Characterization by Next Generation Sequencing Reveals the Molecular Mechanisms Driving the Faster Evolutionary rate of *Cassava brown streak virus* Compared with *Ugandan cassava brown streak virus*

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Abstract

Cassava is a major staple food for 800 million people. Cassava brown streak disease (CBSD), is caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) is suppressing cassava yields in East Africa at an alarming rate. Previous studies have documented CBSV is more devastating than UCBSV. This is because CBSV is harder to breed resistance for, causes more infections and yield losses in cassava, and its species delimitation is more challenging. We set out to characterize the CBSV and UCBSV whole genomes from the 26 previously published genomes and three new from Uganda, using NGS data with the goal of uncovering genetic patterns that explain the observed biological differences. In this paper, we report phylogenetic relationships, rates of synonymous and non-synonymous substitutions, and whole genome-based evolutionary rates for CBSV and UCBSV. Using the whole genome sequences we produced the first coalescent based species tree estimation for CBSV and UCBSV which supports previously published studies pointing to multiple species of both CBSV and UCBSV. This new species framework led to the finding that CBSV has a faster rate of evolution when compared with UCBSV. The genes responsible for CBSV's rapid rate of evolution are NIa, 6K2, NIb and P1. Furthermore, we have discovered that for CBSV, rates of nonsynonomous substitutions are more predominant than synonymous substitution and occur across the entire genome. All comparative analyses between CBSV and UCBSV presented suggests CBSV is outsmarting the cassava immune system, thus is more devastating and harder to control.

Introduction

Cassava (*Manihot esculenta* Crantz) is a major staple food crop for 800 million people in over 100 tropical and sub-tropical countries [1]. In sub-Saharan Africa, it is the main source of dietary calories for approximately 300 million people [2]. The tuberous storage roots of cassava are rich in carbohydrates and can be cooked or processed for human food, animal feeds and a wide range of industrial products. The crop is relatively drought tolerant and can yield well even in less fertile soils, hence, its importance to poor families farming marginal lands [3]. Cultivation of cassava is most adversely affected by two viral diseases; cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) [4], which together were reported to cause production losses of more than US$1 billion every year [5] in Africa.

Serious yield losses due to CMD were first observed on mainland East Africa in the 1920s [6]. Recorded epidemics of CMD later occurred in the 1930s, 1940s and from 1990s to date [7,8]. By contrast, for about 70 years since it was first described [9], CBSD was confined to low altitudes (below 1000 meters above sea level) along coastal eastern Africa in Kenya, Tanzania and Mozambique. However, in the early 2000s, outbreaks of CBSD were reported over 1000 km inland at mid-altitude locations (above 1000m) in multiple countries all around Lake Victoria in Uganda [10], western Kenya [11] and northern Tanzania [4]. Where it is already established in eastern Africa, the current CBSD epidemic prevails as the main cause of losses in cassava production. Over the last 10 years, the CBSD epidemic has expanded to other countries in East and Central Africa such as Rwanda, Burundi, Congo, DR Congo and South Sudan [12-14]. This has significantly increased the risk to countries in central and west Africa which are among the world's leading cassava producers, and where CBSD does not occur.

80 CBSD is caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus*
81 (UCBSV). Both viruses are (+) ssRNA viruses in the genus *Ipomovirus* and family *Potyviridae* [15-18],
82 and are often together referred to as cassava brown streak viruses (CBSVs). The CBSVs have
83 genomic organization of 10 segments, total size of approximately 8.9 to 10.8 kb, and coding for
84 a polypeptide with about 2,900 amino acid residues [15,17,18]. The complete genome of a CBSD
85 causal virus was first sequenced in 2009 [18], and to date there are only 26 publicly available [19].
86 Currently there are two species recognized by the ICTV, but Ndunguru et al. [19] have suggested
87 further speciation in the UCBSV clade. Both viruses are transmitted in a semi-persistent manner
88 by the whitefly *Bemisia tabaci* [20] and mechanically [21]. Symptoms of CBSD on cassava vary with
89 cultivar, virus or plant age, but typically include leaf veinal choloris, brown stem lesions, as well
90 as constrictions, fissures and necrosis of the tuberous storage roots [22,23].
91
92 Although CBSD has become established in eastern Africa, there is limited knowledge on the
93 diversity of causal viruses, their distribution and evolutionary potential. Therefore, it is
94 necessary to obtain several full genome sequences of CBSD viral isolates, better understand the
95 causal viruses and design long term control approaches for the disease.
96
97 In contrast to the growing knowledge on the causal agents of CBSD, host-pathogen interactions
98 are less clear. As such, little is known about specific responses of different cassava varieties to
99 prevailing species or strains of CBSD viral pathogens. Development and dissemination of CBSD-
100 tolerant varieties has been the main means adopted for CBSD control in eastern Africa. With
101 significant efforts geared at breeding for CBSD-resistant varieties, it is of great interest to know
102 if such resistance protects cassava against one or both CBSVs. Such resistance may be
103 expressed as several related features including restricted infection, systemic spread or recovery

104 of infected plants from disease and the possibility that stem cuttings taken from these may give
105 rise to progeny that are virus-free (reversion). Recent studies have shown CBSV to be the more
106 aggressive virus, infecting both tolerant and susceptible cultivars as single or mixed infections
107 with UCBSV [15,24,25]. In contrast, tolerant varieties were infected with only CBSV, but free of
108 UCBSV, suggesting their resistance to the latter. Compared with UCBSV, CBSV isolates have
109 been reported to be more detectable, having higher infection rates by graft inoculation and
110 inducing more severe symptoms [26]. It has also been shown that plants of CBSD tolerant or
111 resistant cultivars graft-inoculated with UCBSV developed milder symptoms and a significantly
112 higher proportion of the progenies were virus-free (reverted) compared to those infected with
113 CBSV [27]. To date, the underlying reasons for this more aggressive nature of CBSV compared
114 with UCBSV are not known.
115
116 In this study, CBSV and UCBSV molecular diversity was investigated by using next generation
117 sequencing to understand new complete genomes of three isolates from Uganda. The
118 sequences obtained were analyzed to determine species composition, CBSV and UCBSV
119 evolutionary rates, role of such changes in virus-host interactions, resulting into cassava cultivar
120 susceptibility or resistance. We set out to answer the following questions:
121 1) How do the three new complete genomes from Uganda compare to those already
122 published [19]?
123 2) Are CBSV and UCBSV distinct species and is there further speciation?
124 3) Why is CBSV more aggressive and harder to breed resistance for than UCBSV?
125
126 **Results**
127 **CBSD Field Symptoms Associated with CBSV and UCBSV Isolates**

128 Categorisation of CBSD foliar symptom distribution on symptomatic plants assessed revealed
129 that the most frequently encountered type was LL - symptoms only on lower leaves (68.4%),
130 followed by SW - systemic and on the whole plant (26.3%), and SL – systemic but localized
131 (5.3%) (table 1). Based on CBSVs detected and CBSD leaf symptom severity scores for 57
132 sampled plants, whereas the majority of plants infected by UCBSV alone as determined by RT-
133 PCR had mild chlorosis (severity score 2), CBSV infections (single or mixture with UCBSV) tended
134 to have moderate to severe symptoms (scores 3-4) in same proportion to those exhibiting score
135 2 (fig. 1, table 1). Regarding the three isolates used here for whole genome sequencing, U8
136 (UCBSV) was from a plant with CBSD score 3 and LL symptom type. Both CBSV isolates (U1 and
137 U4) were from plants with severity scores 2 and 3, symptom types LL and SL, respectively.
138
139 **Next Generation Sequencing**
140 The three samples from Uganda produced raw reads ranging from 21,844,716 to 23,648,990.
141 After trimming for quality using CLCGW, these numbers were reduced to 21,582,374 to
142 23,373,606 (table 2). Following *de novo* assembly of the trimmed reads using CLCGW, the
143 numbers of contigs produced were 621-1,008. The contigs of interest from *de novo* assembly
144 were of lengths 2,214 to 8,954nt, with average coverage 24 to 366. After mapping to a
145 reference genome in Geneious, the lengths of the consensus sequences were 8,893 to 9,563
146 with average coverages of 25 to 393. The final sequences consisted of a consensus between
147 the *de novo* and the mapped consensus with lengths of 8,700 to 8,748.
148
149 **Genomic Variability and Positive Selection**
150 The CBSV genomes included in this study were more variable when compared with those of
151 UCBSV (supplementary figs. S1 and S2). Characterizing amino acid usage at each position in the

152 whole genome revealed that CBSV genomes have non-synonymous substitutions present across
153 their entire genome (fig. 2), and predominating when compared to synonymous substitutions.
154 In contrast, UCBSV had near equal non-synonymous and synonymous substitutions across the
155 entire genome. Genes in the UCBSV genomes with non-synonymous substitutions at a higher
156 frequency were; P1, NIb and HAM1 (fig. 2).
157
158 CBSV had 68 positively selected sites and 66 negatively selected sites, UCBSV had zero
159 positively selected sites (codons) and 558 negatively selected sites (table 3). Analyzed together
160 there are 3 positively selected sites and 1383 negatively selected sites. The coat protein (CP) of
161 CBSV had the highest number of positively selected sites (16) while 6K2 had zero.
162
163 **Rates of Evolution**
164 CBSV and UCBSV have different rates of evolution (table 4). We tested two hypothesis using
165 CODEML. The null hypothesis tested was CBSV and UCBSV have equal rates of evolution while
166 the null hypothesis was that CBSV and UCBSV have different rates of evolution (two omegas;
167 model = 2). The Likelihood Ratio Test was used to test for significance if the difference in
168 likelihood was greater than 3.84 (based on the Chi-squared distribution and one degree of
169 freedom) we rejected the null hypothesis that the rates between CBSV and UCBSV are equal.
170
171 CBSV whole genome sequences showed it is evolving 5 times faster than UCBSV. The genes
172 contributing to this accelerated rate of evolution for CBSV are Nla (D=29.95), followed by 6K2
173 (D=6.74), NIb (D=5.18) and P1 (4.61) (table 4 and fig. 4). The transition/transversion ratios were
174 also estimated using CODEML and show the 6K1 (19.6) and CP (13.2) genes have the highest
175 estimates while the remaining 8 genes ranged from 5.05 – 9.93.

176

177 **Species Tree Estimation - SVDQ**

178 The species phylogeny (fig. 3) shows strong support for a split into two primary viral clades, one
179 consisting of CBSV (fig. 3 clades A and B) and the other consisting of UCBSV (fig. 3 clades E-G),
180 with 100% bootstrap support separating the two clades. Figure 3 shows clades labeled A–G
181 which correspond to; 1) labels A-F from Ndunguru et al [19], and 2) a new clade G defined in this
182 study. Within the CBSV clade, there are several additional clades with 100% bootstrap support,
183 including the two new CBSV whole genomes from Uganda (U1 and U4). These are the first CBSV
184 whole genomes sequences from Uganda. The other CBSV grouping with 100% bootstrap
185 support labeled B in Figure 3 contains 4 Tanzania samples KoR6, Tan 79, Tan 19 1 and Nal 07. In
186 the UCBSV clade there are 6 nodes supported with a 100% bootstrap, including the new UCBSV
187 whole genome added from this study (U8) which is sister to Kab 07 from Uganda. In addition,
188 the CBSV clade had all samples from a given country grouping together while the UCBSV clade
189 had monophyletic clades from different countries (the multi-colored lines in fig. 3).

190

191 **Comparison of Gene Trees to Species Tree**

192 Clades A and B, which partition the CBSV isolates into two groups, are consistently present with
193 high support in all genes except HAM1 and CP (table 5). Clades D and G, which each consist of a
194 pair of UCBSV isolates, have high support across all genes, while clades C and E have relatively
195 high support across a majority of genes. Clade F is strongly supported by the CI gene, which is
196 relatively long, but is not found in the phylogenetic tree estimated for any of the other genes.

197

198 The whole genome concatenated analysis using MrBayes shows strong support (posterior
199 probability 1.0) for all clades (table 5). However, this analysis does not take into account the

200 possibility of variation in the evolutionary processes across the individual genes. The SVDQ
201 analysis, on the other hand, uses a coalescent-based method to estimate the overall species
202 tree, and properly accounts for variation in the evolutionary history for each gene. In viewing
203 the bootstrap support values for each of the clades from the SVDQ analysis, we see that the
204 level of support for each clade across the genome is more accurately represented by the
205 corresponding bootstrap proportion. For example, clade F, which was found only in the
206 phylogeny of the CI gene, shows a bootstrap proportion of 0.44 for the SVDQ analysis (as
207 compared to 1.0 for the MrBayes concatenated analysis) (table 5). Similarly, the SVDQ analysis
208 gives a bootstrap proportion of 0.87 for clade E, which showed posterior probabilities below 0.8
209 for 3 of the 10 genes, as compared to a posterior probability of 1.0 for the concatenated
210 analysis with MrBayes. All other clades are supported with bootstrap values of 1.0, consistent
211 with the MrBayes analysis.
212
213 **Sliding Window SVD Score**
214 The SVD Score Sliding Window analysis (fig. 4) shows several interesting patterns. First, note
215 that the gene boundaries track well with shifts in the magnitude of the SVD Score, indicating
216 that individual genes are subject to specific evolutionary processes that vary from gene to gene.
217 In particular, several genes show strong support for the primary CBSV/UCBSV split, as indicated
218 by their low scores, while other genes show variation from this basic process, as indicated by
219 increases in the scores. In addition, fig. 4 shows the test statistic associated with the hypothesis
220 test of a shift in the rate of evolution between the two groups, with `*' indicating that the rate
221 difference between the two groups is statistically significant. It is readily apparent from the
222 graph that genes that show strong support (low SVD Score) for the primary CBSV/UCBSV split
223 also show strong evidence for statistically significant differences in evolutionary rate. These

224 results support the overall hypothesis that certain genes in CBSV have accelerated rates of

225 evolution that contribute to the increased aggressiveness of the virus.

226
227

228
229 **Discussion**

230 In this study we analyzed the molecular mechanisms underlying the field and laboratory
231 observations that CBSV more readily infects cassava plants and tends to display severe
232 symptoms when compared with those infected with UCBSV. Our analyses included
233 characterizing three new complete CBSV (2) and UCBSV (1) genomes, which were combined
234 with the 26 previously published. Our major findings show further speciation of CBSV and
235 UCBSV, a larger genetic landscape for CBSV, including many nonsynonomous sites, and that
236 CBSV has a faster rate of evolution compared with UCBSV (table 4 and fig. 4).

237

238 **Genes with Accelerated Rates of Evolution in CBSV**

239 We have identified P1, 6K2, NIb and NIa as the genes with accelerated rates of evolution in
240 CBSV. The function of P1 is as an RNA silencing suppressor (RSS), and there is also the
241 suggestion that it may be involved in virion binding to the whitefly stylet via a "bridge"
242 formation by a virus-encoded P1 protein for both CBSV and UCBSV. 6K2 is associated with
243 cellular membrane and is responsible for systemic infection and viral long distance movement
244 [28]. The NIb encodes for a nuclear inclusion polymerase and the NIA for a nuclear inclusion
245 protease [18,29].

246

247 In Potyviruses generally, when NIa and VPg are associated together they are located in the
248 cytoplasm and nucleus of infected cells. When 6K2-VPg-NIa forms a larger product, the VPg
249 plays a role in viral RNA replication [30]. Even though VPg is not one of the genes with a higher
250 evolution rate, both 6K2 and NIa are a part of the complex which affects replication, and this
251 may go some way to explaining their apparent accelerated evolution rate. Is it possible that the

252 accelerated rates of evolution for genes involved in replication could even be a response to the
253 relatively recent interaction of the viruses and cassava? These viruses are not present in South
254 America where cassava originates so the viruses must be native to Africa. It would appear that
255 the adaptation is still occurring and the cassava immune system does not know how to fight
256 these infections yet. Cassava was introduced to East Africa in the 18th century through oceanic
257 movement. The first reports of brown streak disease in Tanzania occurred 1936 [9,13]. There has
258 been little opportunity for the co-evolution of the viruses and the host, therefore a natural
259 resistance would be hard prospect. This raises the possibility of the original host of these
260 viruses, a non-cassava host which may be harboring these viruses or the most recent common
261 ancestor of these viruses. This in turn leads us to wonder just how old these viruses and their
262 ancestors are, and the best way to answer that is to sequence more virus genomes from both
263 cassava and non-cassava hosts wherever they are found.
264

265 **How Can CBSV Still Function with Such a Large Genetic Landscape?**

266 CBSV and UCBSV have different evolutionary patterns as observed by characterizing the whole
267 genome sequences of CBSV and UCBSV separately. CBSV is genetically more diverse when
268 compared with UCBSV, as evident by the greater amino acid usage (supplementary fig. 1), the
269 faster rates of evolution across the entire genome (table 4), and greater number of
270 nonsynonymous sites across the entire genome (Figure 2). How can CBSV still function with
271 such a large genetic landscape? RNA viruses walk a very fine line of having the genetic arsenal
272 to overcome the host immune system and diverging to a point that key functions of genes are
273 lost [31]. Recent studies [32,33] have shown that viruses with a large genetic landscape adapt to host
274 changes much quicker and can overcome the host immune system faster. Viruses that occupy a
275 large portion of the possible sequence space might be less fit but they outcompete the fitter

276 strain when the host immune system shifts and hence these viruses have been described as
277 adapted to "survival of the flattest" [34,35]. This means that a virus that covers the most sequence
278 space will be able to adapt to host immune system faster than those with smaller spaces.
279 Viruses that are adapted in this category ("survival of the flattest") are going to be harder to
280 breed resistance for because the virus has a larger ability to adapt to changes. It is clear that in
281 our case, CBSV is the virus that has a larger sequence space (Supplemental Figure 1) when
282 compared to that of UCBSV, which is clearly smaller (Supplemental Figure 2). CBSV is one of the
283 RNA viruses that can be described as adapted to "survival of the flattest", while UCBSV is not.
284 Therefore, CBSV is more devastating because it has a larger genetic arsenal which it uses
285 overcome the changes breeders are introducing into cassava.
286
287 Not only are the CBSV genomes more genetically diverse, but are also characterized by a large
288 number of nonsynoymous changes in the genome (Figure 2). An excess of nonsynonymous over
289 synonymous substitutions at individual amino acid sites is signifies that positive selection has
290 affected the evolution of a protein between the extant sequences under study and their most
291 recent common ancestor [36]. Positive selection is the process by which new advantageous
292 genetic variants sweep a population and is the mechanism Darwin described to drive evolution.
293 This is further evidence that CBSV has a greater capacity to evade the cassava immune system
294 as compared with UCBSV. CBSV had 66 sites under positive selection (Table 4) while UCBSV had
295 none. The CBSV sites under positive selection are found not only in the regions that have gained
296 the most attention, CP and HAM1-like [13], but are also found in all other genes except 6K2. This
297 is further support for CBSV's ability to outsmart the cassava immune system. Every gene in the
298 CBSV genome (except 6K2) has sites under positive selection indicating effective RNA silencing
299 of the virus will need to encompass many loci.

300

301 Using computational methods combined with field observations we have concluded that CBSV
302 is more devastating than UCBSV. This assertion is also supported by two recent biological
303 studies. The first was a test of reversion in three different cassava varieties (Albert, Kaleso and
304 Kiroba) infected with CBSV and UCBSV. Reversion is a type of resistance mechanism where by
305 virus-infected plants will naturally recover from infection over time, and their progeny from
306 stem cuttings are virus-free. A reversion event infers the host immune system was able to clear
307 or restrict the virus from systemic movement. It was shown that UCBSV infected cassava had a
308 higher rate of reversion when compared to plants infected with CBSV [27] indicating the plants
309 infected with UCBSV recovered more often than those infected with CBSV. This is another line
310 of evidence that CBSV is more devastating and the cassava immune systems of the three
311 varieties tested are struggling to resist the virus.

312

313 The second study that supports the hypothesis that CBSV is more aggressive than UCBSV
314 analyzed virus-derived small RNAs within three cassava varieties (NASE 3, TME204 and 60444).
315 Plants infected with viruses are known to trigger RNAi antiviral defense that can be measured
316 by quantifying the abundance of 21-24 nucleotide (nt) segments produced by the dicer enzyme
317 [37]. Cassava varieties were infected with either CBSV or UCBSV, NGS was used to detect virus-
318 derived small RNAs [24], and the 21-24 nt dicer fragments were mapped to either CBSV or UCBSV
319 depending on which virus was used to infect the plant. The results showed that CBSV infection
320 triggered a stronger immune response as measured by greater abundance of virus derived
321 small RNA fragments across the entire CBSV genome compared with UCBSV. In addition, across
322 all three genotypes they observed that cassava grafted with CBSV-infected buds showed more
323 severe symptoms compared to UCBSV-infected plants [24]. This is further evidence that CBSV is a

324 more aggressive virus and breeding for resistance to CBSV and UCBSV will require different
325 experimental approaches.
326
327 **Implications of the Species Tree for CBSV and UCBSV**
328 We have produced the first species tree estimation of the CBSD causal virus species using whole
329 genome sequences and the coalescent based SVD Quartets species tree estimation algorithm.
330 Differences in the evolutionary history of the two viruses are seen in the branching patterns in
331 Figure 3. CBSV has diverged into two main clades A and B, while UCBSV has several well
332 supported clades but the backbone is still unresolved, indicating more sampling is needed to
333 fully understand the diversity and evolutionary history of UCBSV. The species tree (Figure 3) is
334 similar to the concatenated whole gene tree reported in Ndunguru et al. [19], except addition of
335 the clade labeled "G", and lack of support for clades E and F in the UCBSV species. It is well
336 documented that concatenating genes without using the coalescent based models can produce
337 misleading results [38,39]. In our case, only CI supports clade F, and it is also the longest gene
338 (1,883 bp), therefore swamps signals of other genes. The whole genome concatenation
339 recovers clade F with a posterior probability of 1.00 (Table 4). With regards to clade E, the
340 SVDQ tree was more reflective of the individual gene tree signal by producing a bootstrap value
341 of 0.87 versus 1.00 for the whole genome concatenated tree (Table 4). These results suggest
342 that the topology in the UCBSV species will change as more samples are added.
343
344 Our integrative approach of species tree estimation coupled with analyzing rates of evolution
345 has lead to a new framework for CBSV and UCBSV, which includes analyzing and treating these
346 two groups of viruses as separate species. Multiple putative species of both CBSV and UCBV
347 have been identified which means cassava needs to be resistant to the virus species that are

348 prevalent if farmers' fields. We argue that this genomic diversity and faster rate is what is
349 causing the breeders to struggle with breeding resistant varieties and also why the diagnostic
350 primers are not working consistently. CBSV also has more positively selected sites than UCBSV.
351 It was first thought that CBSD was restricted to the coastal areas and below 1000 m [23] but as
352 more genetic data is gathered CBSV and UCBSV are found at all elevations in many ecozones
353 throughout East Africa [4,10,13,15,19,40]. We are still in the discovery phase with CBSV and UCBSV
354 species as there are only 29 (now with the three new included here) whole genome sequences
355 and other new species of both viruses are likely to be discovered. As we move forward it is
356 important to include all known samples and use appropriate species tree estimation methods
357 such as SVDQ.
358
359 Finally, the traditional gene regions (CP and HAM1-like) used to delimit species and are the
360 targets for diagnostic primers do not recover the species tree (Table 4). We recommend
361 designing new diagnostic regions for other genes that recover the species tree and also do not
362 have an accelerated rate of molecular evolution (Figure 4), such as CI or P3 for species level
363 diagnoses. It is possible that the spread of CBSV and UCBSV could have been exacerbated
364 through dissemination of infected cuttings, as virus indexing with primers targeting CP may
365 have misleadingly returned negative results.
366
367 **Implications of the Results for Cassava Breeding**
368 During the last three decades worldwide, agricultural production has been compromised by a
369 series of epidemics caused by new variants of classic viruses that show new pathogenic and
370 epidemiological properties. An important determinant of the fitness of a virus in a given host is
371 its ability to overcome the defenses of the host. Overcoming plant resistance by changes in the

372 pathogenicity of viral populations represents a specific and important case of emergence, with
373 tremendous economic consequences since it jeopardizes the success and durability of
374 resistance factors in crops as an anti-viral control strategy. In this study, we found CBSV to be
375 more variable, have more positively selected sites and evolving five times faster than UCBSV.
376 These findings have huge implications for cassava improvement efforts in Africa where CBSV is
377 widely present. Field and laboratory results have proven CBSV to be more virulent and more
378 devastating than UCBSV. Knowledge of specific virus species an improved cassava variety is
379 resistant to will determine where to screen, multiply and deploy such varieties. Cassava
380 breeders have to take into consideration the evolutionary and biological differences between
381 CBSV and UCBSV in the breeding programs. For example, cassava breeders can breed varieties
382 that are resistant to CBSV that can be strategically deployed in areas where CBSV is more
383 prevalent, and similarly for UCBSV. Furthermore, it becomes more appropriate to always screen
384 cassava materials against CBSV as a minimum, even if UCBSV is the more prevalent virus. Such
385 strategy will in effect ensure durable resistance as opposed to the indiscriminate screening and
386 distribution of the improved CBSD resistant cassava varieties, without knowledge of the virus
387 species in the area.

388

389 **Methods**

390 **Field Plant Sample Collection**

391 Farmers' fields in Uganda with cassava plants 3-6 months old were surveyed for CBSD in 20
392 districts. In each field, cassava plants were visually assessed to confirm typical CBSD symptoms
393 on leaves and stems. CBSD leaf symptom severity was scored on a 1-5 scale [41,42]; 1 = no visible
394 symptoms, 2 = mild vein yellowing or chlorotic blotches on some leaves, 3 =
395 pronounced/extensive vein yellowing or chlorotic blotches on leaves, but no lesions or streaks

396 on stems, 4 = pronounced/extensive vein yellowing or chlorotic blotches on leaves and mild
397 lesions or streaks on stems, 5 = pronounced/extensive vein yellowing or chlorotic blotches on
398 leaves and severe lesions or streaks on stems, defoliation and dieback. CBSD symptoms were
399 also categorized based on distribution of leaf chlorosis and stem lesions on the plant; systemic
400 and on the whole plant (SW), systemic on leaf or stem parts but localized (SL), only on lower
401 leaves (LL). On selected symptomatic plants, portions of the third fully expanded leaf on a shoot
402 were picked as samples, air-dried by pressing between sheets of newsprint and stored pending
403 RNA extraction.
404
405 **RNA Extraction**
406 About 0.25 g cassava leaf samples were frozen in liquid nitrogen, then ground using a mortar
407 and pestle. 2 ml CTAB lysis buffer (2% CTAB; 100 mM Tris–HCl, pH 8.0; 20 mM EDTA; 1.4 M 134
408 NaCl; 1% sodium sulphite; 2% PVP) was added and samples homogenized. The 1 ml of the
409 homogenate was incubated at 65°C for 15 min, an equal volume of chloroform: isoamyl alcohol
410 (24:1) was added, and the sample was centrifuged for 10 min at approximately 14,500rpm.
411 800µl of the aqueous layer was transferred to a new tube with an equal volume of 4 M LiCl and
412 incubated at -20°C for 2 hrs. The samples were centrifuged for 25 min at 14,500 rpm and the
413 supernatant was poured off. The pelleted RNA was re-suspended in 200 µl TE buffer containing
414 1% SDS, 100 µl of 5M NaCl. 300 µl of ice-cold isopropanol were added and incubated at -20°C
415 for 30 min. The sample was centrifuged at 13,000 rpm for 10 min and the aqueous layer was
416 decanted and RNA pellets washed in 500 µl of 70% ethanol by centrifuging at 13,000 rpm for 5
417 min. The ethanol was decanted off and RNA pellet dried to remove residual ethanol. The RNA
418 was re-suspended in 50 µl nuclease-free water and stored at −80 °C prior to testing.
419

420 **CBSV and UCBSV Detection by RT-PCR**

421 All samples were tested for presence of CBSV and UCBSV by a two-step RT-PCR assay [43]. The
422 PCR mixture consisted of 16.0 µl nuclease free water, 2.5 µl PCR buffer, 2.5 µl $MgCl_2$ (2.5 mM),
423 0.5µl dNTPs (10 mM), 1.0 µl of each primer (10mM) [forward CBSDDF2 5'-
424 GCTMGAAATGCYGGRTAYACAA-3' and reverse CBSDDR 5'-GGATATGGAGAAAGRKCTCC-3'], 0.5
425 µl Taq DNA polymerase and 1.0 µl of cDNA. The PCR thermo profile consisted of: 94°C for 2 min
426 followed by 35cycles of 94°C (30 s), 51°C (30 s) and 72°C (30 s) for denaturation, annealing and
427 extension, respectively. PCR products were analysed by electrophoresis in a x1 TAE buffer on a
428 1.2% agarose gel, stained with ethidium bromide, visualized under UV light and photographed
429 using a digital camera.

430

431 **Sample Selection for Sequencing**

432 From the data obtained in the diagnostic tests, samples for sequencing were selected to
433 represent different geographical regions, symptom types and severities. Three samples that
434 tested positive for either CBSV (2) or UCBSV (1) were selected for this study. The two samples
435 for which presence of CBSV was confirmed (U1 and U4) had been collected from different
436 farmer's fields in Mukono district, central Uganda. The sample with UCBSV (U8) selected for
437 further analysis originated was collected from a field in Mayuge district, eastern Uganda.

438

439 **Generation of the Transcriptomes**

440 The three samples were transported to the laboratory and extracted as detailed above. Total
441 RNA was blotted on to FTA cards and later extracted using methods previously described [44].
442 Total RNA from each sample was sent to the Australian Genome Research Facility (AGRF) for

443 library preparation and barcoding before 100 bp paired-end sequencing on an Illumina
444 HiSeq2000.
445
446 **De novo Sequence Assembly and Mapping**
447 For each sample, reads were first trimmed using CLC Genomics Workbench 6.5 (CLCGW) with
448 the quality scores limit set to 0.01, maximum number of ambiguities to two and removing any
449 reads with <30 nucleotides (nt). Contigs were assembled using the *de novo* assembly function
450 of CLCGW with automatic word size, automatic bubble size, minimum contig length 500,
451 mismatch cost two, insertion cost three, deletion cost three, length fraction 0.5 and similarity
452 fraction 0.9. Contigs were sorted by length and the longest subjected to a BLAST search (blastn
453 and blastx) [45]. In addition, reads were also imported into Geneious 6.1.6 [46] and provided with
454 reference sequences obtained from Genbank (KR108828 for CBSV and KR108836 for UCBSV).
455 Mapping was performed with minimum overlap 10%, minimum overlap identity 80%, allow
456 gaps 10% and fine tuning set to iterate up to 10 times. A consensus between the contig of
457 interest from CLCGW and the consensus from mapping in Geneious was created in Geneious by
458 alignment with MAFFT [47]. Open reading frames (ORFs) were predicted and annotations made
459 using Geneious. Finalized sequences were designated as "complete" based on comparison with
460 the reference sequences used in the mapping process, and "coding complete" if some of the 5'
461 or 3' UTR was missing but the coding region was intact [48,49], and entered into the European
462 Nucleotide Archive (WEBIN ID number Hx2000053576).
463
464 **Genome Alignment and Annotation**
465 Twenty-six whole genomes (12 CBSV and 14 UCBSV) were downloaded from GenBank and
466 imported into Geneious [46], and the MAFFT plugin [47]was used to align them with the 3 new

467 whole genome sequences obtained in this study. Nucleotide alignments were translated into
468 protein using the translate align option in Geneious and then visually inspected for quality.
469 Annotations were transferred to the 3 new genomes from the 26 previously published genomes
470 using the live annotation option in Geneious.
471

472 **Characterizing the Genetic Diversity in CBSV and UCBSV Genomes**

473 CBSV and UCBSV are distinct species (Figure 2) therefore the genomes were treated separately
474 in the analyses in characterizing the genomes. Characterizing the genetic diversity of CBSV and
475 UCBSV was done using the Synonymous Non-synonymous Analysis Program (SNAP v2.1.1)
476 implemented in the Los Alamos National Laboratory HIV-sequence database
477 (http://www.hiv.lanl.gov) [50]. SNAP calculates synonymous and non-synonymous substitution
478 rates based on a set of codon-aligned nucleotide sequences. This program is based on the
479 simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide
480 substitutions of [51], and incorporating a statistic developed for computing variances and
481 covariances of dS's and dN's [52]. An application of the SNAP package in HIV-1 research has also
482 been developed [53].
483

484 **Estimating Rates of Evolution**

485 To further characterize the CBSV and UCBSV genomes, we estimated the rates of molecular
486 evolution using CODEML implemented in PAML (Phylogenetic Analysis by Maximum Likelihood)
487 [54]. PAML is a package of programs for analysis of DNA or protein sequences by using maximum
488 likelihood methods in a phylogenetic framework. The null hypothesis tested was CBSV and
489 UCBSV have equal rates of evolution (one omega; model = 0) while the alternative hypothesis
490 was that CBSV and UCBSV have different rates of evolution (two omegas; model = 2). The

491 Likelihood Ratio Test was used to test for significance if the difference in likelihood was greater
492 than 3.84 (based on the Chi-squared distribution and one degree of freedom) we then rejected
493 the null hypothesis that the rates between CBSV and UCBSV are equal. Initial analyses were
494 carried out for the entire genome and showed CBSV has a higher rate of evolution (Table 4;
495 Figure 4). To identify which gene or genes were contributing to the faster rate of evolution we
496 analyzed the individual genes separately testing the hypotheses and parameters utilized for the
497 complete genome.
498
499 **Testing for Positive Selection**
500 Sites under positive selection were identified using SLAC [55] implemented on the
501 http://www.datamonkey.org web server [56]. The settings used to run SLAC were as follows: the
502 best fitting model (GTR) was specified global dN/dS value was estimated and the significance
503 level was set to 0.01.
504
505 **Gene Tree Estimation**
506 Individual gene trees were estimated using MrBayes 3.2.1 [57] run in parallel on Magnus (Pawsey
507 Supercomputing Centre, Perth, Western Australia) utilizing the BEAGLE library [58]. MrBayes 3.2.1
508 was run utilizing 4 chains for 30 million generations and trees were sampled every 1000
509 generations. All runs reached a plateau in likelihood score, which was indicated by the
510 standard deviation of split frequencies (0.0015), and the potential scale reduction factor (PSRF)
511 was close to one, indicating the MCMC chains converged.
512
513 **Species Tree Estimation**

514 The SVDQ method [59] implemented in PAUP* [60] was used to analyze the whole-genome data.
515 This method allows analysis of multi-locus data in a coalescent framework that allows for
516 variation in the phylogenetic histories of individual genes. The method was run with all possible
517 quartets (23,751) sampled in each of 100 bootstrap replicates, and the consensus across all
518 bootstrap replicates was used as the estimate of the species tree. Bootstrap support values for
519 each node were used to quantify uncertainty in the species tree estimate. The entire analysis
520 took approximately 2.5 minutes on a MacBook Pro running OSX 10.11.2 with a 2.2 GHz Intel
521 Core i7 processor.
522
523 **Comparison of Gene Trees to Species Tree**
524 We compared the single-gene phylogenies constructed using MrBayes with the overall species
525 tree phylogeny estimated using SVDQ and the concatenated phylogeny estimated by MrBayes.
526 For each tree, we evaluated presence or absence of the clades identified by Ndunguru et al.[19]
527 labeled A-F in Figure 3. We identified an additional clade (clade G, Figure 3) that we noticed to
528 be consistently present across genes and methods. For each of these clades present in a
529 particular tree, we recorded the posterior probability (for trees constructed by MrBayes) or the
530 bootstrap proportion (for the tree estimated by SVDQ) in Table 5.
531
532 **Sliding Window SVD Score**
533 The SVD Score [61] was used to quantify support for two viral clades for portions of the genome in
534 a sliding window analysis. Briefly, the SVD Score measures the extent to which the data support
535 a phylogenetic "split" – a division of the taxa into two groups with specified group membership.
536 Low values of the SVD Score indicate strong support for the split of interest, while larger values
537 indicate *either* a lack of support for the split or a shift in the underlying evolutionary process

538 (see Allman et al. (2016) for details and examples). We computed the SVD Score with the split
539 defined by CBSV vs. UCBSV across the genome in windows of 500 bp, sliding in increments of
540 100 bp, and plotted the resulting SVD Scores across the genome, with boundaries between
541 genes marked with vertical lines. The computations took less than one minute on a MacBook
542 Pro running OSX 10.11.2 with a 2.2 GHz Intel Core i7 processor.
543
544

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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719 Table 1: CBSD leaf symptom severities and types on plants infected by *Cassava brown streak*
720 *virus* and *Ugandan cassava brown streak virus*
721

Virus species	Number of plants with CBSD foliar symptom severity score[1]				Number of plants with CBSD foliar symptom type[2]		
	2	3	4	5	SW	LL	SL
CBSV	5	7	1	0	3	8	2
UCBSV	24	5	0	0	7	21	1
CBSV + UCBSV	9	6	0	0	5	10	0
Total	38	18	1	0	15	39	3
Percentage	66.7	31.6	1.7	0.0	26.3	68.4	5.3

722
723
724 [1]Foliar CBSD symptom severity score based on 1-5 scale; 1 = no visible symptoms, 2 = mild vein
725 yellowing or chlorotic blotches on some leaves, 3 = pronounced/extensive vein yellowing
726 or chlorotic blotches on leaves, but no lesions or streaks on stems, 4 = pronounced/extensive
727 vein yellowing or chlorotic blotches on leaves and mild lesions or streaks on stems, 5 =
728 pronounced/extensive vein yellowing or chlorotic blotches on leaves and severe lesions or
729 streaks on stems, defoliation and dieback.
730 [2]Types of foliar CBSD symptoms based on distribution of leaf chlorosis and stem lesions on the
731 plant; systemic and on the whole plant (SW), systemic on leaf or stem parts but localized (SL),
732 only on lower leaves (LL).
733

4 Table 2. Next generation sequencing data for samples from cassava brown streak disease symptomatic plants collected in Uganda
5

Sample ID	Accession number	Virus	No. of reads obtained	No. of reads after trimming	Number of contigs produced (CLC)	Contig length (CLCGW, nt)	Average coverage (CLCGW)	Number of reads mapped to contig of interest	Ref seq. used for mapping	Length of consensus sequence from mapping (Geneious)	No. reads mapped to ref. sequence	Average coverage (Geneious)	Final sequence length (Coding region only)
U1		*CBSV*	23,335,344	23,053,082	726	3919, 2214	31, 24	1264, 549	KR108828	8,893	2,233	25	8,748
U4		*CBSV*	21,844,716	21,582,374	621	8,949	255	23,658	KR108828	8,949	22,987	256	8,748
U8		*UCBSV*	23,648,990	23,373,606	1,008	8,954	366	33,778	KR108836	9,563	178,117	393	8,700

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7

738 Table 3. *Cassava brown streak virus* (CBSV) amino acid (AA) sites under positive selection
739 (analyses method: SLAC Hy-Phy). There were no sites under positive selection for
740 *Ugandan cassava brown streak virus* (UCBSV).
741
742

Gene	CBSV AA site under positive selection
P1	44, 46, 50, 174, 224, 230, 250, 283, 288, 349, 358
P3	415, 455, 467, 472, 499, 525, 618
6K1	658, 678
CI	735, 761, 820, 827, 848, 852, 894, 935, 1218
VPg	1465
NIa	1620, 1645, 1704, 1754, 1785
Nib	1879, 1880, 1890, 1907, 1929, 2109, 2145, 2156, 2161, 2285
HAM1	2320, 2345, 2404, 2432, 2453, 2475, 2519
CP	2550, 2555, 2588, 2611, 2631, 2635, 2640, 2659, 2728, 2745, 2783, 2818, 2843, 2860, 2877, 2884

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758 Table 4. Rates of evolution tested using CODEML implemented in PAML. H_O was CBSV and
759 UCBSV have equal rates of evolution (one omega; model = 0), while H_1 was that CBSV
760 and UCBSV have different rates of evolution (two omegas; model = 2).
761

Gene	Assumptions	K (ts/tv rate ratio)	w (omega Dn/Ds) 0	w (omega Dn/Ds)1	Likelihood Ratio Test (if greater than 3.84 reject H_0) H_0=equal rates
WGS	UCBSV and CBSV equal rates	5.90944	0.06358		
	UCBSV and CBSV different rates	5.9598	0.05518	**0.07622**	26.29*
P1	UCBSV and CBSV equal rates	5.07336	0.10394		
	UCBSV and CBSV different rates	5.05456	0.09047	**0.12203**	4.61*
P3	UCBSV and CBSV equal rates	5.17197	0.08635		
	UCBSV and CBSV different rates	5.20559	0.0764	**0.10198**	2.22
6K1	UCBSV and CBSV equal rates	19.54143	0.00969		
	UCBSV and CBSV different rates	19.69676	**0.01527**	0.00316	3.01
CI	UCBSV and CBSV equal rates	9.9388	0.01722		
	UCBSV and CBSV different rates	8.06276	0.0155	**0.01977**	0.73
6K2	UCBSV and CBSV equal rates	8.40649	0.04684		
	UCBSV and CBSV different rates	8.82738	0.02354	**0.11057**	6.74*
VPg	UCBSV and CBSV equal rates	5.852	0.05759		
	UCBSV and CBSV different rates	5.87009	0.054	**0.06323**	0.29
NIa	UCBSV and CBSV equal rates	8.01105	0.02932		
	UCBSV and CBSV different rates	8.68283	0.01408	**0.06719**	29.95*
NIb	UCBSV and CBSV equal rates	6.07872	0.05329		
	UCBSV and CBSV different rates	6.14047	0.0452	**0.06508**	5.18*
HAM1	UCBSV and CBSV equal rates	7.25177	0.16144		
	UCBSV and CBSV different rates	7.2343	**0.17929**	0.14007	2.23
CP	UCBSV and CBSV equal rates	13.09297	0.06075		
	UCBSV and CBSV different rates	13.26752	0.05606	**0.07155**	1.29
			faster rate UCBSV	**faster rate CBSV**	* Rates are different

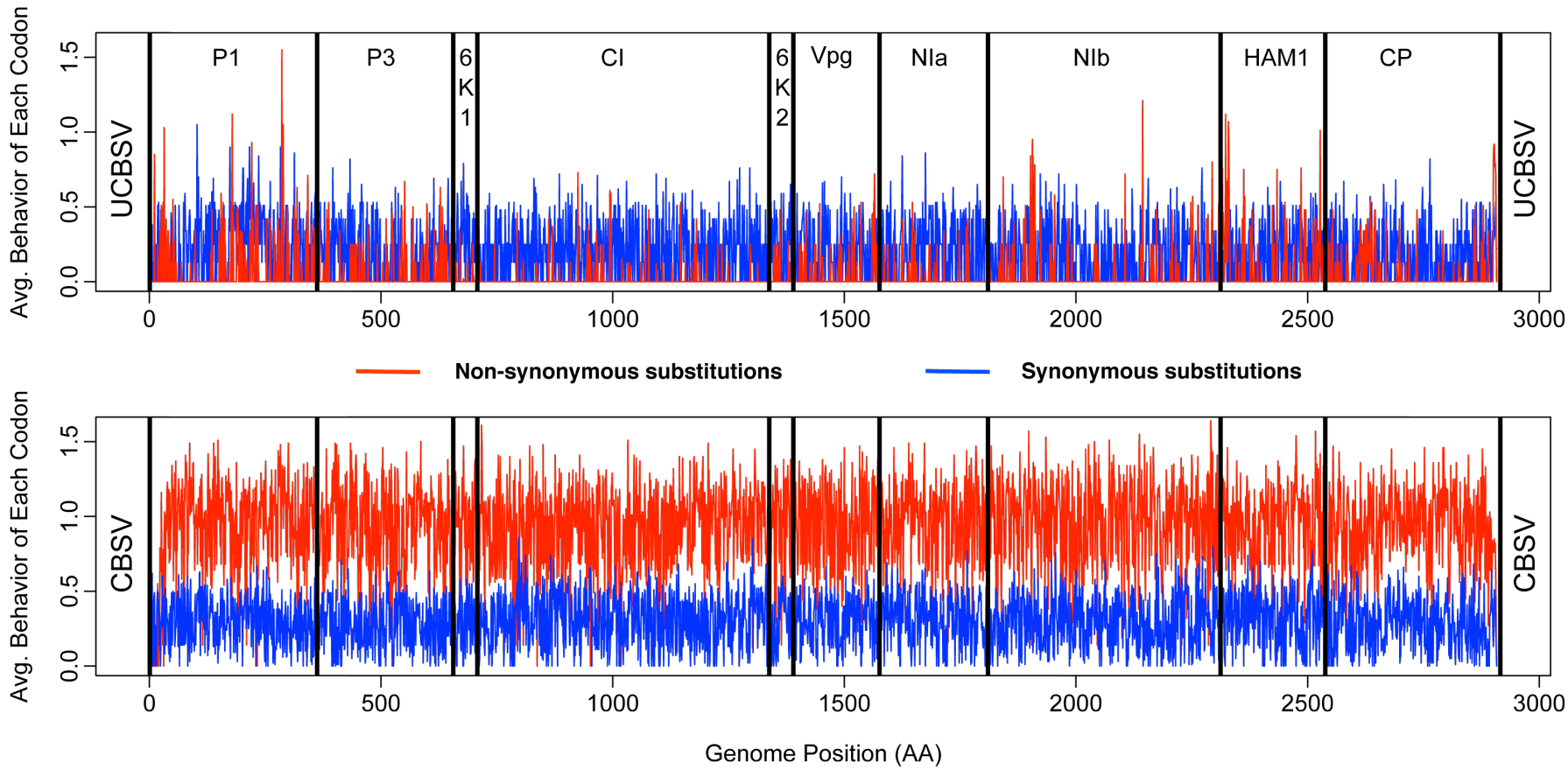
762
763 Table 5. Support for Clades A – G (Figure 3) in individual gene trees and whole genome analyses. Table entries represent
764 posterior probabilities from analysis with MrBayes, except values reported for SVDQ, which are bootstrap
765 proportions. Support values below 95% are indicated in bold, and '--' indicates that the clade was not present.
766
767

Genomic region	Clade A	Clade B	Clade C	Clade D	Clade E	Clade F	Clade G
P1	0.9998	0.9998	0.9698	0.9972	0.9954	--	0.9998
P3	0.9998	0.9998	0.9857	0.9998	0.9998	--	0.9998
6K1	**0.9466**	0.9985	0.9887	0.9995	--	--	0.9842
CI	0.9998	0.9996	0.9976	0.9998	0.9998	0.9999	0.9999
6K2	0.9765	0.9998	**0.7152**	**0.9170**	**0.6646**	--	0.9875
Vpg	0.9998	0.9999	--	0.9918	**0.6455**	--	0.9999
NIa	0.9996	0.9999	0.9998	0.9941	**0.7631**	--	**0.8934**
NIb	0.9999	0.9998	0.9998	0.9999	0.9998	--	0.9998
HAM1-like	--	--	--	0.9989	0.9983	--	0.9855
CP	--	--	--	0.9959	0.9950	--	0.9989
Whole genome	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SVDQ	1.00	1.00	1.00	1.00	**0.87**	**0.44**	1.00

768

Figure 1: Cassava brown streak disease symptoms on leaves and stems of sampled plants; **(a)** Chlorosis along secondary and tertiary leaf veins of CBSV-infected plant of cultivar TME 204 (severity score 3), **(b)** Cultivar TME 14 plant with dual CBSV+UCBSV infection showing chlorosis on secondary or tertiary veins, reverse chlorosis (general chlorosis and green area along veins) (severity score 3), **(c)** UCBSV-infected plant of cultivar TME 204 exhibiting chlorosis on secondary veins, reverse chlorosis, chlorotic spots and mild stem lesions (severity score 3), **(d)** Very severely diseased plant (severity score 5) of cultivar TME 14 infected with both CBSV and UCBSV, and having chlorosis on leaves, severe stem lesions/brown streaks, defoliation, stem dieback.

781 Figure 2. Genetic diversity of CBSV and UCBSV using the Synonymous Non-synonymous Analysis Program (SNAP v2.1.1) implemented in the
782 Los Alamos National Laboratory HIV-sequence database (http://www.hiv.lanl.gov) [50]. UCBSV is on the top panel, CBSV at the
783 bottom. The 10 gene segments are labeled from P1-CP.
784



785

Figure 3. Species tree generated from SVD Quartets using the whole genome sequences. Colors at the tips are based on country of origin. Branches with mixed colors indicate a clade that contains samples with mixed country of origin. For example, the ancestral branch of UCBSV TZ Tan 23 KR108839 and UCBSV UG MI B3 FJ039520 is colored red and orange to indicate a clade with sampled with mixed country of origin.

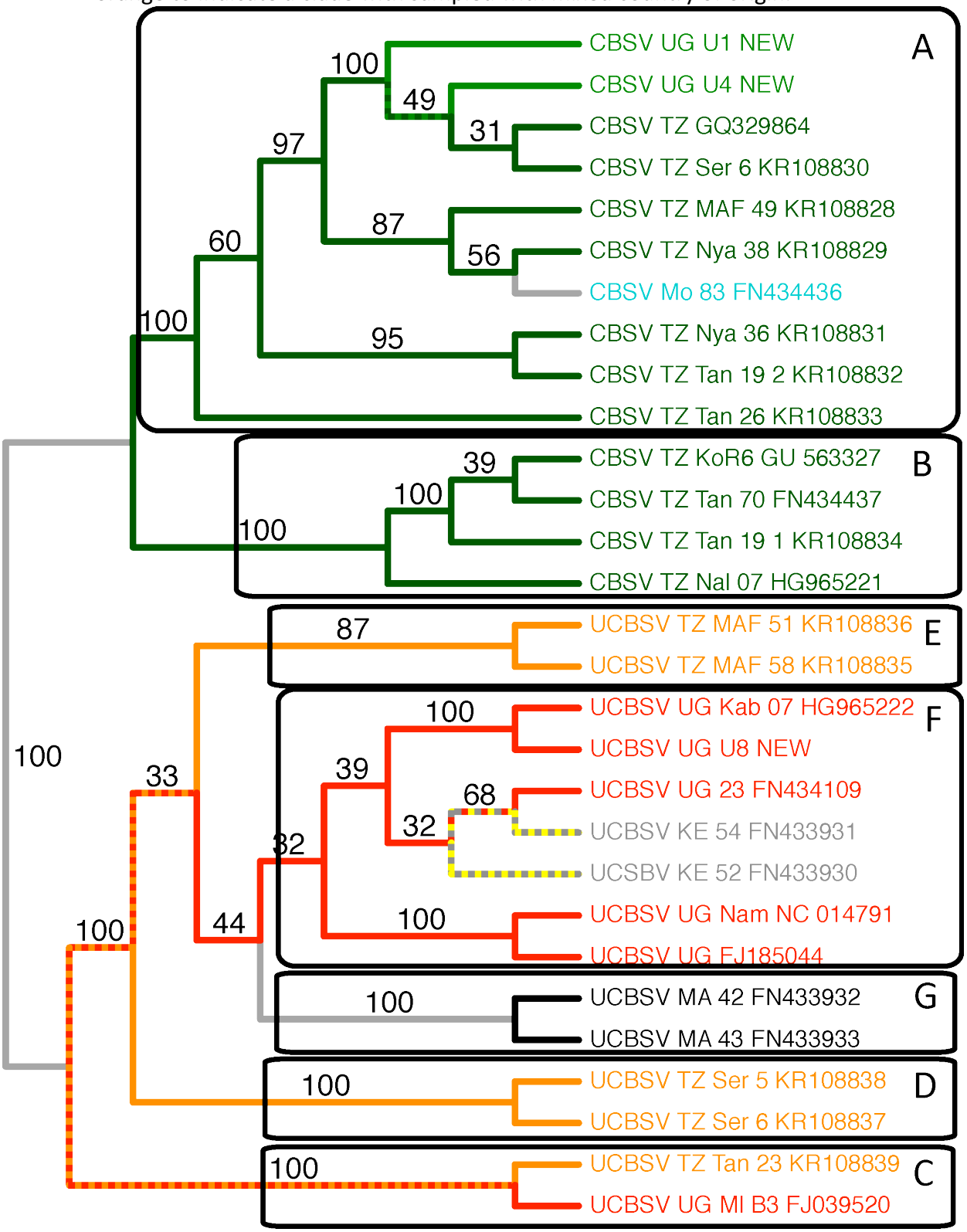
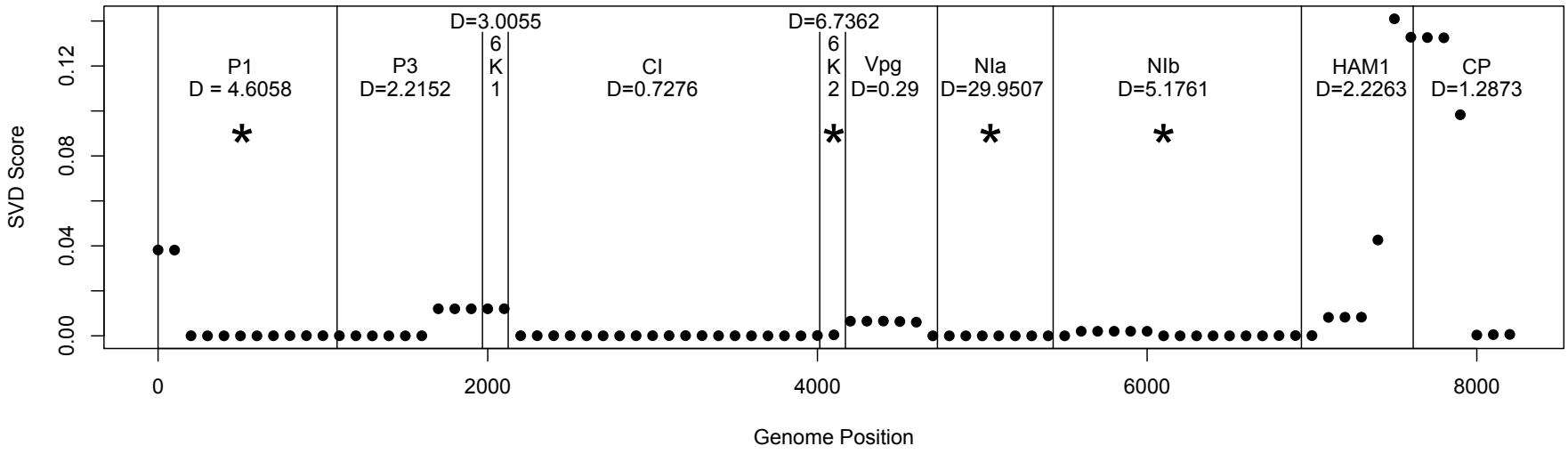
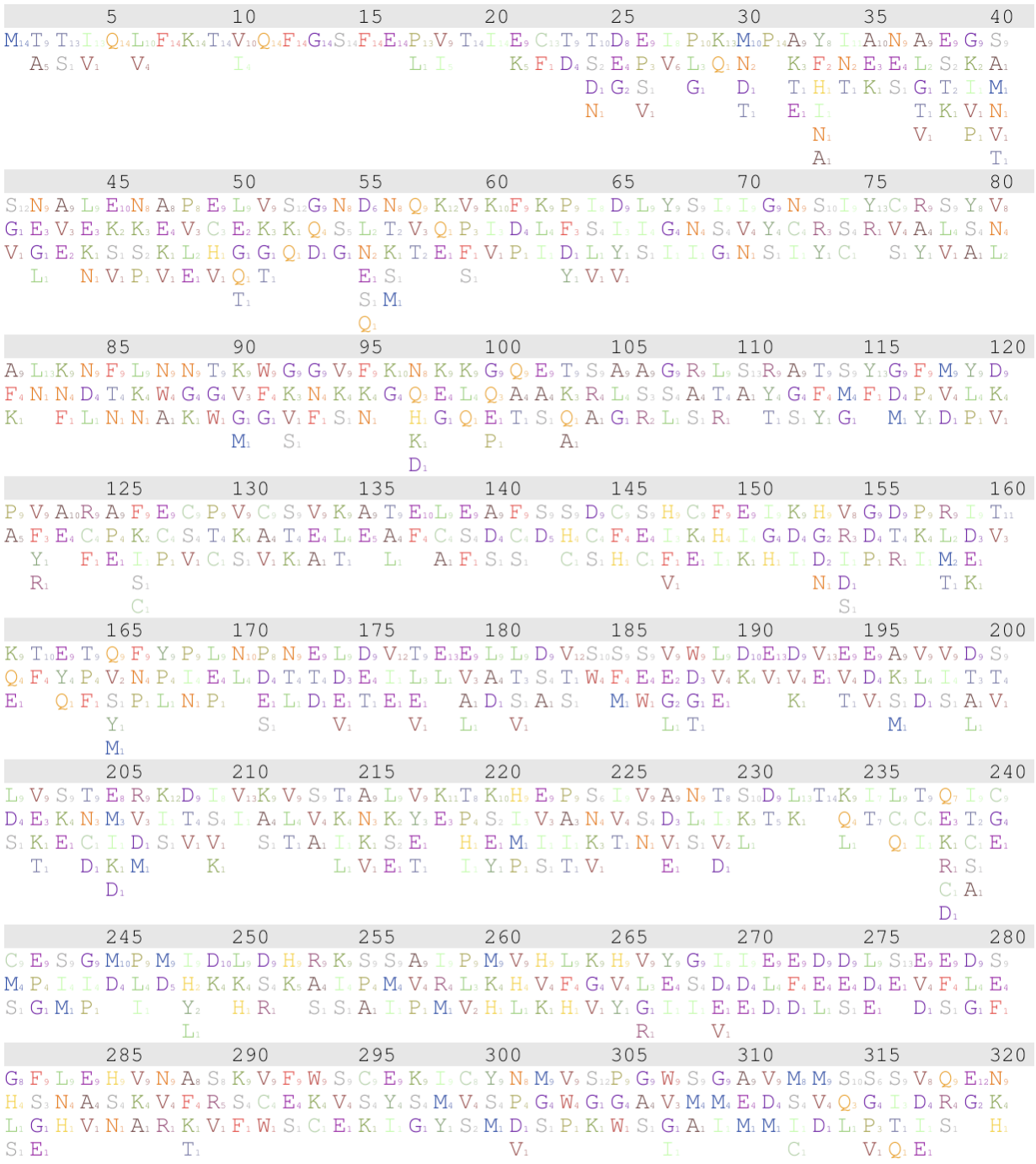
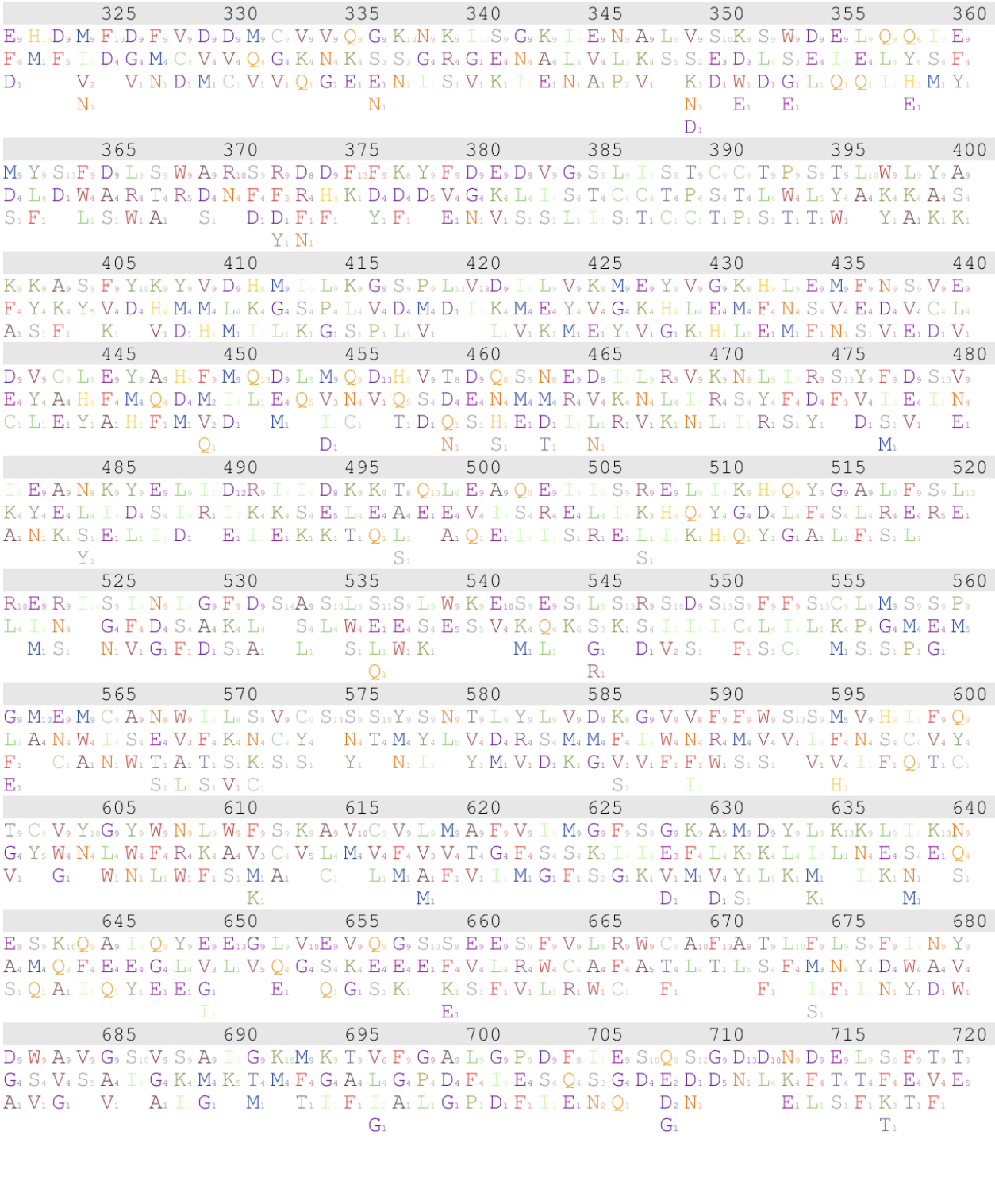


Figure 4. Computed SVD Score with the split defined by CBSV vs. UCBSV across the genome in windows of 500 bp, sliding in increments of 100 bp, and resulting SVD Scores plotted across the genome. Boundaries between genes are marked with vertical lines to further characterize the CBSV and UCBSV genomes. Rates of molecular evolution were estimated using CODEML implemented in PAML (Phylogenetic Analysis by Maximum Likelihood) [54]. The results are shown for each gene and D represents the difference in likelihoods from the null hypothesis (CBSV and UCBSV have equal rates) and the alternative hypothesis (CBSV and UCBSV have different rates).



Supplemental Figure 1. CBSV Amino Acid variability obtained using datamonkey.org. Once files are uploaded to the site, the images below are obtained from the “Information from upload” tab and the pdf is downloaded.





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F4 M1 F5 I2 D4 G4 M4 C4 V4 V4 Q4 G4 K4 N4 K4 S3 S3 G4 R4 G1 E4 N4 A4 L4 V4 L3 K4 S5 S2 E3 D3 L4 S3 E4 I4 E4 L4 Y4 S4 F4
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         N1                                  N1                                      N1    E1    E1            E1
                                                                                     D1
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                                Y1 N1
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                      Q1            D1            N1    S1    T1    N1                                M1
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         Y1                                  S1                          S1
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                                        Q1                          R1
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F1    C1 A1 N1 W1 T1 A1 T1 S1 K1 S1 S1    Y1    N1 I1    Y1 M1 V1 D1 K1 G1 V1 V1 F1 F1 W1 S1 S1    V1 V4 I1 F1 Q1 T1 C1
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                      I1                          E1                                  S1
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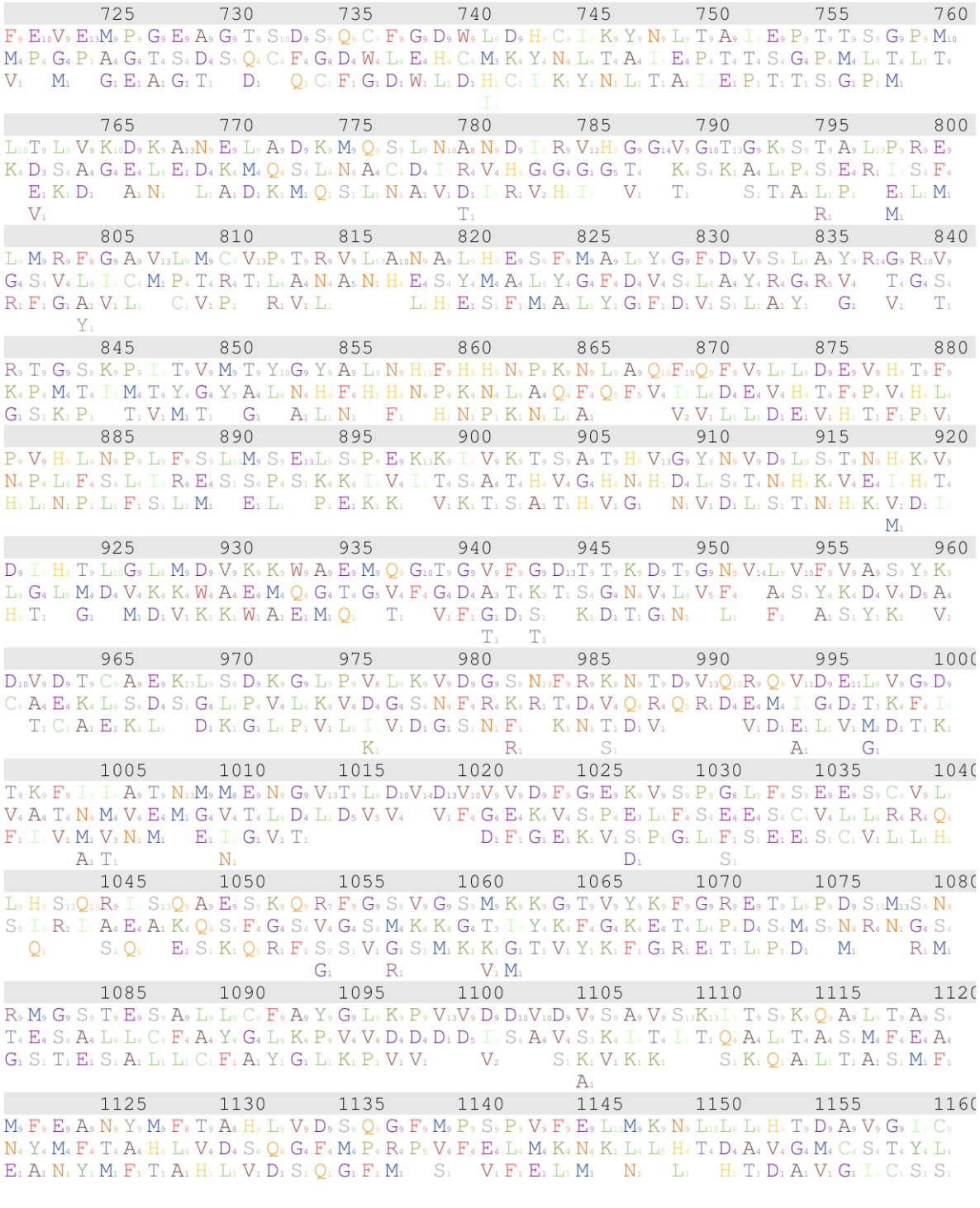

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                                         S1
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                   T1    G1          W1         S1                          S1 Q1                    R1
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                                       L1                I1          D1 S1       E1                    N1
                                                                      S1
         1525      1530      1535      1540      1545      1550      1555      1560
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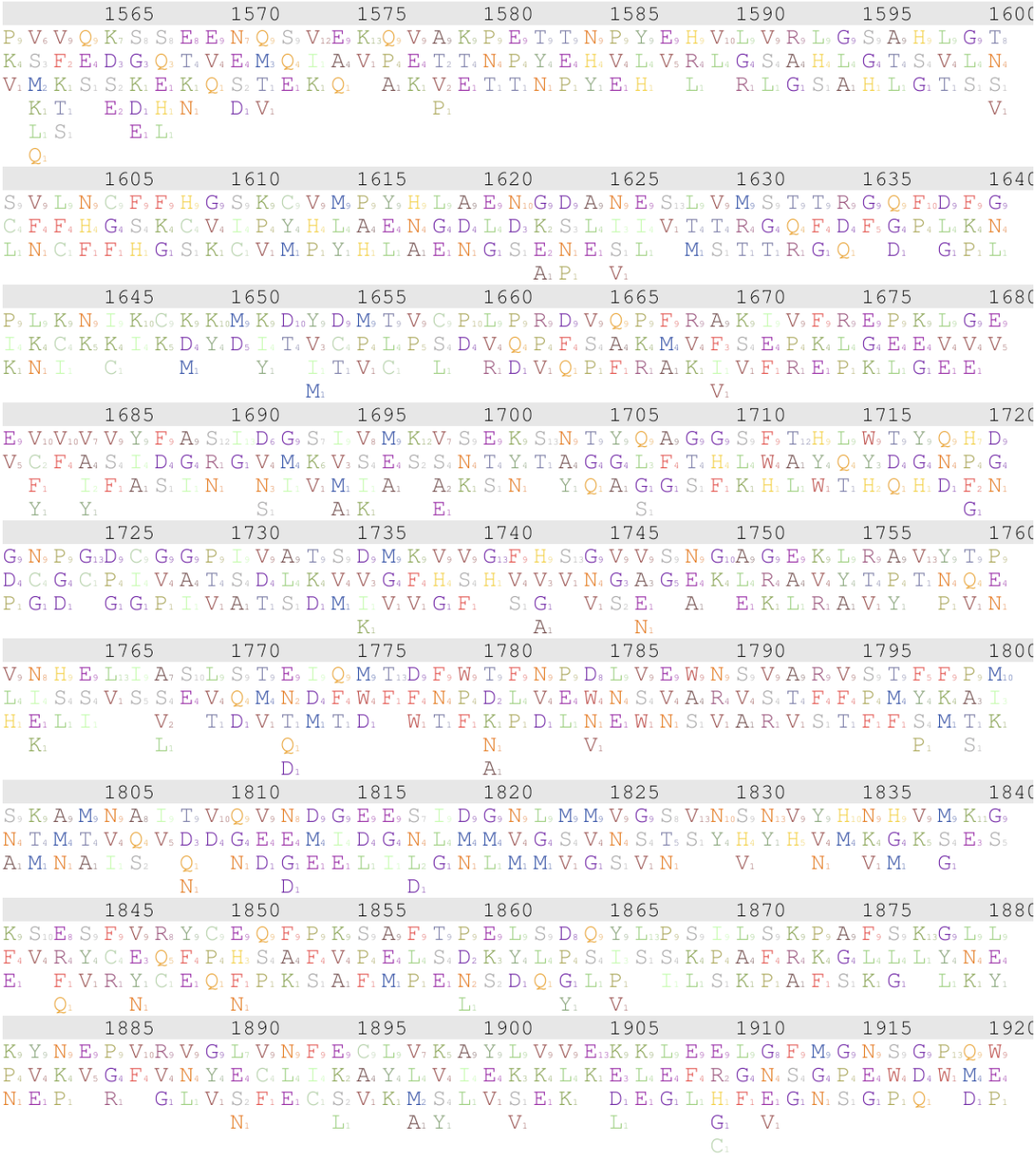

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      N1        D1                                                                                E1    A1
         1965      1970      1975      1980      1985      1990      1995      2000
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F1 K1 H1 L1 A1 G1 G1    V1 G1 I1       G1    L1 K1    E1 L1 R2 P1 V1 E1       S1 D1 M1 K1 T1 R1    F1 T1 G1 A1 P1    D1
                  I1                                   T1 Y1
         2005      2010      2015      2020      2025      2030      2035      2040
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   L1 L1 G1 G1 K1    L1    D1    F1 Y1    H1 F1 Y1 E1 N1 H1 L1 K1 G1 P1 W1 T1 V1 G1 M1 N1 K1 F1 N1 S1 G1 W1 D1 S1 E1 A1
                                                                                                                  L1
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   Y1             M1                                                 V1 H1                                          D1
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      E1 T1       A1 M1                                                F1        A1
                  L1 N1
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                                            E1          I1          M1
                                            V1          M1
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                                                                               S1
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F4 A4 R3 K1 D4 G4 F4 F4 I4 P4 K4 L4 K5 K4 E4 S4 M4 V4 A4 M3 L4 E4 W4 E5 S4 G4 D4 G1 I2 M4 S4 T4 S5 S4 A4 L4 S2 A4 N1 Y4
H1    F1 A1 Q1 R1 D1 G1 G1 Y1 I1 Y2    L1    K1 E1 S1 I1 I1 M2 V4 L1 M2 W1    S1    V2 E1 V1 M1 I1 T1    S1 N3 L1    A1
      Q1                F1       P1                D1 V1 A1                    S2                                A1
                                                     V1                       D1
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                     L1          V1          A1 V1                                                   S1
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                   D1       E1                                   M1          Q1    V1    R1       N3 M1 L1 N1       V1
                   V1
         2325      2330      2335      2340      2345      2350      2355      2360
N9 V8 A7 K8 S9 E9 E7 E9 V9 T9 S9 K9 F7 R9 M8 G9 I10E9 A9 P9 M7 T9 F9 V9 T9 G9 N9 A9 Q8 K10L9 K9 E9 V9 K9 Q9 I9 F9 G9 P9
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T9 I10P9 I9 V10S9 R8 K9 V5 D9 L9 P10E9 S7 Q9 G9 T9 I7 E9 E13I9 I9 K10E9 K9 A11R9 V9 A9 A9 E8 L7 V13G9 G9 P9 V10L9 V9 E9
V3 S4 R3 K4 I3 D4 L4 P4 E4 P5 Q4 G4 T4 V3 E4 E3 I4 V6 K4 I1 K5 A3 R4 V4 A4 S2 E4 L3 V3 G4 G4 P4 G1 L4 V5 E4 D4 T4 S4 L4
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                        L1          A1
         2405      2410      2415      2420      2425      2430      2435      2440
D9 T9 S9 L9 C9 F9 D9 A9 L9 N9 G9 L9 P10G9 P9 Y8 I8 K9 W9 F9 L7 E9 G10I4 G9 L9 E9 G9 L8 Y9 K9 L9 V9 E9 P9 Y9 Q9 N9 S7 M9
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                                                   V1
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                                                 E1                   S1 N1                F1 L1
                                                                      N1
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      R1                                                   L1                    V1
                                                                         A1
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                                           M1 T1 P1       L1 N1    P1 Q1 G1    M1    A1 D1
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                                                          T1
                                                          S1
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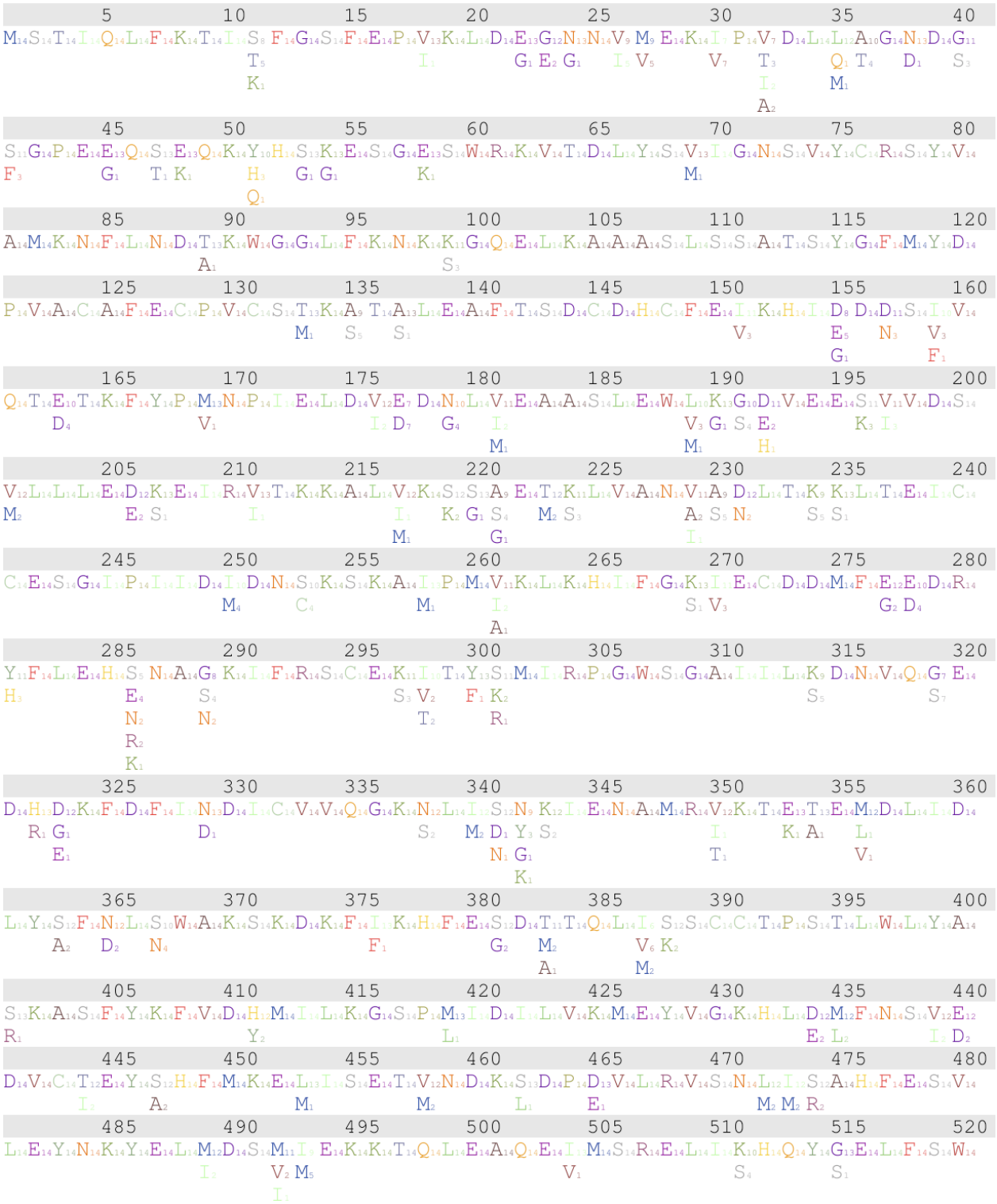
Supplemental Figure 2. UCBSV AA variability determined using datamonkey.org.



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L2 S1

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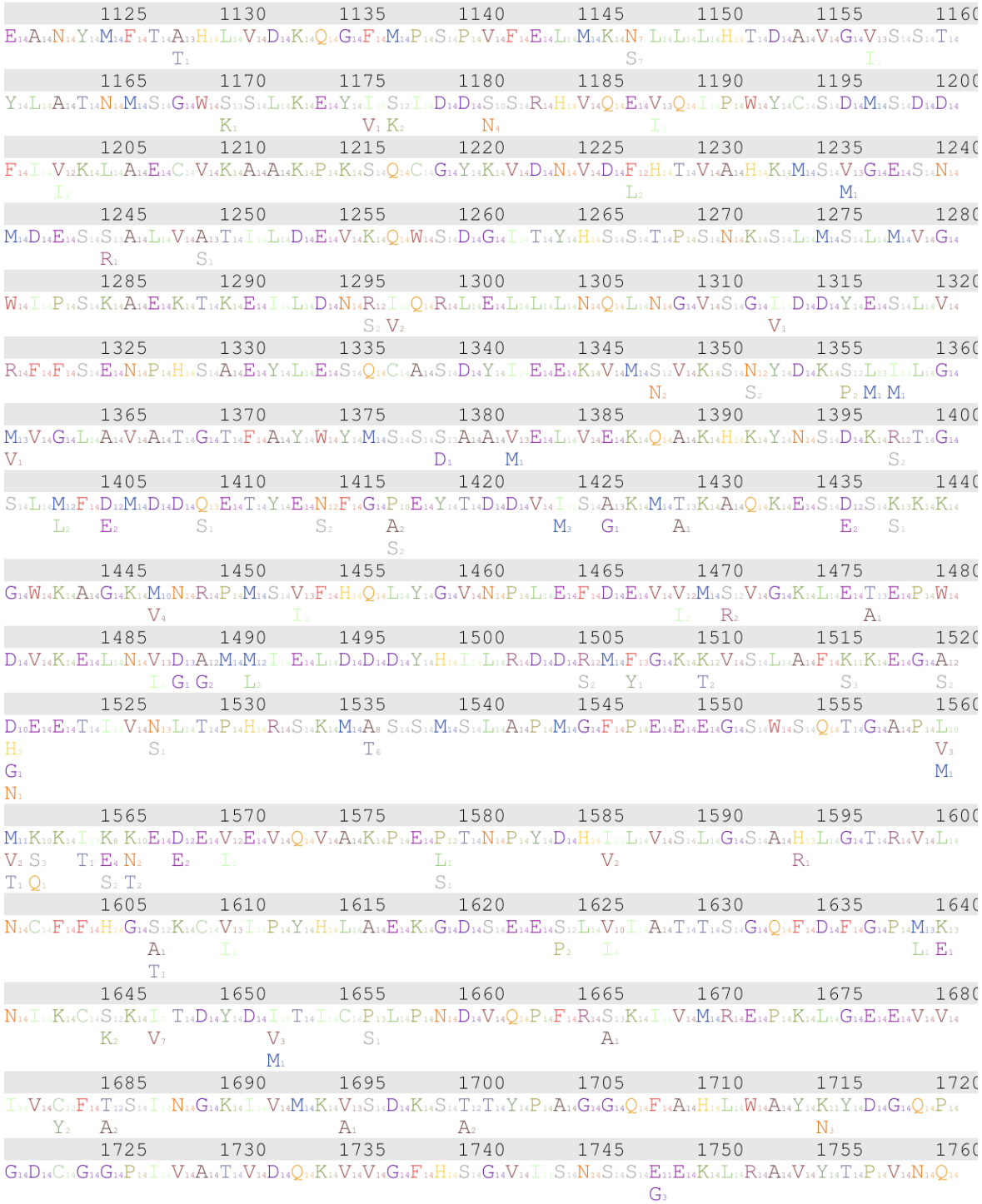

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     M2   Y2 V4   S2
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                                                S1
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V1   K2 T2                                                A4
                                                          M1
                                                          V1
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V14D14N14T14I11M11L14M14I14V14V14E14Y14C14K14A14V14M14E14S12E14G14S13T4 M14Q14F14K14Y14M14C14N14G14D14D14L14I12L14N14V14
          M2 I3                                T2      K1 V4                                    M2
                                                          I2
                                                          E2
                                                          A2
         2165      2170      2175      2180      2185      2190      2195      2200
P14D12D14E14V14S12M14I13Q14S14S14F14S14E11L14F14S14E14C14G14L14D14Y14N13F14D14D14V14H14K14S14M14E14T14I14E14Y14M14S14H14
  N2         N2   V1         D2                   T1
                            N1
         2205      2210      2215      2220      2225      2230      2235      2240
S14F14M14L13K14D14G10V12Y14I14P14K14L14K14K12E14S14I12V14A14I14L14E14W14E14S14G14D14E14I14M14R12T14R12S14A14L14N14A14A14
       M1      D4 L1         S2      V1                                M4   S2   S2
                M1                  M1
         2245      2250      2255      2260      2265      2270      2275      2280
Y14I14E14S14Y14G14Y14E8 D14L13M14V11E14I14E14R14Y14A14V14F14W14A14T8 E13K14G14C14E14Y14P14L14L14D14S14K14R14V14E14G14L14
                D6   P1   I1                                A6 G1
                         T1
                         M1
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          2285      2290      2295      2300      2305      2310      2315      2320
Y14K10D14D14H11T14D14I14N14E14E14W14L14M6 G14I14L14P14P14S14F14E14H14C14Y14V14D14T10Q14T13K12D14L12S13G8 S9 E12K10P12E14
  S4        Y2                          V3                                  M4   I1 E2    V2 K1 E4 G2 D2 Q2 L2
            N1                          I2                                                     S2 E2    E2
                                        S2                                                        K1
                                        T1
          2325      2330      2335      2340      2345      2350      2355      2360
L13S13I5 E12S13H10D13G11V6 P5 Q11M13Q14M14K14F14P14V14T14F14V14T14G14N14L13G14K14L14A14E14V14K13S14I14L14G14I14A13N11D14
F1 G1 V3 G2 N1 C2 N1 S3 T6 S5 R3 L1                                    F2                S1                A13N11D14
      T3      Y2       A1 L2                                                                S1 S3
      P2               M1 T2
      A1
          2365      2370      2375      2380      2385      2390      2395      2400
V14M9 A14K8 N14I14D14L14P14E14V14Q14G14T14P14D11E14I13V13S11K14K14A14Q14L13A14V14K14M14T14N14S12P14V14L14V14E14D14T13C14
  V5    S5                                E2   V1 A1 K2                F1                G2                          A1
        R1                                V1        M1
          2405      2410      2415      2420      2425      2430      2435      2440
L14C14F14N14A14F14N14G14L14P14G14P14Y14I13K14W14F14L14K14E14L14G14L14E8 G14V14V14K12M14L14S14A14F14G8 D14K14S14A14Y14A14
                                        M1                        D6        S2                        E4
                                                                                                    S2
          2445      2450      2455      2460      2465      2470      2475      2480
L14C14T14F14A14Y14V13H14N13E14L7 S14D13P14I13V14F14K14G14V14V14N13G14E14I13V14P14P14R14G14N12N14G14F14G14W14D14P14M9 F14
              A1   S1   S7   E1   V3                K1                          K2                          I5
          2485      2490      2495      2500      2505      2510      2515      2520
K14P14D14G9 C14G7 C14T14F14A14E14M14P9 S14S11I10K14N14D13F14S14H14S14S14S13A14L14E14K14V14K14L11F10L14D14N14L14V10V14K12
        E5   S6                     S5   N2 M3       E1                K1                S3 Y4                M4   S2
             E1                          G1 N1
          2525      2530      2535      2540      2545      2550      2555      2560
Q14E14E12K12K11A13K7 V13A14L14T14I14D12V14Q14A13L14N12Q14E14E14M9 E14A12E14I14T12A14L14K14K14L13W14K9 D14N14G14P14T14S13
      K2 Q1 S3 T1 S6 M1                  E2    V1       D2        V4   E1       A1                M1   S5              G1
         N1          G1                                            I1   T1       S1
          2565      2570      2575      2580      2585      2590      2595      2600
T14R14S14P12F14E14A14S13S14L14S14A14P12Q14V14E14R14V14N14E14L14L14Q14K12L14K14D13E14G14L14Q14T14K14K14S14P14C14G14E14P14
        S2          K1                S2                        Q2       N1
          2605      2610      2615      2620      2625      2630      2635      2640
D14D14G14E14V13V13D13D14D14S14D12D13G12N11N12Q14S14S13G12K14E12V12V8 D12E14S14Q14N13N10Q13Q13P12S12K13P10K13F13K14I11S14
          M1 M1 V1        N2 N1 D1 D3 K1       F1 E2    K2       I4 H1       N2       D1 D4 K2 H1 L1 K2 S1 S2 S1 L1    V2
                            E1    D1                                N1       G1                S1          L1          L1
                                                                                                Q1
          2645      2650      2655      2660      2665      2670      2675      2680
G14D14G13S12A10I9 S12R14D14D14I13D14K13I13P14T14N13A14L14E14I13K14K14T14F14K14P14P14K10V14S14Q14S14A14Y14I13W14I14P14R14
      E1 N3 T4 V5 K2                  S1          S1                                S4
          2685      2690      2695      2700      2705      2710      2715      2720
S14Q14S14D14N14L14T14P14D14V14I13Q14N13F14L13A14Y14M10P14P14S14H13A14M14D14N14Q14L14A14S14G14I13E14V14E14N14W14A14I11E14
                            M1   S1          V4        Y1                                V1                      V2
                                                                                                              M1
          2725      2730      2735      2740      2745      2750      2755      2760
V14A14K14A14Y14G14V14N14M14Q14E14F14Y14R13T14V10L14P14A14W14M10V14N14C14M14V14N14G14T14S14D14E14S14K14N14E14K14S14W14R14
                                        H1   I4          I4
          2765      2770      2775      2780      2785      2790      2795      2800
A14V14E14L14N14S14Q14G14E14D14V14D14D14F14E14Y14P14M14E14P14M14Y14K13F14A14L14P14T14M14S14K14V11M14S14N14F14S14S14Q14A14
                                        S1                                I1
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