and pangenome characterization

After selecting a representative genome from each *Campylobacter* community, we used Orthofinder v.2.5.2 [30] to infer orthologs. Single-copy genes were used to reconstruct the phylogenetic tree using iQtree v.1.6.12 [31] with the TEST argument to find the best fitting model. One thousand bootstrap replicates were generated to assess the significance of internal nodes. *Helicobacter pylori* strain ATCC 43504 (Accession number: GCF_900478295.1) were used as outgroup. We used ggtree [32] to visualize the reconstructed tree. GTDB data (July 2022) were used to analyze different phylogenetic groups within *Campylobacter* [20].

We conducted the pangenome analysis for those communities with more than 20 genomes with Roary v3.13.0 [33]. The Micropan R package v2.1 [34] and the Pagoo package v0.3.9 [35] were used to assess pangenome fluidity values and openness values, respectively.

Declaration of Competing Interest

The authors declare no conflict of interest.

Author contributions

Conceived the study: HP-A and TMV. Data analysis: HP-A, SH-J. Funding, project coordination and infrastructure: TMV. Manuscript writing: HP-A, SH-J, TMV.

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Tables

Table 1: Rate of misclassified genomes per species in the RefSeq database. Genomes originally assigned as belonging to a species and appeared an unexpected community (different from that of their respective type strain genome) were considered misclassified.

Original classification	Reclassification rate (%)
C. coli	7.96 (78/980)
C. concisus	61.86 (133/215)
C. fetus	27.27 (10/35)
C. hepaticus	3.85 (1/26)
C. jejuni	0.39 (6/1551)
C. lanienae	6.45 (2/31)
C. lari	50.00 (9/18)
C. showae	81.82 (9/11)

Table 2: Representative genomes of each community detected in this study. The communities were named after their type strains. Communities without type strains were arbitrarily named with a sequential suffix and received a random representative genome.

Community name	Refseq accession	Community name	Refseq accession
C. armoricus	GCF_013372105.1	*Campylobacter spp57*	GCF_012978795.1
C. avium	GCF_002238335.1	*Campylobacter spp58*	GCF_012978835.1
C. blaseri	GCF_013201895.1	*Campylobacter spp62*	GCF_013416015.1
C. canadensis	GCF_013177655.1	*Campylobacter spp63*	GCF_900573945.1
C. coli	GCF_003590975.1	*Campylobacter spp64*	GCF_900573985.1
C. concisus	GCF_001298465.1	*Campylobacter spp65*	GCF_902795495.1
C. corcagiensis	GCF_013201645.1	*C. sputorum*	GCF_008802035.1
C. cuniculorum	GCF_002104335.1	*C. subantarcticus*	GCF_000816305.1
C. curvus	GCF_013372125.1	*C. upsaliensis*	GCF_900637395.1
C. fetus	GCF_900475935.1	*C. ureolyticus*	GCF_013372225.1
C. fetus testudinum	GCF_002973655.1	*C. volucris*	GCF_008801985.1
C. geochelonis	GCF_013201685.1		
C. gracilis	GCF_001190745.1		
C. helveticus	GCF_002080395.1		
C. hepaticus	GCF_001687475.2		
C. hominis	GCF_900446395.1		
C. hyointestinalis	GCF_013372145.1		
C. iguaniorum	GCF_000736415.1		
C. insulaenigrae	GCF_900637885.1		
C. jejuni	GCF_000254515.1		
C. lanienae	GCF_002139935.1		
C. lari	GCF_013372185.1		
C. lari concheus	GCF_008245025.1		
C. mucosalis	GCF_013372205.1		
C. novaezeelandiae	GCF_004323845.1		
C. ornithocola	GCF_013201605.1		
C. peloridis	GCF_000816785.1		
C. pinnipediorum	GCF_002021925.1		
C. portucalensis	GCF_009690845.1		
C. rectus	GCF_004803795.1		
C. showae	GCF_004803815.1		
Campylobacter spp11	GCF_000313615.1		
Campylobacter spp12	GCF_001489835.1		
Campylobacter spp13	GCF_000344295.2		
Campylobacter spp23	GCF_000816245.1		
Campylobacter spp33	GCF_900446565.1		
Campylobacter spp34	GCF_002139105.1		
Campylobacter spp35	GCF_002139895.1		
Campylobacter spp36	GCF_002179165.1		
Campylobacter spp38	GCF_002738235.1		
Campylobacter spp41	GCF_004357905.1		
Campylobacter spp42	GCF_004378855.1		
Campylobacter spp44	GCF_012978635.1		
Campylobacter spp45	GCF_004803855.1		
Campylobacter spp46	GCF_005406205.1		
Campylobacter spp47	GCF_005406215.1		
Campylobacter spp48	GCF_005406225.1		
Campylobacter spp49	GCF_010092865.1		
Campylobacter spp5	GCF_902460635.1		
Campylobacter spp51	GCF_006864365.1		
Campylobacter spp52	GCF_006864425.1		
Campylobacter spp53	GCF_006864455.1		
Campylobacter spp54	GCF_008633875.1		
Campylobacter spp55	GCF_009362725.1		

Table 3: Pangenome features for each community with 20 or more genomes.

Community name	Fluidity	Openness (α value)	Gene families	Core genes
C. coli	0.209	0.724	12,477	619
C. concisus	0.216	0.770	6682	671
C. fetus	0.125	0.799	4079	1097
C. fetus testudinum	0.080	0.936	2730	1239
C. hepaticus	0.031	1.021	1753	1321
C. hyointestinalis	0.236	1.091	4255	1003
C. jejuni	0.255	0.629	18,350	469
C. lanienae	0.116	0.943	2766	1146
Campylobacter spp12	0.074	0.717	3113	1126
Campylobacter spp33	0.140	0.879	3345	1136
Campylobacter spp5	0.230	0.706	9431	869

Figures

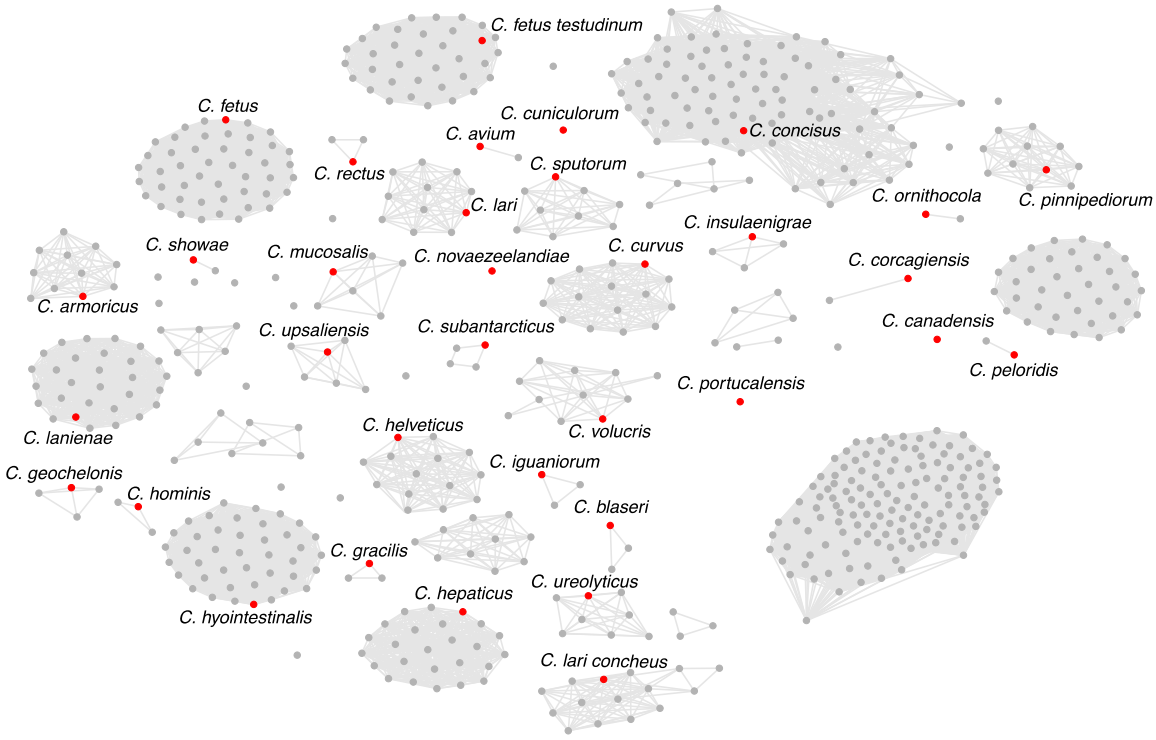


Figure 1. *Campylobacter* network structure. Nodes represent genomes and edges connect those with at least 95% identity. Type strains are highlighted in red. Communities without type strains represent potentially novel species. *C. jejuni* and *C. coli* communities have 1638 and 903 genomes, respectively, and were omitted to improve clarity.

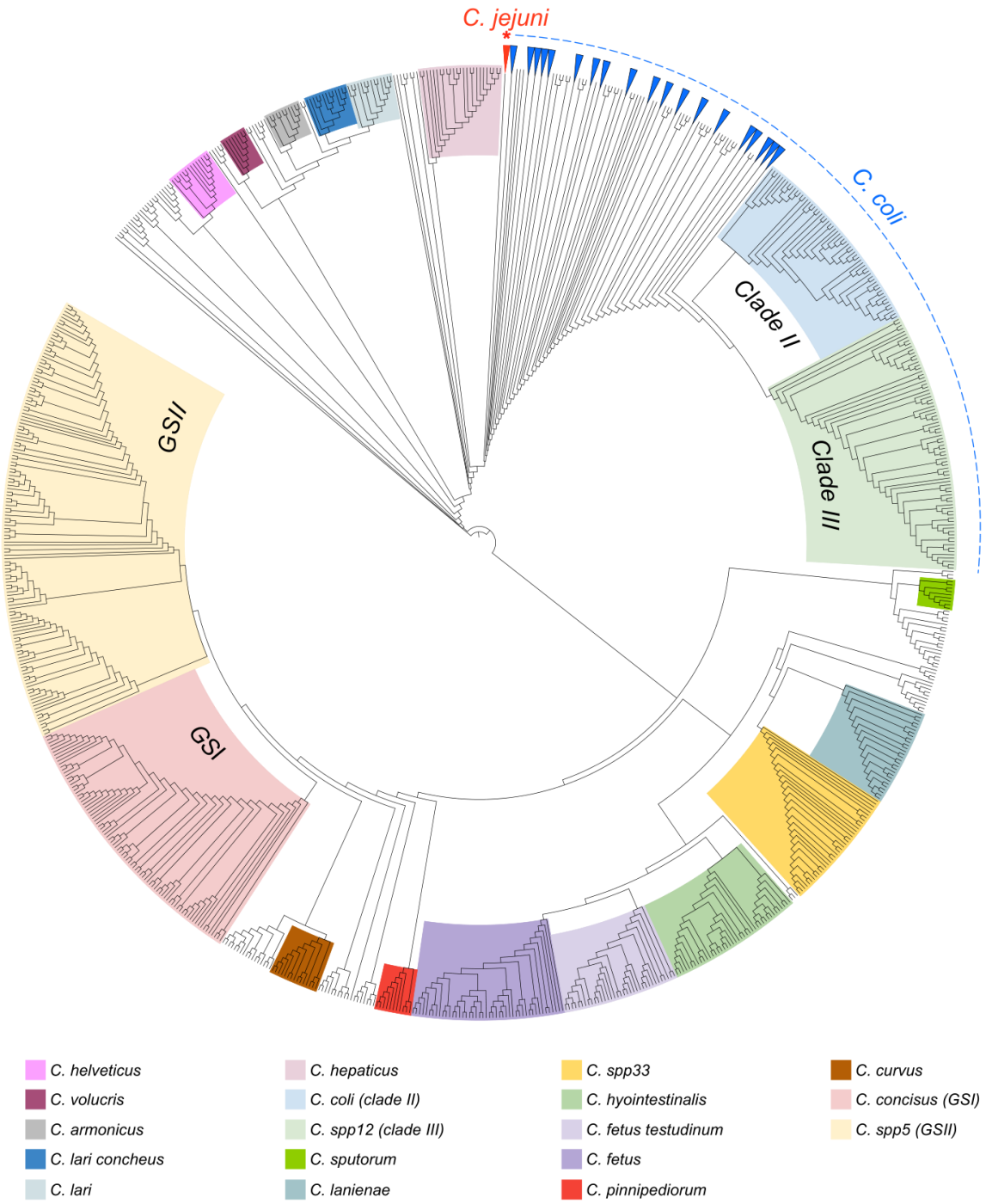
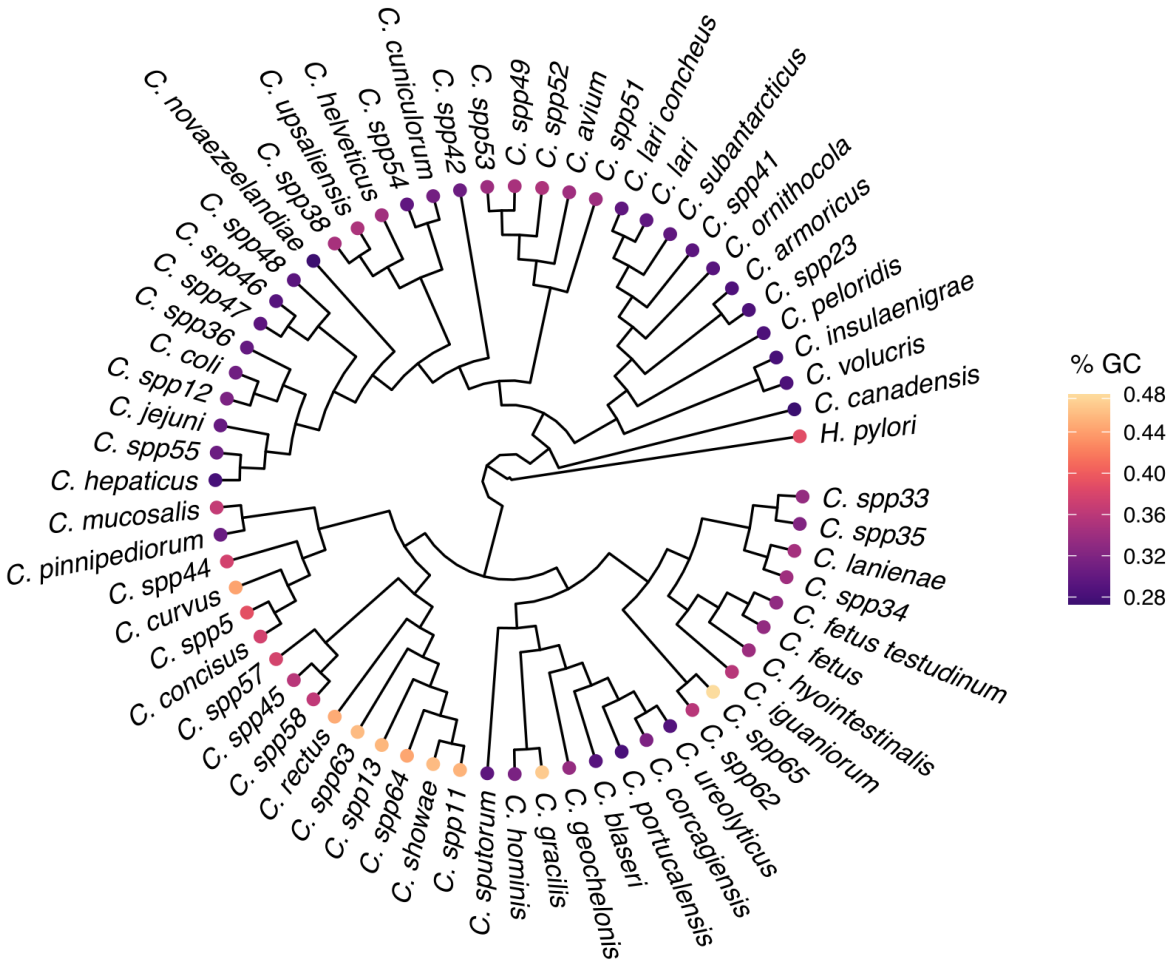


Figure 2. Mash distance tree of *Campylobacter*. Main *Campylobacter* groups are represented in different colors. Branches with many taxa were collapsed to improve visibility.

409



410 **Figure 3.** Phylogenetic reconstruction and GC content in *Campylobacter*. The Maximum likelihood tree
411 was reconstructed using core single-copy genes from one representative genome from each of the 65
412 *Campylobacter* communities. *H. pylori* was used as outgroup.
413

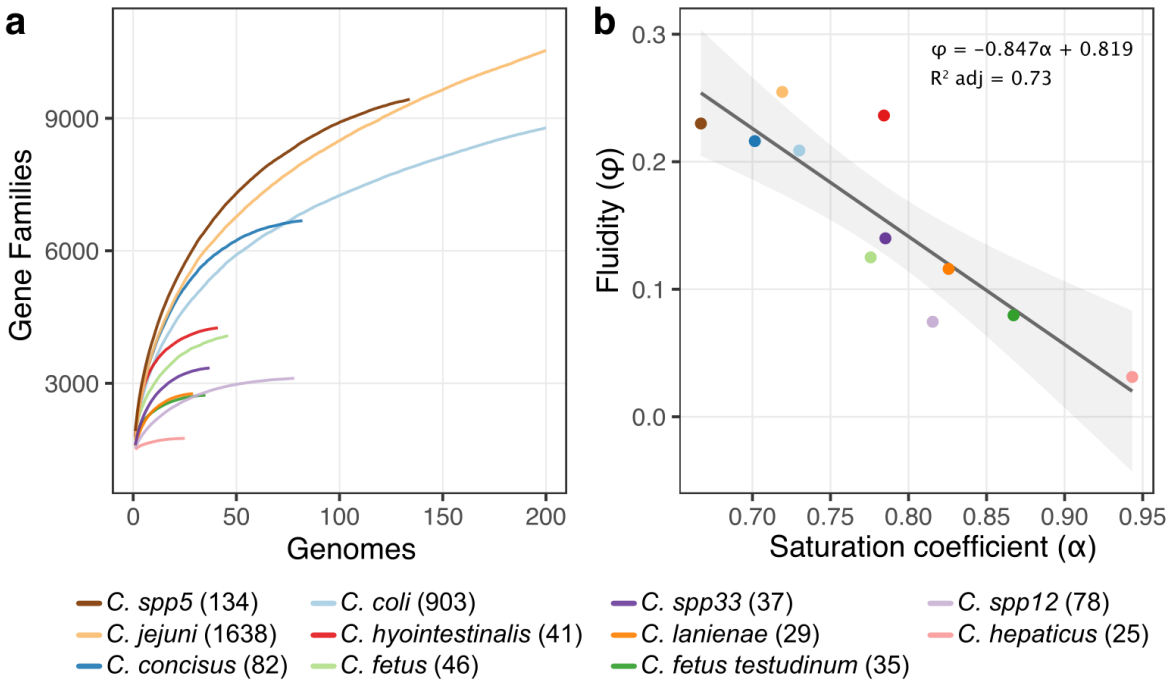


Figure 4. Pangenome openness and fluidity of *Campylobacter* species. **a)** Pangenome cumulative curve from eleven *Campylobacter* species from communities with at least 20 genomes. The number of genomes of each species are indicated within parentheses. Due to the high number *C. jejuni* and *C. coli* genomes, we limited the x-axis to 200 genomes. **b)** Simple linear regression to predict *Campylobacter* pangenome fluidity (ϕ) based on pangenome openness (α). This analysis was conducted with the same communities from panel a.