Genomic surveillance of *Bacillus cereus sensu lato* strains isolated from meat and poultry products in South Africa enables inter- and intra-national surveillance and source tracking

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Abstract

Members of the *Bacillus cereus sensu lato* (*s.l.*) species complex, also known as the *B. cereus* group, vary in their ability to cause illness, but are frequently isolated from foods, including meat products; however, food safety surveillance efforts that employ whole-genome sequencing (WGS) often neglect these potential pathogens. Here, WGS was used to characterize *B. cereus s.l.* strains (n = 25) isolated during surveillance of meat products in South Africa. Strains were collected from beef, poultry, and mixed meat products obtained from (i) retail outlets, processing plants, and butcheries across six South African provinces (n = 15, 7, and 1, respectively), and (ii) imports in cold stores (n = 2). Strains were assigned to *panC* Groups IV, III, II, and V (n = 18, 5, 1, and 1, respectively) and spanned multiple genomospecies, regardless of the taxonomy used. All strains possessed diarrheal toxin-encoding genes, while one sequence type 26 (ST26) strain possessed cereulide (emetic toxin) synthetase-encoding genes. No strains harbored anthrax toxin- or capsule-encoding genes. The 25 strains were partitioned into 15 lineages via *in silico* seven-gene multi-locus sequence typing (MLST), six of which contained multiple strains sequenced in this study, which were identical or nearly identical at the whole-genome scale. Five MLST lineages contained (nearly) identical genomes collected from two or three South African provinces; one MLST lineage contained nearly identical genomes from two countries (South Africa and the Netherlands), indicating that *B. cereus s.l.* can spread intra- and inter-nationally via foodstuffs.

Importance

Nation-wide foodborne pathogen surveillance programs that employ high-resolution genomic methods have been shown to provide vast public health and economic benefits. However, *B. cereus s.l.* are often overlooked during large-scale, routine WGS efforts. Thus, to our knowledge, no studies to date have evaluated the potential utility of WGS for *B. cereus s.l.* surveillance and source

tracking in foodstuffs. In this proof-of-concept study, we applied WGS to *B. cereus s.l.* strains collected via South Africa's national surveillance program of domestic and imported meat products, and we provide strong evidence that *B. cereus s.l.* can be disseminated intra- and internationally via the agro-food supply chain. Our results showcase that WGS can be used for source tracking of *B. cereus s.l.* in foods, although future WGS and isolate metadata collection efforts are needed to ensure that *B. cereus s.l.* surveillance initiatives are on par with those of other foodborne pathogens.

57 **INTRODUCTION**

58 *Bacillus cereus sensu lato* (*s.l.*), also known as the *B. cereus* group, is a complex of closely
59 related, Gram-positive, spore-forming species, which are widespread throughout the environment
60 (1). While some members of *B. cereus s.l.* have important industrial applications or roles (e.g., as
61 biocontrol agents in agricultural settings, as food spoilage organisms) (2-6), others are capable of
62 causing illnesses or death in humans and/or animals (1, 7-9). Illnesses caused by members of *B.*
63 *cereus s.l.* can range in severity from mild to severe/fatal and include anthrax and anthrax-like
64 illness (8, 10, 11), foodborne emetic intoxication (1, 7, 12-15), foodborne diarrheal toxicoinfection
65 (1, 7, 14, 15), and non-gastrointestinal infections (16, 17). As a foodborne pathogen, "*B. cereus*"
66 is estimated to be responsible for more than 256,000 illnesses globally each year (18), although
67 this is likely an underestimate, due to the relatively mild and self-limiting nature of the symptoms
68 that often accompany foodborne illness caused by *B. cereus s.l.* (1).

69 Food safety surveillance efforts around the world have identified *B. cereus s.l.* strains in a
70 wide variety of foodstuffs (1, 7), including raw intact, processed, and ready-to-eat (RTE) meat and
71 poultry products (19-27). In South Africa specifically, previous surveillance efforts have identified
72 *B. cereus s.l.* in (i) retail meats sold at supermarkets in the Pretoria area (i.e., Vienna sausages,
73 salami, and poultry) (27) and (ii) biltong (a spiced intermediate moisture RTE meat product) sold
74 at supermarkets, stalls, kiosks, and butcheries in Bloemfontein, Free State (28). Most recently, in
75 a study of over two thousand meat product samples collected from butcheries, processing plants,
76 abattoirs, and retail outlets across all nine South African provinces, *B. cereus s.l.* was present in
77 4.5 and 2.7% of domestic and imported meat products, respectively (26).

78 Ongoing surveillance efforts in South Africa have indicated that meat and poultry products
79 can harbor *B. cereus s.l.* and may pose a potential food safety risk to South African consumers.

However, it is unclear which *B. cereus s.l.* lineages are present in South African meat and poultry products on a genomic scale. Here, we used whole-genome sequencing (WGS) to characterize 25 *B. cereus s.l.* strains isolated from raw intact, processed, and RTE meat and poultry products collected from processing plants, butcheries, and retail outlets across South Africa, as well as imported meat products. By comparing South African strains sequenced here to all publicly available *B. cereus s.l.* genomes (n = 2,887 total genomes), we identified multiple *B. cereus s.l.* species present among South African meat and poultry products, and we detected multiple potential inter-national and inter-provincial *B. cereus s.l.* dissemination events. Overall, our study serves as the first genome-scale study of South African *B. cereus s.l.* in foodstuffs and showcases the utility of WGS for *B. cereus s.l.* surveillance and source tracking.

RESULTS

***B. cereus s.l.* are present among domestic and imported meat and poultry products in South Africa.** A total of 25 *B. cereus s.l.* strains were isolated from meat and poultry products, which had been collected across South Africa in 2015 and 2016 (Figure 1, Table 1, and Supplemental Table S1). Overall, 19 strains (76.0%) originated from beef products, including: beef wors, a processed South African sausage (n = 7; 28.0%); beef biltong, a South African spiced intermediate moisture RTE meat product (n = 5; 20.0%); processed beef mince (n = 3; 12.0%); RTE beef sausage emulsion (n = 2; 8.0%); and processed beef patties (n = 2; 8.0%, Table 1 and Supplemental Table S1). Five strains (20.0%) were isolated from poultry products, including raw chicken thighs (n = 2; 8.0%), raw chicken quarter-legs (n = 2; 8.0%), and a frankfurter (n = 1; 4.0%, Table 1 and Supplemental Table S1). One strain (4.0%) was isolated from wors, which had been made from a mix of beef, pork, and lamb (Table 1 and Supplemental Table S1).

103 The majority of strains (23 of 25, 92.0%) were obtained from domestic meat and poultry
104 products acquired across six South African provinces (*n* = 5, 5, 4, 4, 3, and 2 strains from Gauteng,
105 Limpopo, Free State, North West, Mpumalanga, and Western Cape, respectively; Figure 1, Table
106 1, and Supplemental Table S1). The remaining two strains (8.0%) were isolated from raw, intact
107 chicken quarter-legs, which had been imported into South Africa from the Netherlands (Figure 1,
108 Table 1, and Supplemental Table S1). Fifteen of the 25 *B. cereus s.l.* strains (60.0%) were isolated
109 from meat or poultry products acquired from retail outlets, while seven (28.0%) were acquired
110 from meat or poultry products obtained from processing plants (Table 1 and Supplemental Table
111 S1). Two strains (from chicken quarter-legs imported from the Netherlands; 8.0%) were derived
112 from poultry in cold stores, while one strain (4.0%) was isolated from the mixed beef-pork-lamb
113 wors acquired from a butchery (Table 1 and Supplemental Table S1).
114 **Multiple species are present among *B. cereus s.l.* from South African meat and poultry**
115 **products.** Species-level taxonomic classification of *B. cereus s.l.* is notoriously challenging (1,
116 29, 30); to avoid taxonomic ambiguities and maximize interpretability, we applied multiple
117 taxonomic assignment and sequence typing methods to the 25 strains sequenced here (Table 1).
118 One such sequence typing framework relies on the pantoate-β-alanine ligase gene (*panC*) to assign
119 *B. cereus s.l.* strains to one of seven or more major phylogenetic groups, which have been proposed
120 to conceptually serve as "species" (31, 32). Using the adjusted eight-group *panC* typing approach
121 implemented in BTyper3 (33), the 25 *B. cereus s.l.* strains sequenced here encompassed four *panC*
122 phylogenetic groups (i.e., "species"; Figure 2 and Table 1). The majority (*n* = 18 of 25, 72.0%) of
123 the strains sequenced here were assigned to *panC* Group IV (Figure 2 and Table 1). The remaining
124 strains were assigned to *panC* Groups III, II, and V (*n* = 5, 1, and 1 strains, representing 20.0%,
125 4.0%, and 4.0% of isolates sequenced here, respectively; Figure 2 and Table 1).

126 Using the Genome Taxonomy Database (GTDB) taxonomy, the 25 *B. cereus s.l.* strains
127 sequenced here encompassed eight genomospecies (Figure 2 and Table 1). The 18 *panC* Group
128 IV strains sequenced here encompassed four GTDB genomospecies, while the five *panC* Group
129 III strains spanned two GTDB genomospecies (Figure 2 and Table 1). The *panC* Group II and
130 Group V strains (*n* = 1 each) were each assigned to separate GTDB genomospecies (Figure 2 and
131 Table 1).

132 Using a standardized genomospecies-subspecies-biovar (GSB) nomenclatural framework
133 proposed for *B. cereus s.l.* in 2020 (34) (referred to hereafter as the "2020 GSB" framework), the
134 25 strains sequenced here encompassed three genomospecies (Figure 2 and Tables 1 and 2). All
135 18 *panC* Group IV strains were assigned to the *B. cereus sensu stricto* (*s.s.*) genomospecies (Figure
136 2 and Tables 1 and 2); genes encoding insecticidal toxins (referred to hereafter as "Bt toxin-
137 encoding genes") were detected within all Group IV *B. cereus s.s.* genomes (using
138 BtToxin_scanner2's "old" gene detection approach), meaning that these 18 strains were predicted
139 to belong to the Thuringiensis biovar (i.e., *B. cereus s.s.* bv. Thuringiensis; Table 2). All five *panC*
140 Group III and the single *panC* Group II strain(s) were assigned to genomospecies *B. mosaicus*
141 within the 2020 GSB framework (Figure 2 and Tables 1 and 2). One *panC* Group III *B. mosaicus*
142 strain (i.e., strain S66) was assigned to PubMLST sequence type 26 (ST26) and possessed
143 cereulide (emetic toxin) synthetase-encoding *cesABCD* and was thus assigned to the *cereus*
144 subspecies and biovar Emeticus (i.e., *B. mosaicus* subsp. *cereus* bv. Emeticus; Table 2). The lone
145 *panC* Group V strain sequenced here was assigned to the *B. toyonensis* genomospecies; Bt toxin-
146 encoding genes were detected in this genome (via BtToxin_scanner2's "old" gene detection
147 approach), and thus this strain was predicted to belong to biovar Thuringiensis (i.e., *B. toyonensis*
148 bv. Thuringiensis; Figure 2 and Tables 1 and 2).

149 Using a rapid, average nucleotide identity (ANI)-based pseudo-gene flow unit assignment
150 scheme, which attempts to assign *B. cereus s.l.* genomes to taxonomic units that mimic *B. cereus*
151 *s.l.* "species" previously delineated using recent gene flow (33), the 25 strains sequenced here were
152 assigned to six pseudo-gene flow units (pseudo-GFUs; Figure 2 and Supplemental Table S1). The
153 18 *panC* Group IV and five *panC* Group III strains sequenced here each spanned two pseudo-
154 GFUs (Figure 2 and Supplemental Table S1). The *panC* Group II and Group V strains (n = 1 each)
155 were each assigned to separate pseudo-GFUs, respectively (Figure 2 and Supplemental Table S1).
156 As mentioned above, considerable phenotypic diversity was predicted among the stains
157 sequenced here, as one strain harbored cereulide synthetase-encoding genes, and 19 strains
158 possessed Bt toxin-encoding genes (detected using BtToxin_scanner2's "old" gene detection
159 approach; Table 2). No anthrax toxin- or capsule-encoding genes were identified within the
160 genomes of the isolates sequenced here (Table 2).
161 Overall, regardless of whether the *panC*, GTDB, 2020 GSB, or pseudo-gene flow unit
162 assignment frameworks were used, *B. cereus s.l.* strains isolated from meat and poultry products
163 in South Africa were considerably diverse and represented multiple genomospecies (Figure 2,
164 Tables 1 and 2, and Supplemental Table S1). Additionally, using PubMLST's seven-gene MLST
165 scheme for *B. cereus*, 17 of 25 strains (68.0%) encompassed 11 STs, with eight strains (32.0%)
166 assigned to unknown STs (Table 1 and Supplemental Table S1). Due to the considerable genomic
167 diversity observed among isolates sequenced here, major lineages represented by strains
168 sequenced in this study are discussed individually in detail below, largely within the context of
169 *panC* Groups and/or MLST STs, as these frameworks are well-established (31, 32, 35, 36) and
170 likely the most interpretable to readers.

171 **Several *B. cereus s.l.* lineages within *panC* Group IV are distributed across multiple South**
172 **African provinces.** Within *panC* Group IV, the 18 strains sequenced here were partitioned into
173 ten lineages using MLST (referred to hereafter as "MLST-based lineages"; Table 3). Based on (i)
174 the whole-genome phylogeny, (ii) pairwise core single-nucleotide polymorphisms (SNPs)
175 identified within MLST-based lineages, and (iii) ANI values calculated within and between
176 MLST-based lineages, four of ten *panC* Group IV MLST-based lineages contained South African
177 strains sequenced in this study, which were highly similar to at least one other strain at the whole-
178 genome level (Figures 3 and 4 and Table 3). One of these lineages (denoted in Table 3 as Lineage
179 IVA) was composed of three South African strains sequenced in this study (S65, S85, and S87),
180 which were assigned to GTDB's "*B. bombysepticus*" genomospecies and belonged to an unknown
181 ST (Figures 3 and 4 and Table 3). Despite all three genomes being nearly identical (pairwise core
182 SNP distance = 0, pairwise ANI > 99.99; Figure 3 and Table 3), the three strains were isolated
183 from (i) three different establishments and provinces (a processing plant in Limpopo, a retail outlet
184 in Gauteng, and a processing plant in Free State), and (ii) two different types of meat products
185 (two strains from beef wors and one from a poultry frankfurter; Figure 4 and Table 1).
186 Similar results were observed for *panC* Group IV ST2721 (Lineage IVB in Table 3), which
187 was also assigned to GTDB's "*B. bombysepticus*" genomospecies and contained three strains
188 sequenced in this study (S56, S79, and New_S84; Figures 3 and 4 and Table 3). The three ST2721
189 genomes were nearly identical (pairwise core SNP distance = 0, pairwise ANI > 99.99; Table 3),
190 despite the fact that the strains originated from three different meat products (one from each of
191 beef wors, beef biltong, and processed beef patties) obtained from three different establishments
192 (from a processing plant in North West province, a retail outlet in North West province, and a retail
193 outlet in Free State, respectively; Figure 4 and Table 1).

194 A third *panC* Group IV lineage of unknown ST, which was assigned to GTDB's *B. cereus*
195 species (Lineage IVD), contained two isolates sequenced in this study (S81 and S88; Figures 3
196 and 4 and Table 3). One strain (S81) had been isolated from beef wors in a processing plant in the
197 North West province; the other (S88) from RTE beef biltong in a retail outlet in Limpopo (Figure
198 4 and Table 1). Both strains were highly similar on a genomic scale (>99.99 ANI) and differed by
199 three core SNPs (Figure 3 and Table 3). For reference, in a previous point source foodborne
200 outbreak caused by *B. cereus s.l.* (37), outbreak isolates could differ by up to seven core SNPs
201 (using the same SNP calling methodology used here) (38).

202 A fourth *panC* Group IV lineage, ST2668, contained three strains sequenced in this study
203 (S53, S63, and S70), which were assigned to GTDB's "*B. thuringiensis_S*" genomospecies
204 (Lineage IVH; Figures 3 and 4 and Table 3). The three strains sequenced here differed by, at most,
205 a single core SNP (Table 3), even though all had been isolated from different meat products
206 (processed beef patties, beef biltong, and processed beef mince) obtained from different
207 establishments (i.e., from retail outlets in each of Gauteng, Free State, and Limpopo, respectively;
208 Figure 4 and Table 1). One publicly available genome associated with a *B. cereus s.l.* strain isolated
209 from grass in KarieDeshe, Israel (39) was additionally assigned to ST2668 (NCBI RefSeq
210 Accession GCF_005217245.1); however, this strain was not closely related to the three nearly
211 identical South African ST2668 strains sequenced in this study (Table 3).

212 The remaining six MLST-based lineages (i.e., Lineages IVC, IVE, IVF, IVG, IVI, and IVJ,
213 corresponding to an unknown ST, ST177, ST2289, ST24, ST1697, and ST1578, respectively;
214 Table 3) contained isolates sequenced in this study, which were not closely related to any other
215 strains at the whole-genome level (Figure 3 and Table 3). Lineages IVC, IVF, and IVI were
216 singleton lineages, which each contained one genome sequenced in this study (S67, S86, and S78,

217 respectively), shared 98.90-99.35 ANI with their closest publicly available neighbors (Figure 3
218 and Table 3). Two lineages (IVG and IVJ) each contained multiple genomes, but only one genome
219 sequenced in this study (i.e., S77 and S51, respectively; Figure 3 and Table 3); for both lineages,
220 the South African genome sequenced here was not identical to any publicly available genomes.
221 The remaining lineage, Lineage IVE (ST177), contained multiple genomes, as well as multiple
222 genomes sequenced in this study (Figure 3 and Table 3). Notably, the two ST177 strains sequenced
223 in this study (S55 and S80), which were assigned to this lineage, were not identical, and were
224 isolated from beef wors from processing plants in the Limpopo and North West provinces,
225 respectively (Table 1), indicating that WGS can be useful for differentiating closely related *B.*
226 *cereus s.l.* genomes within STs.

227 **A *panC* Group III *B. cereus s.l.* lineage with a novel sequence type was detected in beef and**
228 **poultry products from two South African provinces.** Two *panC* Group III *B. cereus s.l.* strains
229 sequenced here were assigned to an unknown ST (S58 and S64; Figure 5 and Table 1). Despite
230 having been isolated from different products (S58 from a raw chicken thigh, S64 from RTE
231 sausage emulsion) from different establishments (retail outlets in Mpumalanga and Western Cape,
232 respectively), the strains were nearly identical (pairwise core SNP distance = 0, pairwise ANI >
233 99.84; Figures 4 and 5 and Table 3), indicating that these two strains share a common source.

234 **A *panC* Group III *B. cereus s.l.* strain with cereulide synthetase-encoding genes belongs to**
235 **the "high-risk" ST26 lineage.** One *panC* Group III *B. cereus s.l.* strain (S66) was assigned to
236 GTDB's *B. paranthracis* genomospecies and possessed cereulide synthetase-encoding genes
237 (Figure 5 and Table 2). This strain, which had been isolated from processed beef mince from a
238 processing plant in Mpumalanga, was a member of ST26 (Figure 5 and Table 2), the ST to which
239 most cereulide-producing *B. cereus s.l.* strains belong (although it should be noted that ST26

240 strains may be capable of producing enterotoxins and causing diarrheal illness, regardless of
241 whether they produce cereulide or not) (38, 40, 41). While members of ST26 are comparatively
242 closely related (>99.52 pairwise ANI), WGS was able to distinguish the South African strain
243 sequenced here from closely related ST26 strains (pairwise core SNP distance >913 relative to
244 publicly available genomes; Figure 5 and Table 3).

245 **A *panC* Group III *B. cereus s.l.* lineage assigned to ST2413 shows evidence of intercontinental**
246 **dissemination.** Two *panC* Group III *B. cereus s.l.* strains sequenced in this study (S59 and S62)
247 were assigned to ST2413 within GTDB's *B. paranthracis* species (Figure 5 and Table 3). Unlike
248 the ST26 strain, which was also assigned to GTDB's *B. paranthracis* genomospecies, neither
249 ST2413 strain possessed cereulide synthetase-encoding genes (Figure 5 and Table 2). Both strains
250 were isolated from raw chicken; however, S59 was isolated from a chicken quarter-leg that had
251 been imported from the Netherlands, and S62 from a chicken thigh sold at a retail outlet in
252 Mpumalanga (Figure 4 and Table 1). Notably, these strains were nearly identical on a genomic
253 scale (pairwise core SNP distance = 1, pairwise ANI = 100.0; Figure 5 and Table 3), despite
254 originating from different continents (i.e., Europe and Africa; Figure 4 and Table 1).

255 **A *panC* Group II *B. cereus s.l.* strain assigned to ST794 is most closely related to a food-**
256 **associated strain responsible for diarrheal illness in Norway.** One *panC* Group II *B. cereus s.l.*
257 strain was sequenced in this study (S57) and was assigned to ST794 within GTDB's *B. wiedmannii*
258 species (Figure 5 and Table 1). Strain S57 had been isolated from RTE beef biltong sold at a retail
259 outlet in Free State (Table 1). When compared to the two publicly available ST794 genomes, S57
260 shared > 99.9 ANI with both publicly available genomes and differed by 31 and 100 core SNPs
261 (relative to genomes with NCBI RefSeq Assembly Accessions GCF_900094845.1 and
262 GCF_007671965.1, respectively; Figure 5 and Table 3). Notably, beef biltong-associated strain

263 S57 sequenced here was most closely related to strain NVH 0674-98, a psychrotolerant strain that
264 had been isolated in Norway from mashed swedes, which were reportedly responsible for diarrheal
265 foodborne illness (42). The other ST794 strain, DE0555, was an environmental isolate collected
266 in 2018 from Durham, North Carolina in the United States (NCBI BioSample Accession
267 SAMN11792715).

268 **A *panC* Group V *B. cereus s.l.* strain from South African mixed-meat wors most closely**
269 **resembles a plant-associated strain from the United States.** One *panC* Group V *B. cereus s.l.*
270 strain was sequenced in this study (S72) and was assigned to ST223 within GTDB's *B. toyonensis*
271 species (Figure 6 and Tables 1-3). S72 had been isolated in a butchery in Gauteng, from a
272 processed wors composed of a mix of beef, pork, and lamb (Table 1). Strain S72 was most closely
273 related to a publicly available genome, strain AFS092321 (NCBI RefSeq Accession
274 GCF_002552615.1), which had been isolated in 2014 from a tree leaf in North Carolina, United
275 States (NCBI BioSample Accession SAMN07598612) (43); the two strains shared >99.9 ANI and
276 differed by 33 core SNPs (Figure 6 and Table 3).

277

278 **DISCUSSION**

279 ***B. cereus s.l.* lineages can be disseminated inter- and intra-nationally via the food supply**
280 **chain.** The movement of commodities (e.g., foods, animals, animal products, agricultural products,
281 consumer products) through inter- and intra-national trade can contribute to the global, regional,
282 and local dissemination of microorganisms, including pathogens (44-48). The international agro-
283 food trade specifically plays an increasingly pivotal role in providing food supplies to communities
284 around the globe but can contribute to the dissemination of foodborne pathogens (47, 48).

285 Consequently, high-resolution technologies such as WGS are being employed increasingly to
286 monitor the spread of pathogens along the food supply chain (49, 50).

287 Using WGS, we identified six South African *B. cereus s.l.* lineages, which showcased
288 evidence of inter-regional dissemination via the trade and transport of meat products (Figure 4 and
289 Table 3). Notably, one *B. cereus s.l.* lineage showed evidence of intercontinental spread between
290 Europe and Africa: a *B. cereus s.l.* ST2413 strain isolated from raw chicken sold in retail outlets
291 in Mpumalanga, South Africa was identical to a ST2413 strain isolated from chicken imported
292 from the Netherlands. We may hypothesize that the raw chicken sold in Mpumalanga originated
293 from the Netherlands, as the Netherlands was the second-largest exporter of chicken meat products
294 to South Africa in 2014-2016 (i.e., the timeframe in which the strains sequenced here were
295 collected) (51); however, this is merely a hypothesis, as we were unable to confirm this with the
296 retail outlet.

297 We additionally identified five *B. cereus s.l.* lineages, which showed evidence of inter-
298 provincial spread within South Africa: four *panC* Group IV lineages and one *panC* Group III
299 lineage were each composed of (nearly) identical strains, which were isolated from meat products
300 in two or more South African provinces (Figure 4 and Table 3). Thus, it is likely that strains within
301 each lineage shared a common source; however, a lack of additional metadata and genomes
302 prevents confirmation of this. Overall, these results showcase the utility of WGS for *B. cereus s.l.*
303 source tracking and surveillance, although future studies relying on additional genomes with
304 detailed metadata are needed.

305 **Nomenclatural frameworks that incorporate both genomic and phenotypic data can improve**
306 **strain-level *B. cereus s.l.* risk assessment.** *B. cereus s.l.* species delineation is notoriously
307 challenging, and numerous *B. cereus s.l.* taxonomic frameworks have been proposed (29).

308 Phenotypic traits historically used for *B. cereus s.l.* species assignment (e.g., motility, colony
309 morphology, ability to cause illness) have long been known to be inconsistent with genome
310 evolution (29, 31, 32). Taxonomies that rely solely on genomic data, however, may lead to
311 incorrect assumptions of a strain's pathogenic potential, particularly when taxonomic labels have
312 deep roots in medicine or industry (29, 34).

313 For example, within some ANI-based taxonomic frameworks (e.g., GTDB), the *B.*
314 *anthracis* genomospecies includes *B. cereus s.l.* strains that, historically, would be referred to as
315 "*B. cereus*" or "Group III *B. cereus*"; these strains possess phenotypic characteristics associated
316 with "*B. cereus*" (e.g., as outlined in the U.S. Food and Drug Administration's Bacteriological
317 Analytical Manual) (52, 53), and like "*B. cereus*", they are incapable of causing anthrax (34).
318 These non-anthrax-causing Group III *B. cereus s.l.* strains have been isolated from diverse
319 environments, including food (e.g., milk, egg, whites, spices), consumer products (e.g., baby
320 wipes), and soil, indicating that these organisms are not uncommon in environmental and industrial
321 settings (34). Thus, as WGS becomes more popular in clinical and industrial settings, it is possible
322 that professionals who rely solely on increasingly popular genomic methods for taxonomic
323 delineation (e.g., GTDB, ANI-based comparisons to species type strains) may incorrectly assume
324 that these strains can cause anthrax, due to the "*B. anthracis*" species labels that some taxonomic
325 classification programs produce (29, 34). Here, during routine surveillance of meat products in
326 South Africa, we identified two *panC* Group III *B. cereus s.l.* strains, which did not possess anthrax
327 toxin- or capsule-encoding genes and did not belong to the classic, "clonal" *B. anthracis* lineage
328 associated with anthrax disease (34, 54). These strains would be classified as "*B. cereus*" or "Group
329 III *B. cereus*" using standard microbiological assays (52, 53); however, these strains were assigned
330 to the "*B. anthracis*" genomospecies using GTDB and similar ANI-based methods (Table 1).

331 Referring to these strains as "*B. anthracis*" would be misleading, as they cannot cause anthrax;
332 thus, this potential miscommunication could have disastrous public health and industrial
333 consequences.

334 We additionally isolated three *B. cereus s.l.* strains from beef and poultry products, which
335 were assigned to the "*B. paranthracis*" genomospecies via GTDB and similar ANI-based methods
336 (Table 1). As noted previously, "*B. paranthracis*" was proposed as a "novel" species in 2017 (55)
337 but was later found to encompass the well-known foodborne pathogen "emetic *B. cereus*" within
338 its genomospecies boundary (29, 37, 38). One of the "*B. paranthracis*" strains isolated here indeed
339 possessed cereulide synthetase-encoding genes and belonged to ST26 (Table 2), the ST that
340 encompasses most *B. cereus s.l.* strains capable of producing emetic toxin (38). This strain thus
341 likely poses a food safety threat and would most likely be referred to as "emetic *B. cereus*" in
342 clinical or industrial settings. Referring to this strain as "*B. paranthracis*" could be misleading to
343 researchers, clinicians, and other professionals who are not well-versed and up to date in *B. cereus*
344 *s.l.* taxonomy (29, 34, 38). However, not all "*B. paranthracis*" strains are capable of producing
345 emetic toxin: here, two ST2413 strains isolated from poultry were assigned to the "*B.*
346 *paranthracis*" genomospecies but did not possess cereulide synthetase-encoding genes (Table 2),
347 indicating that these strains cannot cause emetic intoxication. Thus, differentiating potentially
348 emetic from non-emetic strains is critical for informing public health and food safety efforts.

349 Recently, we proposed a standardized nomenclatural framework for *B. cereus s.l.* (i.e., the
350 2020 GSB framework), which can utilize genomic, genetic, and/or phenotypic information for
351 taxonomic classification (33, 34). Importantly, the 2020 GSB framework relies on a standardized
352 collection of biovars (i.e., biovar Anthracis, Emeticus, and Thuringiensis), which can be applied
353 to individual *B. cereus s.l.* strains to convey phenotypes of clinical and/or industrial importance

354 (i.e., ability to produce anthrax, emetic, and insecticidal toxins, respectively) (33, 34). Within this
355 framework, the absence of the Anthracis biovar term denotes that *B. cereus s.l.* strains sequenced
356 here *cannot* produce anthrax toxin, while the presence/absence of the Emeticus biovar term
357 differentiates cereulide-producing "*B. paranthracis*" from non-cereulide-producing strains (Table
358 2). While the 2020 GSB framework provides a standardized set of *B. cereus s.l.* genomospecies
359 (Tables 1 and 2) (33, 34), researchers and other professionals may prefer to use more established
360 names for lineages (e.g., obtained via MLST, *panC* group assignment); biovar terms can thus be
361 appended to *B. cereus s.l.* lineage names (e.g., the ST26 strain sequenced here, which possesses
362 cereulide synthetase, can be referred to as "*B. cereus s.l.* ST26 biovar Emeticus"). Overall,
363 standardized taxonomic frameworks that can incorporate both genomic/genetic and phenotypic
364 information may improve strain-level risk evaluation of *B. cereus s.l.*

365 **WGS may improve *B. cereus s.l.* genomic surveillance, traceback investigations, and source**
366 **tracking.** WGS has been shown to improve surveillance and source tracking efforts for numerous
367 foodborne pathogens, including *Escherichia coli, Salmonella enterica*, and *Listeria*
368 *monocytogenes* (50, 56). While the amount of publicly available WGS data derived from members
369 of *B. cereus s.l.* is increasing (34), efforts to sequence the genomes of food-associated *B. cereus*
370 *s.l.* strains are lagging relative to other foodborne pathogens. Here, we showed that WGS can
371 conceptually be used for *B. cereus s.l.* surveillance and source tracking; however, our study is
372 limited by a lack of publicly available (i) genomic data and (ii) corresponding metadata associated
373 with *B. cereus s.l.* strains. For example, we identified two identical *B. cereus s.l.* ST2413 strains,
374 which were present in both Dutch and South African raw poultry. However, due to a lack of
375 additional publicly available ST2413 *B. cereus s.l.* genomes, we were unable to gain additional
376 insights into exactly where this lineage originated. Thus, future *B. cereus s.l.* surveillance and

377 WGS initiatives in clinical, industrial, and environmental settings are needed to improve *B. cereus*
378 *s.l.* source tracking and traceback efforts. Furthermore, it is essential that data and metadata
379 obtained in such future initiatives are made publicly available, as international sharing of WGS
380 data can decrease both the amount of time required to solve foodborne outbreaks and the public
381 health burden caused by foodborne pathogens (57). Overall, the proof-of-concept study detailed
382 here highlights the benefits of WGS for *B. cereus s.l.* surveillance and source tracking, even among
383 closely related lineages, and future studies will benefit from increasingly available publicly
384 available WGS data and metadata.
385

386 **MATERIALS AND METHODS**

387 **Isolate selection and whole-genome sequencing.** A subset of 34 isolates were selected from a
388 total of 79 *B. cereus s.l.* isolates from our previous study (26) using simple random sampling
389 without replacement (58) via random numbers generated in Microsoft Excel. Culturing and
390 genomic DNA extraction was performed as described previously (26), using the High Pure PCR
391 Template preparation kit (Roche, Germany). WGS of selected isolates was performed at the
392 Biotechnology Platform, Agricultural Research Council, Onderstepoort, South Africa. DNA
393 libraries were prepared using TruSeq and Nextera DNA library preparation kits (Illumina, San
394 Diego, CA, USA), followed by sequencing on HiSeq and MiSeq instruments (Illumina, San Diego,
395 CA, USA).

396 **Data pre-processing and quality control.** Paired-end reads associated with each of the 34 isolates
397 were supplied as input to Trimmomatic v0.38 (59), which was used to remove Illumina adapters
398 and leading and trailing low-quality/ambiguous bases (LEADING:3 and TRAILING:3,
399 respectively); reads with average per-base quality scores <15 within a 4 bp sliding window

400 (SLIDINGWINDOW:4:15) were additionally cut, and reads with length < 36 bp were removed.
401 FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess
402 the quality of the resulting trimmed paired-end reads.

403 SKESA v2.4.0 (60) was used to assemble each genome, using the trimmed paired-end
404 reads as input and default settings. SPAdes v3.13.1 (61) was additionally used to assemble each
405 genome in "careful" mode, using trimmed paired-end reads as input. QUAST v4.5 (62) was used
406 to assess the quality of each resulting assembly, and CheckM v1.1.3 (63) was used to evaluate
407 genome contamination/completeness. MultiQC v1.8 (64) was used to assess genome quality in
408 aggregate. Assemblies produced using SKESA were used in all subsequent steps, as they were of
409 higher quality based on metrics produced by QUAST (e.g., N50, number of contigs). Genomes
410 with (i) < 95% completeness (via CheckM), (ii) > 5% contamination (via CheckM), and/or (iii)
411 N50 < 20 Kbp were considered to be of low quality and were excluded (n = 4), yielding a
412 preliminary set of 30 genomes used in subsequent analyses.

413 ***In silico* typing and taxonomic characterization.** BTyper3 v3.1.1 (33) was used to characterize
414 each assembled genome (see section "Data pre-processing and quality control" above) using: (i)
415 ANI-based genomospecies, (ii) ANI-based subspecies, and (iii) biovar assignment, using a
416 standardized nomenclatural framework for *B. cereus s.l.* (34) and dependencies FastANI v1.31
417 (54) and BLAST v2.9.0 (65); (iv) ANI-based pseudo-gene flow unit assignment (33) (also via
418 FastANI); (v) *in silico* seven-gene MLST using the PubMLST *B. cereus* database (accessed 25
419 October 2020); (vi) *panC* phylogenetic group assignment, using an adjusted eight-group (Group
420 I-VIII) framework (33). All aforementioned analyses were performed using default settings, as
421 well as with virulence factor minimum coverage thresholds lowered to 0% (--virulence_coverage
422 0) to confirm virulence factor absence. Because BTyper3 uses a conservative approach for Bt toxin

423 gene detection, the command-line implementation of BtToxin_scanner v1.0
424 (BtToxin_scanner2.pl) was used to identify Bt toxin genes in each genome using default settings
425 (66).

426 Taxonomic classification of assembled genomes was additionally performed using GTDB
427 Release 05-RS95 (17 July 2020) and GTDB-Tk v. 1.3.0 (i.e., "GTDB R95"), using GTDB-Tk's
428 "classify_wf" workflow (67-69). Notably, five genomes were assigned to species outside of *B.*
429 *cereus s.l.* (i.e., three genomes classified as *Escherichia flexneri,* one as *Escherichia dysenteriae*,
430 and one as *Staphylococcus saprophyticus* via GTDB-Tk) and were thus excluded, yielding a final
431 set of 25 *B. cereus s.l.* genomes used in subsequent analyses (Table 1 and Supplemental Table S1).

432 **Phylogenomic comparison of South African *B. cereus s.l.* genomes to *B. cereus s.l.* species**
433 **type strains.** Prokka v1.14.6 (70) was used to annotate each of the 25 *B. cereus s.l.* genomes
434 sequenced in this study. Protein coding sequences derived from the type strain genomes of each
435 of the 23 validly published and effective *B. cereus s.l.* species (accessed 28 August 2021) were
436 downloaded from NCBI's RefSeq Assembly database (see Table 1 of Méndez Acevedo, et al. for
437 all type strain accessions) (71). OrthoFinder v2.5.2 (72, 73) was used to identify orthologues
438 among protein coding sequences associated with all 47 genomes (25 *B. cereus s.l.* genomes
439 sequenced in this study, plus 23 *B. cereus s.l.* species type strain genomes), using MAFFT v7.475
440 (74, 75) for sequence alignment and RAxML-NG v1.0.2 (76) for phylogeny construction.

441 The resulting amino acid sequence alignment was supplied as input to IQ-TREE v1.5.4
442 (77), which was used to construct a maximum likelihood (ML) phylogeny, using 1,000 replicates
443 of the ultrafast bootstrap approximation (78), plus the optimal amino acid substitution model
444 selected using ModelFinder (i.e., the general matrix model with empirical amino acid frequencies
445 and the FreeRate model with six categories; JTT+F+R6) (79-82). The resulting phylogeny was

446 rooted using effective species "*B. manliponensis*" (i.e., the most distant recognized member of *B.*
447 *cereus s.l.*) (83) and annotated using the bactaxR package (34) in R v4.1.2 (84).
448 **Acquisition of publicly available *B. cereus s.l.* genomes.** All assembled genomes submitted to
449 the National Center for Biotechnology Information (NCBI) RefSeq database (85, 86) as one of 23
450 validly published or effective *B. cereus s.l.* species (i.e., *albus, anthracis,* "*bingmayongensis*",
451 *cereus,* "*clarus*", *cytotoxicus, fungorum,* "*gaemokensis*", *luti,* "*manliponensis*", *mobilis, mycoides,*
452 *nitratireducens, pacificus, paramycoides, paranthracis, proteolyticus, pseudomycoides,*
453 *thuringiensis, toyonensis, tropicus, weihenstephanensis, wiedmannii*) (1, 29, 55, 71, 83, 87-99)
454 were downloaded (*n* = 2,733; accessed 20 March 2021). QUAST and CheckM were used to assess
455 the quality of each assembled genome (see section "Data pre-processing and quality control"
456 above), and BTyper3 (using default settings) and GTDB-Tk were used for typing and/or taxonomic
457 assignment as described above (see section "*In silico* typing and taxonomic characterization"). The
458 rentrez package (v1.2.3) was used to download metadata associated with each genome's
459 BioSample in R v3.6.1 (84, 100, 101). Publicly available genomes meeting all of the following
460 quality thresholds were used in subsequent analyses (*n* = 2,664; Supplemental Table S2): (i) > 95
461 % completeness (via CheckM), (ii) < 5% contamination (via CheckM), (iii) N50 > 20 Kbp (via
462 QUAST), (iv) composed of < 1,000 contigs (via QUAST).
463 **Acquisition of genomes from a study of *B. thuringiensis* outbreaks.** All sequencing reads
464 associated with isolates from a previous study of outbreaks caused by *B. thuringiensis* in France
465 (102) were downloaded from NCBI's Sequence Read Archive (SRA) database using the SRA
466 Toolkit v 2.8.2 (103, 104). Genomic data for all 171 isolates were pre-processed, assembled, and
467 taxonomically classified as described above, with genomes assembled using SKESA used in
468 subsequent steps (see sections "Data pre-processing and quality control" and "*In silico* typing and

469 taxonomic characterization" above). Four genomes did not meet the quality thresholds employed
470 in this study (see section "Acquisition of publicly available *B. cereus s.l.* genomes" above) and
471 were thus excluded, yielding 167 genomes from the study, which were used in subsequent analyses
472 (Supplemental Table S3).

473 **Acquisition of genomes derived from strains isolated in conjunction with a previous outbreak**
474 **caused by emetic *B. cereus s.l.*** The genomes of 33 *B. cereus s.l.* strains isolated in conjunction
475 with a 2016 emetic outbreak in New York State (United States) were downloaded, preprocessed,
476 and assembled as described previously (37). The quality of each of the 33 genomes was assessed
477 as described above (see section "Data pre-processing and quality control" above), and all genomes
478 underwent taxonomic classification and typing as described above (see section and "*In silico*
479 typing and taxonomic characterization" above). Two genomes did not meet the quality thresholds
480 employed in this study (see section "Acquisition of publicly available *B. cereus s.l.* genomes"
481 above) and were thus excluded, yielding 31 genomes from the study, which were used in
482 subsequent analyses (Supplemental Table S4).

483 **Within-group phylogeny construction.** The 25 *B. cereus s.l.* strains sequenced here spanned four
484 major phylogenetic groups, based on their *panC* sequence (i.e., *panC* Groups II, III, IV, and V;
485 Table 1). Thus, phylogenies were constructed using all genomes assigned to each of the following
486 major lineages: (i) *panC* Group IV (Figure 3), (ii) *panC* Groups II and III (Figure 5), and (iii) *panC*
487 Group V (Figure 6), which are equivalent to the (i) *B. cereus s.s.*, (ii) *B. mosaicus*, and (iii) *B.*
488 *toyonensis* genomospecies within the 2020 GSB taxonomic framework (33), respectively (*panC*
489 Group II and III genomes were aggregated, due to the fact that these lineages are closely related
490 and polyphyletic; Figure 5).

491 For each of the three major lineages, Prokka was used to annotate each genome; the
492 resulting GFF files associated with each genome were supplied as input to Panaroo v1.2.8 (105),
493 which was used to partition genes into core- and pan-genome orthologous gene clusters, using the
494 following parameters (all other parameters were set to their default values): (i) "strict" mode (--
495 clean-mode strict), (ii) core genome alignment using MAFFT (-a core --aligner mafft), (iii) a core
496 genome sample threshold of 95% (--core_threshold 0.95). The resulting core genome (nucleotide)
497 alignment was queried using snp-sites v2.5.1 (106), which was used to identify (i) core SNPs and
498 (ii) constant sites among all genomes in the major lineage. The resulting core SNP alignment was
499 supplied as input to IQ-TREE v1.5.4, which was used to construct a ML phylogeny using the
500 General Time-Reversible (GTR) nucleotide substitution model (107), one thousand replicates of
501 the ultrafast bootstrap approximation (78), and an ascertainment bias correction obtained using
502 constant sites output by snp-sites.

503 For each of the three major lineages, all aforementioned steps were repeated, with the
504 addition of an outgroup genome: for the *panC* Group IV phylogeny, *panC* Group III *B. anthracis*
505 str. Ames Ancestor was used as an outgroup (NCBI RefSeq Accession GCF_000008445.1); for
506 the *panC* Groups II/III and *panC* Group V phylogenies, *panC* Group IV *B. cereus* str. ATCC 14579
507 was used as an outgroup (NCBI RefSeq Accession GCF_006094295.1). Additionally, only
508 genomes with detailed metadata (i.e., a reported year of isolation, isolation source, and geographic
509 location) were included in this analysis (Supplemental Tables S1-S4). Each of the three resulting
510 phylogenies were rooted and scaled using LSD2 v1.4.2.2 (108) and the following parameters: (i)
511 tip dates corresponding to the year of isolation associated with each genome; (ii) constrained mode
512 (-c), with the root estimated using constraints on all branches (-r as); (iii) variances calculated
513 using input branch lengths (-v 1); (iv) 1,000 samples for calculating confidence intervals for

514 estimated dates (-f 1000); (v) a sequence length of 5,500,000 (-s 5500000); (vi) rooting along the
515 outgroup genome (-g -k). The resulting phylogenies were annotated using the bactaxR package in
516 R.

517 **Delineation of MLST lineages and identification of closely related and "identical" genomes.**
518 FastANI v1.31 was used to calculate ANI values between each of the 25 *B. cereus s.l.* genomes
519 sequenced in this study (i.e., as a query genome), and all genomes assigned to the *panC* Group of
520 the query genome (*panC* Groups II and III were aggregated); genomes were then grouped into
521 lineages based on STs assigned using seven-gene MLST (see section "*In silico* typing and
522 taxonomic characterization" above). For each of the resulting MLST lineages, FastANI was used
523 to calculate pairwise ANI values between all genomes within the MLST lineage (Table 3).

524 For each MLST lineage, Snippy v4.6.0 (https://github.com/tseemann/snippy) was used to
525 identify core SNPs among all genomes assigned to the respective MLST lineage, using (i) a
526 genome sequenced in this study as a reference genome (Table 3); (ii) paired-end reads associated
527 with each genome as input (for the genomes sequenced in this study, as well as the genomes from
528 the Bonis, et al. study and the New York State outbreak study) (37, 102) and/or assembled genomes
529 as input (for NCBI genomes); (iii) default settings. For MLST lineages with more than four
530 genomes (i.e., ST24, ST26, ST177, ST223, ST1578), Gubbins v3.1.3 (109) was used to remove
531 recombination, and core SNPs were identified within the resulting filtered alignment using snp-
532 sites. For all MLST lineages, pairwise core SNP distances were calculated within the MLST
533 lineage (i) among all genomes, (ii) among genomes sequenced in this study, and (iii) between
534 genomes sequenced in this study and publicly available genomes (Table 3), using the dist.gene
535 function in the ape package (110, 111) in R.

536 **Data availability.** Paired-end Illumina reads associated with the 25 *B. cereus s.l.* isolates
537 sequenced in this study have been deposited in NCBI's SRA database under BioProject
538 Accession PRJNA798224. Metadata and quality information for all genomes queried in this
539 study are available in Supplemental Table S1 (the 25 isolates sequenced in this study) and
540 Supplemental Tables S2-S4 (all publicly available genomes).
541

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575 **AUTHOR CONTRIBUTIONS**
576
577 LC designed and carried out all computational analyses. IM and LC conceptualized the study. RP
578 and IM sourced the funding for the project. RP supervised the sequencing of the isolates, while
579 MM and AA performed all culturing work and DNA extractions. LC and IM co-wrote the
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899

901 **Table 1.** *B. cereus s.l.* strains sequenced in this study (*n* = 25).

Strain	Year[a]	Meat Sample Type	Meat Category[b]	Food Animal	Establishment	Province[c]	*panC*[d] Group	MLST ST[e]	GTDB Species[f]	2020 GSB Species[g]
S57	2015	Beef Biltong	RTE	Beef	Retail Outlets	Free State	II	794	*wiedmannii*	*mosaicus*
S58	2015	Chicken Thigh	Raw Intact	Poultry	Retail Outlets	Mpumalanga	III	NA	*anthracis*[h]	*mosaicus*
S59	2016	Chicken 1/4 Leg	Raw Intact	Poultry	Cold Store	Import (NL)	III	2413	*paranthracis*	*mosaicus*
S62	2015	Chicken Thigh	Raw Intact	Poultry	Retail Outlets	Mpumalanga	III	2413	*paranthracis*	*mosaicus*
S64	2016	Sausage Emulsion	RTE	Beef	Retail Outlets	Western Cape	III	NA	*anthracis*[h]	*mosaicus*
S66	2015	Beef Mince	Processed	Beef	Processing Plant	Mpumalanga	III	26	*paranthracis*	*mosaicus* subsp. *cereus*
S51	2015	Beef Mince	Processed	Beef	Retail Outlets	Limpopo	IV	1578	*thuringiensis*	*cereus s.s.*
S53	2015	Beef Patties	Processed	Beef	Retail Outlets	Gauteng	IV	2668	*thuringiensis_S*	*cereus s.s.*
S55	2015	Beef Wors	Processed	Beef	Processing Plant	Limpopo	IV	177	*cereus*	*cereus s.s.*
S56	2015	Beef Wors	Processed	Beef	Processing Plant	North West	IV	2721	*bombysepticus*	*cereus s.s.*
S63	2015	Beef Biltong	Processed	Beef	Retail Outlets	Free State	IV	2668	*thuringiensis_S*	*cereus s.s.*
S65	2015	Beef Wors	Processed	Beef	Processing Plant	Limpopo	IV	NA	*bombysepticus*	*cereus s.s.*
S67	2016	Chicken 1/4 Leg	Raw Intact	Poultry	Cold Store	Import (NL)	IV	NA	*bombysepticus*	*cereus s.s.*
S70	2015	Beef Mince	Processed	Beef	Retail Outlets	Limpopo	IV	2668	*thuringiensis_S*	*cereus s.s.*
S77	2015	Beef Wors	Processed	Beef	Retail Outlets	Gauteng	IV	24	*cereus*	*cereus s.s.*
S78	2015	Beef Biltong	RTE	Beef	Retail Outlets	Gauteng	IV	1697	*thuringiensis_S*	*cereus s.s.*
S79	2015	Beef Biltong	RTE	Beef	Retail Outlets	North West	IV	2721	*bombysepticus*	*cereus s.s.*
S80	2015	Beef Wors	Processed	Beef	Processing Plant	North West	IV	177	*cereus*	*cereus s.s*
S81	2016	Beef Wors	Processed	Beef	Processing Plant	North West	IV	NA	*cereus*	*cereus s.s.*
New_S84	2015	Beef Patties	Processed	Beef	Retail Outlets	Free State	IV	2721	*bombysepticus*	*cereus s.s.*
S85	2015	Beef Wors	Processed	Beef	Retail Outlets	Gauteng	IV	NA	*bombysepticus*	*cereus s.s.*
S86	2015	Sausage Emulsion	RTE	Beef	Retail Outlets	Western Cape	IV	2289	*cereus*	*cereus s.s.*
S87	2015	Frankfurter	Raw Intact	Poultry	Processing Plant	Free State	IV	NA	*bombysepticus*	*cereus s.s.*
S88	2015	Beef Biltong	RTE	Beef	Retail Outlets	Limpopo	IV	NA	*cereus*	*cereus s.s.*
S72	2015	Beef-Pork-Lamb Wors	Processed	Mixed	Butchery	Gauteng	V	223	*toyonensis*	*toyonensis*

902 [a]Year of isolation
903 [b]RTE, Ready-to-Eat
904 [c]Two strains were isolated from meat products imported from the Netherlands (NL)
905 [d]*panC* phylogenetic group assigned using BTyper3
906 [e]Sequence type (ST) assigned using the PubMLST seven-gene multi-locus sequence typing (MLST) scheme for *B. cereus* and
907 BTyper3

908 [f]Genome Taxonomy Database (GTDB) species assigned using GTDB-Tk
909 [g]Species and subspecies (where applicable), assigned using the 2020 Genomospecies-Subspecies-Biovar (GSB) nomenclatural
910 framework for *B. cereus s.l.* and BTyper3 (see Table 2 for predicted biovar/phenotypic information)
911 [h]Despite GTDB assigning a species label of "*B. anthracis*", these strains cannot cause anthrax illness, nor do they belong to the classic
912 "*B. anthracis*" lineage most commonly responsible for anthrax illness (34)
913

914 **Table 2.** Predicted phenotypes of *B. cereus s.l.* strains sequenced in this study (n = 25).

Strain	*panC*[a] Group	MLST ST[b]	GTDB Species[c]	Anthrax Toxin & Capsule Genes[d,e]	Emetic Toxin *cesABCD*[d]	Diarrheal Toxin *nheABC*[d]	Diarrheal Toxin *hblACD*[d]	Diarrheal Toxin *cytK-2*[d]	Bt Toxin Genes[f]	2020 GSB Taxonomy[g,h]
S57	II	794	*wiedmannii*	-	-	+	+	+	-/-/+	
S58	III	NA	*anthracis*[i]	-	-	+	-	+	-/-/+	
S59	III	2413	*paranthracis*	-	-	+	-	-	-/-/-	*B. mosaicus*
S62	III	2413	*paranthracis*	-	-	+	-	-	-/-/+	
S64	III	NA	*anthracis*[i]	-	-	+	-	+	-/-/+	
S66	III	26	*paranthracis*	-	+	+	-	-	-/-/+	*B. mosaicus* subsp. *cereus* bv. Emeticus; *B. cereus* bv. Emeticus; *B.* Emeticus
S51	IV	1578	*thuringiensis*	-	-	+	+	+	-/+/+	
S53	IV	2668	*thuringiensis*	-	-	+	+	+	-/+/-	
S55	IV	177	*cereus*	-	-	+	+	+	-/+/+	
S56	IV	2721	*bombysepticus*	-	-	+	+	+	-/+/-	
S63	IV	2668	*thuringiensis*	-	-	+	+	+	-/+/+	
S65	IV	NA	*bombysepticus*	-	-	+	+	-	+/+/-	
S67	IV	NA	*bombysepticus*	-	-	+	+	+	-/+/+	
S70	IV	2668	*thuringiensis*	-	-	+	+	+	-/+/-	*B. cereus s.s.* bv. Thuringiensis; *B.* Thuringiensis
S77	IV	24	*cereus*	-	-	+	+	+	-/+/+	
S78	IV	1697	*thuringiensis*	-	-	+	+	-	+/+/+	
S79	IV	2721	*bombysepticus*	-	-	+	+	+	-/+/-	
S80	IV	177	*cereus*	-	-	+	+	+	-/+/+	
S81	IV	NA	*cereus*	-	-	+	+	+	-/+/-	
New_S84	IV	2721	*bombysepticus*	-	-	+	+	+	-/+/+	
S85	IV	NA	*bombysepticus*	-	-	+	+	-	+/+/+	
S86	IV	2289	*cereus*	-	-	+	+	+	- /+/+	
S87	IV	NA	*bombysepticus*	-	-	+	+	-	+/+/-	
S88	IV	NA	*cereus*	-	-	+	+	+	-/+/+	
S72	V	223	*toyonensis*	-	-	+	+	-*	-/+/+	*B. toyonensis* bv. Thuringiensis; *B.* Thuringiensis

915 [a]*panC* phylogenetic group assigned using BTyper3

916 [b]Sequence type (ST) assigned using the PubMLST seven-gene multi-locus sequence typing (MLST) scheme for *B. cereus* and
917 BTyper3
918 [c]Genome Taxonomy Database (GTDB) species assigned using GTDB-Tk
919 [d]Viurlence factors detected in each genome using BTyper3 and default thresholds (70% amino acid identity and 80% coverage);
920 presence and absence of virulence factors are denoted by "+" and "-", respectively
921 [e]All of *cya, lef, pagA* (toxin), *capABCDE*, *hasABC*, and/or *bpsXABCDEFGH* (capsules)
922 [f]*B. thuringiensis* (Bt) insecticidal toxin-encoding genes detected using (i) BTyper3 (which uses a conservative BLAST-based approach
923 and minimum default amino acid and coverage thresholds of 50 and 70%, respectively), and BtToxin_scanner2's (ii) "old" and (iii)
924 "new" gene detection approaches (detected using default thresholds), each separated by a slash ("/")
925 [g]Species, subspecies (where applicable), and biovar (where applicable) assigned using the 2020 Genomospecies-Subspecies-Biovar
926 (GSB) nomenclatural framework for *B. cereus s.l.* and BTyper3; multiple taxonomic labels are listed for strains that can be referenced
927 using shorted subspecies and/or biovar notation (separated by a semi-colon)
928 [h]Biovar Thuringiensis was assigned to genomes in which Bt insecticidal toxin-encoding genes were detected using
929 BtToxin_scanner2's "old" gene detection approach and default settings (which is less conservative than BTyper3's Bt toxin gene
930 detection approach)
931 [i]Despite GTDB assigning a species label of "*B. anthracis*", these strains cannot cause anthrax illness, nor do they belong to the classic
932 "*B. anthracis*" lineage most commonly responsible for anthrax illness (34)
933
934

935 **Table 3.** Genomic distances within multi-locus sequence typing (MLST) lineages, which contain strains sequenced in this study.

			# of Genomes				Pairwise SNP Range within MLST Lineage		
MLST Lineage[a]	**GTDB Species[b]**	**MLST ST[c]**	**Total**	**Study**	**ANI Range (Mean)[d]**	**Reference Genome[e]**	**Total (Mean)[f]**	**Within-Study (Mean)[g]**	**Study-Public (Mean)[h]**
IVA*	*bombysepticus*	NA	3	3	99.99-100.0 (100.0)	S85	0-0 (0)	0-0 (0)	NA
IVB*	*bombysepticus*	2721	3	3	99.99-100.0 (100.0)	S79	0-0 (0)	0-0 (0)	NA
IVC	*bombysepticus*	NA	1	1	NA	NA	NA	NA	NA
IVD*	*cereus*	NA	2	2	100.0-100.0 (100.0)	S81	3	3	NA
IVE	*cereus*	177	11	2	99.70-100.0 (99.92)	S80	1-531 (239.2)	16	10-531 (258.11)
IVF	*cereus*	2289	1	1	NA	NA	NA	NA	NA
IVG	*cereus*	24[i,j]	12	1	99.75-100.0 (99.91)	S77	1-1,075 (328.6)	NA	94-1,054 (290.3)
IVH*	thuringiensis_S	2668	4	3	98.90-100.0 (99.49)	S53	0-23,098 (11,549)	0-1 (0.67)	23,097- 23,098 (23,097)
IVI	thuringiensis_S	1697	1	1	NA	NA	NA	NA	NA
IVJ	*thuringiensis*	1578[j]	6	1	98.49-99.92 (98.81)	S51	2,461- 40,845	NA	12,642- 36,103 (29,344)
IIA	*wiedmannii*	794	3	1	99.95-100.0 (99.97)	S57	31-105 (78.67)	NA	31-100 (65.50)
IIIA*	*anthracis*[k]	NA	2	2	99.84-100.0 (99.92)	S64	0	0	NA
IIIB*	*paranthracis*	2413	2	2	100.0-100.0 (100.0)	S59	1	1	NA
IIIC	*paranthracis*	26	77	1	99.52-100.0 (99.84)	S66	0-12,716 (4,797)	NA	913-11,406 (3,494)
VA	*toyonensis*	223[j]	41	1	98.95-99.99 (99.51)	S72	14-10,967 (6,600)	NA	33-10,882 (4,971)

936 [a]Lineage identifiers (IDs) pertain to clusters displayed in Figures 3 (Group IV), 5 (Groups II and III), and 6 (Group V); IDs with an
937 asterisk contain two or more strains sequenced in this study, which were highly similar on a genomic scale; NA, not
938 available/applicable
939 [b]Genome Taxonomy Database (GTDB) species assigned using GTDB-Tk
940 [c]Sequence type (ST) assigned using the PubMLST seven-gene MLST scheme for *B. cereus* and BTyper3
941 [d]FastANI average nucleotide identity (ANI) range and mean values between all genomes in the lineage (excludes self-comparisons)
942 [e]Strain sequenced in this study, which was used as a reference genome for single-nucleotide polymorphism (SNP) calling among all
943 genomes within the lineage via Snippy
944 [f]Pairwise SNP distances calculated among all genomes within the lineage (excludes self-comparisons)
945 [g]Pairwise SNP distances calculated among all genomes within the lineage that were sequenced in this study (excludes self-
946 comparisons)
947 [h]Pairwise SNP distances calculated between genomes sequenced in this study and public genomes within the same lineage (excludes
948 self-comparisons)
949 [i]For polyphyletic ST24, one outlier ST24 genome was excluded (NCBI RefSeq Accession GCF_010580595.1)
950 [j]ST was polyphyletic

951 [k]Despite GTDB assigning a species label of "*B. anthracis*", these strains cannot cause anthrax illness, nor do they belong to the classic
952 "*B. anthracis*" lineage most commonly responsible for anthrax illness (34)

953 **FIGURE LEGENDS**

954 **Figure 1.** Geographic origins of *B. cereus s.l.* strains sequenced in this study (*n* = 25).

955

956 **Figure 2.** Maximum likelihood (ML) phylogeny constructed using amino acid sequences derived
957 from the 25 *B. cereus s.l.* isolate genomes sequenced in this study (tip labels colored by
958 geographic origin), plus type strain/species representative genomes of 23 published and effective
959 *B. cereus s.l.* species (gray tip labels). The heatmap to the right of the phylogeny showcases
960 species assignments obtained within the following taxonomic frameworks (from left to right): (i)
961 Genome Taxonomy Database (GTDB) Release 05-RS95 and GTDB-Tk (GTDB R95); (ii)
962 pseudo-gene flow units (GFUs) assigned using BTyper3 (Pseudo-GFU); (iii) genomospecies of
963 the 2020 standardized *B. cereus s.l.* genomospecies/subspecies/biovar (GSB) framework and
964 BTyper3 (2020 GSB); (iv) *panC* Group (I-VIII), assigned using BTyper3 (*panC* Group). The
965 phylogeny was constructed using IQ-TREE, using core orthologs identified among all genomes
966 via OrthoFinder as input. Branch lengths are reported in substitutions per site. Branch labels
967 correspond to branch support percentages obtained using 1,000 replicates of the ultrafast
968 bootstrap approximation (selected for readability). The type strain genome of effective *B. cereus*
969 *s.l.* species "*B. manliponensis*" (the most distant recognized member of *B. cereus s.l.*) was used
970 to root the phylogeny. Heatmap legends for all five taxonomies are colored by their order of
971 appearance in the heatmap, from top to bottom; white heatmap cells denote genomes that could
972 not be assigned to a taxonomic unit within a given taxonomic framework.

973

974

975 **Figure 3**. Maximum likelihood (ML) phylogeny constructed using core SNPs identified among
976 orthologous gene clusters of 1,081 *panC* Group IV *B. cereus s.l.* genomes. The phylogeny was
977 rooted using *panC* Group III *B. anthracis* str. Ames Ancestor as an outgroup (NCBI RefSeq
978 Accession GCF_000008445.1; omitted for readability). Tip label colors and clade labels denote
979 species assigned using GTDB-Tk ("GTDB Species"). The heatmap to the right of the phylogeny
980 denotes (i) whether a strain was sequenced in this study (dark pink) or not (light pink; "Study"),
981 and (ii) multi-locus sequence typing (MLST) sequence types (STs) associated with strains
982 sequenced in this study, where applicable (colored), or not (gray; "ST"). MLST lineages
983 discussed in Table 3 are annotated to the right of the heatmap ("MLST Lineage"). MLST
984 lineages with numerical superscripts contain two or more strains sequenced in this study, which
985 were highly similar on a genomic scale; these lineages are depicted in Figure 4. The tree was
986 rooted and time-scaled using LSD2, with branch lengths reported in years.
987
988 **Figure 4.** *B. cereus s.l.* multi-locus sequence typing (MLST) lineages that contained two or more
989 strains sequenced in this study, which were identical or nearly identical at the whole-genome
990 scale. Lineage names and sequence types (STs), where applicable, are shown in the top right
991 corner of each panel. Geographic and source origins of each strain are displayed in the respective
992 map.
993
994 **Figure 5.** Maximum likelihood phylogeny constructed using core SNPs identified among
995 orthologous gene clusters of 597 *panC* Group II and III *B. cereus s.l.* genomes. The phylogeny
996 was rooted using *panC* Group IV *B. cereus* str. ATCC 14579 as an outgroup (NCBI RefSeq
997 Accession GCF_006094295.1; omitted for readability). Tip label colors and clade labels denote

998 species assigned using GTDB-Tk ("GTDB Species"). The heatmap to the right of the phylogeny
999 denotes (i) whether a strain possesses anthrax toxin-encoding genes *cya, lef,* and *pagA* (dark
1000 orange) or not (light orange; "Anthrax"); (ii) whether a strain possesses cereulide synthetase-
1001 encoding *cesABCD* (dark purple) or not (light purple; "Emetic"); (iii) whether a strain belongs to
1002 *panC* Group II (blue) or III (yellow; *"panC*"*);* (iv) whether a strain was sequenced in this study
1003 (dark pink) or not (light pink; "Study"); (v) multi-locus sequence typing (MLST) sequence types
1004 (STs) associated with strains sequenced in this study, where applicable (colored), or not (gray;
1005 "ST"). MLST lineages discussed in Table 3 are annotated to the right of the heatmap ("MLST
1006 Lineage"). MLST lineages with numerical superscripts contain two or more strains sequenced in
1007 this study, which were highly similar on a genomic scale; these lineages are depicted in Figure 4.
1008 The tree was rooted and time-scaled using LSD2, with branch lengths reported in years.
1009
1010 **Figure 6.** Maximum likelihood phylogeny constructed using core SNPs identified among
1011 orthologous gene clusters of 219 *panC* Group V *B. toyonensis* genomes. The phylogeny was
1012 rooted using *panC* Group IV *B. cereus* str. ATCC 14579 as an outgroup (NCBI RefSeq
1013 Accession GCF_006094295.1; omitted for readability). The heatmap to the right of the
1014 phylogeny denotes (i) whether a strain was sequenced in this study (dark pink) or not (light pink;
1015 "Study"); (ii) multi-locus sequence typing (MLST) sequence types (STs) associated with strains
1016 sequenced in this study, where applicable (colored), or not (gray; "ST"). MLST lineages
1017 discussed in Table 3 are annotated to the right of the heatmap. The tree was rooted and time-
1018 scaled using LSD2, with branch lengths reported in years.

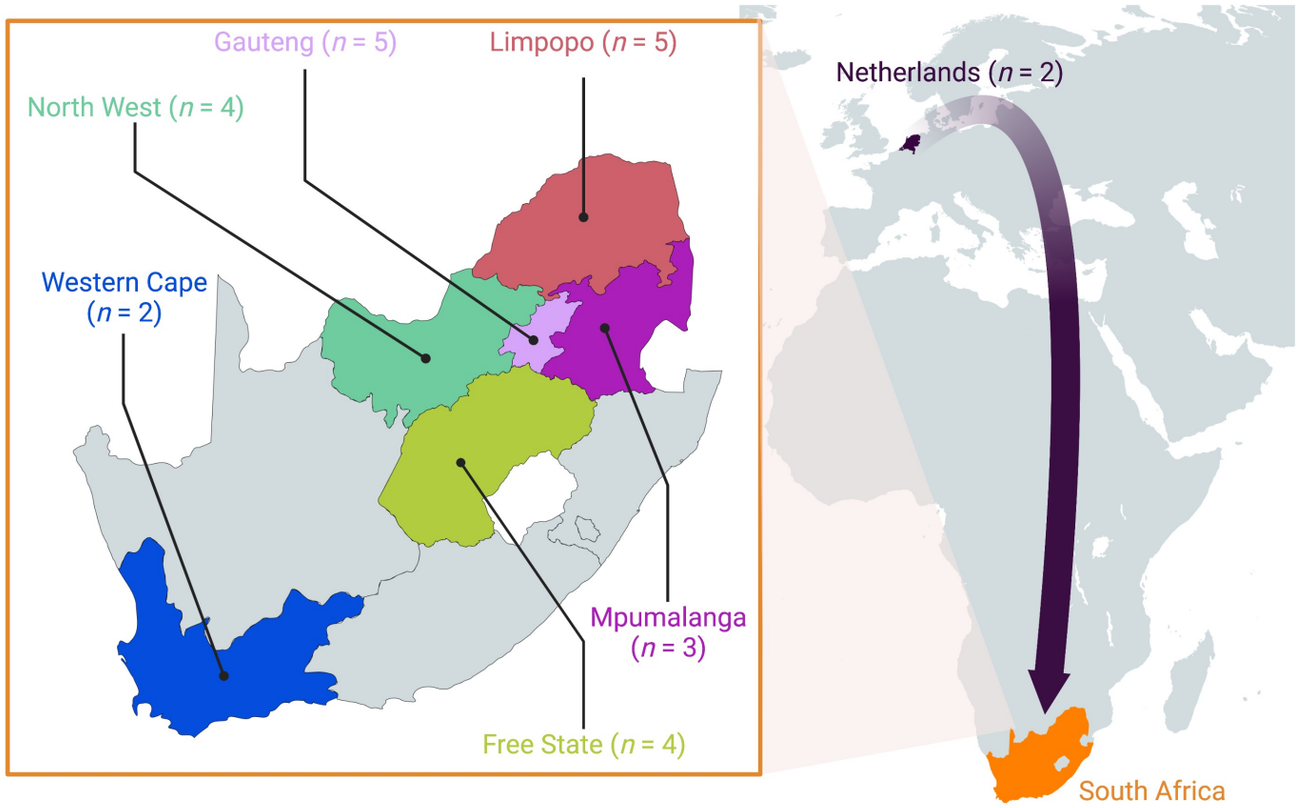
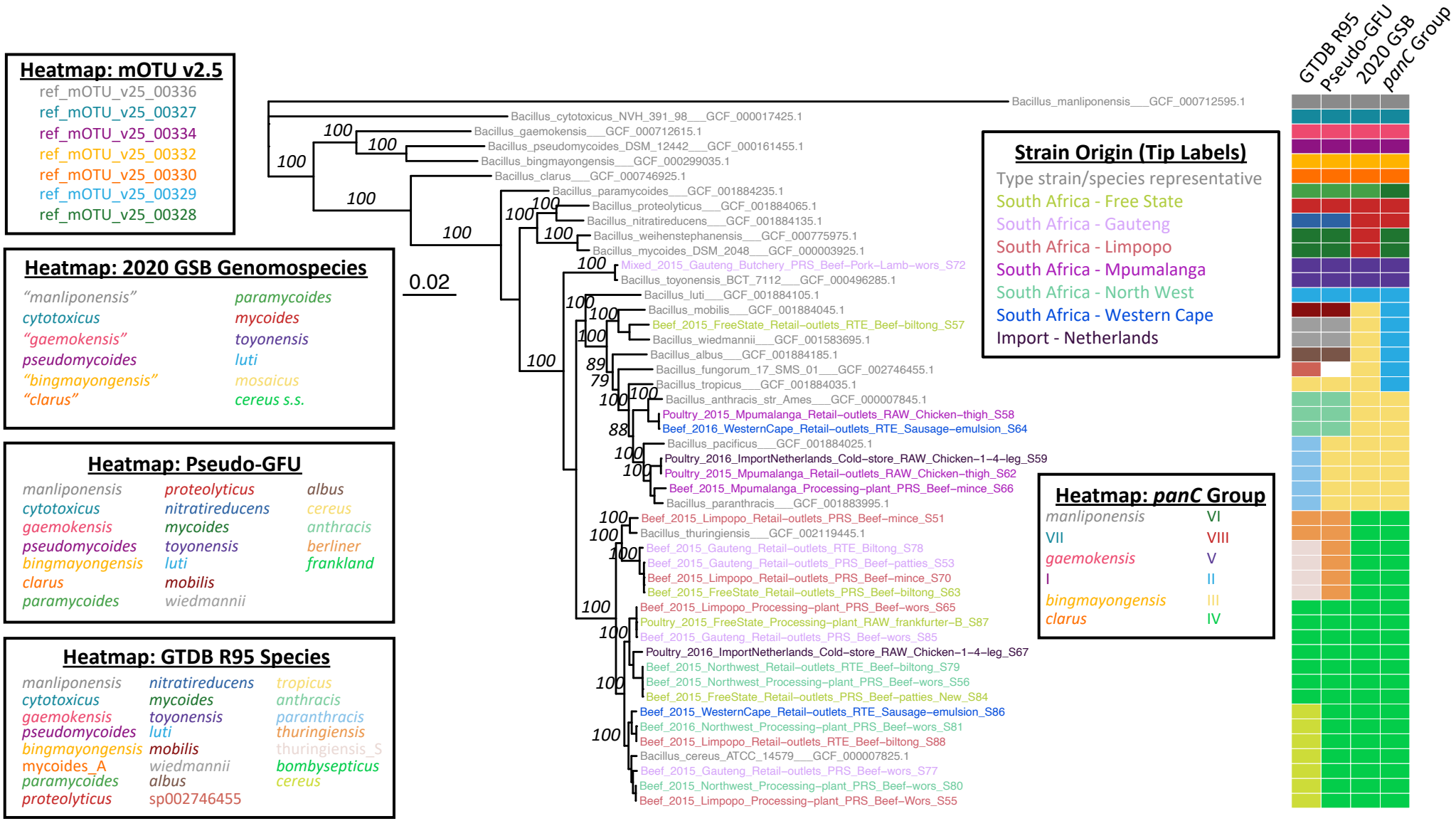


Figure 1. Geographic origins of *B. cereus s.l.* strains sequenced in this study ($n = 25$).



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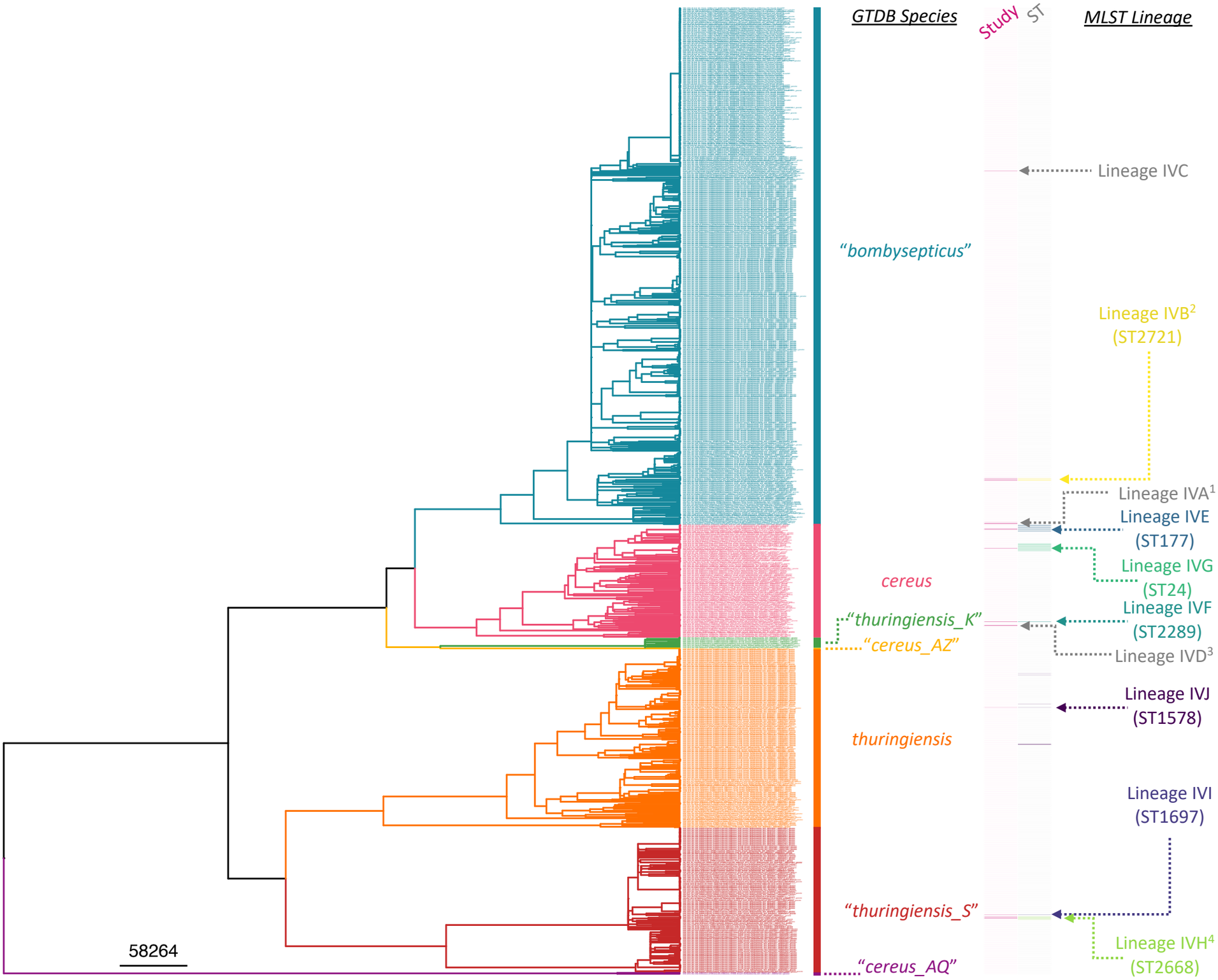

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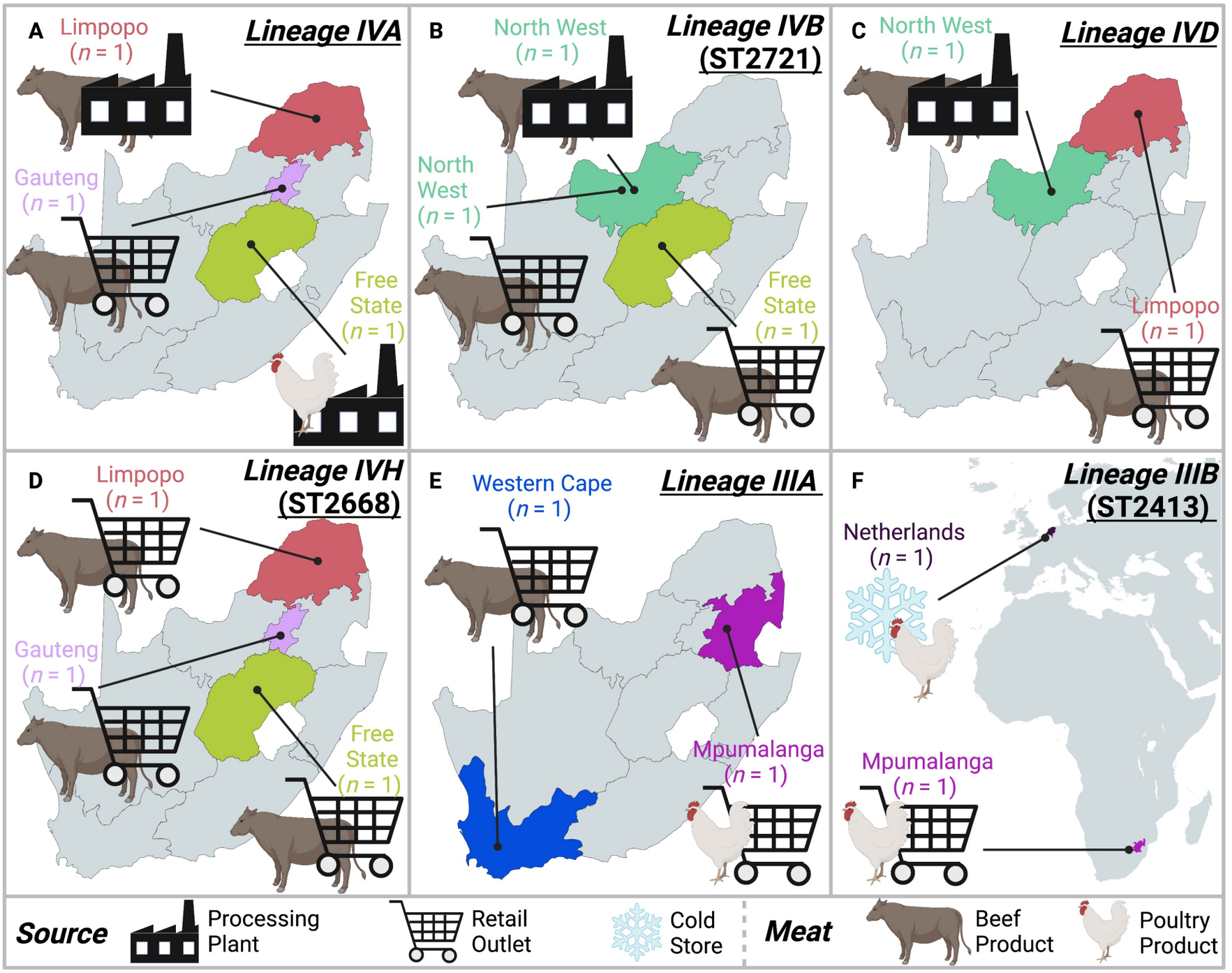

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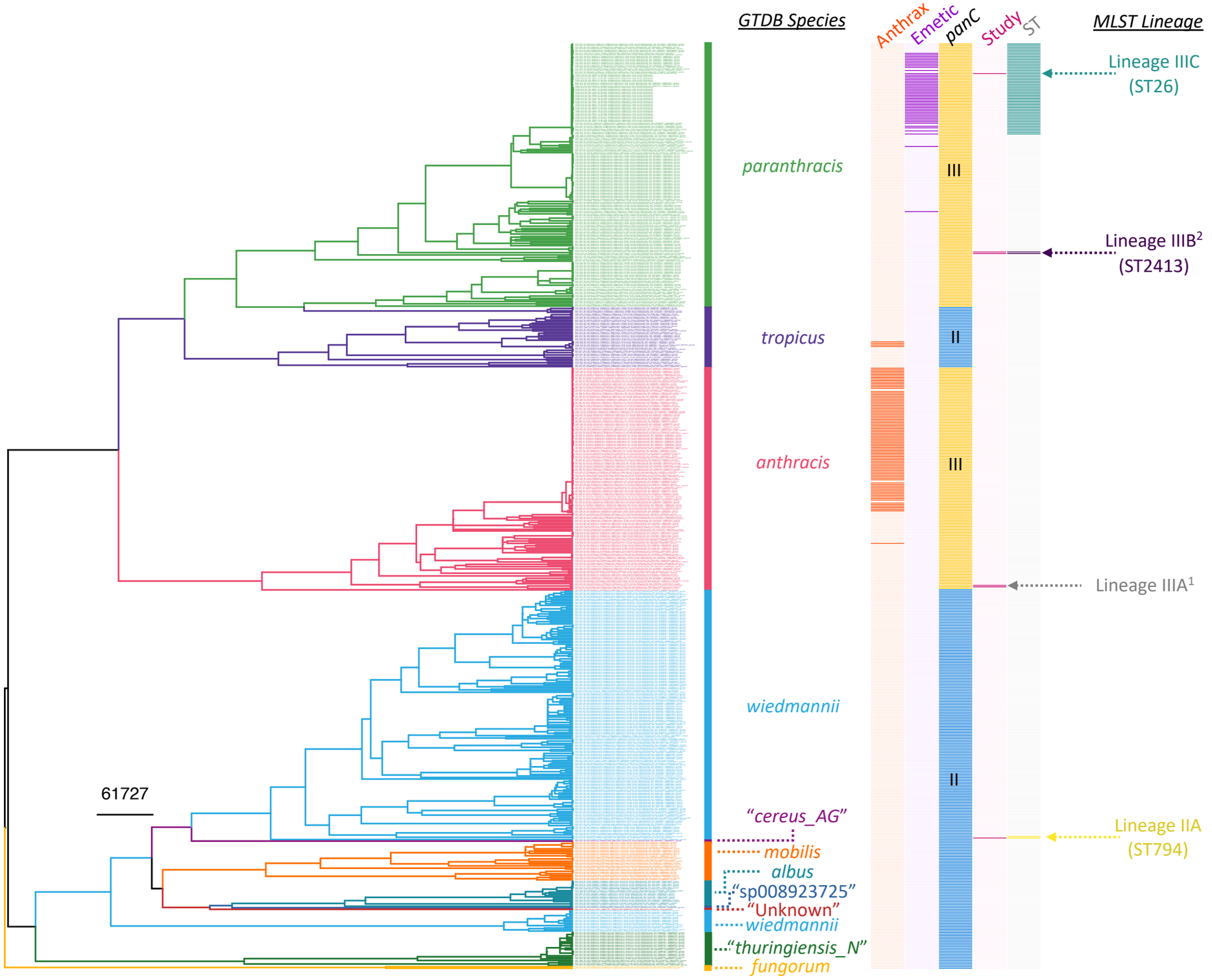

Figure 5. Maximum likelihood phylogeny constructed using core SNPs identified among orthologous gene clusters of 597 *panC* Group II and III *B. cereus s.l.* genomes. The phylogeny was rooted using *panC* Group IV *B. cereus* str. ATCC 14579 as an outgroup (NCBI RefSeq Accession GCF_006094295.1; omitted for readability). Tip label colors and clade labels denote species assigned using GTDB-Tk ("GTDB Species"). The heatmap to the right of the phylogeny denotes (i) whether a strain possesses anthrax toxin-encoding genes *cya*, *lef*, and *pagA* (dark orange) or not (light orange; "Anthrax"); (ii) whether a strain possesses cereulide synthetase-encoding *cesABCD* (dark purple) or not (light purple; "Emetic"); (iii) whether a strain belongs to *panC* Group II (blue) or III (yellow; *"panC"*); (iv) whether a strain was sequenced in this study (dark pink) or not (light pink; "Study"); (v) multi-locus sequence typing (MLST) sequence types (STs) associated with strains sequenced in this study, where applicable (colored), or not (gray; "ST"). MLST lineages discussed in Table 3 are annotated to the right of the heatmap ("MLST Lineage"). MLST lineages with numerical superscripts contain two or more strains sequenced in this study, which were highly similar on a genomic scale; these lineages are depicted in Figure 4. The tree was rooted and time-scaled using LSD2, with branch lengths reported in years.

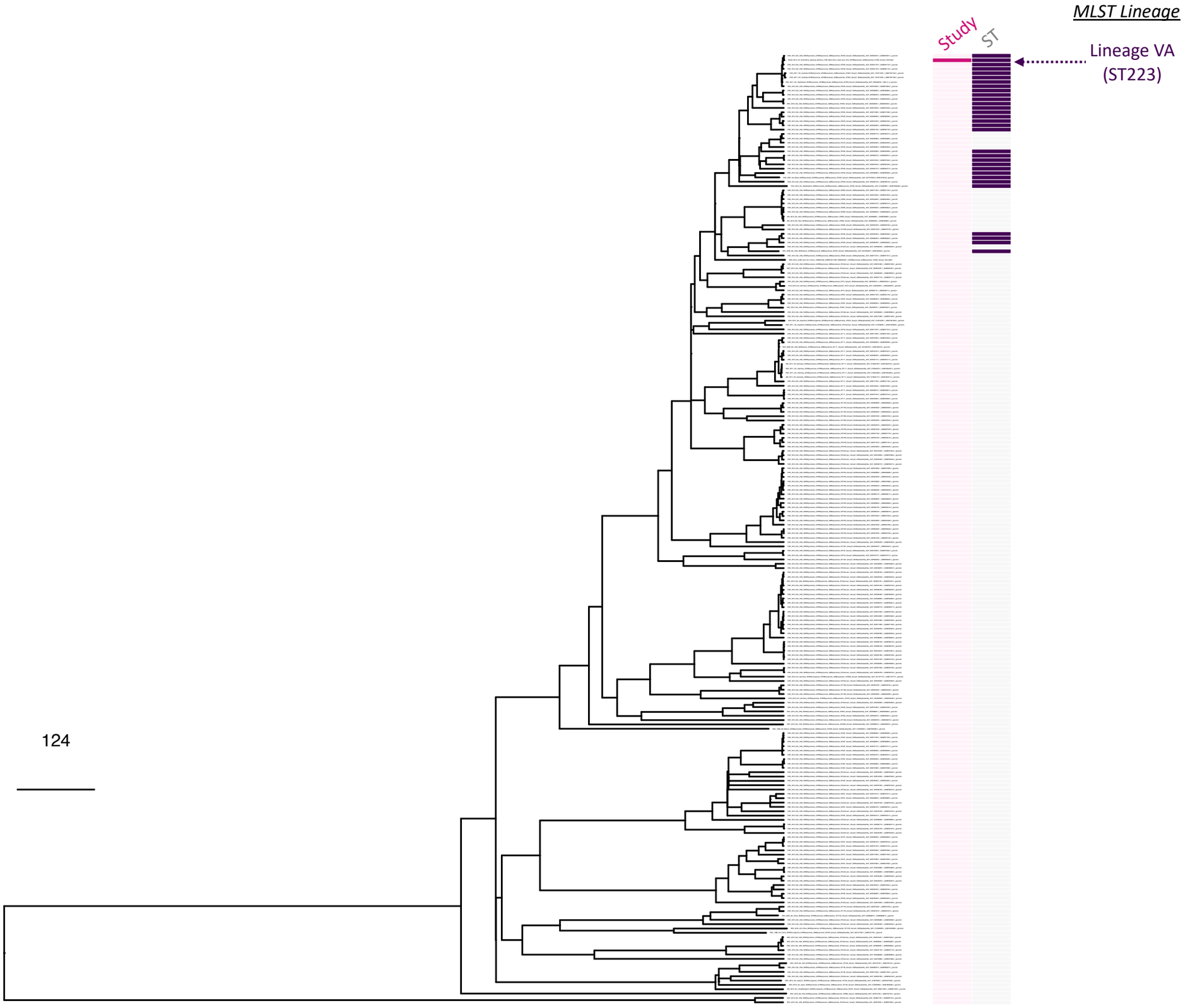

Figure 6. Maximum likelihood phylogeny constructed using core SNPs identified among orthologous gene clusters of 219 *panC* Group V *B. toyonensis* genomes. The phylogeny was rooted using *panC* Group IV *B. cereus* str. ATCC 14579 as an outgroup (NCBI RefSeq Accession GCF_006094295.1; omitted for readability). The heatmap to the right of the phylogeny denotes (i) whether a strain was sequenced in this study (dark pink) or not (light pink; "Study"); (ii) multi-locus sequence typing (MLST) sequence types (STs) associated with strains sequenced in this study, where applicable (colored), or not (gray; "ST"). MLST lineages discussed in Table 3 are annotated to the right of the heatmap. The tree was rooted and time-scaled using LSD2, with branch lengths reported in years.