A natural definition for a bacterial strain and clonal complex.

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Abstract

Large scale surveys of prokaryotic communities (metagenomics) as well as isolate genomes have recently revealed that prokaryotic diversity is predominantly organized in sequence-discrete units that may be equated to species. Specifically, genomes of the same unit (or species) commonly show >95% genome-aggregate average nucleotide identity (ANI) to each other and <90% ANI to members of other species, while genomes showing 90-95% ANI are comparatively rare. However, it remains unclear if such "discontinuities" or gaps in ANI values can be observed within species and thus, be used to define strains or clonal complexes; two cornerstone concepts for microbiology that remain ill-defined. By analyzing 18,123 complete isolate genomes from 330 bacterial species with at least ten genome representatives each, we show that such a natural discontinuity exists at around 99.5% ANI. Further, we show that the 99.5% ANI threshold is largely consistent with how clonal complexes have been defined in previous epidemiological studies but provides clusters with ~20% higher accuracy in terms of evolutionary relatedness of the grouped genomes and greater homogeneity in gene content. Collectively, our results should facilitate future micro-diversity studies across clinical or environmental settings because they provide a more natural definition of a clonal complex and strain.

Main

The strain represents a fundamental unit of microbial diversity that is commonly used across medical or environment studies to designate the smallest distinguishable unit. This term is based on the concept of a pure culture (1), and has been defined by the Bacteriological Code as the group of cultures made "*of the descendants of a single isolation in pure culture*" (2). Accordingly, a strain is expected to represent a genome or a collection of genomes that have no single-nucleotide or gene content differences or, if such differences exist, they are expected to not encode for important phenotypic differences (3). This strain definition, while being commonly used across microbiological fields, remains problematic, however, and often causes confusion in communication. Most notably, it is not clear when two distinct genomes or cells should be considered the same or separate strains since cell ancestry information is often missing, and the existence of phenotypic similarities (or differences) may vary, depending on the

31 growth conditions. Additionally, the isolation of an organism (wild-type) in the laboratory is
32 frequently accompanied by phenotypic changes due to adaptation to the laboratory conditions;
33 yet, the wild-type and the lab-adapted cells or types are typically considered the same strain (1).
34
35 A related term that is also commonly used to catalogue intra-species diversity is the clonal
36 complex (CC). The CC has been used, especially in medical microbiology and epidemiological
37 studies, to identify -for instance- an outbreak caused by a specific pathogenic strain or groups
38 (complexes) of highly related strains. Unlike the more relaxed, context-dependent definition of a
39 "strain", a CC is typically defined as a collection of genomes with no nucleotide sequence diversity
40 (zero single-nucleotide polymorphisms) in 6-7 genetic loci (4). These loci are typically distributed
41 across the genome, to avoid co-selection evolutionary events, and represent fragments
42 (amplicons) of genes shared by most members of a species; that is, core genes. While this
43 definition is pragmatic and operational, it is also problematic because it is arbitrary (e.g.,
44 sometimes CC definitions allow 0- or 1-point mutations), and core genes tend to be more
45 conserved than the genome average. Thus, it remains somewhat speculative how similar (or not)
46 isolates of the same CC may be in the rest of their genome, and this may also depend, at least
47 partly, on the exact loci used in the analysis (5, 6). If the intra-species diversity was organized in
48 discrete units that can be equated to CC or strains, this would have provided for more natural
49 and meaningful definitions for CCs and strains compared to the existing definitions and thus,
50 improved communication about intra-species diversity. While it has been recently recognized
51 that prokaryotic organisms may form such discrete units at the species level (7), it remains
52 unclear whether or not such units also exist within species.
53
54 Specifically, culture-independent (metagenomic) studies of natural microbial populations
55 during the past decade revealed that bacteria and archaea predominantly form sequence-
56 discrete populations with intra-population genomic sequence relatedness typically ranging from
57 ~95% to ~100% genome-aggregate average nucleotide identity (or ANI) depending on the
58 population considered (e.g., younger populations since the last population diversity sweep event
59 show lower levels of intra-population diversity). In contrast, ANI values between distinct

60 populations are typically lower than 90% (8). Intermediate identity genotypes, for example,
61 sharing 85–95% ANI, when present, are generally ecologically differentiated and scarcer in
62 abundance, and thus should probably be considered distinct species (7, 9, 10) rather than
63 representing cultivation or other sampling biases (11). Such sequence-discrete populations have
64 been recovered from many different habitats, including marine, freshwater, soils, human gut,
65 and biofilms, and were usually persistent over time and space [e.g., (12-16)] indicating that they
66 are not ephemeral but long-lived entities. Further, these sequence-discrete populations
67 commonly harbor substantial intra-population gene content diversity (i.e., they are rarely clonal)
68 (12, 15). Therefore, these populations appear to be "species-like" and may constitute important
69 units of microbial communities. Moreover, the 95% ANI threshold appears to be largely
70 consistent with how genomes have been classified into (named) species in the last couple
71 decades; that is, ~97% of named species include only organisms with genomes sharing >95% ANI
72 (17). In summary, it appears that a natural gap in ANI values can be used to define prokaryotic
73 species and has been largely consistent with how species are recognized (17) [Discontinuity or
74 gap here refers to the small number of genome pairs showing 85–95% ANI relative to counts of
75 pairs showing >95% and <85% ANI]. Whether or not a similar ANI gap exists and can be used to
76 define strains or CC has not been evaluated yet.
77
78 **Results/Discussion**
79 **An ANI gap within species around 99.5%**
80 In the process of assessing cultivation biases as a possible explanation for the ANI-based
81 sequence discrete populations (18), we observed another discontinuity (or gap) in ANI values that
82 may be used to define the smallest unit within a species, that of the strain or CC. Specifically, the
83 analysis of 18,123 complete genomes from 330 species available in NCBI's Assembly database
84 with at least 10 such genome representatives each revealed a clear bimodal distribution in ANI
85 values within named species or 95% ANI-defined groups of genomes (genomospecies). That is,
86 there is a scarcity of genomes pairs showing 99.2-99.8% ANI (average around 99.5% ANI) in
87 contrast to genome pairs showing >99.8% or <99.2% ANI. Specifically, among the 18,123
88 complete genomes in our dataset, there are 4,280,133 genome pairs showing >96% ANI, which

89 would translate to about 107,000 pairs per every 0.1 percent unit of ANI if there was no bimodal
90 distribution but the ANI values among these genome pairs were evenly distributed between 96%
91 and 100% ANI. Our analysis revealed only 235,527 genome pairs between 99.2% and 99.8% ANI,
92 which is three-fold fewer data points than expected by chance alone in a uniform ANI value
93 distribution (642,000 pairs expected). No other ANI range within 96-100% had such a strong bias
94 based on our dataset. That is, a pronounced gap in ANI values is observed among very closely
95 related members of a species around 99.5% ANI (Fig. 1). Importantly, this ANI gap appears to be
96 consistent across phylogenetically diverse species from a dozen of distinct bacterial phyla
97 evaluated, including gram-negative and gram-positive, and does not seem to be driven by a
98 couple or a few species based on a sub-sampling of all species to the same number of genomes
99 (n=10) (Fig. S1). Instead, it represents a universal property of the 330 species evaluated (see also
100 Fig. S2 for specific species examples). Therefore, it appears that another important level of
101 genomic differentiation may exist within species that can be used to define strains and CC.
102
103 It is unlikely that this 99.5% intra-species ANI gap is due to cultivation or classification
104 biases due the reasons mentioned previously, such as that cultivation media should not
105 distinguish between members of the same species or closely related groups of organisms (18),
106 and that random subsampling provided similar patterns (Fig. S1). In fact, it is highly likely that the
107 intra-species ANI gap may be even more pronounced in nature because very closely related
108 genomes (e.g., showing >99.8% ANI to each other) are often selected against for genome
109 sequencing (and thus, are likely underrepresented in our collection) based on pre-screening using
110 fingerprinting techniques (e.g., RARP-PCR, MLST) in order to avoid sequencing of redundant
111 genomes. Despite this bias against very closely related genomes, which in all probability exists
112 but we are currently unable to estimate its magnitude, several species have enough very closely
113 related genomes sequenced in our dataset for robust evaluation of patterns in their ANI value
114 distributions (Fig. 1 and S2). We were not able to identify clear exceptions to this 99.5% intra-
115 species ANI gap when examining individual species with enough sequenced representatives,
116 although such exceptions likely exist. For instance, several species in our collection did not have
117 enough very highly related genome representatives (showing >99% ANI to each other) to assess

118 the critical area of ANI value distribution (i.e., the 99-100% range), and this could be due to the
119 pre-screening biases mentioned above or reflect their actual natural diversity patterns (see also
120 Fig. 2, Panel D for an example within the *E. coli*). Further, for a few species (n < 10) such as *Listeria*
121 *monocytogenes* and *Bordetella bronchiseptica*, the intra-species ANI gap appears to exist but is
122 shifted compared to the 99.5% ANI that characterized most well-sampled species (Fig. S2).
123 Therefore, for future studies, we suggest evaluating the ANI value distribution for the species of
124 interest, and if the data indicate so, to adjust the ANI threshold to match the gap in the observed
125 ANI value distribution. The 99.5% ANI should work for most species based on the dataset
126 evaluated here.
127
128 **Gene content diversity within 99.5% ANI clusters**
129 Another notable observation from the data from all species comparisons is that shared
130 gene content decreases, on average, as ANI distance (or genomic divergence) increases within
131 the 95% ANI clusters, but the decrease is biphasic. That is, shared gene content decreases quickly
132 among genome pairs sharing 99.0-100% ANI but then, the decrease is less dramatic in genome
133 pairs sharing between 96.0%-99.0% ANI. In other words, genome pairs sharing between 99.0%-
134 100% ANI (i.e., one ANI unit range) may differ in their total gene content by up to 10% (average
135 values of genome pairs showing ~99.0% ANI) and more divergent genomes of the same species
136 (i.e., showing 96.0%>ANI>99.0%) may differ by up to 20% (average values of genome pairs
137 showing ~96.0% ANI), adding another ~10% of gene content differences for 3 additional units of
138 ANI (vs. 1 unit in the 99-100% range). Collectively, these results show that genome pairs showing
139 >99.5% ANI are also expected to be much more similar in gene-content compared to more
140 divergent genomes of the same species. Not only do these results quantify the amount of gene
141 content diversity expected in comparisons of genomes within the same species, but they are also
142 consistent with the notion that members of the same CC should be highly similar in shared
143 functional gene content and thus, phenotype. While the high gene content diversity within
144 species has been observed previously, even based on the very first few bacterial genomes
145 sequenced (19), the analysis presented here provides robust quantification of shared gene

content as a function of ANI and CC or strain designation, which should be useful in future studies that aim to quantify the size and value of bacterial species pangenomes.

Comparison to clonal complexes (or Sequence Types, STs)

We also assessed how consistent the 99.5% ANI threshold is with the assignment of genomes to the same CC, the latter defined as identical sequences for 6-7 genetic loci. We focus this analysis on the *E. coli* species because it is a good representative of the ANI patterns observed within other species in our dataset, the large number of *E. coli* genomes available (n=975), and the availability of a robust Multi-Locus Sequence Typing/Analysis (MLST/MLSA) scheme (20) that has been used for at least two decades to provide below-species resolution and identify outbreaks of *E. coli* pathogens. Under the *E. coli* MLST scheme, genomes are assigned to the same CC, also commonly called Sequence Type (ST), based on identical sequences in seven *E. coli* core genes (namely, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (20). Our evaluation showed that the 99.5% ANI threshold is largely consistent with how genomes are assigned to STs; that is, ~80% of ST assignments, for the top four most abundant STs (n=615 genomes), were supported by the 99.5% ANI threshold (Fig. 2). In other words, only about 20% of the genomes that were assigned to the same ST showed <99.5% ANI among themselves (high recall). Further, ~2% of the genomes with >99.5% were assigned to different ST, revealing even higher precision (Table S1; Suppl. Fig. S3). The high recall (and precision) of the 99.5% ANI threshold is due, at least in part, to the fact that the core genes used in current MLST schemes tend to be more highly conserved, at the sequence level, than the genome average (represented by ANI), or have been exchanged horizontally very recently among the evaluated genomes. It is also important to note that hundreds, if not thousands (e.g., at least 3,000 for the *E. coli* pairs), of genes are used in each ANI calculation at this level of high relatedness vs. 6-7 genes in MLST schemes and that's another reason for the higher robustness of the former metric against horizontal gene transfer or other evolutionary events that could affect sequence identity. These results are also consistent with our previous conclusion that the 95% ANI threshold should be adjusted upwards if the ANI is based on a few universal or core genes (17). In any case, the 99.5% ANI threshold is a property that emerges from the data themselves as opposed to a manmade, arbitrary identity threshold

175 (i.e., identical sequences in seven loci used in MLST applications) and thus, it should capture the
176 natural diversity patterns better. Consistent with this interpretation, our preliminary results from
177 applying the 99.5% ANI threshold to a collection of *E. coli* isolate genomes collected over a period
178 of 18 months in Northern coastal Ecuador as part of the EcoZUR study (for "*E. coli* en Zonas
179 Urbanas y Rurales") (21) shows that the 99.5%-ANI-defined CCs map well to local outbreaks of
180 pathogenic *E. coli* (Feistel et al., in preparation). Therefore, using the 99.5% ANI to define CCs will
181 prevent inaccurate calls and provide more biologically informed means to define CCs. Another
182 important advantage of the 99.5% ANI is that it can be automatically implemented and thus, does
183 not require manual curation, which is the case when establishing new ST numbers when novel
184 (meaning, not seen previously) sequences become available (4).
185
186 **What are the underlying mechanisms for the 99.5% ANI gap?**
187 The mechanism(s) that underly the 99.5% ANI gap (or the earlier 95% ANI gap for the
188 species level) remain essentially speculative and should be the subject of future research in order
189 to further advance the mechanistic understanding of the microbial diversity patterns observed
190 in nature. Most notable is the idea that members of a population cohere together via means of
191 unbiased (random) genetic exchange which is more frequent within vs. between populations or
192 CCs (i.e., *the biological or sexual species concept*). A competing hypothesis is that several
193 members of the species are functionally differentiated from each other either due to
194 specialization for different growth conditions or different affinities for the same energy substrate
195 and thus, selection over time for these functions purge diversity (i.e., *the ecological species*
196 *concept*) (22-24). It is intriguing to note that the ecological explanation is also consistent with the
197 notion that CCs or different strains of the same species are somewhat ecologically and/or
198 functionally distinguishable from each other. Notably, given an estimated mutation rate of ~$4x10^{-}$
199 ^{10} per nucleotide per generation (25) and between 100 to 300 generations per year (26), it would
200 take two distinct *E. coli* lineages or CCs at least forty thousand years since their last common
201 ancestor to accumulate 1% difference (i.e., fixed mutations) in their core genes or 99% ANI.
202 Therefore, there is enough time, at least theoretically, for the ecological purging of diversity to
203 take place at around the 99.5% ANI level and thus, account for the ANI patterns observed herein.

204 Intriguingly, it has been shown that the explicit inclusion of extinction events in a neutral model
205 of evolution can also result in punctuated distributions of genetic differentiation, opening up a
206 third possibility of historical contingency from stochastic events (27). However, we note that
207 while stochasticity can explain bimodal (or multimodal) distance distributions, a scarcity of ANI
208 values in the exact same range (i.e., around 99.5% ANI) would be unlikely to repeatedly emerge
209 by chance alone across many different species with distinct lifestyles and evolutionary tempo, as
210 opposed to this range varying between species. In any case, the data available in support of one
211 of these (or another) hypotheses remain sparse and/or anecdotal to date, to the best of our
212 knowledge, and the analysis presented in this study did not aim to advance this issue further.
213
214 **Conclusion**
215 Regardless of what the underlying mechanisms are for the 99.5% ANI gap, the results
216 presented here show that the patterns of natural diversity among thousands of sequenced
217 genomes are consistent with a 99.5% ANI threshold that can be used to identify CCs and strains
218 more reliably and precisely compared to the current practice. Regarding the use of this threshold
219 to define CCs vs. strains, we believe that the threshold is highly appropriate, as well as it matches
220 well the intended meaning and use of CCs, and thus its application to CC definition is
221 straightforward. For the strain level, 0.5% or 0.2% difference in ANI (correspondingly, 99.5% and
222 99.8% ANI) represents substantial, non-trivial, genomic divergence that, in most cases, would
223 likely encompass several genomes with at least some phenotypic differences (due to substantial
224 sequence or gene content differences among the genomes; see Fig. 1). Thus, multiple strains will
225 be likely grouped together under the same 99.5% ANI cluster in such cases, and strain, in general,
226 represents a more fine-grained level of resolution than the 99.5% ANI level. That said, we also
227 expect that the latter would somewhat depend on the context of the study and the existence or
228 not of phenotypic differences. For instance, it is possible that all sequence and gene-content
229 differences within some 99.5% ANI genome clusters to be neutral (or not functional), for at least
230 some growth conditions and habitats. Therefore, in such cases, a strain could be defined at the
231 99.5% ANI level and thus, include high(er) intra-strain sequence and/or gene-content diversity.
232 Hence, the 99.5% ANI level is also a good starting point in trying to define strains and

233 subsequently assess their gene and phenotypic differences (or lack of). Collectively, we expect
234 that the findings reported here will advance the molecular toolbox for accurately delineating and
235 following the important units of diversity withing prokaryotic species and thus, would greatly
236 facilitate future epidemiological and micro-diversity studies.
237
238 **Material and Methods**
239 Step by step methods, including how average trendlines were fit to the data, custom
240 Python code, NCBI Assembly accession numbers for selected genomes, and plots for each
241 selected species are available from:
242 https://github.com/rotheconrad/bacterial_strain_definition. Briefly, all genome sequences were
243 obtained from NCBI's RefSeq Assembly database on April 20th, 2022 and were labeled as
244 "complete" and "latest". ANI values and the shared genome fractions were directly obtained
245 from the output of FastANI version 1.32 "One to Many" mode with default settings (Jain et al
246 2018). Results were concatenated to create within species all vs. all output. Self matches were
247 removed, and genome pairs were filtered by minimum ANI values according to the axes of each
248 figure. Selected individual species plots (i.e., all vs. all output) of shared genome fraction vs. ANI
249 are shown in the Supplementary Material. *E. coli* genomes were assigned to sequence types (ST)
250 using the using the command-line tool mlst (https://github.com/tseemann/mlst) version 2.19.0
251 (20) with default settings.
252
253 **Acknowledgments**
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256
257 **Code and data availability**
258 All code and data details are available from
259 https://github.com/rotheconrad/bacterial_strain_definition.
260
261 **Competing interests**

262 The authors declare no competing interests.

263

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271

272 **Figure 1. ANI vs. shared gene content for the 17,283 complete genomes used in this study.** Each
273 datapoint represents a comparison between a pair of genomes. FastANI (17) was used to
274 generate ANI values between the genomes of a pair (x-axis) and their shared genome fraction (y-
275 axis). The shared genome fraction was calculated by dividing the number of bidirectional
276 fragment mappings over the total query fragments determined by FastANI. Only a single set of
277 values is reported per pair, the one that used the longer genome as the reference (and the
278 reverse comparison was omitted). Note that only datapoints representing genome pairs sharing
279 ANI >95% are shown, and that panel B is a zoomed-in version of panel A. The main scatter plot is
280 shaded by density of the points using the Datashader package in Python with Matplotlib. The
281 trendline was calculated using linearGAM from pyGAM and includes the 95% confidence interval.
282 The marginal plots outside the two axes show histograms for the density of datapoints of each
283 axis. Note the low-density region in the ANI value distribution around 99.2-99.8% (Panel A), which
284 becomes more obvious when zooming in to the 98-100% ANI range (Panel B).

285 **Figure 1, panel A**.

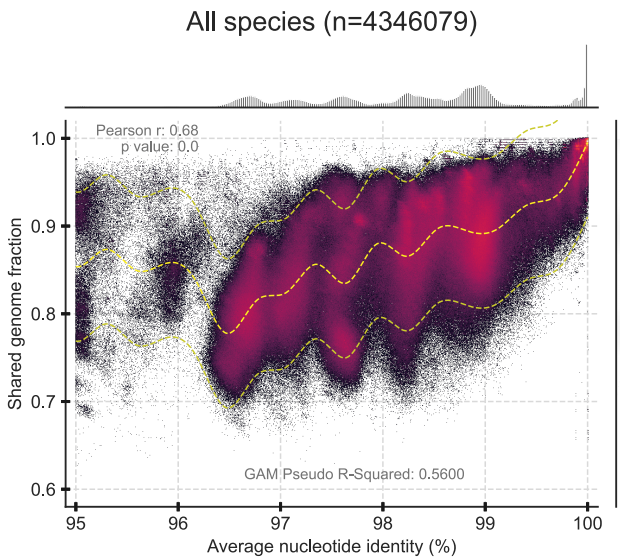


Figure 1, panel B.

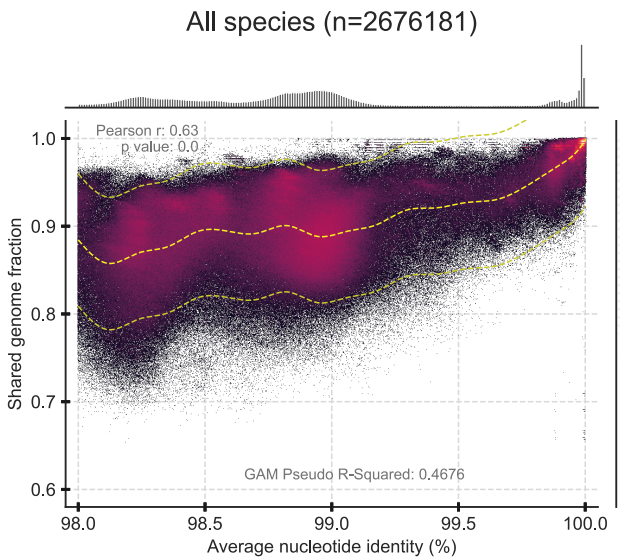
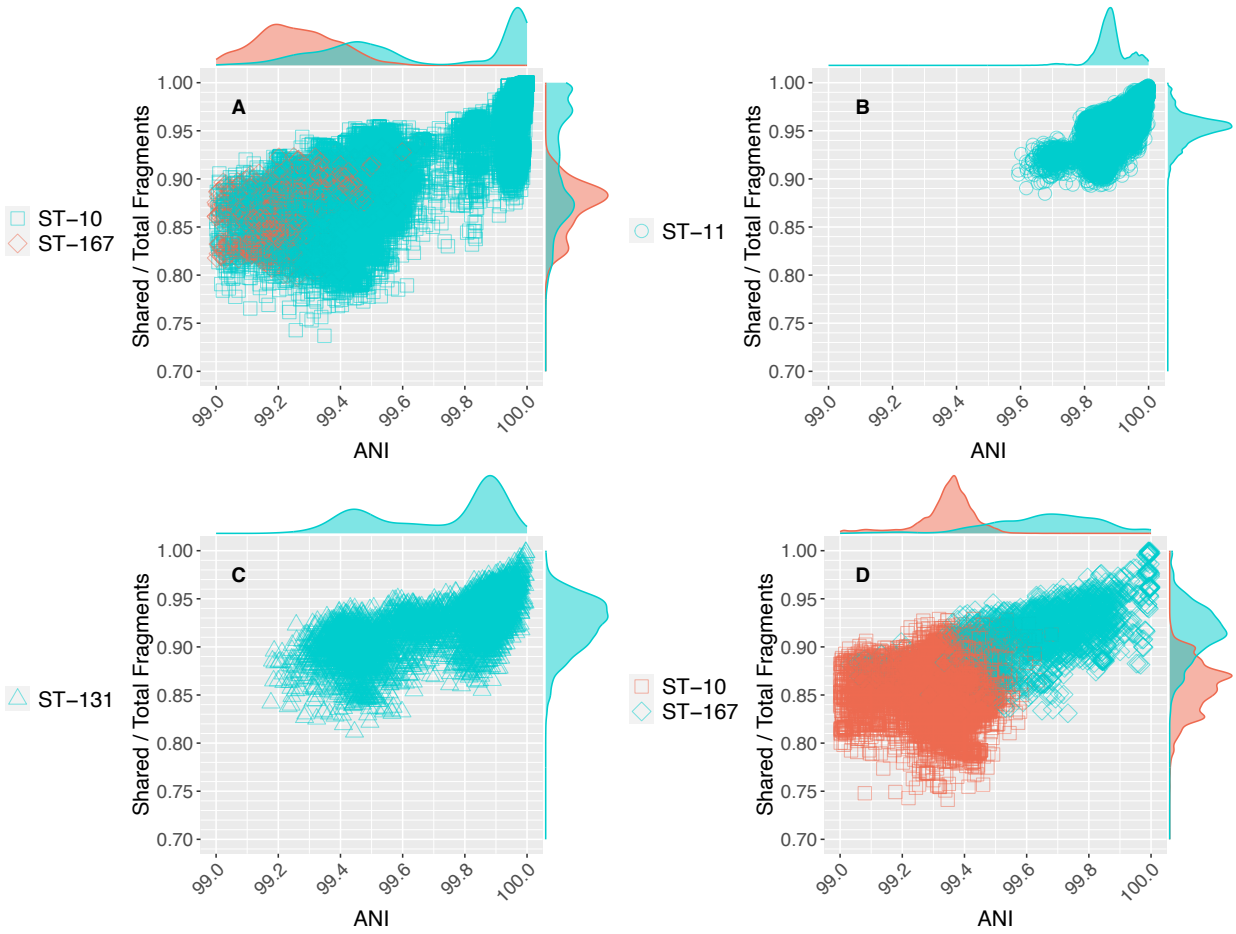


Figure 2. Comparison of the 99.5% ANI threshold to available clonal complexes of *E. coli*. All *E. coli* genomes were assigned to a Sequence Type, ST (or clonal complex) using the command-line tool mlst (https://github.com/tseemann/mlst) version 2.19.0 (20). The four panels show the four most abundant STs based on the number of genomes assigned to them (see Table S1 and Figure S1 for underlying data and F1 statistic, respectively). Each datapoint is a comparison between two genomes, similar to those shown in Figure 1; the marginal plots show the kernel density estimate of datapoints for each axis. Datapoints are cyan if both genomes in the pair were assigned to the same, reference ST; red datapoints represent pairs for which one of the genomes

in the pair is assigned to a closely related, yet distinct ST than the reference ST. Note that for ST-11, recall and precision of 99.5% ANI vs. CC is perfect because there are no genomes, and thus STs, that are closely related to ST-11, which is also consistent with a pronounced ANI gap at 99.5% for ST-11, and the substantial overlap in terms of ANI values between the closely related ST-10 and ST167 (low recall). ST-131 and ST-10 appear to harbor too much genomic diversity and could be split in more than one CC based on the 99.5% ANI criterion (and the bimodal ANI value distribution around 99.6% ANI).



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