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MLVA and SNP analysis identified a unique genetic cluster in Bulgarian strains

M. Antwerpen, D. Ilin, E. Georgieva, H. Meyer, E. Savov, D. Frangoulidis

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1 MLVA- and SNP analysis identified an unique genetic cluster
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4 in Bulgarian *Bacillus anthracis* strains
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16 M. Antwerpen^{1*}, D. Ilin², E. Georgieva², H. Meyer¹, E. Savov² and D. Frangoulidis¹
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19 ¹Bundeswehr Institute of Microbiology, Munich, Germany
20
21
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23 ²Department of Clinical Microbiology, Military Medical Academy, Sofia, Republic of Bulgaria
24
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29 *corresponding author: markusantwerpen@bundeswehr.org
30
31

32 Bundeswehr Institute of Microbiology
33
34
35

36 Neuherbergstr. 11
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38

39 80937 Muenchen
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17 **Abstract**

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3 18 A collection of 40 *Bacillus (B.) anthracis* strains mostly isolated from soil in Bulgaria between 1960
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5 19 and 1980 were investigated. All strains were proven to be *B. anthracis* by culture and amplification of
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7 20 a *B. anthracis*-specific chromosomal marker. PCR demonstrated that in 9 strains both virulence
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9 21 plasmids (pX01+/pX02+) and in 4 strains only one plasmid (pX02+) were present, whereas the
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11 22 majority of strains (n=27) lacked both plasmids (pX01-/pX02-). Multi-Locus-Variable Number of
12
13 23 Tandem Repeat-Analysis (MLVA) using 15 markers differentiated three genotypes. Comparison with
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15 24 typing data of more than 1.000 different *B. anthracis* strains revealed that Bulgarian genotypes
16
17 25 affiliated with the A1.a cluster and form an own unique cluster different from clusters containing
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19 26 strains isolated in geographical proximity like Turkey, Georgia, Hungary, Albania or Italy. In addition,
20
21 27 a new allele of one marker (vrrC2) was identified. Canonical Single Nucleotide Polymorphisms
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23 28 analysis allocated 31 Bulgarian strains into the A.Br.008/009 and 9 strains into the A.Br.WNA group
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25 29 which is the first description of *B. anthracis* strains of the A.Br.WNA group on the Eurasian continent.
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30 Introduction

31 *Bacillus (B.) anthracis*, a gram-positive, aerobe, spore-forming bacterium is found throughout the
32 world. It is the causative agent of anthrax, a disease of wild animals and domestic livestock,
33 predominantly cattle and goats. Anthrax is a zoonotic disease and humans can be infected by virulent
34 strains harboring the virulence plasmids pXO1 and pXO2. Depending on the route of infection,
35 pulmonary or inhalational anthrax, gastrointestinal or cutaneous anthrax can develop and, if left
36 untreated, especially in case of pulmonary anthrax, death may occur [1]. Spores of *B. anthracis* may
37 persist in the soil for decades. Recently, spores have been used in bioterrorist attacks [2-5].
38 DNA-based typing tools have been developed to evaluate the diversity of *B. anthracis* isolates from
39 different geographic areas. These studies have also been undertaken with a view to trace
40 intentionally released strains back to their origins [5-7]. Multi-Locus-Variable Number of Tandem
41 Repeat (VNTR)-Analysis (MLVA) became one of the major assays for molecular typing of *B. anthracis*
42 [8, 9]. Up-to-now, most strains are analyzed by MLVA using 15 loci, representing eleven
43 chromosomal and four plasmid loci. Further progress in differentiating strains is increasingly being
44 achieved by whole genome sequencing which efficiently identifies the rare SNPs present in *B.*
45 *anthracis* [10]. Single nucleotide polymorphisms (SNPs) were identified which define twelve major
46 clonal lineages [9]. A molecular typing strategy was presented which applies canonical-SNPs analysis
47 and VNTR analysis using 15 markers. This strategy had been used to subtype a collection of 1,044 *B.*
48 *anthracis* isolates from 42 countries and an extensive genotype data set was created. These analyses
49 divided the isolates into three previously recognized major lineages (A, B, and C), with further
50 subdivision into 12 clonal groups and, finally, 221 unique MLVA15 genotypes. By this, the global
51 spread and regional diversity of *B. anthracis* became more evident [9].
52 Whereas genotyping data from Western and Central European countries as well as from countries in
53 the South and East of the Black Sea have been published [8, 11], there are no data available from the
54 Bulgarian and Romanian area which forms a bridge between those regions.

56 In this study we analyzed 40 Bulgarian *B. anthracis* strains, mostly isolated from soil, by canSNP
57 typing and MLVA15 and demonstrate that Bulgarian strains form a unique genetic cluster.

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59 **Material and Methods**

60 *Bacterial strains*

61 A collection of 40 *B. anthracis* strains were obtained from the Historical Collection of the Medical
62 Military Academy of Sofia, Bulgaria (table 1). Bulgaria is situated in the Eastern part of Europe on the
63 Western shore of the Black Sea (figure 1). All strains have been isolated between 1960 and 1980 in
64 different districts of Bulgaria from soil with the exception of two strains originating from cattle
65 (isolate 3187 and 3186). The majority of the isolates (n=27) were collected in the South and North-
66 East of Bulgaria (regions of Jambol, Razgrad and Silistra). All strains were cultivated in a biosafety
67 level 3 (BSL) laboratory on Columbia blood agar at 37°C. Colony characteristics were in concordance
68 with a *B. anthracis*-typical phenotype, i. e. flat, dull-grey, irregular bordered, sticky colonies with no
69 hemolysis. DNA isolation was performed as previously described [12]. Strains were confirmed to be
70 *B. anthracis* based on a positive amplification of the chromosomal target *dhp61* [12]. Presence or
71 absence of plasmids pXO1 and pXO2 was determined by amplifying fragments of the target genes
72 *pagA* and *capB* using the RealTime PCR Bacillus anthracis – Detection Kit (Roche Diagnostics,
73 Penzberg, Germany) according to the protocol of the manufacturer.

74 *Multi-Locus-Variable Number of Tandem Repeat-Analysis (MLVA)*

75 MLVA was done using 15 markers according to the protocol published by van Ert *et al* [9]. Platinum
76 Taq polymerase, MgCl₂ and water were purchased from Invitrogen (Karlsruhe, Germany) and
77 Eppendorf (Hamburg, Germany), respectively. Primers were synthesized by Tib-MolBiol (Berlin,
78 Germany). Multiplex-PCR reactions were performed using the Thermocycler GenAmp PCR System
79 9700 (Perkin Elmer, Waltham, USA). PCR products were purified using the PCR Purification Kit
80 (Qiagen, Hilden, Germany) prior to loading on the ABI3100 sequencer (Applied Biosystems,

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81 Darmstadt, Germany). Fragment lengths were determined by comparison to the internal length
82 standard 1200LIZ (Applied Biosystems). Based on the size of the respective repeat unit and the
83 fragment length, a repeat copy number was calculated for each marker according to the algorithm
84 described by Vergnaud *et al* [13]. In order to compare data obtained in this study with typing data
85 described by van Ert *et al.* [9, 14] we determined the repeat copy number of each marker of their
86 strain collection after calibration with data of strains which had been analyzed in parallel in both
87 laboratories (strains A9, A22, A30, A40, A41, A66, A67) and by including the entire genome
88 sequencing data of strains Ames A2010 and Vollum (AAAC01000001.1 and NZ_AAEP00000000,
89 resp.). In some cases sequencing of amplicons was performed using the respective MLVA primers
90 and BigDye Terminator chemistry (Applied Biosystems) on a 3100 ABI sequencer (Applied
91 Biosystems). The sequence of marker vrrC2 of strain 3189 was deposited in Genbank (accession
92 number HM240285). Repeat units were identified by using the software Tandem Repeat
93 (<http://tandem.bu.edu/trf/trf.html>). Data analysis was performed using the Software Bionumerics
94 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was done using categorical
95 coefficients and similarity trees based on the Unweighted Pair Group Method with Arithmetic mean
96 (UPGMA) method.

97 *Canonical Single Nucleotide Polymorphisms (canSNP) Analysis*

98 CanSNP analysis was done according to van Ert *et al.* [9] with slightly modified probe sequences (see
99 supplemental data, table 1), To further sub-classify strains belonging to the WNA cluster SNP analysis
100 was performed according to Kenefic *et al.* [15]. All Primers and probes (supplemental data) were
101 obtained from Tib-Molbiol (Berlin, Germany). PCR was performed with a final concentration of
102 primers (0.3 μ M) and probes (0.1 μ M) using the ABI TaqMan-Mastermix (Applied Biosystems) and
103 approx. 4 ng of template DNA using the MX3000 system (Stratagene, Agilent Technologies,
104 Waldbronn, Germany). Initial denaturation at 96°C for 10 min was followed by 40 cycles, each
105 consisting of denaturation (30 sec at 96°C), annealing (30 sec at 56°C) and elongation (30 sec at
106 72°C). Data evaluation was done using the algorithm published by van Ert *et al.* [9].

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6 109 **Results**

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10 110 All Bulgarian strains could be identified as *B. anthracis* based on amplification of a *B. anthracis*-

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12 111 specific chromosomal sequence targeted by the *dhp61*-PCR assay. 9 out of 40 strains, including strain

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14 112 3187 isolated from cattle, harbored both virulence plasmids (pXO1 and pXO2, respectively), whereas

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16 113 4 strains contained plasmid pXO2 only (see table 1). The 27 others lack both plasmids.

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20 114 *MLVA-Typing*

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23 115 UPGMA cluster analysis of the 9 Bulgarian strains containing both plasmids revealed three different

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25 116 genotypes designated GT1, GT2 and GT3 (table 1 and figure 2). GT2 consisting of 2 strains and GT3

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27 117 consisting of 6 strains differ in the number of repeat copies of marker *vrnA* (10 versus 11 repeat

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29 118 copies) and marker pXO1 (7 versus 10 repeat copies). GT1 is represented by a single strain (3189)

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31 119 with an unique allele size for marker *vrnC2*. Sequencing confirmed that the fragment length of

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33 120 marker *vrnC2* is indeed much lower (333 bp) as compared to already described fragment lengths

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35 121 ranging from 458 bp to 635 bp. This allele (HM240285) is coded 6 in agreement with the currently

36

37 122 used convention in which the 532bp allele present in the sequenced Ames genome when using the

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39 123 same primer set is coded 17 [9].

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42 124 Analysis of four strains containing only plasmid pXO2 (i.e. strains 3188, 3171, 3167 and 3180) allowed

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44 125 differentiation of two genotypes designated GT2a and GT3a, respectively. These strains have

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46 126 identical repeat codes with respect to the 11 chromosomal (the “chromosomal backbone”) and the 3

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48 127 plasmid pXO2-specific markers as strains with both plasmids belonging to GT2 and GT3 (table 1).

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50 128 Likewise, all plasmid-less strains (n=27) belong to 2 genotypes designated GT2b and GT3b because

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52 129 the 11 chromosomal markers are identical as compared to strains of the genotypes GT2 and GT3

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54 130 (table 1).

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131 Clustering resulted in a similarity tree shown in figure 2. Because of identical repeat codes for the 11
132 chromosomal markers all strains clustered closely with either genotype GT2 or GT3. This variation is
133 shown in boxed profiles with either GT2, GT2a and GT2b or GT3, GT3a and GT3b, respectively (figure
134 2).

136 *MLVA-data in a global context*

137 To put typing results of Bulgarian strains in a more global context, a modUPGMA analysis of all MLVA
138 datasets (n=40) was conducted with already published datasets. To this end we included MLVA data
139 of 1,044 *B. anthracis* strains described by van Ert *et al.* and 148 strains of our in-house collection. The
140 analysis demonstrated that the three genotypes GT1, GT2 and GT3 as well as the four “sub-
141 genotypes” GT2a, GT2b, GT3a and GT3b are novel and cluster separately (figure 3). According to the
142 MLVA15 system of van Ert *et al.*, they all represent a new sub-cluster within the A1.a cluster. Strains
143 collected in countries located in the South and East of Bulgaria, such as Georgia, Turkey and Iran,
144 belong to the South Caucasian A3.a cluster [8]. In contrast, strains isolated in countries in the West of
145 Bulgaria, such as Albania, Hungary [9] and Italy [16] form a different sub-cluster within the A1.a
146 cluster (figure 3). The highest similarity of the Bulgarian cluster is seen with the WNA-cluster (figure
147 3) consisting of strains exclusively isolated in Canada and in the Northern States of the US [9, 17].
148 Both clusters differ in the repeat copy number of three markers of the “chromosomal backbone”
149 (VNTR12, VNTR19 and VNTR32). This is shown with a representative strain (A1119USA) of the WNA-
150 cluster in Figure 2.

152 *canSNP-Typing*

153 CanSNP analysis separated the Bulgarian isolates into two different phylogenetic groups. 9 out of 40
154 strains (see table 1) belong to the A.Br.WNA group, whereas 31 strains belong to the A.Br.008/009

155 group. It is interesting to note that genotypes GT2b and GT3b are found in both, the A.Br.WNA and
156 the A.Br.008/009 group. To further sub-classify the Bulgarian WNA-strains we used a SNP-based
157 strategy described by Kenefic *et al.* [15] and determined the nucleotides T, C, and A for the SNPs
158 wna237471, wna1141774 and wna3368524, respectively. Based on this unique signature the 9
159 Bulgarian WNA strains could not be classified in one of those six WNA-sub-clades described by
160 Kenefic *et al.* [15].

161

162 **Discussion**

163 The actual method used for forensic investigations of *B. anthracis* strains and isolates is based on a
164 typing strategy called “progressive hierarchical resolving assay using nucleic acids” (PHRANA)
165 introduced by Keim *et al.* [18]. This strategy proposes in a first step to determine the canSNP group
166 and in a second step to conduct MLVA.

167 *canSNP-Typing*

168 An unexpected result of the canSNP typing described here was that Bulgarian *B. anthracis* strains
169 could be divided into two distinct canSNP groups, i.e. A.Br.008/009 and A.Br.WNA. According to our
170 findings 9 out of 40 Bulgarian strains (all derived from soil) belong to canSNP group A.Br.WNA which
171 is the first description of A.Br.WNA strains on the Eurasian continent. Based on typing data described
172 in the literature for South-Caucasian strains from Georgia and Turkey [8] and for strains from
173 Kazakhstan [11] we had assumed that Bulgarian strains would belong to the canSNP group
174 A.Br.008/009. To further sub-classify WNA strains Kenefic *et al.* [15] had investigated DNA of 352
175 WNA-strains (according to van Ert *et al.*) and showed that 351 WNA-strains could be sub-classified
176 into 6 distinct WNA-sub-clades. One strain (#2000032989), however, derived from a historical
177 collection hold at CDC could not be grouped [15]. SNP analysis of the Bulgarian WNA-strains revealed
178 identical SNPs as this strain for the investigated three nucleotide positions. The explanation that
179 trading of contaminated animal products from North America has established a focus of A.Br.WNA

180 strains in Bulgaria is highly unlikely. Instead, the most parsimonious explanation is homoplasy of the
181 WNA-specific point mutation: The same A.Br.WNA-specific mutation has occurred in the Bulgarian
182 area as it had led to the formation of canSNP group A.Br.WNA in North America from its ancestor
183 group A.Br.008/009. Differences in three chromosomal MLVA-markers observed between
184 “American” representatives of the A.Br.WNA-SNP-group and Bulgarian-WNA-isolates support this
185 theory and genome sequencing of a Bulgarian strain of the WNA cluster might improve our
186 knowledge with respect to classification.

187 Homoplasy, which is very rarely seen in *B. anthracis* (1 in >25.0000 data points), has already been
188 postulated to have occurred for strain A0303: this strain has been allocated to the WNA-clade
189 although canSNP 2589947 shows the ancestral A type instead of the G expected in the WNA clade
190 [15, 19].

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192 *MLVA-Typing*

193 Historically, MLVA typing of *B. anthracis* strains started in the year 2000 and was based on eight
194 markers (6 chromosomal and one plasmid marker each) which allowed the discrimination of 89
195 different genotypes out of 400 isolates [20]. Based on this 1st generation typing system, all Bulgarian
196 strains have the identical MLVA profile with regard to the 6 chromosomal marker as strain A1119USA
197 of the WNA-cluster [9], recently published Kazakh strains [11] and five genotypes of the MLVA8
198 system [20] (GT1, 2, 4, 5, 13 and 14). A few years later two different extensions of the MLVA8 system
199 were developed by Le Fléche *et al.*, Lista *et al.* and van Ert *et al.* [9, 21, 22] and are still in use for
200 typing. The MLVA25 system described by Lista *et al.* showed a high discriminatory power especially
201 for strains of their European collection. The MLVA15 system introduced by van Ert *et al.* was applied
202 to 1,044 strains and contributed significantly to the understanding of the geographical diversity of *B.*
203 *anthracis* in the world. Analysis of the Bulgarian strains with the MLVA15 system yielded a higher
204 discriminatory power as compared to the MLVA8 system: Differences could be observed in three

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2 205 chromosomal markers (*vrpA*, *vrpC2*, *BaVNTR32*) between the Bulgarian and the WNA-cluster, whereas
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4 206 the patterns only show only up to two differences dependent on the strain investigated (figure 2).

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6 207 In a given geographic location strains of *B. anthracis* may have different origins. Either the strains are
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8 208 indeed genuine for this region or they had been imported into the region from other areas by animal
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10 209 or human factors, such as trading. Investigation of spores collected in a Belgian wool factory [23] and
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12 210 retrospective investigations of outbreaks in Switzerland in 1979 [24] show, that the outbreaks could
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14 211 be linked to spore-contaminated wool either from Kazakhstan or the Middle East. These examples
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16 212 highlight that only accurate and detailed information about an outbreak can identify its real origin.
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18 213 Typing data derived from a strain with no further information do not necessarily contribute to the
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20 214 understanding of the global diversity of anthrax.
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25 215 An interesting option to identify genuine strains of a given geographical region is to analyze soil-
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27 216 associated *B. anthracis* strains. It is known that strains may lack one or even both plasmids [25] after
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29 217 long-term-storage based on repeated passage, instead of lyophilization. We assume that with
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31 218 Bulgarian collection, a loss of plasmids had occurred over the years resulting in 27 plasmid-less
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33 219 strains out of 40 strains.
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38 220 However, using the existing algorithms based on a categorical coefficient and an “unweighted pair
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40 221 group mean arithmetic-analysis”, plasmid-less strains or strains lacking one plasmid would be
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42 222 grouped into different clusters distinct to strains with the identical chromosomal backbone and
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44 223 plasmids (data not shown).
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47 224 This results in false clustering of plasmid-less strains in the UPGMA dendrogram. Up to now this
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49 225 mathematical problem has not been discussed yet, because only a few genotypes of plasmid-less
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51 226 strains are part of analyses described , e.g. 7 GTs out of 221 GT [9]. In the context of the “global” *B.*
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53 227 *anthracis* collection of van Ert *et al.* [9] consequences of false clustering were not obvious for the
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55 228 authors. In our geographically rather limited collection consequences of false clustering stand out. By
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57 229 using the feature of the categorical coefficient clustering of Bionumerics 5.1 missing markers are
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230 ignored and the data analysis is based on chromosomal markers only. As a consequence, all strains
231 with the same chromosomal background cluster together (boxed genotypes in figure 2) and are
232 assigned as subgroups of the respective genotypes. A typing system relying on chromosomal markers
233 only seems to be useful for epidemiology and interpretation of diversity, in forensic investigations of
234 virulent *B. anthracis* strains all typing data have to be taken into account.

235 *Molecular evolution in Bulgaria*

236 Data interpretation of epidemiologically relevant MLVA profiles in combination with phylogenetically
237 relevant results of the canSNP typing revealed an interesting finding. *B. anthracis* strains with
238 genotypes GT2b and GT3b, lacking plasmid markers, are present in both canSNP groups A.Br.008/009
239 and A.Br.WNA. A similar finding has been reported by Simonson *et al.* [17]. They identified the
240 identical MLVA15 genotype for Chinese strains A0576CHI and A0731CHI, which belong to two
241 different canSNP groups (A.Br.Ames and A.Br.001/002, respectively). With regard to phylogeny this
242 points to two different populations existing in the same area. Against the background of different
243 mutation frequencies of MLVA markers and canSNPs, it has to be assumed, that first the mutation of
244 the SNP occurred resulting in two different populations in Bulgaria. Lateron, MLVA markers evolved
245 in parallel. The Bulgarian and the Chinese data demonstrate that MLVA and canSNP typing do not
246 always led to the same result. Therefore, interpretation of only MLVA-based typing results with
247 regard to phylogeny has to be performed with caution.

248 **Conclusion:**

249 In this study we demonstrate that Bulgarian *B. anthracis* strains form a geographically separated
250 cluster with 3 unique MLVA genotypes different from genotypes found in neighboring countries. In
251 addition, canSNP analysis differentiated Bulgarian *B. anthracis* strains into two groups, i.e. the
252 A.Br.008 and the A.Br.WNA group, represented by a new WNA sub-clade. This the first description of
253 A.Br.WNA strains on the Eurasian continent. The genetic typing data provided here, including data of
254 strains lacking either one or both plasmids, increase our knowledge of the diversity of *B. anthracis* in

255 the Black Sea-region and may help to extend the understanding of the global distribution and genetic
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2 256 relationship of *B. anthracis* in the world.
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9 258 **Acknowledgment**

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16
17
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19 338
20 339 Table 1: Variable Number of Tandem Repeat sizes for 40 Bulgarian *Bacillus anthracis* strains.

21
22 340 #Analysis was performed according to van Ert *et al.* [9] and the repeat codes were calculated
23
24 341 according to Vergnaud *et al.* [13].

25
26 342 *fragment length which has not been described so far for allele *vrrC2*.

27
28
29 343 § isolated from cattle

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31 344

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34 345 Figure 1: Map of Europe with Bulgaria located on the Western shore of the Black Sea.

35
36 346

37
38
39 347 Figure 2: MLVA based dendrogram of Bulgarian *Bacillus anthracis* strains. One representative strain
40
41 348 each belonging to one of the 12 canSNP group was included for comparison. Bulgarian strains cluster
42
43 349 separately from the A.Br.WNA canSNP group. Boxed MLVA patterns highlight the three Bulgarian
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45 350 genotypes with respective sub-branches.

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52 352 Figure 3: Dendrogram based on MLVA typing data of 1.191 *Bacillus anthracis* strains and isolates
53
54 353 using the modUPGMA-clustering method. MLVA clusters were assigned normal letters, geographical
55
56 354 associated clusters are in italic letters.

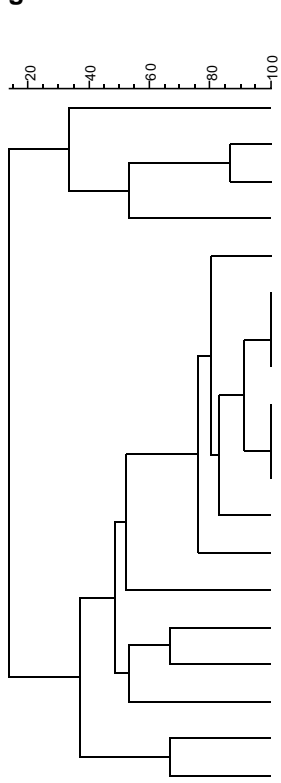
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Figure



Figure 2 Categorical MLVA15

modUPGMA



| | vtrA | vtrB1 | vtrB2 | vtrC1 | vtrC2 | CG3 | pX01 | pX02 | Bavntr12 | Bavntr16 | Bavntr17 | Bavntr19 | Bavntr23 | Bavntr32 | Bavntr35 | | |
|----|------|-------|-------|-------|-------|-----|------|------|----------|----------|----------|----------|----------|----------|----------|--------------------|--------------|
| 11 | 23 | 8 | 28 | 21 | 2 | | | 4 | 5 | 24 | 6 | 5 | 2 | 22 | 5 | A1055USA | C.Br.A1005 |
| 9 | 19 | 8 | 53 | 17 | 2 | | 7 | 10 | 5 | 24 | 3 | 5 | 3 | 11 | 3 | A0382SOA | B.Br.001/002 |
| 9 | 19 | 8 | 53 | 17 | 2 | | 6 | 9 | 5 | 24 | 3 | 5 | 3 | 11 | 3 | A0442SOA | B.Br.Kruger |
| 9 | 15 | 5 | 53 | 17 | 2 | | 12 | 6 | 5 | 22 | 3 | 5 | 3 | 14 | 5 | A0333GER | B.Br.CNEVA |
| 10 | 16 | 7 | 57 | 21 | 1 | | 8 | 7 | 6 | 21 | 4 | 5 | 4 | 9 | 4 | A1119USA | A.Br.WNA |
| 11 | 16 | 7 | 57 | 21 | 1 | | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | 3177 + 5 isolates | GT3 |
| 11 | 16 | 7 | 57 | 21 | 1 | | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | 3180 + 2 isolates | GT3a |
| 11 | 16 | 7 | 57 | 21 | 1 | | | | 6 | | | 5 | 4 | 13 | 4 | 3151 + 17 isolates | GT3b |
| 10 | 16 | 7 | 57 | 21 | 1 | | 7 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | 3187 + 1 isolate | GT2 |
| 10 | 16 | 7 | 57 | 21 | 1 | | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | 3188 | GT2a |
| 10 | 16 | 7 | 57 | 21 | 1 | | | | 6 | | | 5 | 4 | 13 | 4 | 3150 + 8 isolates | GT2b |
| 10 | 16 | 7 | 57 | 6 | 1 | | 10 | 11 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | 3189 | GT1 |
| 10 | 16 | 7 | 57 | 21 | 1 | | 7 | 7 | 6 | 20 | 4 | 4 | 4 | 13 | 4 | A0280ITA | A.Br.008/009 |
| 8 | 16 | 6 | 48 | 21 | 2 | | 10 | 8 | 6 | 20 | 4 | 5 | 2 | 13 | 4 | A0416ZIM | A.Br.Vollum |
| 8 | 16 | 7 | 57 | 17 | 2 | | 9 | 9 | 6 | 20 | 5 | 4 | 4 | 12 | 4 | A03881ARG | A.Br.003/004 |
| 10 | 16 | 7 | 57 | 17 | 2 | | 8 | 11 | 6 | 20 | 3 | 4 | 4 | 16 | 4 | A0406IND | A.Br.Aust94 |
| 10 | 16 | 7 | 57 | 17 | 2 | | 7 | 8 | 5 | 4 | 5 | 6 | 4 | 13 | 4 | A0421IDO | A.Br.005/006 |
| 10 | 16 | 7 | 53 | 17 | 2 | | 6 | 7 | 6 | 4 | 4 | 4 | 3 | 16 | 5 | A1045USA | A.Br.001/002 |
| 10 | 16 | 6 | 53 | 17 | 2 | | 7 | 9 | 6 | 20 | 4 | 4 | 4 | 16 | 5 | A2012USA | A.Br.Ames |

Figure 3
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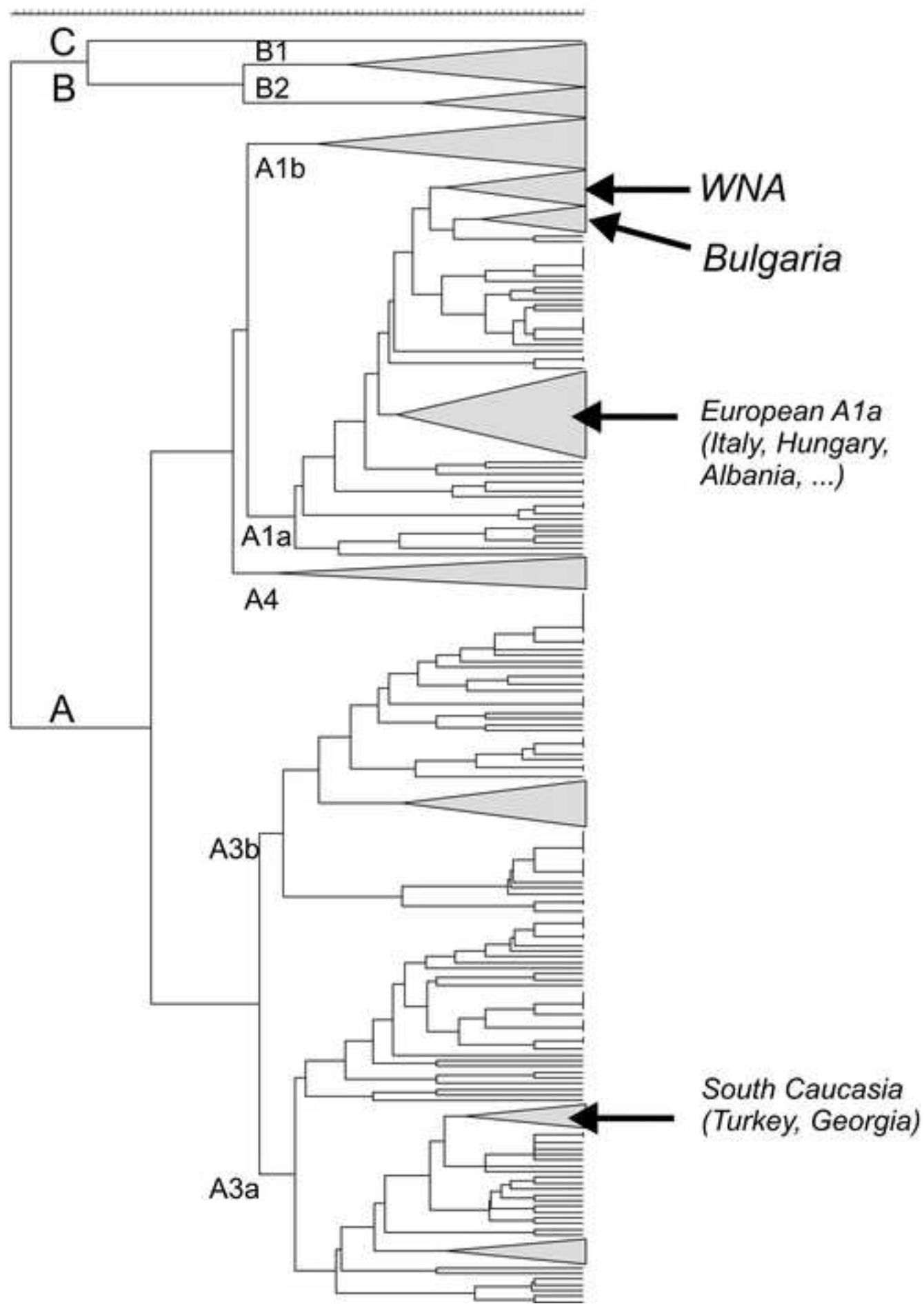


Table 1

| designation | district | canSNP | MLVA-Genotype | vrrA | vrrB1 | vrrB2 | vrrC1 | vrrC2 | CG3 | pXO1 | pXO2 | BaVNTR12 | BaVNTR16 | BaVNTR17 | BaVNTR19 | BaVNTR23 | BaVNTR32 | BaVNTR35 | |
|-------------|------------|--------------|---------------|------|-------|-------|-------|-------|-----|------|------|----------|----------|----------|----------|----------|----------|----------|---|
| 3189 | Sofia | A.Br.008/009 | GT1 | 10 | 16 | 7 | 57 | 6* | 1 | 10 | 11 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | |
| §3187 | Kjustendil | | GT2 | 10 | 16 | 7 | 57 | 21 | 1 | 7 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 | |
| 3185 | Sofia | | | | 10 | 16 | 7 | 57 | 21 | 1 | 7 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3188 | Sofia | | | GT2a | 10 | 16 | 7 | 57 | 21 | 1 | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3170 | Silistra | | | GT2b | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3179 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3177 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3168 | Tarnovo | | | GT3 | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3166 | Tarnovo | | | | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3165 | Jambol | | | | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3161 | Jambol | | | | 11 | 16 | 7 | 57 | 21 | 1 | 10 | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3171 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3180 | Silistra | | | GT3a | 11 | 16 | 7 | 57 | 21 | 1 | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3167 | Tarnovo | | | | 11 | 16 | 7 | 57 | 21 | 1 | | 8 | 6 | 21 | 4 | 5 | 4 | 13 | 4 |
| 3184 | Sofia | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3183 | Sofia | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3182 | Sofia | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3176 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3175 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3178 | Silistra | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3164 | Jambol | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3163 | Jambol | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3162 | Jambol | | | GT3b | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3181 | Sofia | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3160 | Razgrad | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3159 | Razgrad | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3158 | Razgrad | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3156 | Jambol | | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3154 | Razgrad | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3152 | Jambol | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3151 | Jambol | | | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3174 | Silistra | A.Br.WNA | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3173 | Silistra | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3172 | Silistra | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |
| 3150 | Jambol | | | GT2b | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3157 | Jambol | | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3155 | Jambol | | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3153 | Razgrad | | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| §3186 | Sofia | | | | 10 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | |
| 3169 | Tarnovo | | GT3b | 11 | 16 | 7 | 57 | 21 | 1 | | | 6 | | 5 | 4 | 13 | 4 | | |

Electronic Supplementary Material

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