1   **Genetic and environmental influences on the evolution of virulence in the HIV-**

2   **associated opportunistic human fungal pathogen *Cryptococcus neoformans***

3   Yiwu Yu[1,10]¶, Yuanyuan Wang[2,6,7]¶, Linghua Li[3]¶, Xiaoqing Chen[2,7]¶, Xinhua Huang[2],

4   Huaping Liang[5], Tong Jiang[2,7], Guojian Liao[8], Min Chen[9], Liping Zhu[10], Muyuan Li[8],

5   Tao Zhou[8], Qinyu Tang[1]，Jingjun Zhao[1,4]* and Changbin Chen[2,6]*

6   [1] Department of Dermatology, Tongji Hospital, Tongji University School of Medicine,

7   Shanghai 200065, China

8   [2] The Center for Microbes, Development and Health, Key Laboratory of Molecular

9   Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences,

10   Shanghai 200031, China

11   [3] Institute of Infectious Disease, Guangzhou No.8 People's Hospital, Guangzhou 510060,

12   Guangdong, China

13   [4] Gusu School, Nanjing Medical University, Suzhou Municipal Hospital, The Affiliated

14   Suzhou Hospital of Nanjing Medical University, Suzhou 215002, Jiangsu, China

15   [5] State Key Laboratory of Trauma, Burns and Combined Injury, Department of Wound

16   Infection and Drug, Army Medical Center (Daping Hospital), Army Medical University,

17   Chongqing 400042, China

18   [6] Nanjing Advanced Academy of Life and Health, Nanjing 211135, Jiangsu, China

19   [7] University of Chinese Academy of Sciences, Beijing 100049, China

20   [8] College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China

21   [9] Department of Dermatology, Shanghai Key Laboratory of Medical Mycology,

22   Changzheng Hospital, Second Military Medical University, Shanghai, China

23   [10] Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai

24   200040, China

25   ***** Address correspondence to Jingjun Zhao, zhaojingjun2015@aliyun.com

26   Changbin Chen, cbchen@ips.ac.cn

27   ¶These authors have contributed equally to this work.

28   **Affiliations**

29   Yiwu Yu: 784888698@qq.com

30   Yuanyuan Wang: yywang@ips.ac.cn

31   Linghua Li: llheliza@126.com

32   Xiaoqin Chen: xqchen@ips.ac.cn

33   Xinhua Huang: xhhuang@ips.ac.cn

34   Huaping Liang: 13638356728@163.com

35   Tong Jiang: tjiang@ips.ac.cn

36   Guojian Liao: gjliao@swu.edu.cn

37   Min Chen: chenmin9611233@163.com

38   Liping Zhu: zhulp@fudan.edu.cn

39   Muyuan Li: 18434390502@163.com

40   Tao Zhou: 455810979@qq.com

41   Qinyu Tang: 623082932@qq.com

42 **Abstract**

43 The fungus *Cryptococcus neoformans* is considered the leading cause of death in

44 immunocompromised patients. Despite numerous investigations concerning its

45 molecular epidemiology, there are only a few studies addressing the impacts of varying

46 factors on genotype-phenotype correlations. It remains largely unknown whether

47 genetic and environmental variabilities among isolates from different sources may have

48 dramatic consequences on virulence. In this study, we analyzed 105 Chinese *C.*

49 *neoformans* isolates, including 54 from HIV-infected patients, 44 from HIV-uninfected

50 individuals and 7 from a natural environment, to investigate factors influencing the

51 outcome of *C. neoformans* infection. MLST analysis clearly identified sequence type

52 (ST) 5 as the prevalent sequence type in all clinical isolates and interestingly, genotypic

53 diversities were observed in isolates from both HIV-uninfected individual and natural

54 environment but not those from HIV-infected patients. Moreover, we found that

55 compared to those from HIV-infected patients, the isolates from HIV-uninfected

56 individuals exhibited enhanced virulence-associated traits including significantly

57 elevated capsule production and melanin formation, increases in survival in human

58 cerebrospinal fluid (CSF), less effective uptake by host phagocytes, and higher

59 mortality in a mouse model of cryptococcosis. Consistently, pathogenic phenotypes

60 were associated with CD4 counts of patients, implying environmental impact on within-

61 host *C. neoformans* virulence. Importantly, a large-scale whole-genome sequencing

62 analysis revealed that genomic variations within genes related to specific functions may

63 act as a vital driving force of host intrinsic virulence evolution. Taken together, our

64 results support a strong genotype-phenotype correlation suggesting that the pathogenic

65   evolution of *C. neoformans* could be heavily affected by both genetic and

66   environmental factors.

67   **Key words:** *Cryptococcus neoformans*; Virulence; Genotype-phenotype correlation;

68   Environmental factors; Genetic variants.

69   **Introduction**

70   Cryptococcosis refers to a major invasive fungal infection caused by the encapsulated

71   yeast species of the genus *Cryptococcus*, particularly *Cryptococcus neoformans* and

72   *Cryptococcus gattii*. *C. neoformans* contains two varietal forms, *C. neoformans var.*

73   *grubii* and *C. neoformans* var. *neoformans* [1, 2], and is distributed worldwide. In

74   comparison, *C. gattii* was originally thought to be restricted only in tropical and

75   subtropical districts but now this species was recognized in expanded temperate regions

76   due to an outbreak of cryptococcosis on Vancouver Island, Canada [3]. The prevalence

77   of cryptococcosis has increased in the past decade, and nearly a million cases of

78   cryptococcal meningitis are diagnosed annually around the world, mainly in

79   immunocompromised patients due to Human Immunodeficiency Virus (HIV) infection,

80   organ transplantation, cytotoxic chemotherapy, and corticosteroid use [4, 5]. Clinical

81   isolates of the *C. neoformans* and *C. gattii* species complexes are responsible globally

82   for 15 to 30% of deaths in HIV/AIDS patients due to cryptococcal meningitis [6].

83   Meanwhile, *C. neoformans* accounts for the most common cause of meningitis in HIV

84   adults in sub-Saharan Africa [6].

85   Phylogenetic analyses, as well as a number of genotyping studies, are being conducted

86   using clinical and environmental *C. neoformans* isolates collected all over the world, in

87   order to lay the basis for a comprehensive picture of the global genetic structure of this

88   fungus [7, 8], and significant genetic diversities are observed in the *C. neoformans*

89   species complex. All clinical *Cryptococcus* strains were initially treated as a single

90   species with a conserved name *C. neoformans* [9]. It was later classified into four

91   serotypes (A, B, C and D) based on Cryptococcal antigenic heterogeneity [10, 11].

92   Revised taxonomy further categorized the serotype B and C isolates to *C. gattii* [12],

93   while *C. neoformans* encompassed three major serotypes including *C. neoformans* var.

94   *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and the hybrid

95   serotype AD [1]. Moreover, the *C. neoformans* species complex consists of two

96   evolutionary divergent species, *C. neoformans* and *C. deneoformans*, as well as their

97   associative hybrids (*C. neoformans* x *C. deneoformans*). Of the two lineages, *C.*

98   *neoformans* exhibited a worldwide distribution causing among 95% of cryptococcal

99   infections and >99% in AIDS individuals, whereas *C. deneoformans* is more frequent

100   in Europe and less virulent [13-15]. Due to a rapid development of molecular biology

101   techniques including PCR fingerprinting, amplified fragment length polymorphism

102   (AFLP) analysis, multilocus sequencing MLST and whole-genome sequencing [16-19],

103   the relatedness of isolates at a molecular level has revolutionized our ability to

104   differentiate among molecular types of the genus *Cryptococcus*. It is now well

105   appreciated that *C. neoformans* can be divided into at least five major molecular types

106   (AFLP1/VNI, AFLP1A/VNB/VNII, AFLP1B/VNII, AFLP3/VNIII and AFLP2/VNIV)

107   [2, 18, 20]. Among them, *C. neoformans var. grubii* (serotype A/VNI) was found to

108   naturally reside on avian excreta and trees, have a worldwide distribution, and

109   contribute to over 80% of cryptococcosis [21]. Similar to the VNI clade, the VNII clade

110   is also globally distributed. However, *C. neoformans var. grubii* (serotype A/VNB) was

111   primarily found in sub-Saharan Africa and South America [22, 23]. Moreover, *C.*

112   *neoformans var. neoformans* (serotype D/VNIV) was mostly found in Western Europe

113   and South America and the *C. neoformans* hybrid (serotype AD/VNIII) showed a

114   higher prevalence in the Mediterranean area of Europe [2, 24]. Collectively, these

115   observations strongly suggest that molecular types of the *C. neoformans* isolates not

116   only differ in their serological, epidemiological and ecological characteristics, but also

117 exhibit diverse features on clinical presentations, antifungal susceptibility and

118 therapeutic outcomes [16, 25, 26].

119 Recently, a growing body of evidence highlights the impact of *C. neoformans* genotype

120 on the host disease outcome. For example, an epidemiological study by Wiesner *et al*.

121 found that genotypes of *C. neoformans* isolates from HIV/AIDS patients in Uganda

122 could be grouped into three distinct clonal clusters within the VNI clade and among

123 them, both ST93 and ST77 isolates showed the highest mortality risk [27].

124 Comparatively, a similar study using clinical isolates from South African HIV/AIDS

125 patients identified that ST32 isolates in the VNB clade exhibit worse patient outcome

126 [28] and moreover, genotypic analysis of clinical *C. neoformans* isolates from Brazil

127 revealed that ST93 in VNI clade is the most prevalent sequence type in HIV-infected

128 patients [23]. However, a strikingly different link between isolate genotype and disease

129 outcome was found in Asian countries, where *C. neoformans* infections are often

130 observed in immunocompetent individuals [29] and ST5 represents the major sequence

131 type in *C. neoformans* isolates from East Asian countries including China, Japan,

132 Vietnam and South Korea [30, 31]. For example, a study using 136 Vietnamese clinical

133 isolates of *C. neoformans var. grubii* revealed that ST5 isolates are responsible for 82%

134 of infections in HIV uninfected patients, compared to only 35% cases in HIV infected

135 patients [31]. In addition, MLST analysis of Chinese clinical *C. neoformans* isolates by

136 different research groups also identified ST5 as the predominant sequence type [32].

137 Of course, notable exceptions exist. For instance, studies have indicated that ST4 and

138 ST6 are the major MLST types in *C. neoformans var. grubii* isolates of Thailand

139 whereas ST93 turns to be the dominant isolates from India and Indonesia [30, 33].

140 Interestingly, detailed information regarding genetic diversity of the isolates suggests

141   that those from Thailand posit an evolutionary origin in African and the strains from

142   China may have the same African origin but were expanded more flexibly and globally

143   [5, 33]. Taken together, these studies highlight the presence of global genetic diversities

144   in *C. neoformans* isolates and argue the correlation between pathogen genotype and

145   patient phenotype. Of course, it is important to note that the patho-phenotypic variations

146   of *C. neoformans* isolates cannot be fully explained by genotypic diversity, and other

147   factors should also be considered.

148   Factors determining disease prevalence and species specificity are relatively unknown,

149   but are speculated to be associated with the host immune response, genetic varieties,

150   and virulence factors. For example, the type 1 helper T-cell (Th$_1$) response could

151   stimulate classical activation of macrophages and eliminate internalized cryptococcal

152   cells, however, the type 2 helper T-cell (Th$_2$) response was found to promote the

153   disseminated, uncontrolled cryptococcal infection [34, 35], suggesting that patients

154   exhibiting different immune responses to cryptococcal infections could yield varied

155   clinical outcomes. Moreover, the distribution and prevalence of the molecular types

156   appear to be highly relevant to geographical locations, the size of samples and host

157   characteristics [1, 2]. For example, previous studies suggested that *C. neoformans*

158   serotype A is one of the most common varieties and account for the majority of

159   cryptococcal infections in Asia, especially in HIV-AIDS patients [34, 35]. In addition,

160   studies showed that a list of virulence factors including the presence and size of the

161   polysaccharide capsule, melanin production by laccase, cell size variation, growth at

162   37 ℃ and secretion of enzymes such as phospholipase, proteinase and urease,

163   sphingolipid utilization, contribute to *C. neoformans* pathogenicity [36, 37]. However,

9

164   it remains unclear whether these factors were correlated, and if so, how to affect the

165   evolution of *C. neoformans* pathogenicity?

166   In an attempt to interpret the molecular epidemiology of *Cryptococcus* species, the

167   strain differences in genotype and phenotype, as well as the impacts of genetic and

168   environmental correlations on the evolution of fungal virulence, we analyzed a

169   collection of 105 clinical and environmental isolates of *C. neoformans* in China,

170   including those isolated from HIV-infected patients and HIV-uninfected individuals.

171   The genotype of each isolate was determined by MLST and the evaluation of

172   pathogenic phenotypes was performed *in vitr*o and *in vivo*. Moreover, the genotype-

173   phenotype correlations were further assessed by genetic variations through the genome-

174   wide linkage and association analyses. Our results compare the impacts of genetic and

175   environmental factors on affecting the correlation between genotype and phenotype of

176   *C. neoformans* isolates, and provide *in vitro* and *in vivo* data to support the influence of

177   genetic and environmental changes on genotypic and pathogenic variations.

178   **Materials and methods**

179   **Ethics statement**

180   All of animal experiments were performed in compliance with the Regulations for the

181   Care and Use of Laboratory Animals issued by the Ministry of Science and Technology

182   of the People's Republic of China, which enforces the ethical use of animals. The

183   protocol was approved by IACUC at the Institut Pasteur of Shanghai, Chinese Academy

184   of Sciences (Permit Number: 160651A).

185   **Strains**

10

186 A total of 105 isolates of *C. neoformans* strains were assayed in this work, including

187 44 from the HIV-uninfected patients (HIV-u group), 54 from the HIV-infected patients

188 (HIV-i group) and 7 from the nature (Env group). Clinical and laboratory records of all

189 patients were obtained from Guangzhou No.8 People's Hospital, Huashan Hospital,

190 Changzheng Hospital and Southwest University. The data collected for analysis

191 included age, gender, initial symptoms, HIV infection status and CD4$^+$ T cell count (at

192 the time of diagnosis). The detailed information about each of the samples is presented

193 in **S1 Table**. Among these, 98 strains were isolated from cerebrospinal fluid (CSF)

194 samples (n = 90) and blood cultures (n = 8). *C. neoformans* clinical isolates were single

195 colony purified on YPD medium and then maintained as glycerol stocks at -70°C for

196 long-term storage. A detailed information about each sample is listed in **S2 Table**. Each

197 strain was streaked and grown as a single colony on yeast peptone dextrose (YPD)

198 medium prior to use.

199 A list of reference strains was included in this study, including international strains used

200 for phylogenetic analysis (H99 in USA, WM148 and WM626 in Australia, ST93 in

201 Brazil) and standard strains for *in vitro* and *in vivo* assays (JEC20 and H99). All strains

202 were maintained on yeast peptone dextrose (YPD) medium prior to use.

203 **DNA extraction**

204 Isolates were grown on Sabouraud dextrose agar slants for 48 h. Single colonies were

205 isolated, re-inoculated in10ml of liquid medium, and grown at 30°C for 24 h. The cells

206 were collected by centrifugation and genomic DNA was extracted using the

207 cetyltrimethylammonium bromide (CTAB) DNA isolation method as previously

208 described [38].

Multilocus Sequence Typing (MLST)

Multilocus sequence type analysis was carried out using the seven ISHAM consensus loci (*CAP59*, *GPD1*, *IGS1*, *LAC1*, *PLB1*, *SOD1*, *URA5*), following a standard procedure [18, 22]. All primers used in this study were described previously [27] and listed in **S6 Table** .

Phylogenetic analysis

The sequences were aligned with the computer program CLUSTAL W and a phylogenetic tree was drawn based on the neighbor-joining (NJ) model. The evolutionary distances were computed based on the *p*-distance, and all gaps were eliminated. A bootstrap analysis was performed with 1,000 replicates [39].

Assays for melanization and capsule formation

Melanin production was measured by comparing pigmentation of strains grown on L-DOPA media to reference strains with strong (H99) and weak (JEC20) melanization, using a method described previously [40]. Relative melanization scores of zero (equal to JEC20), one to four (between JEC20 and H99) and five (more than or equal to H99) were assigned to the strains based on comparison with the reference strains grown on the same plate.

The capsule induction assay was performed using a method described previously [41], with some modifications. In brief, stationary-phase fungal cultures were washed and resuspended in PBS. Cells were diluted 1/100 in capsule induction medium [10% Sabouraud dextrose medium in 50mM MOPS buffer (pH 7.3)] and incubated at 30 °C and 180rpm for 48h. The size of capsule was measured by staining the cells with India ink and imaging at a magnification of ×63 under a light microscope. As described by

232   Fernandes *et al*. [42], the diameters of the whole cell (yeast cell + capsule) and the cell

233   body (the cell wall only) were each measured using Image J software, and the mean of

234   the values was calculated for 10-20 cells for each isolate.

235   The identification of mating types was carried out in a classical PCR analysis using the

236   mating type and serotype specific primers [43]. All primers are listed in **S6 Table**.

237   **Infection of macrophages with *Cryptococcus***

238   Phagocytosis assays were performed with the murine macrophage-like cell line J774,

239   using a method previously described [44, 45]. Briefly, macrophage cells in DMEM

240   supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine,

241   and 1% penicillin-streptomycin (Sigma-Aldrich) were plated into each well of a 24-

242   well tissue culture plate for 24 hours at 37°C with 5% CO_2. Macrophage cells ($1.5 \times$

243   10^5) were incubated in serum-free DMEM medium for 2 hours, activated with 15μg/ml

244   phorbol myristate acetate (PMA) (Sigma-Aldrich) for 30-60 minutes, and then co-

245   incubated with *C. neoformans* yeast cells opsonized by a monoclonal antibody

246   (C66441M, purchased from Meridian Life Science, Inc) for 2 hours at 37°C with 5%

247   CO_2 (MOI=1:10). Extracellular yeast cells were removed by extensive washes with

248   prewarmed PBS. The extent of *Cryptococcus* phagocytosis was calculated as the

249   number of cryptococci internalized by macrophages 2 hours after infection. Results

250   were expressed as the mean of 3 to 4 experimental repeats.

251   **CSF survival assay**

252   Human cerebrospinal fluid (CSF) was pooled from Shanghai Changzheng Hospital

253   patients receiving serial therapeutic lumbar punctures, collected anonymously from

254   populations of at least 15 patients. The human CSF was fully examined for parameters,

255   including white cell count, protein and glucose levels, within the normal ranges and for

256   the absence of antifungal drugs. Clinical isolates of *C. neoformans* were replicated in

257   48-well plates in Sabouraud dextrose broth (SDB) and incubated at 37°C for 3-4 days

258   until saturation. Cultures were then diluted, inoculated into CSF at a concentration of

259     $1\sim2 \times 10^6$ cells/ml, and incubated at 37°C for 96 hours. As described previously [45],

260   aliquots were collected at different time points (0,12, 24, 36,72 and 96 hours after

261   inoculation) and plated on Sabouraud dextrose agar (SDA) media for CFU counts. The

262   survival slope was determined as the mean rate of increase or decrease in cryptococcal

263   counts after CSF treatment, by averaging the slope of the linear regression of $\log_{10}$

264   CFU/ml over time for each strain.

265   **Virulence studies**

266   A well characterized murine inhalation model of cryptococcosis was used [46] . Briefly,

267   three isolates were randomly picked from each of the three groups and individually

268   grown overnight in liquid YPD cultures at 30 ℃. All strains tested are ST5, except for

269   one environmental strain (Env #103; ST39). Cells were counted using a hemocytometer

270   and the yeast suspension with a final concentration of 1×10^7 cells/ml was prepared.

271   6-8 week-old female C57BL/6 mice were anesthetized by intraperitoneal injection of

272   ketamine (75 mg/kg) and medetomidine (0.5-1.0 mg/kg) and a 50 μL volume of the

273   yeast suspension (1×10^5 cells) was intranasally injected. 8 mice were infected per

274   inoculum. Mice were monitored several times a week until the observance of disease

275   symptoms (weight loss, ruffled fur, shallow breathing, abnormal gait and lethargy) and

276   then monitored daily. Mice were sacrificed by CO_2 inhalation followed by cervical

277   dislocation when signs of severe morbidity, including significant weight loss, abnormal

278   gait, hunched posture and swelling of the cranium, were clearly displayed. A Kaplan-

279   Meier method was employed to analyze survival curves using GraphPad Prism 6.0

280   software. Differences in the median survival among species were determined by

281   performing the log-rank test [38, 47].

Whole Genome Sequencing

283   28 C. neoformans strains isolated from HIV-infected patients and HIV-uninfected

284   patients were subjected to whole-genome sequencing. In order to minimize the

285   genotype impact on the sequencing, all 28 strain were selected ST5. Next generation

286   sequencing library preparations were constructed following the manufacturer's

287   protocol (NEBNext® Ultra™ DNA Library Prep Kit for Illumina®). For each sample,

288   1 µg genomic DNA was randomly fragmented to <500 bp by sonication (Covaris S220)

289   and DNA fragments were treated with End Prep Enzyme Mix for end repairing, 5'

290   Phosphorylation and dA-tailing in one reaction, followed by a T-A ligation to add

291   adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using

292   AxyPrep Mag PCR Clean-up (Axygen) and fragments of ~410 bp (with the

293   approximate insert size of 350 bp) were recovered. Each sample was then amplified by

294   PCR for 8 cycles using P5 and P7 primers, with both primers carrying sequences which

295   can anneal with flowcell to perform bridge PCR and P7 primer carrying a six-base index

296   allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag

297   PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent

298   Technologies, PaloAlto, CA, USA), and quantified by Qubit2.0 Fluorometer

299   (Invitrogen, Carlsbad, CA, USA). The whole genome of each strain was sequenced

300   using Illumina NovaSeq6000 PE150 at the Beijing Novogene Bioinformatics

301   Technology Co., Ltd.

Data analysis

The raw data was removed the sequences of adaptors, polymerase chain reaction (PCR) primers, content of N bases more than 10% and bases of quality lower than 20 by using Cutadapt (V1.9.1). Then the clean data was mapped to the *C. neoformans* H99 reference genome FungiDB (http://fungidb.org/fungidb/) by using BWA (V0.7.17) and mapping results were processed by Picard (V1.119) to remove duplication. SNPs/InDels were called by the GATK Unified Genotyper (V3.8.1) and Annotated by Annovar (V21 Apr 2018). GO-term analysis of processes enriched among specific gene sets of HIV-i and HIV-u group was performed using the topGO (V2.34.0).

Statistics

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) in GraphPad Prism 6.0 software (San Diego, CA). The following *p*-values were considered: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files, or are available upon request to the corresponding authors. Whole-genome sequences are available at NCBI under BioProject ID: PRJNA680424.

Results

Characterization of clinical presentation and outcome

323   In order to study the correlations amongst environmental conditions, immune

324   inflammatory syndrome and other clinical outcomes, we collected a total of 98

325   clinical isolates of *C. neoformans* that were recovered from cerebrospinal fluid (CSF)

326   samples (n=90) and blood cultures (n=8) of 98 patients hospitalized at different

327   locations of China. Among them, 54 strains are from HIV-infected patients and 44 are

328   from HIV-uninfected individuals. Clinical laboratory data was investigated from 95

329   patients (three patients' data was somehow missed). Both **Table 1** and **S1 Table**

330   showed the detailed information about the influence of common symptoms on clinical

331   outcome that could be used to Fig out the relationships between isolate origin and

332   clinical consequences. The majority of patients were male (74; 77.9%), the age ranged

333   from 21 to 68 years and the mean age was 41 ± 11 years.

334   Among them, the most common presenting symptom was headache (70%), especially

335   in HIV-infected patients (73%). Comparatively, the HIV-uninfected patients were less

336   frequently found to have high fever but they had more severe symptoms such as seizure,

337   cerebral herniation and cerebellar signs. Moreover, 39 out of 51 HIV-infected patients

338   presented $CD4^+$ T-cell counts below 50 /mm^3. However, the mean $\pm$ SD of $CD4^+$ T-

339   cell counts from 44 HIV-uninfected individuals was 381 ± 92 mm^3/ml. These results

340   are consistent with a recent study that the HIV-associated cryptococcal meningitis

341   normally occurs in patients with higher CD4 counts [48].

342   **MLST and phylogenetic analyses**

343   The 98 clinical isolates, together with 7 environmental strains collected from pigeon

344   excreta, were classified into three groups (HIV-u group: 44 strains from HIV-

345   uninfected individuals; HIV-i group: 54 strains from HIV-infected patients; Env group:

17

346   7 environmental strains from the pigeon excreta). After colony purification, all 105

347   isolates were analyzed to determine their genotypes by Multilocus Sequence Typing

348   (MLST), based on a formal study that described a consensus sequence-based

349   epidemiological typing scheme, using seven housekeeping genes (*CAP59*, *GPD1*, *IGS1*,

350   *PLB1*, *LAC1*, *SOD1*, *URA5*) [18]. For each strain, locus allele identifiers were used and

351   combined to generate a sequence type (ST) representing the strain genotype [49].

352   MLST analysis of all 105 isolates demonstrated the presence of 9 sequence types (ST),

353   including ST5 (n = 94; 89.5%), ST359 (n = 2; 1.9%), ST2 (n = 2; 1.9%), and ST39 (n

354   = 2; 1.9%), ST360 (n=1; 0.9%), ST194 (n=1; 0.9%), ST31 (n=1; 0.9%), ST93 (n=1;

355   0.9%), ST195 (n=1; 0.9%) (**Fig 1A**). To our surprise, we found in **S2 Table** that all

356   isolates from HIV-i group were restricted to a single ST (ST5). In comparison, STs

357   identified in isolates from HIV-u and Env groups were more diverse, as we observed

358   that strains from HIV-u group contain 8 STs, including ST5 (n=36), ST359 (n=2), ST2

359   (n=1), ST360 (n=1), ST194 (n=1), ST31 (n=1), ST93 (n=1) and ST195 (n=1), and

360   strains from the Env group also harbor 3 STs, including ST5 (n=4), ST39 (n=2) and

361   ST2 (n=1).

362   In addition, a phylogenetic analysis was performed among strains from different

363   geographic locations (China, Australia, Brazil and USA), based on the concatenated

364   sequences of MLST loci (**Fig 1B**). The phylogenetic tree revealed that ST5 was quite

365   close to ST359 and ST360, while ST39 was far from other identified STs. Moreover,

366   we concluded from this study that the major epidemic clone of *C. neoformans var.*

367   *grubii* in China was ST5 regardless of their origins. Actually, similar patterns were

368   observed in previous studies using strains isolated from HIV-uninfected individuals of

369   China [7, 50].

370   As shown in **Fig 1A**, we found that the majority of the isolates in this collection were

371   of one sequence type (ST5), which was 54 out of 54 in the HIV-i group, 36 out of 44

372   in the HIV-u group and 4 out of 7 in the Env group, prompting us to evaluate the

373   potential relevance of the isolates to virulence according to their origins other than the

374   STs.

375   ***In vitro* and *ex vivo* phenotyping**

376   *C. neoformans* produces several important virulence factors, most notably the mating

377   type, polysaccharide capsule, and melanin production. Studies have shown that mating

378   type can influence virulence through cell type (*MAT*α or *MAT***a**) and the function of

379   specific genes such as those related to MAP kinase pathway [51-53]. The mating types

380   and serotypes of all isolates were determined by multiplex PCR using specific primers

381   described previously [18]. Among the 105 strains evaluated, 104 were characterized as

382   serotype A MATα (Aα) and only one strain was found to be serotype A MAT**a** (A**a**)

383   (**S1 Fig**).

384   Melanin is another major virulence factors in *C. neoformans*. We carried out *in vitro*

385   assays to evaluate the ability of all 105 isolates to produce melanin by patching on

386   medium containing L-DOPA (L-3,4-dihydroxyphenylalanine) [54]. A scoring method,

387   based on K-means clustering analysis, was employed to evaluate melanin production

388   of all tested isolates. Two reference strains (JEC20 and H99) were used as experimental

389   controls, given the fact that the strain JEC20 has been reported to produce almost no

390   melanin whereas the clinical isolate H99 exhibits strong melanization [55]. As shown

391   in **Fig 2 and S2 Fig**, we found that strains in HIV-u group produce significantly higher

392   levels of melanin, as showed by dark pigments, when compared to those in HIV-i group

19

393   ($p < 0.0005$ by Tukey adjusted t-test), implying that the clinical isolates from the two

394   groups may have differences in pathogenicity.

395   This notion was further supported by assaying the polysaccharide capsule formation of

396   each isolate. Capsule induction of each isolate was achieved under nutrient-limiting

397   conditions (capsule induction medium; 10% Sabouraud dextrose medium in MOPS

398   buffered at pH 7.3) and measured by staining with India ink, following a protocol

399   described in Materials and Methods. The capsule size of each strain was determined by

400   Adobe Photoshop software (Adobe Inc, USA) and the data was further analyzed by a

401   K-means clustering algorithm. We observed that after induction, strains in HIV-u group

402   generate much larger capsules than those in HIV-i group, although their sizes are almost

403   indistinguishable under un-induced condition (**Fig 2 and S3 Fig**).

404   The ability of *C. neoformans* to survive in the cerebrospinal fluid (CSF) has been

405   found to contribute to the cryptococcal virulence due to its presence in clinical

406   specimens and production of life-threatening disease in the central nervous system

407   (CNS). To further evaluate the pathogenic variabilities between cryptococcal strains

408   from HIV-infected and uninfected individuals, we assayed the survival of all 98

409   clinical isolates in human CSF. When compared to those from the HIV-I group,

410   isolates from the HIV-u group exhibited more resistant to killing by human CSF (**Fig**

411   **3A**). Moreover, we found a positive correlation between CSF survival and capsule

412   size ($r = 0.6737$, $p < 0.0001$; **Fig 3B**). These data sustain the proposition that strains in

413   HIV-u group appear to be more virulent than host in HIV-I group.

414   In addition, many studies have emphasized the role of fungal internalization by

415   macrophages as a critical virulence factor in cryptococcal disease [56, 57].

20

416   Interestingly, when the macrophage uptake of *C. neoformans* cells was evaluated in

417   the established murine macrophage-like cell line J774, we observed that isolates from

418   the HIV-i group were phagocytosed at a higher rate than those from the HIV-u group

419   (**Fig 3C**), and this trait could be explained by differences in capsule size, since

420   phagocytic uptake and capsule size is inversely correlated in this set of isolates ($r = -$

421   0.5345, $p < 0.0001$; **Fig 3D**). Actually, our results are consistent with previous reports

422   that the capsule passively inhibits phagocytosis of *C. neoformans* by macrophages

423   and the capsule-dependent anti-phagocytic activity represents a major virulence

424   attribute [45, 58, 59].

425   Taken together, both *in vitro* and *ex vivo* phenotypic assays suggest that compared to

426   those from the HIV-infected patients, the clinical isolates derived from the HIV-

427   uninfected individuals exhibit significantly enhanced capsule production and melanin

428   formation, higher increases in survival in CSF, and less effective uptake by host

429   phagocytes, which represent key factors associated with *Cryptococcus* pathogenicity

430   [60, 61].

431   ***In vivo* virulence analyses**

432   Given the observed differences in capsule and melanin production between strains from

433   HIV-uninfected and infected groups, we sought to ask whether strain origins may affect

434   *C. neoformans* virulence. We conducted an *in vivo* virulence analysis using a mouse

435   model of cryptococcosis [46]. Groups of 6-8-week female C57BL/6 mice (8 mice per

436   group) were inoculated intranasally with strains from the three groups (we randomly

437   picked three strains in each group), as well as the H99 strain serving as a control. All

438   isolates tested are ST5, except for one environmental strain (Env #103; ST39). Mice

439 were sacrificed by CO_2 inhalation followed by cervical dislocation when signs of severe

440 morbidity, including weight loss, abnormal gait, hunched posture and swelling of the

441 cranium, were clearly observed. Consistent with a previous study [46], the control strain

442 H99 caused a lethal infection by day 20. As expected, we observed that mice inoculated

443 with the strains of HIV-u group showed similar or even worse signs of disease as the

444 control strain. In contrast, mice inoculated with strains from both HIV-i and Env groups

445 showed almost identical survival rates (**Fig 3E**), and the strains in these two groups are

446 much less virulent than those in HIV-u group.

447 Thus, consistent with the results obtained from *in vitro* and *ex vivo* assays, our *in vivo*

448 analysis also indicated that clinical isolates of *C. neoformans* from different hosts

449 exhibit differentiation of virulence phenotypes, implying the importance of

450 environmental factors on the evolution of virulence.

451 **Genetic variations**

452 Studies have shown that microbial pathogens evolve a broad range of intrinsic and

453 extrinsic strategies to acquire and modulate existing virulence traits in order to achieve

454 successful colonization in the host [62]. In addition to the impact of environmental

455 factors, pathogenicity of *C. neoformans* must be also influenced at the genetic level,

456 since genetic variation in the genome is recognized as a fact of life in microbes,

457 allowing pathogens to adapt to their chosen host and also to resist clearance by the host.

458 Moreover, whole genome-resequencing technology has been used as a powerful tool to

459 explore the genetic mechanism in fungi and fungal-host interaction [63]. Hence, we

460 sequenced and assembled the whole genomes of 28 strains in the collection (ST5), 14

461 from HIV-u group and 14 from HIV-i group (the strains were chosen based on the

462   patients' parameters, including close age, sex and several other factors within the same

463   group), and performed a comparative study to identify potential correlations between

464   genetic variation and virulence, by comparing the genome difference of 28 isolates

465   against the finished reference strain H99. Quality control analyses of our sequencing

466   data were summarized in **S3 Table**. Overall, sequencing the 28 isolates yielded a total

467   of 65 Gb containing 436 million raw reads, with an average of 15.6 million reads for

468   each sample. The raw reads were further filtered by removing the low quality reads and

469   duplicate reads, producing 52 Gb containing 349 million high quality reads.

470   Approximate 94.8% of the clean reads could be mapped to the H99 reference genome,

471   varied from 30.77 to 99.08% among different strains (**S4 Table**). These mapped

472   sequences were used for further analyses.

473   **S5 Table** lists the number of mutations observed in the 28 clinical isolates comparing

474   with the reference genome of H99. We identified a total of 65,106 variants, including

475   57,401 SNPs (single nucleotide polymorphism; 13,133 (22.9%) synonymous and

476   10,755 (18.7%) nonsynonymous) and 7,705 indels (insertions/deletions; 4,149 (53.8%)

477   InDel-Ins and 3,556 (46.2%) InDel-Del), across the genomes of 14 HIV-u isolates. In

478   comparison, our analysis across the genomes of 14 HIV-i isolates generated a total of

479   64,138 variants, with 56,655 SNPs (13,064 (23.1%) synonymous and 10,683 (18.9%)

480   nonsynonymous) and 7,483 indels (4,058 (54.2%) InDel-Ins and 3,425 (45.8%) InDel-

481   Del). The relationship between number of variants and gene was identified in **Fig 4A**

482   and the inset panel was magnified to show genes with at least 20 variants for

483   visualization purposes. The results showed that there was no significant difference in

484   both groups. As shown in **Fig 4B**, the majority of genes in both groups harbor relatively

485   few variants and there is no correlation between gene length and the number of variants,

486   indicating that the relative frequencies of genetic variations in these clinical isolates are

487   quite similar and appear to be independent of strain origins. In addition, we surprisingly

488   found in **Fig 4C** that the genes harboring more than 80 variants were almost identical

489   in all sequenced isolates of both groups, suggesting that differential virulence may not

490   be generalized to regions encompassing extreme genomic instability, instead, may be

491   attributed to genomic mutations of genes associated with specific functions.

492   Indeed, extensive analysis about the genome comparison between H99 and the clinical

493   isolates identified polymorphisms in these two groups. **Fig 5A** showed variants that

494   were common or specific in the isolates from HIV-u and HIV-i groups. We identified

495   55,292 common SNPs (we defined common variants as the ones that are found in more

496   than 7 isolates) in both groups, and only 628 and 648 variants specific for HIV-u and

497   HIV-i groups, respectively. Moreover, we found that compared to those specific SNPs

498   of HIV-i group, the isolates of HIV-u group exhibited a significant higher level of

499   variant enrichment at the intergenic region (54.05% vs 41.90%) but much less

500   abundance in both upstream and exonic regions (4.98% vs 9.49% and 19.16% vs

501   28.18%, respectively). Other locations, such as intronic, downstream and

502   ncRNA_exonic regions, showed no difference in variant distribution between the two

503   groups **(Fig 5B)**. Furthermore, the 648 unique variants of isolates in HIV-u group were

504   associated with 329 genes whereas the 628 unique variants in HIV-i group counts for

505   206 genes.

506   Importantly, when we determined the functional categories of the unique genes

507   identified in each of the two groups using Gene Ontology (GO) Database, surprising

508   findings were obtained. Overall, the functions of the specific genes of each group could

509   be classified into three general directions (**Fig 6A**, **B** and **C**). In the biological process

510 category, the top highly enriched GO terms in HIV-i group include protein

511 glycosylation activity while those in HIV-u group were enriched in signal transduction

512 activity. In the cellular component, the GO terms of RISC complex and riboflavin

513 synthase complex were significantly enriched in HIV-i and HIV-u groups, respectively.

514 As for the molecular function category, genes with specific variations in strains of HIV-

515 i group showed a high percentage of metal/zinc ion binding process whereas those in

516 HIV-u group dominated in the process of oxidoreductase activity. Although isolates

517 from both HIV-i and HIV-u groups exhibit a large number of overlapping variants in

518 the genomes, there is a subset of genes in each group that harbor unique mutations and

519 account for different functions. The related functions and names of major mutated

520 genes differed in two groups were listed in **Table 2**. We observed that the functions of

521 HIV-i strain specific genes are mainly linked to microbial metabolism while those in

522 HIV-u group impinge their roles in stress response, signal transduction and drug

523 resistance. For example, the isolates from the HIV-u group harbored specific genetic

524 variants in the genes *SSK1*, *TCO2* and *PBS2* who have been found to be key players in

525 the fungal two-component system and the HOG signaling pathway and reported to

526 regulate stress responses, drug sensitivity, sexual development, differentiation and

527 virulence of *C. neoformans* [64, 65]. Moreover, specific mutations were also identified

528 in the genes like *RHO104/RIM20* and *APT4* in the isolates from HIV-uninfected

529 individuals but not in those from HIV-infected patients. Rho104/Rim20 are effectors

530 of the PKA signaling pathway and required for many virulence-associated phenotypes

531 such as titan cell formation in *C. neoformans* [66], and Apt4 is the P4-ATPase subunit

532 of the Cdc50 family and regulates iron acquisition and virulence in *C. neoformans* [67].

533 In comparison, most genes harboring specific mutations in the isolates of HIV-i group

534 are involved in metabolic processes, such as the MAPK signaling pathway (*SSK2*),

535   tryptophan biosynthesis (*TRP1*), riboflavin biosynthesis (*RIB3*), cell wall protein

536   glycosylation (*KTR3*) and cellular metabolism and compound biosynthesis (*KIC*, *HRK1*,

537   *KIN1*, *GPA3*, *FZC45*, *SNF102*, *URE7*, *DST1* and *BCK1*).

538   These data strongly suggest that compared to those isolated from HIV-infected patients,

539   *C. neoformans* strains from HIV-uninfected individuals need to evolve genetic

540   variations in a list of specific genes related to environmental adaptation, since microbes

541   have to confront with more rigor host environment, such as immune clearance.

542   **Discussion**

543   Cryptococcosis, primarily caused by *Cryptococcus neoformans* and *Cryptococcus*

544   *gattii*, is a primary opportunistic fungal infection that has been found to be associated

545   with patients with HIV infection. Of course, this fungal infection also occurs in other

546   underlying disorders, including immunosuppressant usage, transplantation, cancers and

547   diabetes mellitus *etc*. So far, studies on cryptococcosis in China have been carried out

548   mainly in HIV-uninfected patients [29]. In this study, we used a large number of *C.*

549   *neoformans* strains isolated from different sources, including those from the HIV-

550   infected and HIV-uninfected patients, as well as natural environment, and

551   systematically evaluated impacts of genetic and environmental factors on genotypes

552   and virulence-related phenotypes of these isolates. We observed strong correlations and

553   trends among the three different environments, which suggest that host environments

554   appear to play important roles in affecting the virulence of *C. neoformans* isolates.

555   Overall, strains isolated from the HIV-infected patients showed much lower virulence

556   when compared to those from the HIV-uninfected individuals and a natural

557   environment, indicating that *C. neoformans* pathogenicity has a great plasticity during

558   interaction with different hosts. Interestingly, the information from whole-genome

559   sequencing suggests that the high incidence of hotspot mutations may not be the major

560   factor that accounts for the differential virulence among the isolates derived from either

561   the HIV-i or HIV-u group. Instead, genomic variations within genes related to specific

562   functions may act as a major driving force of host intrinsic virulence evolution.

563   Broad epidemiology and molecular typing studies of *C. neoformans* and *C. gattii*

564   species complex have been reported all over the world, showing that genotypes are

565   distinctly related to geographical locations [2]. Originally we planned to study the

566   genotype-phenotype correlation using strains harboring different sequence types (ST).

567   Unexpectedly, our results identified that all of the 105 isolates belong to the molecular

568   type AFLP1/VNI and ST5 was the most common sequence type (n = 94; 89.5%),

569   although other STs were also identified using MLST, making it impossible to evaluate

570   the difference in the production of virulence factors between isolates from different STs.

571   Consistently, the observation that ST5 was the predominant sequence type that caused

572   human cryptococcosis in China was found to be the same case in most other Asian

573   countries [68]. A distinct genotypic characteristic of Chinese *C. neoformans* isolates

574   was apparent that ST5 represents the most prevalent genotype in clinical isolates from

575   both HIV-infected and -uninfected samples and comparatively, the genotypes of those

576   natural isolates (from pigeon droppings) are much more diverse. Furthermore, the data

577   currently available imply that the genotypic diversity in isolates from HIV-infected

578   patients appear to be more limited than those from HIV-uninfected individuals, and of

579   course, supporting this notion requires larger isolate collections and decent work in data

580   analyses.

581 Although strains with different genotypes harbor almost the same mating type, we

582 observed differences of these strains in melanin production and capsule formation.

583 Apparently, strains isolated from the HIV-uninfected patients produced significantly

584 more melanin than those isolated from the HIV-infected patients and the nature,

585 suggesting that once infected, a higher melanin biosynthesis of cryptococcal strains

586 might be triggered in response to a relatively stronger immune system, given that host

587 immune system has completely been dampened in HIV-infected patients. Considering

588 the prevalence of L-DOPA in the central nervous system [27], our results also proposed

589 that an increased melanin production in strains isolated from the HIV-uninfected

590 patients could potentially occur *in vivo* and promotes virulence, leading to an increase

591 in the mortality. Moreover, capsule generation in different strains yields similar results.

592 A previous study showed that capsule size in cryptococcal cells was found to differ

593 significantly between species and individual situation [69]. As expected, we observed

594 the smallest size of capsule in strains from HIV-infected patients, more likely reflecting

595 the fact that these strains might be more easily to cross biological barriers and to

596 disseminate in the brain in immunocompromised patients. However, the relationships

597 between capsule size and virulence have found controversial issues. One literature

598 reported that *C. neoformans* isolates with higher capsule formation were associated

599 with lower fungal clearance rates and increased intracranial pressure [70]. However,

600 this notion was disagreed by other groups, as their studies showed that *C. neoformans*

601 isolates with less capsule were more virulent and resulted in a higher fungal load in the

602 brain [54]. In order to verify that *in vitro* capsule and melanin production does reflect

603 the degree of pathogenicity, we test more virulence-related traits, including the ability

604 of *C. neoformans* to survive in human CSF, the level of macrophage uptake, and

605 morbidity in mouse model of cryptococcosis. These *in vitro*, *ex vivo* and *in vivo* data

606 sustain the importance of host environment on the virulence evolution of *C. neoformans*.

607 Actually, our findings are well consistent with the study by Robertson *et al*. [70].

608 Considering an impaired immune system in the HIV-infected patients, it is reasonable

609 to conclude that virulence may not be an important factor in establishment of a

610 successful infection. However, for colonization in a relatively healthy individual, such

611 as HIV-negative patients, the cryptococcal strains must evolve to enhance their traits in

612 virulence, in order to fight against the host immune system.

613 Mutation represents the ultimate source of the genetic variation required for adaptation

614 and most of time the genomic mutation rate is adjusted to a level that best promote

615 adaptation. We hypothesized that changes due to highly frequent genetic variations may

616 contribute to the observed virulence variabilities among strains derived from different

617 groups. To our surprise, we found that it is not the case, the basis of virulence

618 attenuation and the multiple phenotypic changes of strains from HIV-infected group

619 was unable to be largely addressed by the genomic changes at a high frequency.

620 Consistently, a previous whole-genome sequencing study in 32 isolates from 18 South

621 African patients with recurrent cryptococcal meningitis also revealed that only a few

622 genetic changes, including single nucleotide polymorphism (SNPs), small

623 insertions/deletions (indels) and variation in copy number, were identified between

624 incident and replapse isolates [71]. However, we did observe genetic variations for a

625 few specific genes in these two groups. For example, the isolates from HIV-uninfected

626 group harbor frequent genomic mutations identified in a list of genes specifically

627 related to environmental adaptation, which could explain the increased virulent

628 phenotypes of this group when compared to the strains from HIV-infected group.

629 Genetic mutations actively respond to the change of host environment and thereby drive

630   the adaptive evolution of fungal virulence. In contrast, we observed in HIV-infected

631   group that genetic variations were mostly occurred in genes associated with metabolic

632   processes, implying that the mutations may significantly correlate with survival with

633   respect to the possible role of these genes in receiving nutrients from the host, which is

634   expected to have almost no immune stresses.

635   In conclusion, through successful combination of genotyping, pathogenic phenotyping

636   and comparative genomics, our work has for the first time performed phenotypic

637   characterization and evaluated virulence-related properties of *C. neoformans* strains

638   isolated from three different host environments, including the HIV-uninfected

639   individuals, HIV-infected patients and the natural resources. Our studies highlighted

640   that host environmental factors may majorly account for the attenuation of virulence

641   and various other phenotypic changes in the strains from HIV-infected patients that are

642   different from their HIV-uninfected counterparts. Moreover, our work also supports an

643   essential role of the genetic variations in driving the evolution of fungal virulence. This

644   might be extremely important for *C. neoformans* to avoid clearance of host immunity

645   and establish successful colonization. Further biochemical, molecular genetics and

646   immunological studies are required to confirm and assess the relative importance of

647   both environmental and genetic factors in regulating pathogenicity and causing

648   invasive infection of *C. neoformans*. Such studies will not only improve our

649   understanding of pathogenic mechanisms of this fungus but also facilitate design of

650   new therapeutic approaches based on these factors.

651   **Acknowledgments**

672   **Conflict of interest**

673   The authors declare that they have no conflict of interest.

674   **References**

675 1.   Kwon-Chung KJ, Varma A. Do major species concepts support one, two or more species within
676   Cryptococcus neoformans? FEMS Yeast Res. 2006;6(4):574-87. doi: 10.1111/j.1567-
677   1364.2006.00088.x. PubMed PMID: 16696653.

678 2.   Cogliati M. Global Molecular Epidemiology of Cryptococcus neoformans and Cryptococcus gattii:
679   An Atlas of the Molecular Types. Scientifica (Cairo). 2013;2013:675213. doi: 10.1155/2013/675213.
680   PubMed PMID: 24278784; PubMed Central PMCID: PMCPMC3820360.

681 3.   Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, et al. A rare genotype of
682   Cryptococcus gattii caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada).
683   Proc Natl Acad Sci U S A. 2004;101(49):17258-63. doi: 10.1073/pnas.0402981101. PubMed PMID:
684   15572442; PubMed Central PMCID: PMCPMC535360.

685 4.   Guo LY, Liu LL, Liu Y, Chen TM, Li SY, Yang YH, et al. Characteristics and outcomes of
686   cryptococcal meningitis in HIV seronegative children in Beijing, China, 2002-2013. BMC Infect Dis.
687   2016;16(1):635. doi: 10.1186/s12879-016-1964-6. PubMed PMID: 27814690; PubMed Central PMCID:
688   PMCPMC5097362.

689 5.   Chen M, Xu Y, Hong N, Yang Y, Lei W, Du L, et al. Epidemiology of fungal infections in China.
690   Front Med. 2018;12(1):58-75. doi: 10.1007/s11684-017-0601-0. PubMed PMID: 29380297.

691 6.   Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, et al. Global burden of
692   disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis.
693   2017;17(8):873-81. doi: 10.1016/S1473-3099(17)30243-8. PubMed PMID: 28483415; PubMed Central
694   PMCID: PMCPMC5818156.

695 7.   Wu SY, Lei Y, Kang M, Xiao YL, Chen ZX. Molecular characterisation of clinical Cryptococcus
696   neoformans and Cryptococcus gattii isolates from Sichuan province, China. Mycoses. 2015;58(5):280-
697   7. doi: 10.1111/myc.12312. PubMed PMID: 25808662.

698 8.   Alves GS, Freire AK, Bentes Ados S, Pinheiro JF, de Souza JV, Wanke B, et al. Molecular typing
699   of environmental Cryptococcus neoformans/C. gattii species complex isolates from Manaus, Amazonas,
700   Brazil. Mycoses. 2016;59(8):509-15. doi: 10.1111/myc.12499. PubMed PMID: 27005969.

701 9.   Benham RW. Cryptococcosis and blastomycosis. Ann N Y Acad Sci. 1950;50(10):1299-314. doi:
702   10.1111/j.1749-6632.1950.tb39828.x. PubMed PMID: 14783320.

703 10. Evans EE. The antigenic composition of Cryptococcus neoformans. I. A serologic classification by
704   means of the capsular and agglutination reactions. J Immunol. 1950;64(5):423-30. PubMed PMID:
705   15415610.

706 11. Wilson DE, Bennett JE, Bailey JW. Serologic grouping of Cryptococcus neoformans. Proc Soc Exp
707   Biol Med. 1968;127(3):820-3. doi: 10.3181/00379727-127-32812. PubMed PMID: 5651140.

708 12. Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M. (1557) Proposal to conserve the name
709   Cryptococcus gattii against C. hondurianus and C. bacillisporus (Basidiomycota, Hymenomycetes,
710   Tremellomycetidae). Taxon. 2002;51(4):804-6.

711 13. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the
712   current global burden of cryptococcal meningitis among persons living with HIV/AIDS. Aids.
713   2009;23(4):525-30.

714 14. Dromer F, Mathoulin S, Dupont B, Letenneur L, Ronin O, Group FCS. Individual and
715   environmental factors associated with infection due to Cryptococcus neoformans serotype D. Clinical
716   infectious diseases. 1996;23(1):91-6.

717 15. Kwon-Chung KJ, Edman JC, Wickes BL. Genetic association of mating types and virulence in
718   Cryptococcus neoformans. Infection and immunity. 1992;60(2):602-5.

719   16.   Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E, IberoAmerican Cryptococcal Study G.
720   Molecular typing of IberoAmerican Cryptococcus neoformans isolates. Emerg Infect Dis.
721   2003;9(2):189-95. doi: 10.3201/eid0902.020246. PubMed PMID: 12603989; PubMed Central PMCID:
722   PMCPMC2901947.

723   17.   Boekhout T, Theelen B, Diaz M, Fell JW, Hop WCJ, Abeln ECA, et al. Hybrid genotypes in the
724   pathogenic yeast Cryptococcus neoformans. Microbiology (Reading). 2001;147(Pt 4):891-907. doi:
725   10.1099/00221287-147-4-891. PubMed PMID: 11283285.

726   18.   Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, et al. Consensus multi-
727   locus sequence typing scheme for Cryptococcus neoformans and Cryptococcus gattii. Med Mycol.
728   2009;47(6):561-70. doi: 10.1080/13693780902953886. PubMed PMID: 19462334; PubMed Central
729   PMCID: PMCPMC2884100.

730   19.   Cuomo CA, Rhodes J, Desjardins CA. Advances in Cryptococcus genomics: insights into the
731   evolution of pathogenesis. Mem Inst Oswaldo Cruz. 2018;113(7):e170473. doi: 10.1590/0074-
732   02760170473. PubMed PMID: 29513784; PubMed Central PMCID: PMCPMC5851040.

733   20.   Hagen F, Colom MF, Swinne D, Tintelnot K, Iatta R, Montagna MT, et al. Autochthonous and
734   dormant Cryptococcus gattii infections in Europe. Emerg Infect Dis. 2012;18(10):1618-24. doi:
735   10.3201/eid1810.120068. PubMed PMID: 23017442; PubMed Central PMCID: PMCPMC3471617.

736   21.   Antinori S. New Insights into HIV/AIDS-Associated Cryptococcosis. ISRN AIDS.
737   2013;2013:471363. doi: 10.1155/2013/471363. PubMed PMID: 24052889; PubMed Central PMCID:
738   PMCPMC3767198.

739   22.   Litvintseva AP, Thakur R, Vilgalys R, Mitchell TG. Multilocus sequence typing reveals three
740   genetic subpopulations of Cryptococcus neoformans var. grubii (serotype A), including a unique
741   population in Botswana. Genetics. 2006;172(4):2223-38. doi: 10.1534/genetics.105.046672. PubMed
742   PMID: 16322524; PubMed Central PMCID: PMCPMC1456387.

743   23.   Andrade-Silva LE, Ferreira-Paim K, Ferreira TB, Vilas-Boas A, Mora DJ, Manzato VM, et al.
744   Genotypic analysis of clinical and environmental Cryptococcus neoformans isolates from Brazil reveals
745   the presence of VNB isolates and a correlation with biological factors. PLoS One. 2018;13(3):e0193237.
746   doi: 10.1371/journal.pone.0193237. PubMed PMID: 29505557; PubMed Central PMCID:
747   PMCPMC5837091.

748   24.   Viviani MA, Cogliati M, Esposto MC, Lemmer K, Tintelnot K, Colom Valiente MF, et al.
749   Molecular analysis of 311 Cryptococcus neoformans isolates from a 30-month ECMM survey of
750   cryptococcosis in Europe. FEMS Yeast Res. 2006;6(4):614-9. doi: 10.1111/j.1567-1364.2006.00081.x.
751   PubMed PMID: 16696657.

752   25.   Altamirano S, Jackson KM, Nielsen K. The interplay of phenotype and genotype in Cryptococcus
753   neoformans disease. Biosci Rep. 2020;40(10). doi: 10.1042/BSR20190337. PubMed PMID: 33021310;
754   PubMed Central PMCID: PMCPMC7569153.

755   26.   Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, et al. Clinical practice
756   guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society
757   of america. Clin Infect Dis. 2010;50(3):291-322. doi: 10.1086/649858. PubMed PMID: 20047480;
758   PubMed Central PMCID: PMCPMC5826644.

759   27.   Wiesner DL, Moskalenko O, Corcoran JM, McDonald T, Rolfes MA, Meya DB, et al. Cryptococcal
760   genotype influences immunologic response and human clinical outcome after meningitis. mBio.
761   2012;3(5). doi: 10.1128/mBio.00196-12. PubMed PMID: 23015735; PubMed Central PMCID:
762   PMCPMC3448160.

763   28.   Beale MA, Sabiiti W, Robertson EJ, Fuentes-Cabrejo KM, O'Hanlon SJ, Jarvis JN, et al. Genotypic
764   Diversity Is Associated with Clinical Outcome and Phenotype in Cryptococcal Meningitis across

33

765   Southern Africa. PLoS Negl Trop Dis. 2015;9(6):e0003847. doi: 10.1371/journal.pntd.0003847.
766   PubMed PMID: 26110902; PubMed Central PMCID: PMCPMC4482434.

767   29.   Chen J, Varma A, Diaz MR, Litvintseva AP, Wollenberg KK, Kwon-Chung KJ. Cryptococcus
768   neoformans strains and infection in apparently immunocompetent patients, China. Emerg Infect Dis.
769   2008;14(5):755-62. doi: 10.3201/eid1405.071312. PubMed PMID: 18439357; PubMed Central PMCID:
770   PMCPMC2600263.

771   30.   Khayhan K, Hagen F, Pan W, Simwami S, Fisher MC, Wahyuningsih R, et al. Geographically
772   structured populations of Cryptococcus neoformans Variety grubii in Asia correlate with HIV status and
773   show a clonal population structure. PLoS One. 2013;8(9):e72222. doi: 10.1371/journal.pone.0072222.
774   PubMed PMID: 24019866; PubMed Central PMCID: PMCPMC3760895.

775   31.   Day JN, Qihui S, Thanh LT, Trieu PH, Van AD, Thu NH, et al. Comparative genomics of
776   Cryptococcus neoformans var. grubii associated with meningitis in HIV infected and uninfected patients
777   in Vietnam. PLoS Negl Trop Dis. 2017;11(6):e0005628. doi: 10.1371/journal.pntd.0005628. PubMed
778   PMID: 28614360; PubMed Central PMCID: PMCPMC5484541.

779   32.   Dou HT, Xu YC, Wang HZ, Li TS. Molecular epidemiology of Cryptococcus neoformans and
780   Cryptococcus gattii in China between 2007 and 2013 using multilocus sequence typing and the
781   DiversiLab system. Eur J Clin Microbiol Infect Dis. 2015;34(4):753-62. doi: 10.1007/s10096-014-2289-
782   2. PubMed PMID: 25471194.

783   33.   Simwami SP, Khayhan K, Henk DA, Aanensen DM, Boekhout T, Hagen F, et al. Low diversity
784   Cryptococcus neoformans variety grubii multilocus sequence types from Thailand are consistent with an
785   ancestral African origin. PLoS Pathog. 2011;7(4):e1001343. doi: 10.1371/journal.ppat.1001343.
786   PubMed PMID: 21573144; PubMed Central PMCID: PMCPMC3089418.

787   34.   Perfect JR. Cryptococcus neoformans: a sugar-coated killer with designer genes. FEMS Immunol
788   Med Microbiol. 2005;45(3):395-404. doi: 10.1016/j.femsim.2005.06.005. PubMed PMID: 16055314.

789   35.   Beenhouwer DO, Shapiro S, Feldmesser M, Casadevall A, Scharff MD. Both Th1 and Th2
790   cytokines affect the ability of monoclonal antibodies to protect mice against Cryptococcus neoformans.
791   Infect Immun. 2001;69(10):6445-55. doi: 10.1128/IAI.69.10.6445-6455.2001. PubMed PMID:
792   11553589; PubMed Central PMCID: PMCPMC98780.

793   36.   Kozel TR. Virulence factors of Cryptococcus neoformans. Trends Microbiol. 1995;3(8):295-9.
794   PubMed PMID: 8528612.

795   37.   Zaragoza O. Basic principles of the virulence of Cryptococcus. Virulence. 2019;10(1):490-501. doi:
796   10.1080/21505594.2019.1614383. PubMed PMID: 31119976; PubMed Central PMCID:
797   PMCPMC6550552.

798   38.   Mayer FL, Kronstad JW. Disarming Fungal Pathogens: Bacillus safensis Inhibits Virulence Factor
799   Production and Biofilm Formation by Cryptococcus neoformans and Candida albicans. MBio. 2017;8(5).
800   doi: 10.1128/mBio.01537-17. PubMed PMID: 28974618; PubMed Central PMCID: PMCPMC5626971.

801   39.   Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive
802   multiple sequence alignment through sequence weighting, position-specific gap penalties and weight
803   matrix choice. Nucleic Acids Res. 1994;22(22):4673-80. PubMed PMID: 7984417; PubMed Central
804   PMCID: PMCPMC308517.

805   40.   Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM. Systematic genetic analysis of
806   virulence in the human fungal pathogen Cryptococcus neoformans. Cell. 2008;135(1):174-88. doi:
807   10.1016/j.cell.2008.07.046. PubMed PMID: 18854164; PubMed Central PMCID: PMCPMC2628477.

808   41.   García-Rodas R, Cordero RJ, Trevijano-Contador N, Janbon G, Moyrand F, Casadevall A, et al.
809   Capsule growth in Cryptococcus neoformans is coordinated with cell cycle progression. MBio.
810   2014;5(3):e00945-14.

811   42.   Fernandes OdFL, Costa CR, Junior RdSL, Vinaud MC, e Souza LKH, de Paula JAM, et al. Effects
812   of Pimenta pseudocaryophyllus (Gomes) LR Landrum, on melanized and non-melanized Cryptococcus
813   neoformans. Mycopathologia. 2012;174(5-6):421-8.

814   43.   Shulman JJ, Hontz A, Sedlis A, Walters AT, Balin H, LoSCUITO L. The Pap smear: take two.
815   American journal of obstetrics and gynecology. 1975;121(8):1024-8.

816   44.   Hansakon A, Mutthakalin P, Ngamskulrungroj P, Chayakulkeeree M, Angkasekwinai P.
817   Cryptococcus neoformans and Cryptococcus gattii clinical isolates from Thailand display diverse
818   phenotypic interactions with macrophages. Virulence. 2019;10(1):26-36.

819   45.   Sabiiti W, Robertson E, Beale MA, Johnston SA, Brouwer AE, Loyse A, et al. Efficient
820   phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis. The Journal of
821   clinical investigation. 2014;124(5):2000-8.

822   46.   Denham ST, Verma S, Reynolds RC, Worne CL, Daugherty JM, Lane TE, et al. Regulated release
823   of cryptococcal polysaccharide drives virulence and suppresses immune infiltration into the central
824   nervous system. Infect Immun. 2017. doi: 10.1128/IAI.00662-17. PubMed PMID: 29203547; PubMed
825   Central PMCID: PMCPMC5820953.

826   47.   Guess T, Lai H, Smith SE, Sircy L, Cunningham K, Nelson DE, et al. Size Matters: Measurement
827   of Capsule Diameter in Cryptococcus neoformans. J Vis Exp. 2018;(132). doi: 10.3791/57171. PubMed
828   PMID: 29553511.

829   48.   Tugume L, Rhein J, Hullsiek KH, Mpoza E, Kiggundu R, Ssebambulidde K, et al. HIV-associated
830   cryptococcal meningitis occurring at relatively higher CD4 counts. The Journal of infectious diseases.
831   2019;219(6):877-83.

832   49.   Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics
833   Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725-9. doi: 10.1093/molbev/mst197. PubMed PMID:
834   24132122; PubMed Central PMCID: PMCPMC3840312.

835   50.   Dou H, Wang H, Xie S, Chen X, Xu Z, Xu Y. Molecular characterization of Cryptococcus
836   neoformans isolated from the environment in Beijing, China. Med Mycol. 2017;55(7):737-47. doi:
837   10.1093/mmy/myx026. PubMed PMID: 28431114.

838   51.   Hull CM, Heitman J. Genetics of Cryptococcus neoformans. Annu Rev Genet. 2002;36:557-615.
839   doi: 10.1146/annurev.genet.36.052402.152652. PubMed PMID: 12429703.

840   52.   Wickes BL. The role of mating type and morphology in Cryptococcus neoformans pathogenesis.
841   Int J Med Microbiol. 2002;292(5-6):313-29. doi: 10.1078/1438-4221-00216. PubMed PMID: 12452279.

842   53.   Nielsen K, Heitman J. Sex and virulence of human pathogenic fungi. Adv Genet. 2007;57:143-73.
843   doi: 10.1016/S0065-2660(06)57004-X. PubMed PMID: 17352904.

844   54.   Pool A, Lowder L, Wu Y, Forrester K, Rumbaugh J. Neurovirulence of Cryptococcus neoformans
845   determined by time course of capsule accumulation and total volume of capsule in the brain. J Neurovirol.
846   2013;19(3):228-38. doi: 10.1007/s13365-013-0169-7. PubMed PMID: 23733307.

847   55.   Eisenman HC, Chow SK, Tse KK, McClelland EE, Casadevall A. The effect of L-DOPA on
848   Cryptococcus neoformans growth and gene expression. Virulence. 2011;2(4):329-36. doi:
849   10.4161/viru.2.4.16136. PubMed PMID: 21705857; PubMed Central PMCID: PMCPMC3173677.

850   56.   Johnston SA, May RC. Cryptococcus interactions with macrophages: evasion and manipulation of
851   the phagosome by a fungal pathogen. Cellular microbiology. 2013;15(3):403-11.

852   57.   Smith LM, Dixon EF, May RC. The fungal pathogen C ryptococcus neoformans manipulates
853   macrophage phagosome maturation. Cellular microbiology. 2015;17(5):702-13.

854   58.   García-Rodas R, Zaragoza O. Catch me if you can: phagocytosis and killing avoidance by

35

855   Cryptococcus neoformans. FEMS Immunology & Medical Microbiology. 2012;64(2):147-61.

856   59.   Casadevall A, Coelho C, Cordero RJ, Dragotakes Q, Jung E, Vij R, et al. The capsule of
857   Cryptococcus neoformans. Virulence. 2019;10(1):822-31.

858   60.   Gish SR, Maier EJ, Haynes BC, Santiago-Tirado FH, Srikanta DL, Ma CZ, et al. Computational
859   Analysis Reveals a Key Regulator of Cryptococcal Virulence and Determinant of Host Response. MBio.
860   2016;7(2):e00313-16. doi: 10.1128/mBio.00313-16. PubMed PMID: 27094327; PubMed Central
861   PMCID: PMCPMC4850258.

862   61.   Wang ZA, Li LX, Doering TL. Unraveling synthesis of the cryptococcal cell wall and capsule.
863   Glycobiology. 2018;28(10):719-30. doi: 10.1093/glycob/cwy030. PubMed PMID: 29648596; PubMed
864   Central PMCID: PMCPMC6142866.

865   62.   Casadevall A, Pirofski LA. Host-pathogen interactions: redefining the basic concepts of virulence
866   and pathogenicity. Infect Immun. 1999;67(8):3703-13. doi: 10.1128/IAI.67.8.3703-3713.1999. PubMed
867   PMID: 10417127; PubMed Central PMCID: PMCPMC96643.

868   63.   Urban M, Pant R, Raghunath A, Irvine AG, Pedro H, Hammond-Kosack KE. The Pathogen-Host
869   Interactions   database   (PHI-base):   additions   and   future   developments.   Nucleic   Acids   Res.
870   2015;43(Database issue):D645-55. doi: 10.1093/nar/gku1165. PubMed PMID: 25414340; PubMed
871   Central PMCID: PMCPMC4383963.

872   64.   Bahn YS, Kojima K, Cox GM, Heitman J. A unique fungal two-component system regulates stress
873   responses, drug sensitivity, sexual development, and virulence of Cryptococcus neoformans. Mol Biol
874   Cell. 2006;17(7):3122-35. doi: 10.1091/mbc.e06-02-0113. PubMed PMID: 16672377; PubMed Central
875   PMCID: PMCPMC1483045.

876   65.   Bahn YS, Kojima K, Cox GM, Heitman J. Specialization of the HOG pathway and its impact on
877   differentiation and virulence of Cryptococcus neoformans. Mol Biol Cell. 2005;16(5):2285-300. doi:
878   10.1091/mbc.e04-11-0987. PubMed PMID: 15728721; PubMed Central PMCID: PMCPMC1087235.

879   66.   Okagaki LH, Wang Y, Ballou ER, O'Meara TR, Bahn YS, Alspaugh JA, et al. Cryptococcal titan
880   cell formation is regulated by G-protein signaling in response to multiple stimuli. Eukaryot Cell.
881   2011;10(10):1306-16. doi: 10.1128/EC.05179-11. PubMed PMID: 21821718; PubMed Central PMCID:
882   PMCPMC3187071.

883   67.   Hu G, Caza M, Bakkeren E, Kretschmer M, Bairwa G, Reiner E, et al. A P4-ATPase subunit of the
884   Cdc50 family plays a role in iron acquisition and virulence in Cryptococcus neoformans. Cell Microbiol.
885   2017;19(6).   doi: 10.1111/cmi.12718.   PubMed   PMID: 28061020;   PubMed   Central   PMCID:
886   PMCPMC5429215.

887   68.   Choi YH, Ngamskulrungroj P, Varma A, Sionov E, Hwang SM, Carriconde F, et al. Prevalence of
888   the VNIc genotype of Cryptococcus neoformans in non-HIV-associated cryptococcosis in the Republic
889   of Korea. FEMS Yeast Res. 2010;10(6):769-78. doi: 10.1111/j.1567-1364.2010.00648.x. PubMed PMID:
890   20561059; PubMed Central PMCID: PMCPMC2920376.

891   69.   Fernandes KE, Brockway A, Haverkamp M, Cuomo CA, van Ogtrop F, Perfect JR, et al. Phenotypic
892   Variability Correlates with Clinical Outcome in Cryptococcus Isolates Obtained from Botswanan
893   HIV/AIDS Patients. MBio. 2018;9(5). doi: 10.1128/mBio.02016-18. PubMed PMID: 30352938;
894   PubMed Central PMCID: PMCPMC6199498.

895   70.   Robertson EJ, Najjuka G, Rolfes MA, Akampurira A, Jain N, Anantharanjit J, et al. Cryptococcus
896   neoformans ex vivo capsule size is associated with intracranial pressure and host immune response in
897   HIV-associated cryptococcal meningitis. J Infect Dis. 2014;209(1):74-82. doi: 10.1093/infdis/jit435.
898   PubMed PMID: 23945372; PubMed Central PMCID: PMCPMC3864387.

899   71.   Chen Y, Farrer RA, Giamberardino C, Sakthikumar S, Jones A, Yang T, et al. Microevolution of
900   Serial Clinical Isolates of Cryptococcus neoformans var. grubii and C. gattii. mBio. 2017;8(2). doi:

901   10.1128/mBio.00166-17. PubMed PMID: 28270580; PubMed Central PMCID: PMCPMC5340869.

902
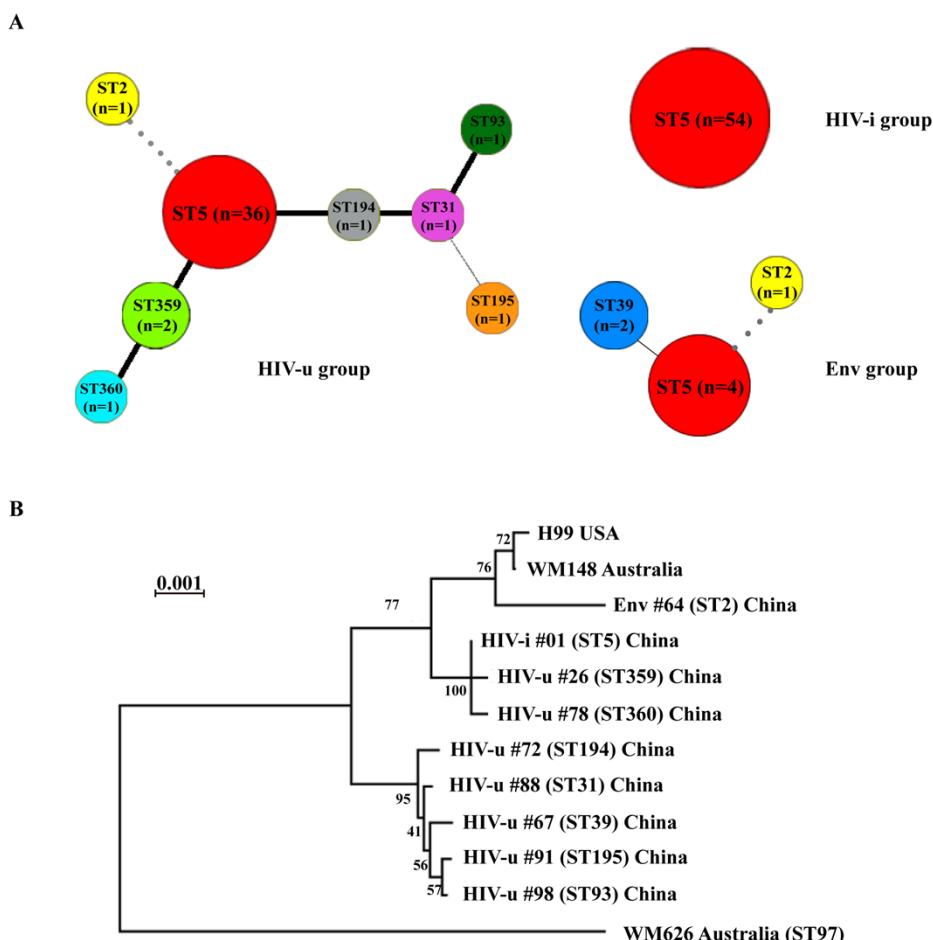
Fig 1. Genetic and evolutionary relationships among *C. neoformans* strains derived

from different sources. **(A)** Hypothesis of the evolution of the 105 C. neoformans

strains, based on phylogenetic relationships described by sequence analysis of the

MLST scheme. Representative STs labelled with different colors. The number of

isolates sharing the same ST is listed in each node, whereas the lines between STs

indicate inferred phylogenetic relationships and are denoted by bold black, plain black,

or grey line, depending on the number of allelic mismatches between profiles (bold

black: < 3; Plain black: 3-4; Grey: > 4). **(B)** Phylogeny of selected *C. neoformans* strains

911 based on DNA sequences from eight housekeeping gene loci. The tree was constructed

912 with the allelic frequencies using the neighbor-joining (NJ) model and 1,000 bootstrap

913 replicates were performed. Numbers labeled with HIV-u, HIV-i or Env are strains from

914 105 Chinese isolates in this study; Three reference strains, one from the United States

915 and the other two from Australia, are also included.

916
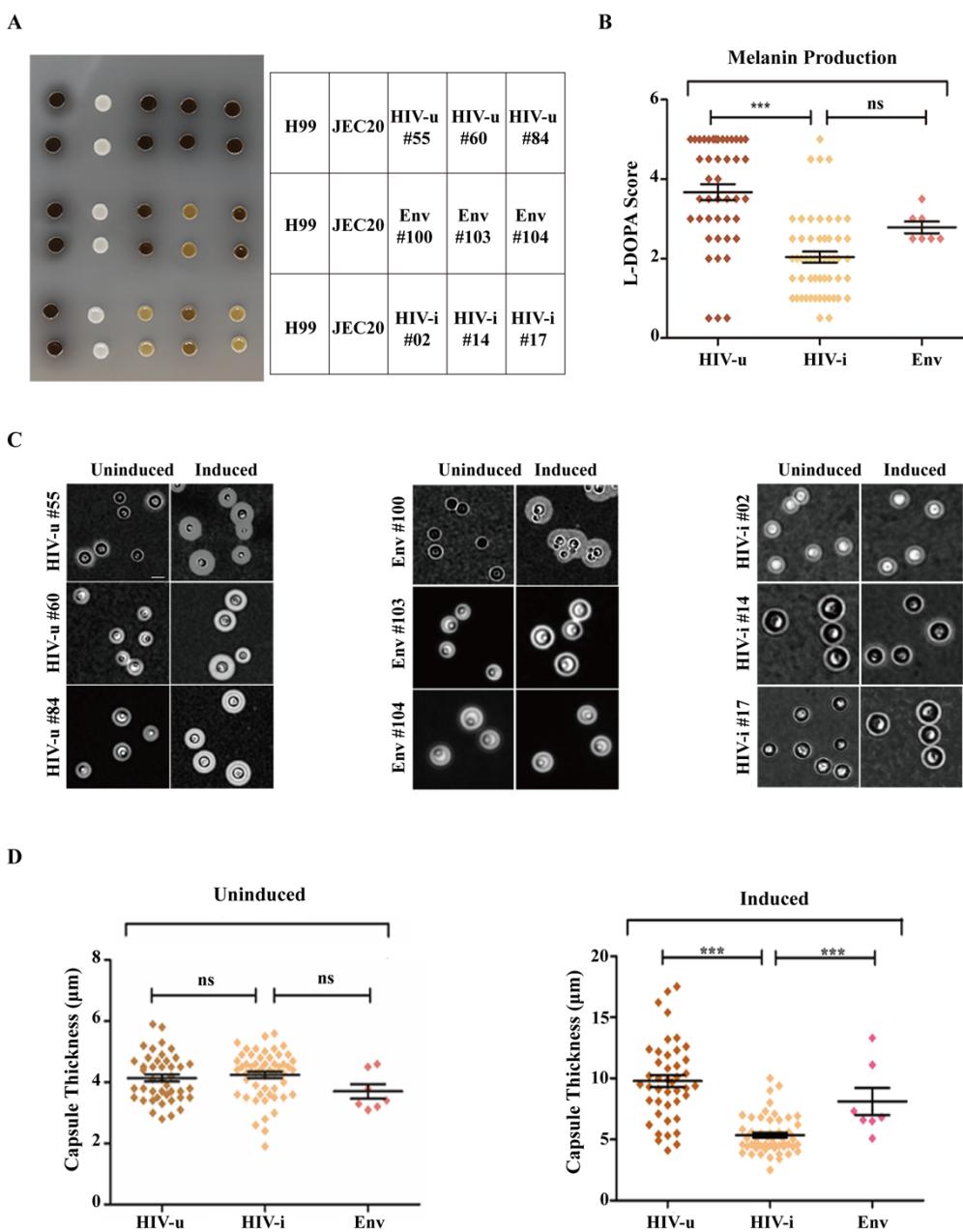
917 **Fig 2.** *In vitro* characterization of melanin biosynthesis and the formation of

918 polysaccharide capsule in *Cryptococcus* isolates from each of the three groups. **(A)**

919 Three representative isolates were randomly selected from each group and melanin

920 phenotypes were verified by growth at 30 ℃ on plates containing L-DOPA. Pictures

921 were taken after incubation for 3 days. *C. neoformans* strains H99 and JEC20 were used

922 as positive and negative control, respectively. There were two replicates for each strain.

923 **(B)** Statistical comparison of melanin biosynthesis in all 105 isolates of *C. neoformans*.

924 The scores were calculated based on a K-Means cluster analysis. The vertical bars

925 represent standard errors of the means in each group. ns $p > 0.05$, *** $p < 0.001$. **(C)**

926 As in A, the same isolates were analyzed for capsule formation by India ink staining

927 under capsule-inducing and non-inducing growth conditions. A single colony from

928 each isolate was resuspended in PBS containing India ink, subjected to a short vortexing

929 and examined under a light microscope at ×63 magnification. Scale bars, 10 μm. (D)

930 Statistical comparison of capsule formation in all 105 isolates of *C. neoformans*. As

931 described in Materials and Methods, stationary-phase fungal cultures were washed and

932 resuspended in PBS, and diluted 1/100 in capsule induction medium (10% Sabouraud

933 dextrose medium in MOPS buffered at pH 7.3). After incubation at 30 ℃ and 180rpm

934 for 48h, suspensions of India ink were photographed and the capsule thickness of each

935 isolate was measured. Each symbol represents the average capsule thickness of 10-20

936 cells. * $p < 0.05$, *** $p < 0.001$, by unpaired two-tailed Student's test.
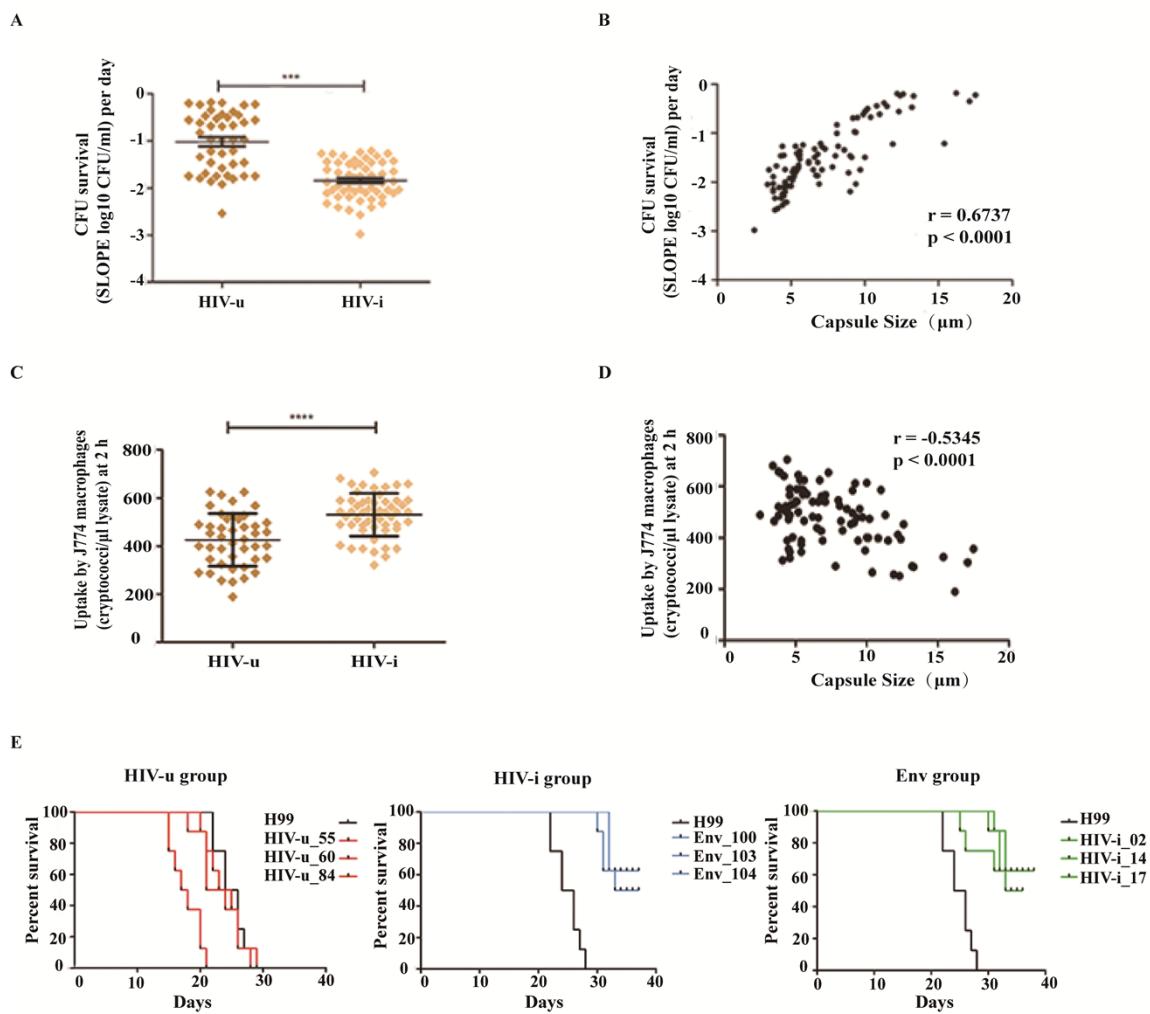
39

Fig 3. *Ex vivo* and *in vivo* assays investigating virulence-associated characteristics in *Cryptococcus* isolates from each of the three groups. **(A, B)** Cryptococcal survival in human CSF. Cells of each clinical isolate were inoculated into human CSF at a concentration of $1\sim2 \times 10^6$ cells/ml and aliquots were collected at different time points (0, 12, 24, 36, 72 and 96 hours after inoculation) and plated on Sabouraud dextrose agar (SDA) media for CFU counts. The survival slope was determined as the mean rate of increase or decrease in cryptococcal counts after CSF treatment, by averaging the slope of the linear regression of $\log_{10}$ CFU/ml over time for each strain. A. Survival

40

947   comparison of clinical *C. neoforman*s strains between HIV-u and HIV-i group after

948   incubation with human CSF; B. Association of capsule size with *ex vivo* cryptococcal

949   survival in human CSF. **(C, D)** Phagocytic uptake of clinical *C. neoforman*s strains by

950   macrophage-like cell line J774. Macrophage cells (1.5×10^5) were incubated in serum-

951   free DMEM medium for 2 hours, activated with 15μg/ml phorbol myristate acetate

952   (PMA) for 30-60 minutes, and then co-incubated with *C. neoformans* yeast cells pre-

953   opsonized by a monoclonal antibody for 2 hours at 37°C with 5% CO_2 (MOI=1:10).

954   The extent of *Cryptococcus* phagocytosis was calculated as the number of cryptococci

955   internalized by macrophages 2 hours after infection. Results were expressed as the

956   mean of 3 to 4 experimental repeats. C. Phagocytic uptake comparison of clinical *C.*

957   *neoforman*s strains between HIV-u and HIV-i group after incubation with J774; D.

958   Association of capsule size with *ex vivo* phagocytic uptake. **(E)** Kaplan-Meier survival

959   curves of mice infected with individual strains. Groups of female C57BL/6 mice (8 for

960   each group) were inoculated intranasally with 1×10^5 CFUs of the indicated strain and

961   monitored for progression to severe morbidity. As in Fig 2, the same three

962   representative isolates from each of the three groups were used. For comparison, the

963   strain H99 was used as a control. (Left) HIV-u group; (Middle) HIV-i group; and (Right)

964   Env group. Note: All *C. neoformans* strains, including the control strain H99 and all 9

965   tested isolates, were tested for pathogenicity in one experiment. ***$p < 0.001$, by Log-

966   rank test.

967
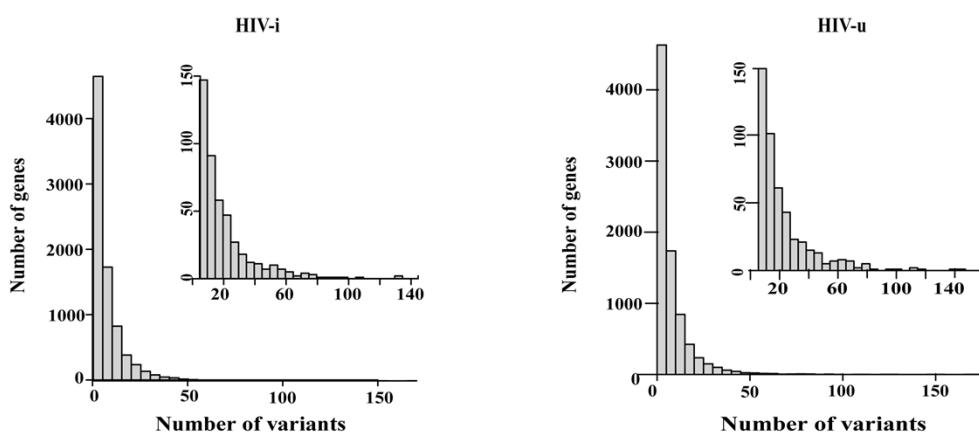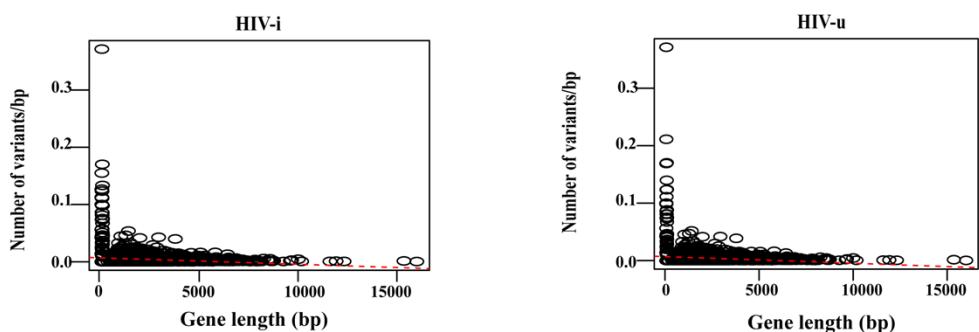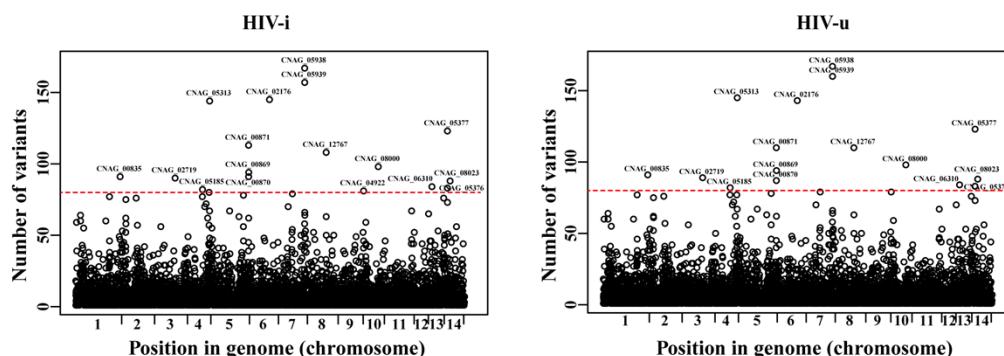
Fig 4. Summary of genomic variants identified from whole-genome sequencing of selected *C. neoformans* isolates. **(A)** A total of 28 clinical isolates, including 14 from the HIV-i group and another 14 from the HIV-u group, were sequenced. Shown is the number of variants that were identified in each gene locus. Genes with at least 20 variants were selected and presented in the magnified inset panel. **(B)** All sequenced

42

973   isolates from both groups showed no correlation between the number of variants and

974   gene length per base pair in each gene ($p < 0.5$). **(C)** Distribution of variants across the

975   genomes of sequenced isolates. Notably, a cluster of genes that were identical in both

976   groups had significantly high numbers of variants. Genes harboring more than 80

977   variants are indicated with their names.

978
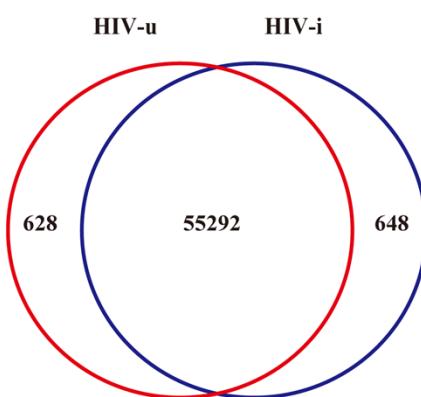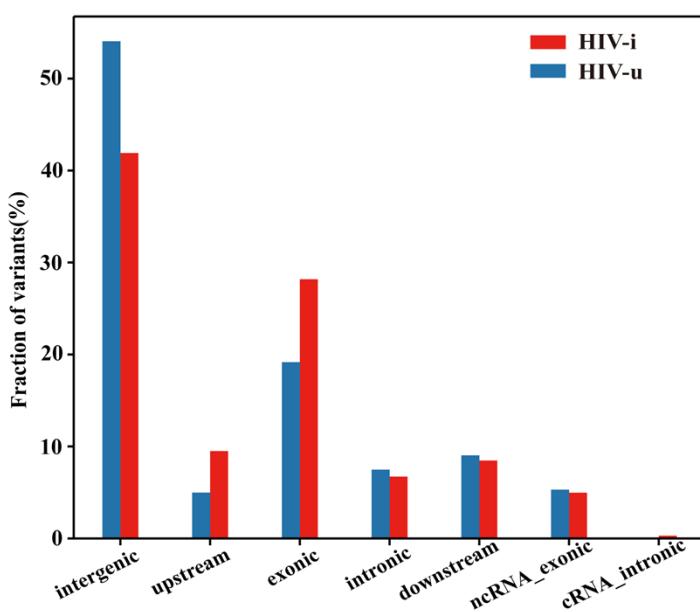
979

980

981   **Fig 5.** Common and group-specific variants identified in the sequenced isolates. **(A)**

982   Venn diagram showing a summary of the number of common or group-specific variants.

983   A common variant was defined as the one that are found in more than 7 isolates of each

984   group. **(B)** Genomic distribution of the group-specific variants.

985

Fig 6. Characterization of the genes harboring group-specific variants. **(A)** Venn diagram showing the number of genes that were associated with group-specific variants.

988   It has to be noted that 43 genes are common in both groups only because the variants

989   in each group were mapped to different positions of the same gene. **(B)** Significantly

990   enriched Gene Ontology (GO) categories (*p*-value <0.05) with group-specific genes.

991   **(C)** Functional classification pie chart showing the annotated genes that harbor group-

992   specific variants, according to the Gene Ontology (GO) Term analysis

993   **Table 1.** Comparison of clinical data for HIV-uninfected and -infected groups

| | Mean ± SD (*n*)[a] | | | |
| | Patients | | | |
Parameter	**Total subjects**[b]	**HIV-u** 44 isolates	**HIV-i** 51 isolates	***p*-value**[c]
Age (yr)	41±11	38±9	43±12	0.0679
Sex (males/ females)	74/21	33/11	41/10	0.5792
CD4 Count (no. of cells/mm3)	193±188	381±92	34±48	<0.001
Temperature (°C)	37.4±0.9	37.1±0.6	37.6±1.1	0.0037
Fever (Y/N)	45/50	12/32	33/18	<0.001
Headache(Y/N)	66/29	29/15	37/14	0.5105
Seizure(Y/N)	24/71	16/28	8/43	0.0207
Cerebral herniation(Y/N)	47/48	12/32	4/47	0.0143
Cerebellar Signs(Y/N)	16/79	28/16	20/31	0.0176

994   [a] Values, including age, CD4 counts and temperature, were represented by means ±

995   SD for the statistic characteristics and clinical parameters. *n* indicates the number of

996   patients.

997   [b] Total subjects indicate the mean ± SD of patients.

998   [c] *p* values were calculated by one-way ANOVA or Fisher's exact test as appropriate.

999   **Table 2.** Characterization of major genes harboring specific variations in HIV-u and

1000   HIV-i groups, respectively

Function	Broad annotation	Gene name	Description	Classic fisher
Genes harboring specfic variations in HIV-u group				
Response to stress	CNAG_03818	SSK1	Response to fungicide/UV/radiation/light stimulus	0.011
	CNAG_00769	PBS2	Cellular response to stimulus	0.013
Signaling	CNAG_05590	TCO2	Signaling, phosphorelay signal transduction system	0.001
	CNAG_06606	RHO104	Intracellular signal transduction	0.046
	CNAG_03751	Unknown	Cation(calcium) channel activity	0.022
	CNAG_02796	Unknown	Transferase activity, transferring alkyl or aryl groups	0.022
	CNAG_04599	Unknown	3-methyl-2-oxobutanoate hydroxymethy	0.022
	CNAG_05712	RIB4	Intramolecular transferase activity, transferring acyl groups	0.022
	CNAG_06806	ETF1alpha		

	CNAG_03582	RIM20	Macromolecule localization	0.023
	CNAG_05282	APT4		
	CNAG_05395	VAM6		
Antifungal resistance	CNAG_02565	Unknown	Organic acid biosynthetic process Carboxylic acid biosynthetic process	0.006
	CNAG_02796	Unknown		
	CNAG_05194	Unknown		
	CNAG_06421	Unknown		
	CNAG_04087	Unknown	Intrinsic and integral component of organelle membrane	0.032
	CNAG_04556	Unknown		
	CNAG_04604	Unknown	Tryptophan-tRNA ligase activity	0.022
	CNAG_06421	Unknown	Ccetolactate synthase activity	0.044
Autophagy	CNAG_02796	Unknown	Cellular amino acid metabolic and biosynthetic process	0.020
	CNAG_04599	Unknown		
	CNAG_06421	Unknown		
	CNAG_04484	Unknown	Organelle membrane fusion	0.022

Genes harboring specfic variations in HIV-i group

MAPK	CNAG_05063	SSK2	MAPK/ protein serine/threonine/tyrosine kinase activity	0.036
	CNAG_02853	Unknown	Amidophosphoribosyltransferase activity	0.036
PHO pathway	CNAG_04501	TRP1	Indole-3-glycerol-phosphate synthase activity, Phosphoribosylanthranilate isomerase activity	0.036
	CNAG_02506	RIB3	3,4-dihydroxy-2-butanone-4-phosphate synthase activity	0.0361
Glycosylation	CNAG_03832	KTR3	Glycoprotein metabolic and biosynthetic	0.011

			process, macromolecule glycosylation	
Cellular metabolism and biosynthesis	CNAG_00405	KIC1	Regulation of cellular and biosynthetic metabolic process	0.128
	CNAG_00745	HRK1	Regulation of biosynthetic process	0.015
	CNAG_01938	KIN1	Biosynthetic process	0.015
	CNAG_02090	GPA3	Carbohydrate derivative metabolic process	0.012
	CNAG_02305	FZC45	Organic substance biosynthetic process	0.006
	CNAG_06490	SNF102	Organic cyclic compound biosynthetic process	0.007
	CNAG_00678	URE7	Regulation of biological process	0.010
	CNAG_07679	DST1	Positive regulation of metabolic process	0.015
	CNAG_04755	BCK1	Aromatic compound biosynthetic process	0.019

1001