

1 **Population genomics of *Cryptococcus neoformans* var. *grubii* reveals new**
2 **biogeographic relationships and finely maps hybridization**

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49 **Abstract**

50 *Cryptococcus neoformans* var. *grubii* is the causative agent of cryptococcal meningitis, a
51 significant source of mortality in immunocompromised individuals, typically HIV/AIDS
52 patients from developing countries. Despite the worldwide emergence of this ubiquitous
53 infection, little is known about the global molecular epidemiology of this fungal pathogen.
54 Here we sequence the genomes of 188 diverse isolates and characterized the major
55 subdivisions, their relative diversity and the level of genetic exchange between them. While
56 most isolates of *C. neoformans* var. *grubii* belong to one of three major lineages (VNI, VNII,
57 and VNB), some haploid isolates show hybrid ancestry including some that appear to have
58 recently interbred, based on the detection of large blocks of each ancestry across each
59 chromosome. Many isolates display evidence of aneuploidy, which was detected for all
60 chromosomes. In diploid isolates of *C. neoformans* var. *grubii* (serotype A/A) and of hybrids
61 with *C. neoformans* var. *neoformans* (serotype A/D) such aneuploidies have resulted in loss
62 of heterozygosity, where a chromosomal region is represented by the genotype of only one
63 parental isolate. Phylogenetic and population genomic analyses of isolates from Brazil
64 revealed that the previously 'African' VNB lineage occurs naturally in the South American
65 environment. This suggests migration of the VNB lineage between Africa and South America
66 prior to its diversification, supported by finding ancestral recombination events between
67 isolates from different lineages and regions. The results provide evidence of substantial
68 population structure, with all lineages showing multi-continental distributions demonstrating
69 the highly dispersive nature of this pathogen.

70 **Author Summary**

71 *Cryptococcus neoformans* var. *grubii* is a human fungal pathogen of immunocompromised
72 individuals that has global clinical impact, causing half a million deaths per year. Substantial
73 genetic substructure exists for this pathogen, with two lineages found globally (VNI, VNII)
74 whereas a third has appeared confined to sub-Saharan Africa (VNB). Here, we utilized
75 genome sequencing of a large set of global isolates to examine the genetic diversity,
76 hybridization, and biogeography of these lineages. We found that while the three major
77 lineages are well separated, recombination between the lineages has occurred, notably
78 resulting in hybrid isolates with segmented ancestry across the genome. In addition, we
79 showed that isolates from South America are placed within the VNB lineage, formerly
80 thought to be confined to Africa, and that there is phylogenetic separation between these
81 geographies that substantially expands the diversity of these lineages. Our findings provide
82 a new framework for further studies of the dynamics of natural populations of *C. neoformans*
83 var. *grubii*.

84 **Introduction**

85 The environmental basidiomycetous yeast *Cryptococcus neoformans* is capable of causing
86 invasive fungal infections primarily in immunocompromised individuals. Meningitis is the
87 most serious manifestation of cryptococcosis. The HIV/AIDS pandemic increased the
88 population of these susceptible individuals and led to an increase in *C. neoformans* infection
89 rates (Day, 2004). *C. neoformans* is the leading cause of mortality in HIV/AIDS patients
90 worldwide, particularly in sub-Saharan Africa, where approximately half a million deaths
91 occur annually (Park et al., 2009). While cryptococcal infection rates in HIV positive
92 individuals have declined due to highly active antiretroviral therapy (HAART), new estimates
93 continue to suggest there are more than 100,000 deaths/year (Rajasingham et al., 2017);
94 recent data also suggests that the incidence of cryptococcosis has plateaued at a high
95 number despite HAART availability. Furthermore, the increasing number of people living with
96 other immunodeficiencies, including transplant and cancer patients, represents a growing
97 population at risk for cryptococcosis (Maziarz and Perfect, 2016).

98 There are three major serotypes of *C. neoformans* distinguished by different capsular
99 antigens, which include two separate varieties (*Cryptococcus neoformans* var. *grubii* and
100 *Cryptococcus neoformans* var. *neoformans*, serotypes A and D respectively) and a hybrid
101 between the two (serotype AD). While *C. neoformans* isolates are primarily haploid, diploid
102 AD hybrid isolates consisting of both serotype A (*Cryptococcus neoformans* var. *grubii*) and
103 serotype D (*Cryptococcus neoformans* var. *neoformans*) have been isolated from both
104 clinical and environmental sources mostly in Europe (Cogliati, 2013; Desnos-Ollivier et al.,
105 2015; Franzot et al., 1999). Serotype A isolates are the most common cause of infection,
106 accounting for 95% of all *C. neoformans* infections globally (Casadevall and Perfect, 1998;
107 Heitman et al., 2011). Genomes of serotype A and D isolates differ by 10-15% at the
108 nucleotide level (Janbon et al., 2014; Kavanaugh et al., 2006; Loftus et al., 2005), and
109 laboratory crosses of A and D isolates are possible but show reduced viability of meiotic
110 spores (Lengeler et al., 2001; Vogan and Xu, 2014).

111 *Cryptococcus neoformans* var. *grubii* can be divided into three molecular types, or lineages:
112 VNI, VNII and VNB (Litvintseva et al., 2006; Meyer et al., 1999, 2009). The VNI and VNII
113 lineages are isolated globally, while the VNB lineage is predominantly located in sub-
114 Saharan Africa (Litvintseva et al., 2006), although there is some evidence for VNB occurring
115 in South America (Bovers et al., 2008; Ngamskulrungrroj et al., 2009) and in the USA, Italy,
116 and China in AD hybrid isolates (Litvintseva et al., 2007). Apart from clinical isolation, the
117 VNI lineage is primarily associated with avian excreta (Lugarini et al., 2008; Nielsen et al.,
118 2007) while the VNB lineage is found mostly in association with specific tree species

119 predominantly mopane trees (Litvintseva and Mitchell, 2012; Litvintseva et al., 2011). These
120 and recent studies have shown that VNI infections are associated with urbanized
121 populations where an avian-associated reservoir, pigeon guano, is also found, while the
122 VNB lineage is widely recovered in the African arboreal environment (Litvintseva et al.,
123 2011; Vanhove et al., 2017).

124 Mating in *C. neoformans* occurs between cells of opposite mating types (*MATa* and *MAT α*)
125 (Kwon-Chung, 1975, 1976), although unisexual mating can also occur (Lin et al., 2005).
126 *MAT α* isolates are capable of unisexual mating both within and between the two serotypes
127 (Lin et al., 2005, 2007), and recombination was shown to occur at similar levels in bisexual
128 and unisexual mating in serotype D isolates (Desnos-Ollivier et al., 2015; Sun et al., 2014).
129 Due to the rarity of *MATa* isolates of both serotypes in the environment (Lengeler et al.,
130 2000a; Litvintseva et al., 2003; Viviani et al., 2001), unisexual mating may have evolved to
131 enable meiotic recombination and genetic exchange between isolates. Several studies have
132 found evidence of recombination within VNI, VNII, and VNB populations although not
133 between these lineages (Bui et al., 2008; Litvintseva et al., 2003, 2005).

134 An additional level of genome diversity detected in *Cryptococcus neoformans* var. *grubii*
135 includes the presence of cryptic diploid isolates and variation in the copy number of
136 individual chromosomes or regions. Close to 8% of *Cryptococcus neoformans* var. *grubii*
137 global isolates appear diploid; these isolates contain the *MAT α* locus and many appear
138 autodiploid, thought to result either from endoreduplication or self-mating (Lin et al., 2009).
139 While the vast majority of serotype A or D isolates appear haploid, individual chromosomes
140 can be present at diploid or triploid levels (Hu et al., 2011). For chromosome 1, a specific
141 advantage of aneuploidy is copy number amplification of the azole drug targets or efflux
142 transporters, associated with drug resistance (Sionov et al., 2010). While the specific
143 selective advantage of other chromosomal aneuploidies is unknown, same-sex mating of
144 *MAT α* isolates generates aneuploid progeny at high frequency, some of which also exhibit
145 azole resistance (Ni et al., 2013). Titan cells, polyploid yeast cells produced in the lung of
146 infected animals, also generate aneuploid progeny under stress conditions (Gerstein et al.,
147 2015).

148 Previous studies examining the global population structure of *Cryptococcus neoformans* var.
149 *grubii* have used typing methods for a few genetic loci or focused on particular geographical
150 regions or countries (Hiremath et al., 2008; Khayhan et al., 2013; Litvintseva et al., 2006;
151 Oliveira et al., 2004). Recent approaches have applied whole genome sequencing (WGS) to
152 trace the microevolution of *Cryptococcus*, identifying variation that occurs during the course
153 of infection (Chen et al., 2017; Ormerod et al., 2013; Rhodes et al., 2017) or in the

154 environment (Vanhove et al., 2017). Here, we use WGS of 188 isolates to provide a
155 comprehensive view of the population variation between the three major lineages; the
156 sequenced isolates were selected to represent the diversity of *Cryptococcus neoformans* var.
157 *grubii* including each of the three major lineages and global geographical sampling. We
158 identify contributions to genomic diversity generated through inter-lineage meiotic exchange
159 to create haploid hybrids, generation of AD diploid hybrids, and regional copy number
160 amplification. Furthermore, we finely analyze the phylogenetic relationships and trace the
161 evolution of *Cng* at the global population level.

162 **Results**

163 **Population subdivisions and detection of genetic hybrids**

164 To examine the evolution of *C. neoformans* var. *grubii*, we sampled the population by
165 sequencing the genomes of 188 isolates (**Table 1, Table S1**) representing each of the three
166 major genetic subpopulations (VNI, VNII, and VNB) previously defined using multi-locus
167 sequence typing (MLST) (Litvintseva et al., 2006; Meyer et al., 2009). These isolates are
168 geographically diverse, originating from North America, South America, the Caribbean, Asia,
169 Europe, and Africa (**Table S1**). The VNI global lineage is the most geographically diverse,
170 whereas VNII is represented by a smaller number of locations and VNB appears most highly
171 prevalent in southern Africa. For VNI and VNB, both clinical and environmental isolates were
172 included, with 25 VNI isolates originating from avian guano or trees and 7 VNB isolates from
173 trees or other environmental sources (**Table S1**). For each isolate we identified SNPs using
174 GATK by aligning Illumina reads to the H99 reference genome assembly (**Methods**, (Janbon
175 et al., 2014)). Whereas 164 isolates appeared haploid, 24 isolates were determined to be
176 heterozygous diploids (**Methods, Table 1**) and analyzed separately. An initial phylogeny of
177 the 164 haploid isolates separated the three lineages but intermediate placement of five
178 isolates suggested the presence of hybrid haploid genotypes (**Figure S1**). As the
179 phylogenetic placement of such hybrid isolates is complicated by recombination, we
180 removed these isolates from the phylogenetic analysis and analyzed them using alternative
181 approaches (see below).

182 A phylogeny inferred from the SNPs for all non-hybrid isolates strongly supports the
183 three major lineages of *C. neoformans* var. *grubii*: VNI, VNII, and VNB (**Figure 1**). Of these
184 159 isolates, only 6 (4%) contain the rare *MATa* allele, including four VNB isolates (Bt63,
185 Bt85, Bt206, and CCTP15) and two VNI isolates (125.91 and Bt130). Based on these whole
186 genome SNP comparisons, none of these *MATa* isolates appeared highly related to each
187 other or to any *MAT α* isolate. The two VNI *MATa* isolates are well separated within this
188 group, with Bt130 found in a subgroup of African isolates and 125.91 most closely related to

189 a pair of isolates from Africa and North America (**Figure 1**). Phylogenetic analysis showed
190 that VNB has the highest diversity between isolates, showing the longest tip branches
191 compared to VNI or VNII. In addition, VNB consisted of two diverged subgroups, VNBI and
192 VNBI, as suggested previously by MLST (Chen et al., 2015; Litvintseva et al., 2006, 2011)
193 and genomic analysis (Desjardins et al., 2017; Vanhove et al., 2017).

194 To better understand the population structure of the three lineages and identify potential
195 inter-lineage recombination, we compared results of two independent approaches. First, we
196 used principle components analysis (PCA) to identify the major groups in the population
197 using the SNP data. By comparing the SNP variants across isolates using PCA, we found
198 there are three major clusters corresponding to the VNI, VNII, and VNB lineages (**Figure 2**).
199 The five isolates that showed intermediate positions in phylogenetic analysis (**Figure S1**)
200 also appeared at intermediate positions by PCA, placed between VNI and VNB. In addition,
201 two isolates were separated from the VNII cluster and shifted towards the VNB cluster. All of
202 these seven isolates were collected from southern Africa, and all had a clinical origin except
203 isolate Ftc260-1, which was isolated from the environment (**Table S1**). Of the seven, two
204 sets of isolates share nearly identical ancestry ratios and are closely linked on the
205 phylogenetic tree. Isolates Bt131, Bt162, and Bt163 differed by an average of only 39 SNP
206 positions; similarly CCTP51 and MW_RSA852 differed by 200 SNP positions, suggesting
207 these five isolates are descended from two hybridization events. Therefore, four unique
208 hybridization events were detected in total, three for VNI-VNB and one for VNII-VNB. While
209 the basal branching VNB isolates from Brazil could suggest a hybrid ancestry, all appear to
210 be uniformly VNB (>99% of sites).

211 Next, we identified the ancestry contribution of each isolate using STRUCTURE with three
212 population subdivisions. This confirmed that most isolates have a single dominant ancestry
213 assigned to the VNI, VNB, and VNII lineages. In addition, the isolates with intermediate
214 positions indicated by PCA were found to have mixed ancestry contributions by
215 STRUCTURE. SNP sites for the VNI-VNB hybrids contain an average of 40.8% VNI
216 ancestry and 59.2% VNB ancestry whereas the VNII-VNB hybrids have 85.8% VNII and
217 14.2% VNB ancestry (**Table S2**). The similar fraction of ancestry in the VNI-VNB hybrids
218 suggests they could be recent mixtures of the two lineages, whereas the VNII-VNB hybrids
219 may be more ancient mixtures with additional crosses to VNII isolates biasing the final ratio
220 of parental SNPs.

221 **Evidence of recent meiotic exchange generating haploid hybrids**

222 To examine the degree of intermixing of ancestry for these hybrid genotypes across the
223 genome, we identified the most likely ancestry for each SNP site using the site-by-site mode

224 in STRUCTURE. Selecting positions where the ancestry assignment was most confident
225 (0.9 or greater, **Methods**), we examined the distribution of these sites by ancestry across
226 the fourteen chromosomes (**Figure 3**). Each of the three VNI-VNB hybrids displayed
227 different patterns of large regions corresponding to a single ancestry. For example,
228 chromosome 1 has three large blocks of different ancestry in Bt125, four in Bt131, and two
229 in Ftc260-1 (**Figure 3A-C**). While all chromosomes contained regions of both VNI and VNB
230 ancestry groups in Bt125 and Ftc260-1, two chromosomes of Bt131, chromosome 6 and 9,
231 have only large regions of VNB ancestry. By contrast, CCTP51, which contains a lower
232 fraction of the second ancestry (VNB), appears more highly intermixed with smaller ancestry
233 blocks (**Figure 3D**). Notably, three of the four unique genotypes (Bt131, CCTP51, and
234 Ftc260-1) contain the rare *MATa* locus; in all *MATa* isolates, the mating type locus region is
235 of VNB ancestry, whereas the mating locus region in the *MATα* isolate (Bt125) is of VNI
236 ancestry (**Methods**). Overall these patterns suggest a recent hybridization of VNI and VNB
237 isolates, with recombination during meiosis generating chromosome-wide intermixing
238 resulting in distinct parental haplotype blocks. In Bt125, a 205 kb region of scaffold 6 is
239 present at nearly twice (1.92 fold) the average depth. Otherwise this isolate, and the other
240 six hybrid isolates, was found to contain even levels of ploidy across the 14 chromosomes
241 based on read depth.

242 For the three VNI-VNB hybrids showing large ancestry blocks, we also utilized the site
243 ancestry predictions to finely map the genotypes within each population. Given the roughly
244 equal contribution of the two ancestry sites and the large block size for each in these
245 genomes, we hypothesized that these hybrids could have resulted from recent mating of one
246 genotype of each lineage, which we could reconstruct using separate phylogenies of each
247 site class. For each genotype, sites mapped to either the VNI or VNB ancestry were
248 selected and a separate phylogeny constructed for each of these two sets of sites. For VNI
249 ancestry sites, these isolates had very different genotypes, with Ftc260-1 most closely
250 related to a diverse set of African isolates in VNI, whereas both Bt125 and Bt131 are more
251 closely related to highly clonal clades of VNI isolates (**Figure S2A,C,E**). Similarly for a
252 separate phylogenetic analysis of VNB ancestry sites, Bt125 and Bt131 were placed within
253 one of the two major subclades of VNB while Ftc260-1 was placed in the other (**Figure**
254 **S2B,D,F**). This supports that these three hybrids originated from very different genotypes of
255 VNI and VNB parental isolates.

256 **Diploid isolates and genome plasticity**

257 As noted above, a total of 24 sequenced isolates displayed heterozygous SNP positions
258 across the genome. Four of these isolates had higher rates of polymorphism overall and

259 appear to be hybrids within or between VN lineages (Bt66, Cng9, PMHc.1045.ENR.STOR,
260 and 102-14) (**Figure S3**). Each of these isolates contain two copies of the *MAT α* mating type
261 locus, suggesting they arose from same sex mating of two *MAT α* parental isolates. In
262 addition, 11 serotype A diploids showed very low rates of heterozygosity (**Figure S3**),
263 consistent with AFLP and MLST-based evidence that they arose from endoreduplication or
264 self-mating (Lin et al., 2009). The remaining isolates include eight serotypeA/serotypeD
265 diploids, of which seven contain both *MAT α* and *MAT α* mating types and one is homozygous
266 for the *MAT α* locus, and one serotype A/*Cryptococcus gattii* hybrid containing two copies of
267 *MAT α* .

268 All types of diploid isolates in our set, including A/A diploids, exhibit regions of loss of
269 heterozygosity (LOH) in the genome, where alleles of only one parental isolate are present.
270 Three of the A/A diploids (Bt66, Cng9, and 102-14) are heterozygous throughout nearly all of
271 the genome; Cng9 exhibited only a small LOH region at the start of chromosome 2, which
272 also has haploid levels of genome coverage. Isolate PMHc1045 by contrast has large LOH
273 regions on six scaffolds, including a 1.1 Mb region of chromosome 6 (**Figure S3**). Some of
274 these regions of LOH in PMHc1045 are linked to aneuploid chromosome segments,
275 including a region of chromosome 12 reduced to haploid levels and or triploid levels of the
276 region adjacent to a LOH on chromosome 6. All LOH regions are telomere-linked,
277 reminiscent of what has previously been reported across diverse isolates of *Candida*
278 *albicans* (Hirakawa et al., 2015).

279 We next inferred the ancestry of the two parental isolates contributing to the A/A hybrids by
280 examining the frequency of SNP alleles that are highly predictive for VNI, VNII, or VNB
281 (**Methods**). Three of the isolates (Cng9, PMHc1045, and 102-14) have similar frequencies
282 of such VNII and VNB alleles, whereas Bt66 is comprised of VNI and VNB predictive alleles
283 (**Table S3**). Comparing Cng9 and PMHc1045 directly, 89.2% of variant sites are identical;
284 this fraction increases to 97.3% when LOH regions are excluded and a similar fraction of
285 sites are shared with 102-14. By comparison, both isolates share 23% of variant positions
286 with Bt66 when LOH regions are excluded from both. Notably, LOH has resulted in a mixing
287 of genotypes; examining predictive alleles for each of the eight LOH regions of PMHc1045
288 revealed regions corresponding to each lineage. Two regions encompassing 1.4% of the
289 genome share the highest fraction of private alleles with other VNB isolates whereas the
290 remaining six regions encompassing 10.2% of the genome share most private alleles with
291 other VNII isolates. Thus, LOH has led to large differences between otherwise highly similar
292 Cng9 and PMHc1045 isolates and resulted in blended ancestry by converting regions to
293 each of the two parents in PMHc1045.

294 The eight AD hybrids also showed evidence of even more extreme aneuploidy and LOH
295 related to loss of one of the two parental chromosomes. All isolates displayed evidence of
296 aneuploidy, by examining read coverage across both the H99 serotype A and JEC21
297 serotype D reference genomes (**Figure S4**). While some isolates have retained
298 chromosomes of both A and D origin, others have lost a chromosome from one parent and
299 duplicated the corresponding chromosome of the other (**Figure 4, Figure S4**). For example
300 in RCT14, two copies of chromosome 1 are present but both have serotype A origin;
301 similarly in IFNR21, both copies of chromosome 10 have serotype D origin. Both of these
302 isolates display additional aneuploidies, with 3 copies of some chromosomes. Notably,
303 CCTP50 appears mostly triploid, with either 2:1 or 1:2 ratios of the A:D ratio for each
304 chromosome (**Figure 4**); this pattern is also observed in IFN26 (**Figure S4**). In IFN-R26, loss
305 of chromosome 4 in JEC21, balanced by gain of chromosome 5 in H99 (**Figure S4**), has
306 resulted in a *MATa/MATa* genotype. While the mating type of the original JEC21 parent can
307 not be determined, this suggests that generation of *MATa/MATa* diploids can occur via
308 chromosome loss and duplication. All other isolates are *MATa/MATα*, suggesting that they
309 originated from opposite sex mating. While diploid AD hybrids have been isolated from both
310 environmental or clinical sources (Litvintseva et al., 2006), all eight AD hybrids in our set are
311 of clinical origin.

312 To examine the diversity of these AD hybrids, SNPs were identified by comparison to a
313 combined A (H99) and D (JEC21) genome reference. Phylogenetic analysis of A and D
314 genome SNPs revealed that both the A and D copies of each hybrid are closely related for
315 these isolates (**Figure S5**). On average, the A genomes differ by 6,108 SNP positions and
316 the D genomes by 3,935 SNP positions. The A genomes are from the VNB lineage, most
317 closely related to Bt206 in our analysis (**Figure S5**). The low diversity of both the A and D
318 genomes between isolates suggests that this set of 8 AD hybrids may have originated from
319 a single hybrid isolate or from a set of closely related A and D parental isolates.

320 **Chromosomal copy number variation**

321 On a smaller scale than whole-genome hybridization, chromosomal copy number variants
322 appear to be common in *C. neoformans* and may be an adaptive mechanism for virulence
323 (Rhodes et al., 2017). In the set of 164 primarily haploid isolates, 25 exhibited whole or
324 partial chromosomal aneuploidies (**Figure S6**). In 13 of the 25 isolates, an entire
325 chromosome or region thereof showed a doubling of sequencing coverage, consistent with a
326 diploid chromosome in an otherwise haploid isolate. The remaining 12 isolates show a 50%
327 gain in coverage better explained by a diploid isolate with a triploid chromosome or region.

328 These likely diploid isolates do not display heterozygous base calls, suggesting a recent
329 endoreduplication of the genome and associated aneuploidy of additional chromosomes.

330 Aneuploidies of particular chromosomes may provide a specific biological advantage or
331 alternatively be better tolerated. In general, the smallest chromosomes (12 and 13) are the
332 most frequently observed to exhibit aneuploidy (**Figure S6**). Several isolates have an
333 increased copy number of chromosome 1; amplification of the lanosterol-14- α -demethylase
334 *ERG11* and the major efflux transporter *AFR1* located on chromosome 1 can confer
335 resistance to azole drugs (Sionov et al., 2010). Of the four isolates that contain chromosome
336 1 aneuploidies, either *ERG11* (CCTP34) or *AFR1* (IFN-R11 and RCT6) or both genes
337 (CCTP9) are present at elevated copy number. The elevated copy number of *AFR1* appears
338 correlated with increased drug resistance; both CCTP9 and RCT6 displayed fluconazole
339 MIC values of 256 ug/ml, whereas CCTP34 appeared more susceptible at an MIC of 8 ug/ml
340 (**Methods**). Notably, all of the isolates with chromosome 1 aneuploidies are of clinical origin,
341 as are 24 of all 25 isolates with detected aneuploidies (**Figure S6, Table S1**). Of the seven
342 isolates with hybrid ancestry, only Bt125 included a small region of chromosome 6 at higher
343 copy number; otherwise this and the other hybrid isolates appeared to be haploid. Across
344 the diploid and haploid isolates, we detected aneuploidies affecting all chromosomes
345 (**Figures S3, S4, and S6**).

346

347 **Conservation of gene content and structure across lineages**

348 To examine the extent of gene content variation across the three major lineages of *C.*
349 *neoformans* var. *grubii*, we assembled and annotated genomes of 39 representative isolates
350 (**Methods**). Previously a high quality reference genome was produced for the H99 VNI
351 isolate (Janbon et al., 2014); our data set includes new annotated assemblies for 9 diverse
352 VNB isolates, 27 VNI isolates, and three VNII isolates (**Table S4**). The gene sets across all
353 40 assemblies (including H99) were compared to each other and to those of four *C. gattii*
354 (representing VGI, VGII, VGIII, and VGIV) and one *C. neoformans* var. *neoformans*
355 (serotype D) reference genomes (**Methods**) in order to evaluate gene conservation. Based
356 on orthologs identified across these genomes (**Methods**), an average of 4,970 genes are
357 conserved across all 45 compared *Cryptococcus* gene sets; within serotype A, an average
358 of 5,950 genes are conserved in all 40 genomes (**Figure S7**). A phylogeny inferred from
359 4,616 single copy genes supports VNII in an ancestral position relative to the more recently
360 diverging VNI and VNB (**Figure S7**).

361 Gene content is highly conserved across *C. neoformans* var. *grubii* with few examples of
362 genes specific to the separate lineages (**Supplementary Note**). Based on ortholog profiling,

363 a total of 11 genes are specific to VNI, three specific to VNB, and 25 specific to VNII (**Table**
364 **S5**). These include two clusters of genes specific to VNI or VNII located within otherwise
365 syntenic regions of the genome (**Figure 5**). The cluster of five genes unique to VNI genomes
366 include a predicted haloacid dehydrogenase, an amidohydrolase and an allantoinate
367 permease, which could be involved in uptake of uric acid products. The cluster of six genes
368 unique to the VNII genomes includes a predicted transcription factor, amino acid transporter,
369 hydrolase, dihydropyrimidinase, and oxygenase superfamily protein. While both clusters are
370 also missing from the JEC21 *C. neoformans* var *neoformans* genome, the more distantly
371 related *C. gattii* genomes contain syntenic orthologs of all of the VNII-specific cluster genes
372 and between 1 and 3 non-syntenic orthologs of the VNI-specific cluster. These patterns
373 suggest gene loss and perhaps lateral transfer in some species and lineages account for
374 these differences. There was little other evidence of lineage-specific gene loss; orthologs
375 missing in only one lineage included only hypothetical proteins. In addition, we further
376 searched for genes with loss-of-function mutations in all members of each lineage using
377 SNP data, to find genes that may be disrupted but still predicted in the assemblies. However,
378 we found no convincing evidence of disrupted genes with known functions in any of the
379 three lineages (**Supplementary Note**).

380 Given the high level of gene conservation between lineages, we sought to identify rapidly
381 evolving genes that might be involved in phenotypic differences between *C. neoformans*
382 lineages. For each gene, we built a consensus sequence for each lineage and then
383 calculated pairwise d_N and d_S of these fixed sites. As d_S was uniformly low throughout the
384 dataset due to limited genetic diversity, we identified differences in d_N , which measures both
385 the mutation rate and selection. The top 10 annotated genes with the largest d_N for each
386 pairwise comparison are shown in Table 2, and the three comparisons in total include 18
387 unique genes. The set is dominated by transcription factors (*GLN3*, *PDR802*, *SXI1 α* ,
388 *YOX101*, and *ZNF2*) and transferases (*ATG2602*, *CDC43*, *GPI18*, *HOC1* and *RAM1*), many
389 of which have already been implicated in virulence (Esher et al., 2016; Jung et al., 2015; Lee
390 et al., 2015; Selvig et al., 2013; Wang et al., 2012) or resistance to oxidative stress (Jung et
391 al., 2015). In particular, *CDC43* and *RAM1* are both rapidly evolving; these genes represent
392 the two major independent methods of prenylation, key in proper subcellular localization of
393 many proteins, often to the membrane (Esher et al., 2016; Selvig et al., 2013). Other rapidly
394 evolving genes include β -glucan synthase *KRE63*, superoxide dismutase *SOD1*, and mating
395 regulator *SXI1 α* , the latter of which is highly divergent between VNII and both VNI and VNB,
396 and could play a role in reproductive isolation of the VNII lineage.

397 **Population measures and biogeography**

398 Strikingly, recently identified VNB genotypes from South America are placed in the
399 phylogeny as basally branching clades for each VNB subgroup, which otherwise consist of
400 genotypes from Africa (**Figure 1**). All of the six South American VNB isolates contain the
401 *MAT α* genotype. By contrast, both VNI and VNII consist of more closely related though more
402 geographically diverse sets of isolates; one large clonal group is found in VNII, whereas
403 several are observed for VNI, which is oversampled owing to its higher prevalence in
404 patients and environments worldwide. Overall, VNB showed the highest average pairwise
405 diversity ($\pi=0.00736$), nearly four times the level in VNI ($\pi=0.00200$), with the lowest value
406 for VNII ($\pi=0.00105$) (**Table 3**). Genetic diversity within the VNB lineage was similar
407 between the South America and African isolates ($\pi=0.00727$ and 0.00736 , respectively).
408 However, genetic diversity of VNI isolates in India was lower than VNI isolates in Africa
409 ($\pi=0.00146$ and 0.00337). VNB also contained the largest fraction of private alleles
410 compared to VNI and VNII, reflecting the higher variation within VNB (**Table 4**). By contrast,
411 VNI and VNII had the highest number of fixed differences, reflecting the long branches
412 leading to these clades. The average divergence (d_{XY}) between lineages ranges is 0.012
413 comparing isolates from VNI and VNB and 0.015 for comparison of either to VNII (**Table 4**),
414 highlighting the low nucleotide divergence between the lineages. VNI and VNII were the
415 most differentiated of the three lineages as shown by pairwise whole genome fixation
416 indexes (F_{st}) (Weir and Cockerham 1984). The highest average chromosome F_{st} value is
417 0.874 between VNI and VNII isolates, while the average chromosome F_{st} values of VNI-VNB
418 and VNB-VNII are 0.595 and 0.707, respectively (**Table 4**).

419
420 To further examine the evolutionary history of the novel South American VNB isolates, we
421 subdivided VNB into four subclades (VNBI-South America, VNBI-Africa, VNBII-South
422 America, and VNBII-Africa) and calculated alleles unique to each subclade and shared
423 across VNB groups or geography (Methods). These subclades represent all combinations of
424 the two previously identified VNB groups (VNBI and VNBII) and the two geographies (South
425 America and Africa). One South American VNB isolate (V53), nested deeply within African
426 isolates on the phylogeny, was excluded from the analysis. All four of the subclades
427 contained more unique alleles than were shared across either VNB group or geography
428 (**Figure 7**), suggesting both a high level of genetic diversity within each subclade and some
429 degree of reproductive isolation between each subclade. Furthermore, there was greater
430 number of unique alleles shared within the VNB groups from different geographic regions
431 than were shared across VNB groups within the same geographic region (**Figure 7**). This
432 geographically and phylogenetically segregated diversity suggests that one or more ancient
433 migration events occurred between South America and Africa during the diversification of
434 VNB, followed by geographic isolation. In contrast, the VNI and VNII lineages showed a

435 pattern consistent with more rapid current migration, where isolates from different
436 geographic regions in many cases differed by fewer than 200 SNPs.

437 We next evaluated whether VNI and VNB showed a signal of genetic isolation by distance
438 using the Mantel test. In both VNI and VNB, genetic distance was significantly positively
439 correlated with geographic distance ($p = 0.0001$ and $p = 0.042$, respectively). When VNB
440 was separated into VNBI and VNBII, each lineage showed an even stronger signal ($p =$
441 0.0051 and $p = 0.0009$, respectively), suggesting much of the correlation seen within VNB is
442 representative of isolation within each subclade. Therefore, despite VNB showing signals of
443 more ancient migration while VNI shows signals of recent migration, both demonstrate
444 genetic substructure according to geography.

445 **Recombination between and within lineages**

446 The basal branching of Brazilian VNB isolates revealed in the phylogenetic analysis
447 suggested that South America could be a global center of *C. neoformans* var. *grubii* diversity.
448 To further investigate this hypothesis, and to explore recombination in the context of
449 population structure, we implemented the chromosome painting approach of fineStructure
450 (Lawson et al., 2012), which identifies shared genomic regions between individuals and
451 thereby ancestral relationships among individuals and populations. Our linked co-ancestry
452 model found the highest level of sharing among VNB isolates; in addition, there is evidence
453 of strong haplotype donation from South American VNB isolates (V2, V31, and V87) to all
454 other lineages and continents, suggestive of ancestral recombination (**Figure 6**).

455 Independent confirmation of ancestry using STRUCTURE confirmed that V87 includes
456 primarily VNB ancestry with ~1% VNI alleles (**Table S7**). Interrogating the chunk counts,
457 which are lengths of DNA shared by a donor to other individuals, and lengths produced by
458 fineStructure revealed that the haplotype chunks donated by these 'ancestral' isolates were
459 substantially higher than seen for other isolates, with other African VNB isolates receiving
460 significant chunks and lengths (Bt102, Bt63, Bt85, Tu229-1, Tu360-1, Tu369-1, and Tu401-
461 1) from the South American VNB isolates. Isolate V53 donated less strongly than these
462 three isolates to all lineages. Other South American VNB isolates (WM 1408 and V17)
463 donated strongly to specific lineages: WM 1408 to VNII and VNB, whilst V17 donated to VNI
464 and VNB. However, these findings for WM 1408 and V17 were not corroborated using
465 STRUCTURE. Despite their allocation to separate VNB subpopulations, V2 and V17 (VNB-I
466 and VNB-II respectively) donate the most genetic material (when interrogating the chunk
467 counts) to VNI isolates in Africa, India, and Thailand.

468

469 Within the VNI lineage, fineStructure analysis identified a subset of isolates with a high
470 frequency of haplotype sharing (**Figure 8**). Notably, a group of African (Tu259-1, 125.91,
471 RCT52, Bt100, Bt207 and Bt30) and Indian (INCr213 and INE071) isolates show strong
472 haplotype donation with many other VNI isolates, suggestive of ancestral recombination
473 events. These isolates are dispersed over four subpopulations within the VNI lineage.
474 Though the geographical distance between these populations should preclude frequent
475 intermixing, these isolates from Africa and India may include a higher fraction of ancestral
476 alleles, leading to a lack of phylogeographic structure among these highly geographically
477 distinct populations.

478

479 Finding that ancestral recombination in the VNB lineage contributed to VNI lineage diversity
480 suggested that there will be a signature of admixture linkage disequilibrium (LD) in these two
481 populations. Linkage disequilibrium differs between lineages (**Figure S8**), with VNII LD
482 decaying slowly with physical distance, and manifesting an LD50 (where linkage
483 disequilibrium has decayed to half its maximum value) at >150 kb. However, this value may
484 reflect the highly clonal nature and relatively small number of sequenced VNII isolates. LD
485 decay is relatively slow for VNI with an LD50 of 4,500 bp, whereas LD decays more rapidly
486 in the VNB lineage, with an LD50 of 1,500 bp. When separated into geographical origin of
487 isolation (**Figure S8 (b)**), LD50 for South American VNB appears greater (> 150 kbp) than
488 that seen in African VNB (2,000 bp). The slower decay of LD in VNI and VNII relative to VNB
489 may reflect a predominately asexual mode of reproduction owing to the rarity of the *MATa*
490 idiomorph.

491

492 **Discussion**

493 This population genomic analysis of *Cryptococcus neoformans* var. *grubii* has revealed new
494 biogeographic relationships and highlighted a complex history of hybridization events
495 between groups. Analysis of genome-wide variation of 188 geographically diverse isolates
496 greatly increases the resolution of the VNI, VNII, and VNB phylogenetic groups and
497 precisely measures the level of genetic differentiation between isolates within each group
498 and across geographic scales. This data supports a much higher diversity of isolates in the
499 VNB group compared to VNI and VNII isolates. Notably, we show that hybridization between
500 these groups can result in genome mixing suggestive of recent and ongoing meiotic
501 exchange. A recent taxonomic proposal to divide the *C. neoformans* and *C. gattii* species
502 complexes into seven monophyletic species did not subdivide *C. neoformans* var *grubii* into
503 separate species; although VNI, VNII, and VNB were strongly supported clades in a
504 multilocus phylogeny, coalescent based approaches did not clearly support these three

505 lineages as separate species (Hagen et al., 2015). Our genome-wide analysis has
506 uncovered new biogeographic structure and ongoing hybridization between lineages of *C.*
507 *neoformans* var. *grubii*, suggesting that further subdivision is not straightforward. In addition,
508 such hybridization events may be a biological feature that extends across other lineages
509 within the *C. neoformans* and *C. gattii* species complexes (Farrer et al., 2015; Hagen et al.,
510 2015), prompting a need for wider investigation of the population genomic structure of this
511 whole complex is needed to support formal changes in taxonomy (see perspective by
512 Kwon-Chung'17).

513

514 The placement of isolates from Brazil at basal branching positions of the two VNB subclades
515 phylogenetically separates the South American and African isolates within both the VNBI
516 and VNBII groups. This finding, along with the presence of a large number of unique alleles
517 in each of these four subclades and strong haplotype sharing seen with fineStructure
518 analysis (**Figure 7**), suggests that there was ancient migration of the VNB group between
519 Africa and South America following the initial divergence of VNBI and VNBII but prior to each
520 group's diversification. This finding appears consistent with a prior report of diverse isolates
521 from Brazil in a new VNI genotype 1B (Oliveira et al., 2004). While the lack of a reliable
522 molecular clock combined with substantial rates of recombination prevents dating the time of
523 divergence between VNB from South America and Africa with confidence, this division
524 clearly occurred after these continents split over 110 million years ago, and also after VNB
525 itself subdivided into two lineages – VNBI and VNBII. As is the case with VNI, cross-Atlantic
526 migration events may have also vectored VNB between these two continents. However,
527 despite evidence for these migration events, the majority of VNI and VNBII migrations were
528 likely much more recent than is seen with VNB, with nearly clonal isolates of VNI and VNBII
529 found in disparate geographic regions. The presence of one South American VNB isolate
530 (V53) nested within African isolates on the phylogeny does, however, suggest a limited
531 number of more recent migration events may be occurring between the two regions even
532 within VNB despite the large degree of reproductive isolation that we observed. Identification
533 of additional South American VNB isolates is necessary to determine their diversity and
534 relationship to isolates from African continental regions. Although the sequenced isolates all
535 contain the *MATa* genotype, our sample size was small and likely under-represents the true
536 diversity of this lineage in South America and the ecological reservoirs that it occupies.

537 Given the propensity of *Cryptococcus neoformans* var. *grubii* VNI and VNBII for having an
538 environmental reservoir in bird excreta (unlike VNB which is principally associated with
539 arboreal reservoirs (Litvintseva et al., 2011; Vanhove et al., 2017)), it has been proposed
540 that pigeons globally dispersed *Cryptococcus neoformans* var. *grubii* from a genetically

541 diverse population in southern Africa (Litvintseva et al., 2011), resulting in an expansion of
542 the *Cng* VNI out of Africa. Litvintseva et al. (2011) hypothesized that this “out-of-Africa”
543 model for the evolution of VNI explains the origin of the global VNI population. Other studies
544 showing lower genetic diversity of VNI populations in Southeast Asia (Simwami et al., 2011)
545 and in South America (Ferreira-Paim et al., 2017), further supporting an African origin of
546 *Cryptococcus neoformans* var. *grubii*. An alternative explanation for the higher diversity of
547 African VNI could be that this lineage originated elsewhere and became more diverse in this
548 continent by mating with the ‘native’ VNB population or due to other factors. Our analysis did
549 not find a large subset of VNB alleles within the African VNI isolates based on ancestry
550 analysis. In addition, we found one VNI subclade composed mostly of African isolates that
551 appears to be recombining at higher frequency than other VNI groups. The phylogenetic
552 intermixing of isolates from India and Africa strongly support the hypothesis that there is
553 long-range dispersal and ancient recombination in environmental populations in India and
554 Africa, indicative of multiple migratory events across time and into the present. Did VNI
555 therefore evolve ‘Out of Africa’? Further sampling of environmental isolates from across
556 South America as well as more diverse regions of Africa, as well as estimation of the
557 mutation rate in *Cryptococcus neoformans* var. *grubii* to allow calibration of a molecular
558 clock, is needed to further test this hypothesis.

559 While gene content is very similar across the *Cryptococcus neoformans* var. *grubii* lineages,
560 we found examples of lineage specific genes including clusters unique to VNI or VNII. While
561 this suggests that the *Cryptococcus neoformans* var. *grubii* gene inventory based on H99
562 (Janbon et al., 2014) is largely representative of all lineages, additional genes specific to
563 VNII and VNB are important to consider in studies focusing on isolates of these lineages.
564 Differences in gene expression may also differentiate the lineages, and it is important to note
565 that these will include lineage-specific genes that may contribute to variation in clinical
566 profiles and virulence that occur among lineages of *Cryptococcus neoformans* var. *grubii*
567 (Beale et al., 2015). In addition, we found the most rapidly evolving genes between each of
568 the lineages include transcription factors and transferases, suggesting phenotypic diversity
569 may be generated through transcriptional reprogramming and protein modification rather
570 than changes in gene content. The *SX11* gene detected in comparisons of VNII with both
571 VNI and VNB appears to be highly substituted in the VNII lineage; this sequence divergence
572 of *SX11* in VNII could contribute to differences in mating with this group. Truncated alleles of
573 *SX11* are frequently observed in the serotype D *MAT α* chromosome of AD hybrids and
574 suggested to contribute to increased mating efficiency (Lin et al., 2007).

575 Our analysis revealed that hybrid isolates originate from multiple lineages, and resolved the
576 parental genotypes. Prior analysis with MLST loci suggested that some isolates contain a

577 mix of multiple genotypes (Chen et al., 2015; Litvintseva et al., 2003). However the
578 sensitivity and precision of these methods is limited by the small number of loci examined,
579 the use of genes involved in virulence that may be under different selective pressure, as well
580 as incomplete lineage sorting in some groups. Analysis of genome-wide variation revealed
581 that some isolates appear to be a recent mix of different ancestries, based on the detection
582 of large blocks of sites with each ancestry; this could result from a small number of crossing
583 over events for each chromosome during meiosis. Other isolates contain more highly
584 intermixed ancestry across the genome and predominantly of a single ancestry; these may
585 have occurred by more historical hybridization followed by subsequent mating within a single
586 lineage group. The demonstration of genome mixing in hybrid isolates raises interesting
587 questions about whether such fundamentally new assortments of the three lineages could
588 generate genotypes with new phenotypes, which perhaps have a fitness or selective
589 advantage.

590 Analysis of hybrids between serotypes A and D revealed a remarkable degree of genome
591 reassortment. All of the 8 sequenced AD isolates show evidence of aneuploidy, affecting the
592 copy number of 12 of 14 serotype A derived chromosomes and all 14 serotype D derived
593 chromosomes. This is consistent with the high rate of AD isolate aneuploidy previously
594 reported using flow analysis of DNA content (Lengeler et al., 2001) or comparative genome
595 hybridization (Hu et al., 2008). For some chromosomes, only one parental genotype was
596 detected in a subset of five isolates; this includes a loss of the serotype D copy of
597 chromosome 1, as previously observed in analysis of three AD hybrid isolates (Hu et al.,
598 2008). However, we further find that loss of heterozygosity (LOH) in some cases is due to
599 partial copies of several chromosomes, suggesting that genomic instability in AD hybrids
600 may result in chromosomal breakage. LOH was also observed for smaller regions in diploid
601 AA hybrids. Similar LOH events are frequently observed in diploid fungi including *Candida*
602 *albicans* (Hirakawa et al., 2015) and may contribute to the generation of genetic diversity in
603 both species.

604 Aneuploidy was also commonly observed in the haploid *Cryptococcus neoformans* var.
605 *grubii* isolates. Additional copies of regions of Chromosome 1 that include *AFR1* or *ERG11*
606 are associated with drug resistance, though aneuploidies of additional chromosomes are
607 also observed (Sionov et al., 2010). Although functional significance of aneuploidy of other
608 chromosomes is less well understood, most of the isolates exhibiting aneuploidy are of
609 clinical origin, suggesting increased copy of other genes may provide an advantage or that
610 there is higher genome instability during infection. An isochromosome of the left arm of
611 chromosome 12 that arose during infection has been reported (Ormerod et al., 2013) and
612 chromosome 12 aneuploidy is widely seen in African patients with relapsed infections (Chen

613 et al., 2017; Rhodes et al., 2017) although the specific role of this duplication is unclear. Our
614 data suggests that there could be additional isochromosomes based on the detection of
615 partial chromosomes using sequencing read depth; alternatively these regions could be
616 represented in the genome as fusions with other chromosomes.

617 Previous studies of *Cryptococcus gattii* have pointed towards South America as a source of
618 the diversity for the *C. gattii* VGII lineage (Engelthaler et al., 2014; Hagen et al., 2013).
619 Given the shared evolutionary history of *C. gattii* and *Cryptococcus neoformans* var. *grubii*
620 (Xu et al., 2000), South America could also represent a major diversity center of
621 *Cryptococcus neoformans* var. *grubii*. Our data suggests that *Cryptococcus neoformans* var.
622 *grubii* VNB isolates in both subgroups from South America have undergone ancestral
623 recombination events, donating genetic material to all lineages across multiple geographical
624 locations. Our study also provides clear evidence for recombination within the VNI and VNII
625 lineages, where nearly all the isolates contain the *MAT α* mating type. This suggests that
626 mating likely occurs between *MAT α* isolates, as is found in *C. neoformans* var. *neoformans*
627 (Sun et al., 2014). Previous studies have hypothesized that *Cryptococcus neoformans* var.
628 *grubii* can complete its sexual reproductive life cycle in environmental niches, such as plants
629 (Xue et al., 2007) and pigeon guano (Nielsen et al., 2007; Vanhove et al., 2017). Our
630 observations that all lineages of *Cryptococcus neoformans* var. *grubii* show the ability to
631 widely disperse, to recombine, and to hybridize, illustrates that this pathogen has a high
632 degree of evolutionary plasticity that is likely related to its success in infecting the
633 immunosuppressed 'human environment', thereby causing a high burden of mortality
634 worldwide (Armstrong-James et al., 2014).

635

636 **Methods**

637 Isolate selection

638 A total of 188 *C. neoformans* var. *grubii* isolates were selected from previous studies, which
639 include 146 clinical isolates, 36 environmental isolates, 4 animal isolates and 2 isolates of
640 unknown isolation source; these isolates were collected from 14 different countries:
641 Argentina, Australia, Botswana, Brazil, China, Cuba, France, India, Japan, South Africa,
642 Tanzania, Thailand, Uganda and USA (**Table S1**). Most of the clinical isolates were isolated
643 from the cerebrospinal fluid of patients. Eight of the 36 environmental isolates were isolated
644 from pigeon guano, and most of the remaining isolates were collected from Mopane and
645 other tree species.

646 Details of clinical trials and ethical review

647 French isolates were collected during the Crypto A/D study (Dromer et al., 2007). The study
648 was approved by the local ethical committee and reported to the French Ministry of Health
649 (registration # DGS970089). For clinical trials undertaken in South Africa (Bicanic et al.,
650 2007, 2008; Jarvis et al., 2012; Loyse et al., 2012) and Thailand (Brouwer et al., 2004),
651 ethical approval was obtained from the Wandsworth Research Ethics Committee covering St.
652 George's University of London. Local ethical approval was obtained from the University of
653 Cape Town Research Ethics Committee in South Africa the ethical and scientific review
654 subcommittee of the Thai Ministry of Public Health. Clinical isolates from India were
655 collected during routine diagnostic service; local ethical approval was obtained from the
656 Institutional Ethical Committee of Vallabhbhai Patel Chest Institute, University of Delhi, India.

657 Fluconazole sensitivity testing

658 Fluconazole MICs were determined for two isolates by the NHLS laboratory in Green Point,
659 Cape Town using the E-test method (Biomerieux) (Bicanic et al., 2006).

660

661 DNA isolation and sequencing

662 Each yeast isolate was recovered from a freezer stock and purely cultured on an YPD or SD
663 agar plate for 48-60 h. Next, a single colony was inoculated to another YPD plate and
664 cultured for 24 h. Approximately 100 µl of yeast cells were used for DNA isolation using the
665 MasterPure yeast DNA purification kit (Epicenter, Madison, WI) according to the
666 manufacturer's instructions. Alternatively, a single colony was inoculated into 6ml YPD broth
667 supplemented with 0.5M NaCl and cultured for 40 hours at 37°C, prior to extraction using the
668 MasterPure Yeast DNA purification kit (Epicentre) as previously described (Rhodes et al.,
669 2017).

670 DNA was sequenced using Illumina technology; for each isolate, a small insert library was
671 constructed and used to generate between 14 and 150 Million paired-end reads with 101bp
672 per isolate, which results in 56 to 603 fold average coverage of reads aligned to the H99
673 genome. In addition, large insert libraries were constructed for 15 isolates (**Table S4**) and
674 also used to generate 101bp paired-end reads. Isolates were sequenced at Imperial College
675 London and the Broad Institute (**Table S1**).

676 Read alignment, variant detection, and ploidy analysis

677 Illumina reads were aligned to the *Cryptococcus neoformans* var. *grubii* reference genome
678 H99 (Janbon et al., 2014) using the Burrows-Wheeler Aligner (BWA) 0.7.12 mem algorithm

679 (Li, 2013) with default parameters. BAM files were sorted and indexed using Samtools (Li et
680 al., 2009) version 1.2. Picard version 1.72 was used to identify duplicate reads and assign
681 correct read groups to BAM files. BAM files were locally realigned around INDELs using
682 GATK (McKenna et al., 2010) version 3.4-46 'RealignerTargetCreator' and 'IndelRealigner'.

683 SNPs and INDELs were called from all alignments using GATK version 3.4-46
684 'HaplotypeCaller' in GVCF mode with ploidy = 1, and genotypeGVCFs was used to predict
685 variants in each isolate. All VCFs were then combined and sites were filtered using
686 variantFiltration with QD < 2.0, FS > 60.0, and MQ < 40.0. Individual genotypes were then
687 filtered if the minimum genotype quality < 50, percent alternate allele < 0.8, or depth < 10.

688 In examining isolates with a high proportion of sites that were removed by these filters,
689 inspection of the allele balance supported that these isolates were diploid. For heterozygous
690 diploid isolates, haplotypeCaller was run in diploid mode. VariantFiltration was the same,
691 with the added filter of ReadPosRankSum < -8.0. Then for individual genotype filtration there
692 was no allele depth filter but otherwise was the same. The filters were kept as similar as
693 possible to maximize combinability. For AD hybrids, a combined reference of H99 (Janbon
694 et al., 2014) and JEC21 (Loftus et al., 2005) was used for alignment and SNP identification.

695 To examine variations in ploidy across the genome, the depth of bwa alignments at all
696 positions was computed using Samtools mpileup, and then the average depth computed for
697 5kb windows across the genome.

698 MAT locus determination

699 To evaluate the mating type alleles present in each isolate, Illumina reads were aligned
700 using bwa mem to a multifasta of both versions of the mating type locus (AF542529.2 and
701 AF542528.2 (Lengeler et al., 2000b)). Depth at all positions was computed using Samtools
702 mpileup, and then the average depth computed for the *SXI1* and *STE20* genes for both
703 idiomorphs. Nearly all isolates showed unique mapping to either the *MATa* or *MATα* alleles
704 of both genes; one isolate, Ftc158, showed significant mapping to both *MATa* and *MATα*,
705 though 2-fold more to *MATα*. For the hybrid haploid isolates, the ancestry of the *MAT* locus
706 was determined from the Structure site by site output.

707 Genome assembly and annotation

708 Illumina sequence for each isolate was assembled using Allpaths for 36 isolates (see Table
709 S4 for release numbers for each assembly) or SPAdes 3.6.0 (with parameter -careful) for
710 the remaining 3 isolates. Assemblies with both fragment and jump libraries were more
711 contiguous than those with fragment only data (average of 84 or 561 scaffolds, respectively,

712 **Table S4**). However there was little difference in the total contig length between assemblies
713 with or without jump data (average 18.4Mb and 18.5Mb, respectively, **Table S4**).

714 The predicted protein coding gene set for each assembly was generated by combining three
715 primary lines of evidence. Genes were transferred to each new assembly from the well
716 annotated H99 assembly (Janbon et al., 2014) based on whole genome nucleotide
717 alignments from nucmer. Genemark-ES (Ter-Hovhannisyan et al., 2008) was run on each
718 assembly to generate a de novo set of calls. These two sets were combined and improved
719 using PASA (Haas et al., 2008) with RNA-Seq data of three in vitro conditions (YPD, Limited
720 media, and Pigeon guano) generated for H99 (Janbon et al., 2014) and for the VNB isolate
721 Bt85 also input. Repetitive elements were removed from the gene set based on
722 TransposonPSI (<http://transposonpsi.sourceforge.net/>) alignments or PFAM domains found
723 only in transposable elements. The filtered set was assigned sequential locus identifiers
724 across each scaffold. The average number of 6,944 predicted genes across all assemblies
725 (**Table S4**) is close to the 6,962 predicted on the H99 reference.

726 Ortholog identification and comparison

727 To identify orthologs across the set of 45 *Cryptococcus* genomes (**Table S4**), proteins
728 clustered based on BLASTP pairwise matches with $\text{expect} < 1e-5$ using ORTHOMCL v1.4 (Li
729 et al., 2003). To identify orthologs specific to each of the serotype A lineages, we required
730 that genes were present in 90% of the assembled genomes for VNI (36 or more) or VNB (8
731 or more) or all VNII (3 genomes). To confirm that orthologs were missing in the other two
732 lineages, synteny was examined around each gene; in some cases this identified candidate
733 orthologs missed by OrthoMCL, which were confirmed by BLASTP similarity and removed.

734 Phylogenetic analysis

735 A phylogeny for the sets of 159 or 164 isolates was inferred from SNP data using RAXML
736 version 8.2.4 (Stamatakis, 2014) with model GTRCAT and 1,000 bootstrap replicates. A
737 separate analysis of the phylogenetic relationship based on gene content included 40
738 *Cryptococcus neoformans* var. *grubii* serotype A genomes (28 VNI, 3 VNII, and 9 VNB), 1
739 *Cryptococcus neoformans* var. *neoformans* serotype D genome (JEC21), and 4 *C. gattii*
740 genomes (WM276, R265, CA1873, and IND107) (**Table S4**). The total of 4616 single copy
741 orthologs identified in all genomes were aligned individually with MUSCLE (Edgar, 2004) at
742 the protein level, converted to the corresponding nucleotide sequence to maintain reading
743 frame alignment, poorly aligning regions removed trimal (Capella-Gutiérrez et al., 2009), and
744 invariant sites removed. A phylogeny was inferred using RAXML version 7.7.8 in rapid
745 bootstrapping mode with model GTRCAT and 1,000 bootstrap replicates.

746 Population structure

747 To examine major population subdivisions, we examined how isolates clustered in a
748 principal components analysis (PCA). SNP calls for all the isolates were compared using
749 SMARTPCA (Patterson et al., 2006). To identify the major ancestry subdivisions and their
750 contributions to the isolates appearing at intermediate positions in the PCA, a total of
751 338562 randomly subsampled positions containing variants in at least two isolates and less
752 than 5% missing data were clustered using the Bayesian model-based program
753 STRUCTURE v2.3 (Pritchard et al., 2000) in the site-by-site mode. Ancestry was plotted
754 across the genome for each isolate using the matplotlib plotting package in Python.

755 For analysis of *Cryptococcus neoformans* var. *grubii* diploid isolates (**Table S3**), diagnostic
756 SNPs for VNB and VNII were present exclusively in the respective group, and called for all
757 VNB, VNII, and ≥ 100 VNI isolates. Diagnostic SNPs for VNI were present exclusively in
758 VNII and VNB, and called for all VNB, VNII, and ≥ 100 VNI isolates.

759 Population genetic measures including π , F_{st} , and Tajima's D were calculated using
760 popGenome (Pfeifer et al., 2014). d_N and d_S measures were calculated from fixed SNPs in
761 each lineage using codeml version 4.9c (Yang, 2007). To examine the distribution of the
762 alleles within VNB, we first identified 445,193 alleles private to VNB (present in at least 1
763 VNB isolate and no VNI or VNII isolates). We subdivided VNB into four clades (VNBI-South
764 America, VNBI-Africa, VNBII-South America, and VNBII-Africa) and calculated the number
765 of those private alleles unique to each clade (present in that one clade and no others) and
766 shared across VNB groups or geography (present in the two compared clades but no others).
767 The Mantel test was conducted using the center-point of each country to determine
768 distances between isolates and the number of SNPs between each pairwise set of isolates.
769 The test was conducted using available Python software
770 (<https://github.com/jwcarr/MantelTest>) with 1000 permutations and the upper tail test of
771 positive correlation.

772 Linkage disequilibrium

773 Linkage disequilibrium was calculated in 500 bp windows of all chromosomes except for the
774 ~ 100 kb mating type locus on chromosome 5 with vcftools version 1.14 (Danecek et al.,
775 2011), using the --hap-r2 option with a minimum minor allele frequency of 0.1.

776

777 Population inference by fineStructure

778 Model-based clustering by fineStructure (Lawson et al., 2012) assigns individuals to
779 populations based on a coancestry matrix created from SNP data, using either Markov chain
780 Monte Carlo or stochastic optimisation. The algorithm uses chromosome painting, which is

781 an efficient way of identifying important haplotype information from dense data, such as SNP
782 data, and efficiently describes shared ancestry within a recombining population. Each
783 individual is painted using all the other individuals as donors. For example, if an isolate *x* is
784 clonal and a donor, the clonally related recipients will receive almost all of their genetic
785 material from isolate *x*, and its closest relatives. This approach has been applied to analyze
786 recombination in fungal (Engelthaler et al., 2014) and bacterial studies (Yahara et al., 2013).
787
788 fineStructure analysis (Lawson et al. 2012) was performed using an all lineage SNP matrix,
789 with one representative of each clonal VNI population in order to infer recombination,
790 population structure, and ancestral relationships of all lineages. A separate analysis of all
791 VNI lineage isolates was also performed. This approach was based on the presence or
792 absence of shared genomic haplotypes. ChromoPainter reduced the SNP matrix to a
793 pairwise similarity matrix under the linked model, which utilises information on linkage
794 disequilibrium, thus reducing the within-population variance of the coancestry matrix relative
795 to the between-population variance. Since the MAT idotypes introduce large bias into SNP
796 analysis, they were removed to enable characterisation of more defined populations. There
797 was no significant loss of sharing of genetic material when compared to retaining the MAT
798 locus.

799

800 **Acknowledgments**

801 We thank the Broad Institute Genomics Platform for generating DNA sequence for this study
802 and Jose Munoz for helpful comments on the manuscript.

803 **Funding Statement**

804 This project has been funded in whole or in part with Federal funds from the National
805 Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of
806 Health and Human Services, under grant number U19AI110818 and by the National Human
807 Genome Research Institute grant number U54HG003067 to the Broad Institute. Support to
808 J.R.P. came from Public Health Service Grants AI73896, AI93257. JR and MAB were
809 supported by a UK Medical Research Council Grant awarded to MCF, TB and TH (MRC
810 MR/K000373/1). MV was supported by a UK Natural Environment Research Council PhD
811 studentship. JH was supported by NIH grants AI39115-19 and AI50113-13. The funders had
812 no role in study design, data collection and analysis, decision to publish, or preparation of
813 the manuscript.

814 **Data access**

815 All sequence data from this study have been submitted to GenBank under BioProject ID
816 PRJNA 384983 (<http://www.ncbi.nlm.nih.gov/bioproject>); individual accession numbers are
817 listed in Supplemental Tables S1 and S4.

818 **Author contributions**

819 **Investigation:** JR, CAD, SMS, SS, CAC
820 **Validation:** JR, CAD, SMS, CAC
821 **Visualization:** JR, CAD, SMS, SS, CAC
822 **Writing – Original Draft Preparation:** JR, CAD, CAC
823 **Writing – Review & Editing:** JR, CAD, MCF, CAC, AA, MAB, DME, WM, FH, JMV, JH, AL,
824 JP
825 **Resources:** MCF, CAC, MV, YC, JP, TB, TH, VP, ALC, AC, FH, MTI-Z, WM, DME, AA, JMV,
826 JH
827 **Supervision:** CAC, MCF
828 **Funding Acquisition :** CAC, MCF
829 **Conceptualization :** CAC, MCF, AL

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833 **Tables**

Table 1. Properties of Sequenced isolates.

Haploid isolates

Population	Isolates (#)	<i>MAT</i> α	<i>MATa</i>
VNI	111	109	2
VNII	23	23	0
VNB	25	21	4
VNI/VNB	5	1	4
VNII/VNB	2	2	0

Diploid isolates

Population	Isolates (#)	<i>MAT</i> α / <i>MAT</i> α	<i>MATa</i> / <i>MATa</i>	<i>MATa</i> / <i>MAT</i> α
VNI/VNB	1	1	0	0
VNII/VNB	2	2	0	0
VNB/Cnn	8	0	1	7
VNB/Cng	1	1	0	0

834

835

836 **Table 2.** Rapidly evolving genes in the three lineages of *C. neoformans* var. *grubii*.
 837 Consensus sequences were built for each lineage, and d_N and d_S were calculated for each
 838 lineage pair. As d_S was uniformly low throughout the dataset due to limited genetic diversity,
 839 for each pair of lineages we identified the 10 genes with assigned names (Inglis et al., 2014)
 840 with the highest d_N , which measures both the mutation rate and selection.

Comparison	d_N	Locus	Gene	Annotation
VNI vs VNB	0.0181	CNAG_01841	<i>GLN3</i>	transcription factor, deletion sensitive to organic peroxides (Jung et al., 2015)
	0.0155	CNAG_03894	<i>PDR802</i>	transcription factor, deletion with reduced virulence (Jung et al., 2015)
	0.0095	CNAG_03213	<i>UVE1</i>	UV damage endonuclease
	0.0092	CNAG_02756	<i>CDC43</i>	geranylgeranyltransferase-I, essential for virulence (Selvig et al., 2013)
	0.0090	CNAG_06655	<i>GPI18</i>	GPI-anchor transamidase
	0.0089	CNAG_01908	<i>HEM4</i>	uroporphyrinogen-III synthase
	0.0085	CNAG_03133	<i>ATG2602</i>	UDP-glucose sterol transferase
	0.0084	CNAG_03617	<i>CLP1</i>	clampless protein 1
	0.0076	CNAG_05740	<i>RAM1</i>	farnesyltransferase β -subunit, essential for virulence (Esher et al., 2016)
	0.0068	CNAG_03637	<i>YKU80</i>	Double strand break repair factor and silencing regulator, deletion has reduced virulence (Liu et al., 2008)
VNI vs VNII	0.0610	CNAG_05836	<i>HOC1</i>	α 1,6-mannosyltransferase (Lee et al., 2015)
	0.0408	CNAG_05838	<i>RGD1</i>	Rho GTPase activating protein, deletion has increased virulence (Liu et al., 2008)
	0.0214	CNAG_06031	<i>KRE63</i>	β -glucan synthase, involved in capsule and cell wall formation, deletion has decreased virulence (Gilbert et al., 2010)
	0.0149	CNAG_06814	<i>SXI1α</i>	α cell type transcription factor, required for mating (Hull et al., 2002)
	0.0142	CNAG_01841	<i>GLN3</i>	see above
	0.0135	CNAG_03229	<i>YOX101</i>	transcription factor, deletion sensitive to organic peroxides (Jung et al., 2015)
	0.0127	CNAG_03398	<i>ZIP2</i>	zinc ion transporter
	0.0113	CNAG_03133	<i>ATG2602</i>	see above
	0.0110	CNAG_03366	<i>ZNF2</i>	transcription factor, overexpression results in reduced virulence (Wang et al., 2012)
	0.0104	CNAG_01019	<i>SOD1</i>	superoxide dismutase
VNB vs VNII	0.0617	CNAG_05836	<i>HOC1</i>	see above
	0.0402	CNAG_05838	<i>RGD1</i>	see above
	0.0171	CNAG_06031	<i>KRE63</i>	see above
	0.0128	CNAG_03366	<i>ZNF2</i>	see above
	0.0122	CNAG_06814	<i>SXI1α</i>	see above
	0.0114	CNAG_03213	<i>UVE1</i>	see above
	0.0104	CNAG_01019	<i>SOD1</i>	see above
	0.0104	CNAG_03398	<i>ZIP2</i>	see above
	0.0102	CNAG_01841	<i>GLN3</i>	see above
	0.0102	CNAG_02756	<i>CDC43</i>	see above

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Table 3. Comparison of VN group statistics

Populations	Isolates (#)	Segregating sites	Pi	Tajima's D
VNI	111	190,716	0.00200	-0.107179
VNII	23	337,990	0.00105	-1.005950
VNB	25	613,991	0.00736	-0.232596

844

845

Table 4. Pairwise comparison of VN group statistics

Comparisons	Fixed	Shared	Private_A	Private_B	dXY	Fst
VNB vs VNI	54,719	52,536	446,566	102,817	IvB: 0.0119	IvB: 0.595
VNB vs VNII	118,329	68,211	405,406	78,444	BvII: 0.0154	BvII: 0.707
VNI vs VNII	188,590	38,501	116,845	83,802	IvII: 0.0152	IvII: 0.874

846

847

848 **Figure Legends**

849 Figure 1. Phylogenetic analysis supports three major lineages of *C. neoformans* var. *grubii*.
850 Using a set of 876,121 SNPs across the 159 non-hybrid isolates, the phylogenetic
851 relationship was inferred using RAxML. The percentage of 1,000 bootstrap isolates that
852 support each node is shown for major nodes with at least 90% support. For each isolate, the
853 geographical site of isolation is noted by colored boxes.

854 Figure 2. Ancestry characterization of three major groups highlights hybrid isolates. A. The
855 fraction of ancestry ($k=3$) estimated by STRUCTURE is shown within a column for each
856 isolate. B. Principal components analysis separates the 3 major lineages, with the hybrid
857 isolates showing a mix of VNB ancestry with either VNI or VNII.

858 Figure 3. Large blocks of ancestry suggest recent recombination between lineages. For
859 each of the four isolates depicted (A-D), the STRUCTURE assigned ancestry for each site
860 along each chromosome is depicted as a colored bar corresponding to VNI, VNII, and VNB
861 ancestry. Locations of centromeres are marked with black bars.

862 Figure 4. Chromosome ancestry and ploidy variation of AD hybrids. For three AD hybrid
863 isolates (RCT14, IFNR21, and CCTP50), the contribution and copy number of A (green) and
864 D (blue) ancestry chromosomal regions was measured by aligning all sequence reads to a
865 combined AD reference (H99, left and JEC21, right). The copy number of each chromosome
866 is depicted, with either full or partial chromosomal regions shown; see Figure S4 for detailed
867 coverage plots for all AD hybrid isolates.

868 Figure 5. Lineage-specific gene clusters. Two large-lineage specific clusters were detected
869 in the VNI genomes or VNII genomes; these are depicted using a representative genome
870 from each lineage. A. Insertion of CNAG_06649 to CNAG_06653 in H99 (blue, VNI);
871 syntenic genes in Bt85 (pink, VNB) and MW_RSA852 (green, VNII) are connected with grey
872 bars. B. Insertion of C358_04097 to C358_04102 in MW_RSA852.

873 Figure 6. Genome-sharing analysis of *Cryptococcus neoformans* var. *grubii* using
874 fineStructure was performed on a SNP matrix using a representative of each clonal
875 population within the VNI lineage. These genomes were reduced to a pairwise similarity
876 matrix, which facilitates the identification of population structure based on haplotype sharing
877 within regions of the genome. The x-axis represents the “donor” of genomic regions, while
878 the y-axis represents the recipient of shared genomic regions. The scale bar represents the
879 amount of genomic sharing, with black representing the largest amount of sharing of genetic
880 material, and white representing the least amount of shared genetic material (no sharing).
881 The geographical site of isolation is illustrated with coloured boxes as in Figure 1, and
882 lineages are also shown.

883 Figure 7. VNB alleles in population subdivisions and across geography. A. Phylogeny of
884 VNB lineage showing major subdivisions (VNB-I and VNB-II) and inferred ancestral
885 geography (South America or Africa, depicted as continent shapes). B. Classification of all
886 445,193 private VNB alleles (present in at least 1 VNB isolate and no VNI or VNII isolates)
887 by subdivisions and geography. Most VNB alleles are specific for the each VNB subdivision
888 and for the geographic subdivisions within each group. More alleles are shared between
889 geographic locations in the same subdivision (VNB-I or VNB-II) than are shared within
890 geographic locations across subdivisions.

891 Figure 8. Genome-sharing analysis of the *Cng* VNI lineage using fineStructure on a SNP
892 matrix of 111 genomes. The x-axis represents the “donor” of genomic regions, whilst the y-
893 axis represents the recipient of shared genomic regions. The scale bar represents the
894 amount of genomic sharing, with blue representing the largest amount of sharing of genetic
895 material, and yellow representing the least amount of shared genetic material (no sharing).

896

897 **Supplementary Figure Legends**

898 Figure S1. Phylogenetic analysis of all sequenced isolates. Using a set of 876,121 SNPs
899 across 165 isolates, the phylogenetic relationship was inferred with RAxML. The percentage
900 of 1,000 bootstrap replicates that support each node is shown for major nodes with at least
901 90% support. Isolates with hybrid ancestry based on Structure are colored red.

902 Figure S2. Phylogenetic analysis of ancestry typed SNPs in hybrid isolates. SNPs with VNI
903 and VNB ancestry were separated for each hybrid isolate, and combined with the wider set
904 of SNPs for sequenced VNI or VNB isolates. Phylogenies were inferred using RAxML and
905 the percentage of 1,000 bootstrap replicates that support each node is shown. The hybrid
906 isolate in each phylogeny is highlighted with red text.

907 Figure S3. Loss of heterozygosity in AA diploid isolates. For each of the three diploid
908 heterozygous isolates of AA ancestry, the frequency of heterozygous SNPs/kb within each
909 isolate and the sequence depth is depicted for each of 14 chromosomes of H99. A. Bt66
910 has no apparent loss of heterozygosity. B. Cng9 shows a small LOH region at the start of
911 scaffold 2, which also appears haploid in sequence depth. C. PMHc1045 shows more
912 extensive LOH on multiple chromosomes, some of which are also associated with
913 aneuploidy regions.

914 Figure S4. AD hybrids show high chromosomal aneuploidy. For each AD hybrid isolate the
915 normalized depth of reads aligned to a combined AD reference is depicted across the
916 chromosomes of the H99 and JEC21 genomes. In some isolates, the loss of a chromosome
917 of one ancestry appears compensated by the gain of an extra copy in the other ancestry,
918 noted by brackets, resulting in homozygosity of some regions of the hybrid genome.

919
920 Figure S5. Phylogenetic analysis of AD hybrid isolates. For each isolate, SNPs called
921 against the H99 or JEC21 reference genome were separated, and those for H99 were
922 combined with those from other VNB isolates. Phylogenies were inferred using RAxML.

923
924 Figure S6. Chromosomal aneuploidy highlighted by normalized read depth. The average
925 depth of Illumina reads aligned to the 14 scaffolds of the H99 genome was plotted across
926 the genome; whole and partial chromosomal aneuploidies were detected for 25 isolates.

927
928 Figure S7. Phylogenetic relationship and gene conservation for selected *Cryptococcus*.
929 Using de novo assemblies and associated gene calls for the genome each isolate, orthologs
930 were identified using OrthoMCL, and the aligned protein sequences of 4,616 single-copy
931 genes were used to infer a phylogeny using RAxML. Bar graphs represent the numbers of
932 core protein clusters shared across all genomes (green), conserved protein clusters found in
933 a subset (grey), species specific protein clusters blue or orange) and clusters specific to a
934 single genome (yellow).

935

936 Figure S8. Linkage disequilibrium as a function of distance expressed as the correlation
937 coefficient r^2 for a) the three lineages, VNB, VNII and VNI and b) South American and
938 African VNB.

939

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943 **Supplementary Tables Legends**

944 Table S1. Metadata for sequenced isolates.

945 Table S2. Ancestry of Hybrid Strains from Structure

946 Table S3. Shared allele counts of diploid strains

947 Table S4. Genome assembly and annotation statistics.

948 Table S5. Lineage-specific genes.

949 Table S6. Genes conserved in *C. neoformans* var. *grubii* but absent in *C. gattii* (WM276,
950 R265, CA1873, IND107) and *C. neoformans* var *neoformans* (JEC21)

951 Table S7. Ancestry for selected haploid isolates from Structure.

952

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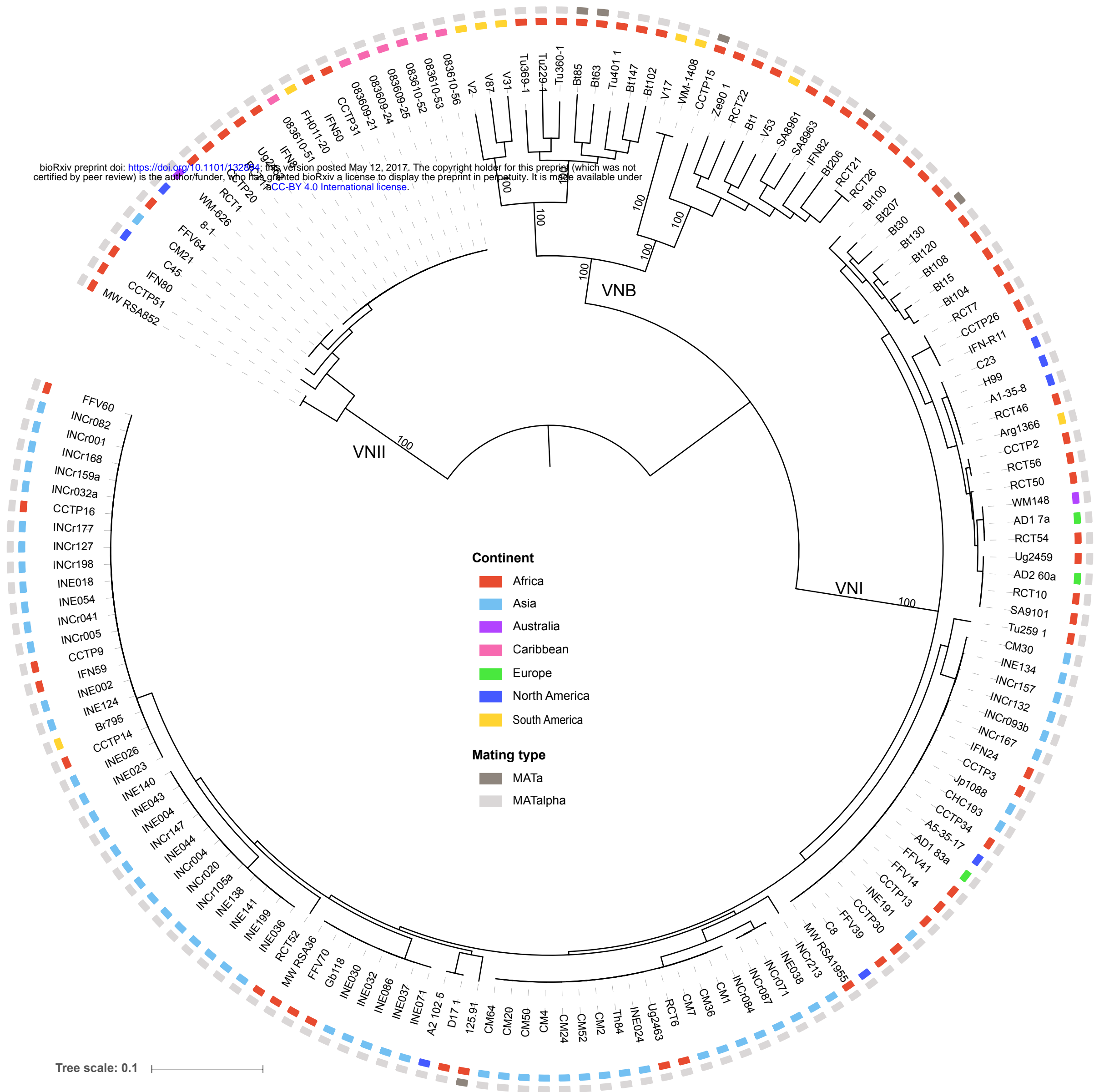
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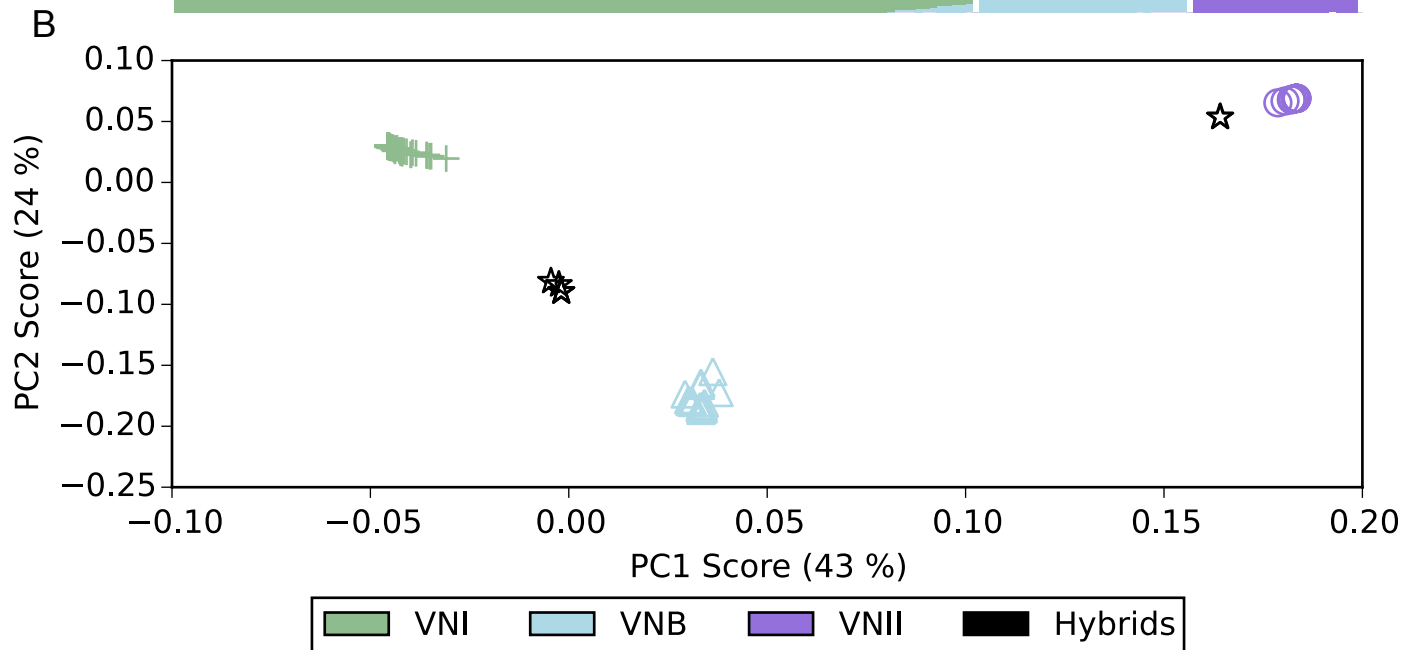
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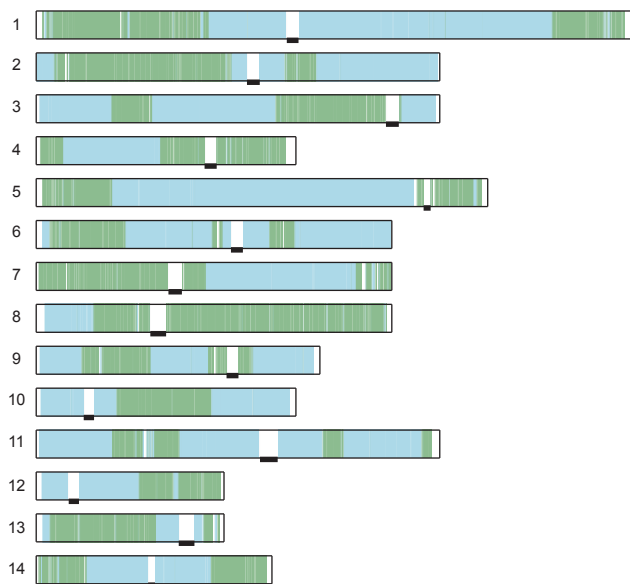
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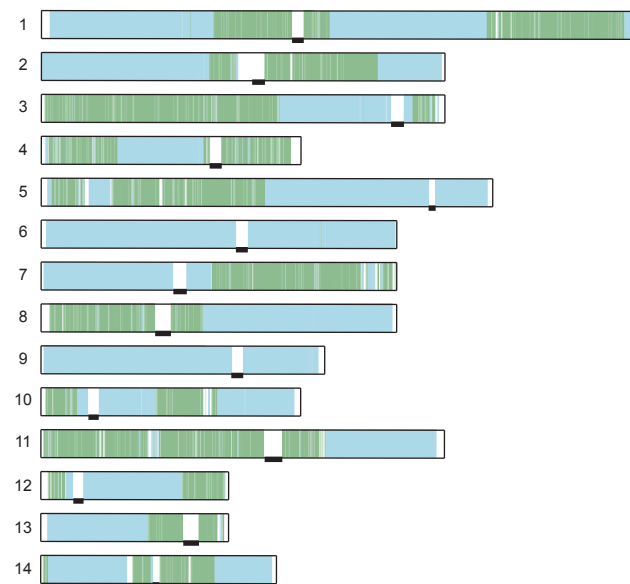
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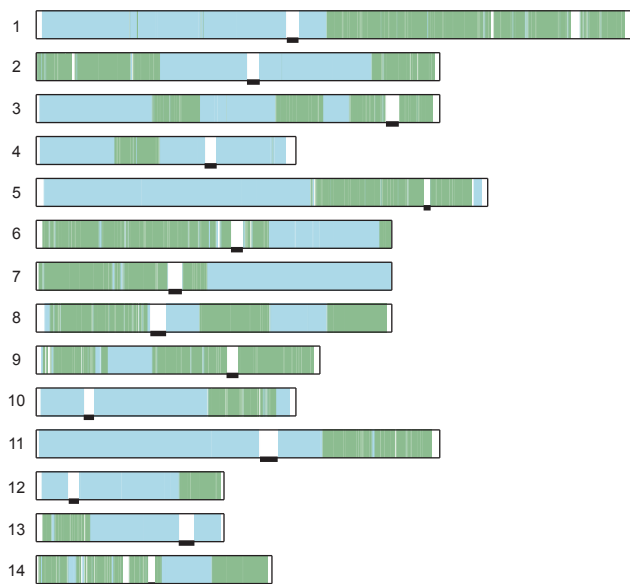
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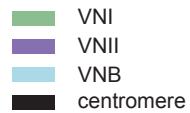
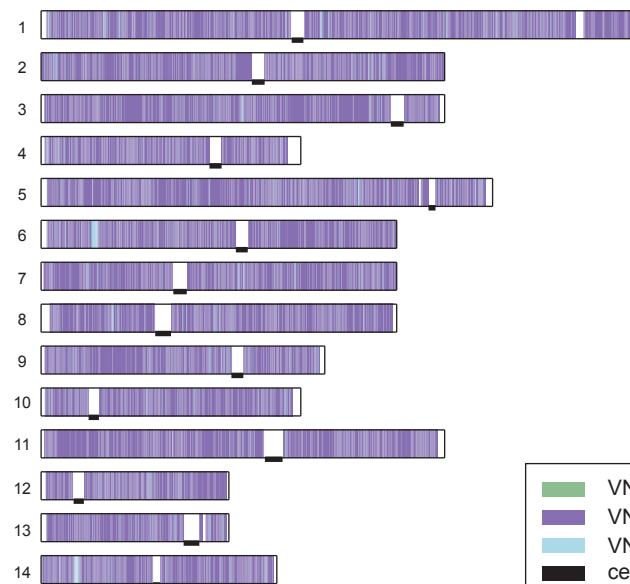
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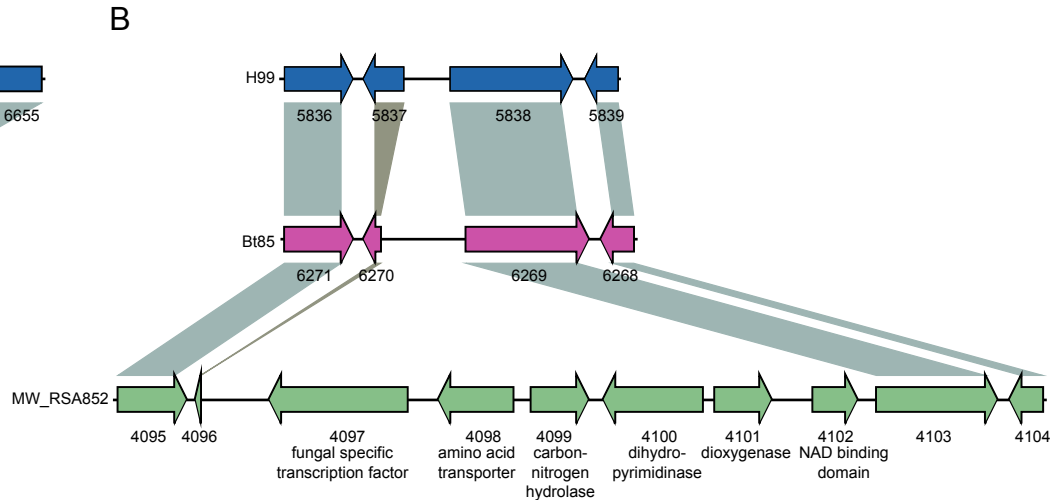
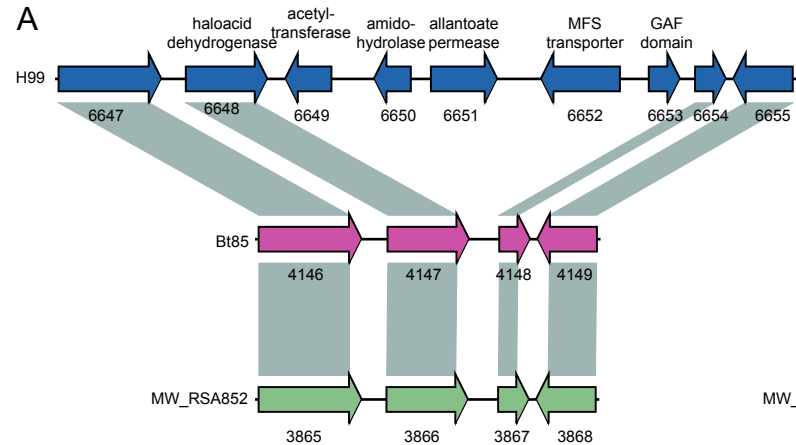


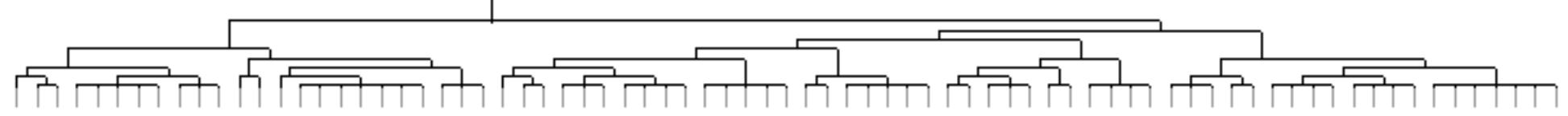
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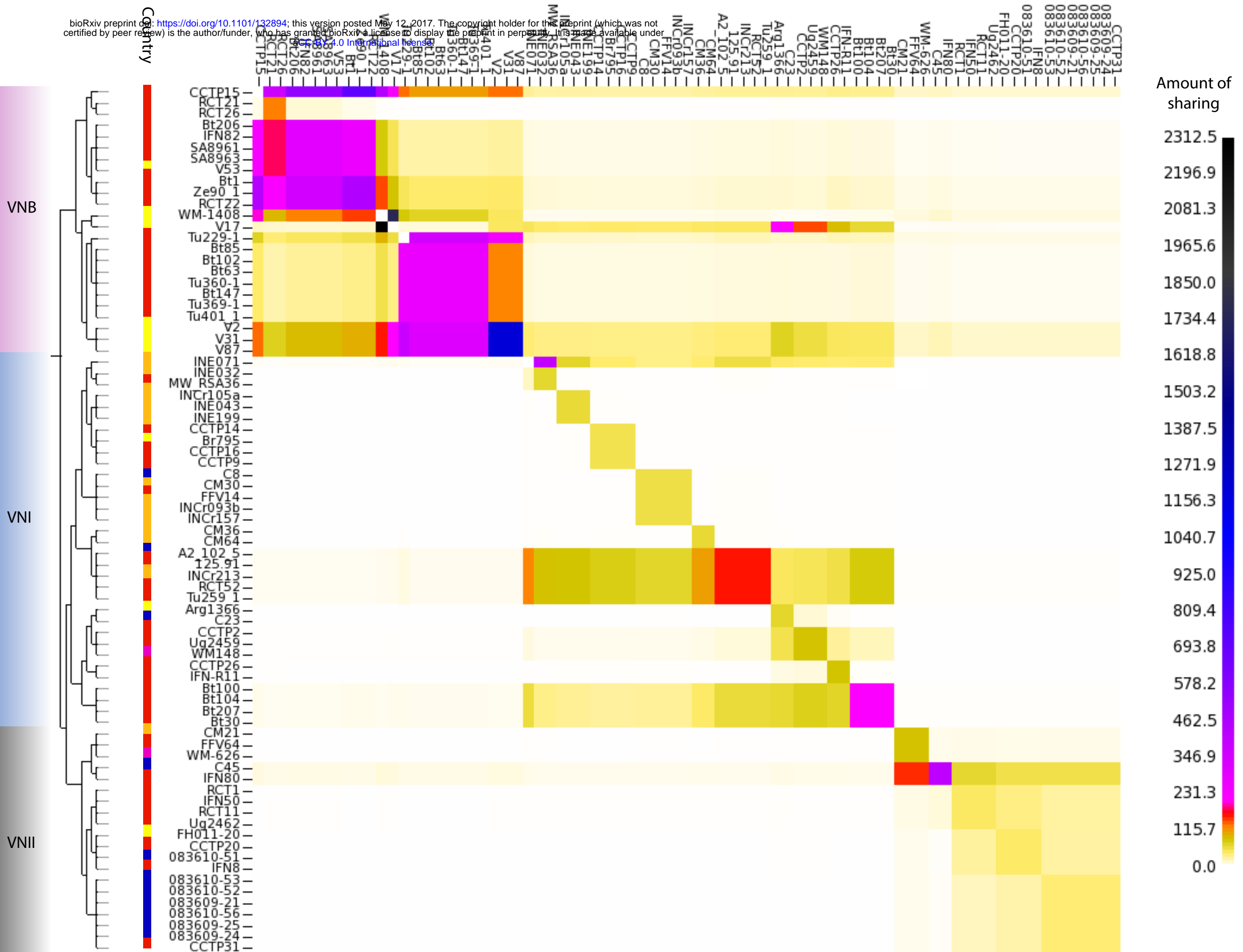


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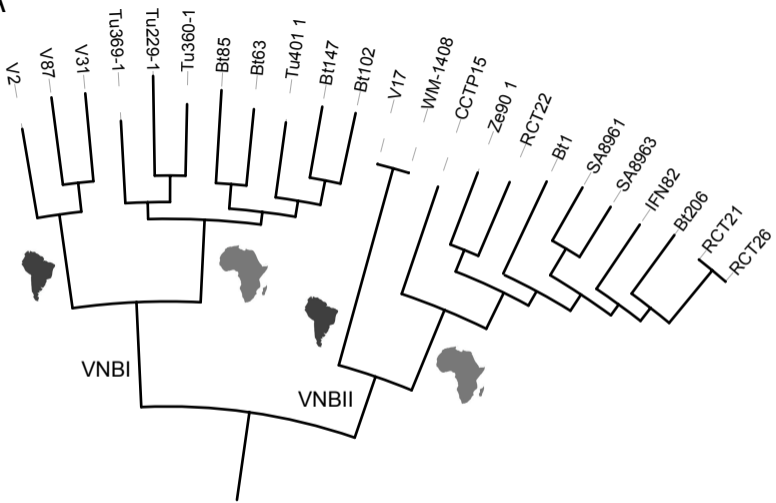




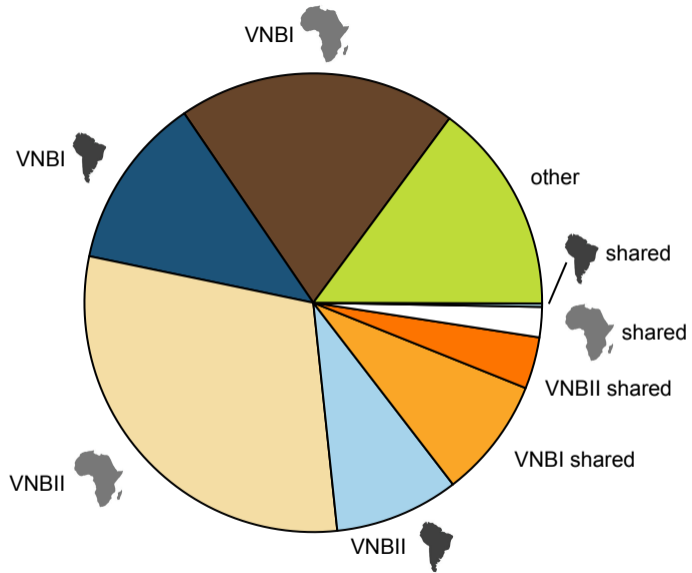
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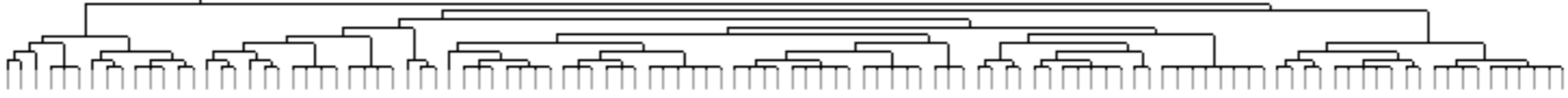


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