Tracing the evolution and genomic dynamics of mating-type loci in *Cryptococcus* pathogens and closely related species

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Abstract

Sexual reproduction in basidiomycete fungi is governed by *MAT* loci (*P/R* and *HD*), which exhibit remarkable evolutionary plasticity, characterized by expansions, rearrangements, and gene losses often associated with mating system transitions. The sister genera *Cryptococcus* and *Kwoniella* provide a powerful framework for studying *MAT* loci evolution owing to their diverse reproductive strategies and distinct architectures, spanning bipolar and tetrapolar systems with either linked or unlinked *MAT* loci. Building on recent large-scale comparative genomic analyses, we generated additional chromosome-level assemblies uncovering distinct evolutionary trajectories shaping *MAT* loci organization. Contrasting with the small-scale expansions and gene acquisitions observed in *Kwoniella*, our analyses revealed independent expansions of the *P/R* locus in tetrapolar *Cryptococcus*, possibly driven by pheromone gene duplications. Notably, these expansions coincided with an enrichment of AT-rich codons and a pronounced GC-content reduction, likely associated with recombination suppression and relaxed codon usage selection. Diverse modes of *MAT* locus linkage were also identified, including three previously unrecognized transitions: one resulting in a pseudobipolar arrangement and two leading to bipolarity. All the three transitions involved translocations. In the pseudobipolar configuration, the *P/R* and *HD* loci remained on the same chromosome but genetically unlinked, whereas the bipolar transitions additionally featured rearrangements that fused the two loci into a nonrecombining region. Mating assays confirmed a sexual cycle in *C. decagattii*, demonstrating its ability to undergo mating and sporulation. Progeny analysis in *K. mangrovensis* revealed substantial ploidy variation and aneuploidy, likely stemming from haploid-diploid mating, yet evidence of recombination and loss of heterozygosity indicates that meiotic exchange occurs despite irregular chromosome segregation. Our findings underscore the importance of continued diversity sampling and provides further evidence for convergent evolution of fused *MAT* loci in basidiomycetes, offering new insights into the genetic and chromosomal changes driving reproductive transitions.

Introduction

Sexual reproduction is a cornerstone of eukaryotic biology, generating genetic variation through recombination and allele segregation, which fuels adaptation in changing environments [1-3]. The Fungal Kingdom is amongst the most ecologically diverse, with species thriving in nearly every environment, and fulfilling roles as decomposers, symbionts, commensals, as well as pathogens of plants, animals, and other fungi [4-6]. Their ecological versatility is mirrored in a wide range of dispersal stages and reproductive strategies, ranging from heterothallism, requiring genetically distinct individuals of opposite mating types, to homothallism, which allows self-fertilization by a single individual as well as outcrossing [7, 8].

In heterothallic basidiomycete fungi, sexual compatibility is typically governed by two mating-type (*MAT*) loci. The pheromone/receptor (*P/R*) locus encodes at least one mating pheromone and one G-protein-coupled pheromone receptor, enabling mate recognition and fusion. The *HD* locus encodes two homeodomain transcription factors (HD1/Sxi1 and HD2/Sxi2) that heterodimerize post-mating to control dikaryotic growth and sexual development [9]. Importantly, successful mating and offspring production can only occur between mating partners carrying different, compatible alleles of both *MAT* loci.

Most basidiomycetes exhibit a tetrapolar breeding system, where the *P/R* and *HD* loci are unlinked, located on separate chromosomes, and may harbor multiple alleles, allowing each meiotic event to generate up to four distinct mating types among haploid progeny [10]. Some lineages, however, have evolved a bipolar system, in which sexual compatibility is controlled by a single *MAT* locus. This transition can occur either through loss of function of one locus in mating-type determination, as observed in *Coprinellus disseminatus* [11] and other mushroom-forming *Agaricomycetes* [12-14], or via the physical linkage and subsequent fusion (i.e., genetic linkage) of the *P/R* and *HD* loci. In the latter case, translocations or chromosome fusion bring the two loci together onto the same chromosome, followed by additional rearrangements that suppress

recombination, resulting in a single, fused, nonrecombining *MAT* region that is often expanded, highly rearranged, and biallelic [15-18]. Additionally, some basidiomycetes exhibit a pseudobipolar configuration, where the two *MAT* loci are on the same chromosome but remain genetically unlinked because they are sufficiently distant to allow recombination, as seen in skin-commensal/pathogenic *Malassezia* species [19, 20]. Beyond heterothallism, several basidiomycetes reproduce without a partner. This strategy is broadly classified as homothallism but encompasses diverse genetic mechanisms, including primary homothallism, where a single individual carries all of the *MAT* alleles necessary for sexual development [21-24], and unisexual reproduction, where mating occurs between cells of the same mating type [25].

Evidence suggests that tetrapolarity is ancestral in basidiomycetes, with bipolarity via *P/R-HD* fusion arising independently multiple times [15, 16, 18, 26, 27]. The bipolar configuration, often observed in fungal species associated with plants or animals as commensals or pathogens, increases mating success among siblings from a single parental pair from 25% (tetrapolar) to 50% (bipolar), thereby facilitating inbreeding [9, 28, 29]. This may be particularly advantageous during host colonization, where spore dispersal and access to unrelated mating partners may be inherently more limited. Bipolar species with fused *MAT* loci are found across the three basidiomycete subphyla, including smut fungi of grasses [15, 30, 31], anther-smut *Microbotryum* infecting *Caryophyllaceae* [17, 27, 32, 33], and two *Tremellomycetes* lineages: *Trichosporonales* [18] and the human-pathogenic *Cryptococcus* species [16, 34, 35].

An important aspect of fused *MAT* loci is their tendency to incorporate additional genes involved in sexual development, thereby influencing key morphological processes (e.g., dikaryotic hyphal growth) and dispersal mechanisms (e.g., spore production) [36]. Additionally, recombination suppression often extends beyond the core mating-type genes, encompassing larger regions of the *MAT*-containing chromosome [37]. While this may preserve beneficial allele combinations, it also contributes to genomic degeneration, including gene loss, accumulation of

transposable elements (TEs), and extensive structural rearrangements. These features share similarities with sex chromosome evolution in other eukaryotes, highlighting potential convergent evolution in sexual reproduction regulation [34, 38].

The genus *Cryptococcus* includes both human-pathogenic species of critical importance and nonpathogenic saprobic members. The pathogenic clade consists of *Cryptococcus neoformans*, *C. deneoformans*, and six species within the *C. gattii* complex [39, 40], with *C. neoformans* ranking first on the World Health Organization's list of critical fungal pathogens [41, 42]. Nonpathogenic species include *C. wingfieldii*, *C. amylolentus*, *C. floricola*, *C. depauperatus*, and *C. luteus* [23, 43-46] along with newly identified lineages [47, 48], many associated with insects, particularly bark beetles [49]. In contrast, its sister genus, *Kwoniella*, consists solely of saprophytic species found in diverse environments, including soil, seawater, plant material, and insect frass [45, 47, 48, 50, 51].

Sexual reproduction is well-characterized in *Cryptococcus* and has been linked to pathogenicity in medically relevant species [52-54]. A hallmark of the *Cryptococcus* sexual cycle is the yeast-to-hypha transition, which occurs after mating between cells of opposite mating types (**a** and α), or during unisexual reproduction [25]. This morphological switch appears to aid in foraging for nutrients and mating partners [55, 56] and in enhancing survival against environmental stressors and microbial predators, thought to be major selective forces in *Cryptococcus* evolution [52, 57-61]. Eventually, the resulting hyphae differentiate into basidia, where diploid nuclei undergo meiosis, producing basidiospores that are more resilient to environmental stresses and significantly smaller than yeast cells. These spores facilitate alveolar deposition upon inhalation and subsequent dissemination within the host [62-64]. In contrast, sexual reproduction in *Kwoniella* has only been documented in *K. mangrovensis* and *K. heveanensis* [51, 65, 66]. These species do not form aerial basidia with spore chains, which likely makes them less efficient at environmental dispersal than *Cryptococcus*.

Recent surveys have identified several isolates that likely represent novel *Cryptococcus* and *Kwoniella* species [40, 47, 48, 67, 68]. However, these discoveries are often based on single strains, limiting direct mating tests and hindering the characterization of reproductive strategies and compatibility systems. In such cases, high-quality genome assemblies offer a powerful alternative, enabling comparative analyses of *MAT* loci to elucidate reproductive systems. While our recent work generated chromosome-level genome assemblies for several *Cryptococcus* and *Kwoniella* species, providing insights into their genomic architecture and evolution [48], this was restricted to single strains per species, precluding in-depth analyses of *MAT* locus variation.

Here, we consider a broader diversity of species than previously available and, where possible, strains of opposite mating types within the same species, allowing for a systematic characterization of *MAT* loci structure, their chromosomal organization, and key evolutionary transitions across the two genera. Our findings support that tetrapolarity is the ancestral state of both *Cryptococcus* and *Kwoniella*, with *P/R-HD* loci fusion (i.e., genetic linkage) evolving independently three times: (i) in the common ancestor of pathogenic *Cryptococcus* species, (ii) in a recently identified nonpathogenic *Cryptococcus* species, and (iii) in a single *Kwoniella* species. Additionally, our analyses revealed that the *P/R* locus itself is highly dynamic, sharing only a core set of four genes across both genera, and has undergone lineage-specific expansions, particularly in *Cryptococcus* where increased pheromone gene copy number appears to have driven substantial rearrangements. Experimental crosses also confirmed a previously unobserved sexual cycle in *C. decagattii* and provided the first direct evidence of recombination in *K. mangrovensis*, revealing meiotic exchange in progeny and non-haploid offspring with potential for self-fertility. Collectively, our findings contribute to the growing body of evidence that fused *MAT* loci have evolved multiple times independently in basidiomycetes, reinforcing the role of convergent evolution in shaping reproductive strategies and ecological adaptation.

Results

Chromosome-level assemblies and updated phylogenetic relationships of *Cryptococcus* and *Kwoniella*

To enable detailed analyses of *MAT* locus structure and evolution, we expanded our previous genomic dataset [48] by incorporating genome sequences for 12 *Cryptococcus* and 7 *Kwoniella* strains, including opposite mating-types where available. Among the *Cryptococcus* strains, four represent three currently undescribed species (*Cryptococcus* sp. 3, sp. 4, and sp. 5; **Fig 1A**), while the remaining eight are opposite mating types of pathogenic *Cryptococcus* species previously sequenced [48]. For *Kwoniella*, three strains belong to the recently described species *K. ovata*, *K. endophytica*, and *K. fici* [47, 69, 70], while the remaining four strains are different mating types of *K. mangrovensis*, *K. europaea*, *K. botswanensis*, and *K. heveanensis* [51, 65, 66]. All but three genomes were assembled using a combination of long-read (PacBio or Oxford Nanopore) and short-read (Illumina) sequencing, yielding chromosome-level genome assemblies. The exceptions, *K. europaea* PYCC6162, *K. botswanensis* CBS12717, and *K. heveanensis* BCC8398 were sequenced solely with Illumina, resulting in more fragmented assemblies. In total, the expanded dataset comprises 27 *Cryptococcus* strains (spanning 17 species) and 22 *Kwoniella* strains (spanning 18 species) (see **S1 Appendix** for additional details).

To establish evolutionary relationships across species and determine the placement of the newly sequenced taxa, we identified 3,086 single-copy orthologous genes shared across all *Cryptococcus* and *Kwoniella* species, as well as three outgroup species (*Tremella mesenterica*, *Saitozyma podzolica*, and *Bullera alba*) following a previously described approach [48]. Phylogenetic analyses employing both Maximum Likelihood (ML) and coalescent-based methods yielded largely congruent results, except for the variable placement of *Cryptococcus* clades B and C relative to clade A (**Fig 1A** and **S1 Fig**). This inconsistency, noted in earlier studies [23, 44, 46] remains unresolved with the current dataset. Despite this, our analyses resolved the phylogenetic

178 placement of the newly identified *Cryptococcus* species. Among them, *Cryptococcus* sp. 3,
179 currently known from a single strain (CMW60451) isolated from a bark beetle (*Lanurgus* sp.)
180 infesting twigs of the endangered conifer *Widdringtonia cedarbergensis* in the Cederberg
181 Mountains of South Africa, was found to be more closely related to *C. depauperatus*, albeit
182 positioned on a relatively long branch. Additionally, *Cryptococcus* sp. 4 [71] and *Cryptococcus* sp.
183 5 clustered with the previously identified *Cryptococcus* sp. 6 (OR918) [48, 72], together forming
184 an early-branching clade within the genus. Among *Kwoniella*, *K. ovata* and *K. endophytica* were
185 identified as sister species closely related to *K. dendrophila*, while *K. fici* grouped more closely
186 with *Kwoniella* sp. 4. The new *Cryptococcus* and *Kwoniella* species will be formally described
187 elsewhere.

188

189 **Sexual reproduction, breeding systems, and *MAT* gene identification and organization**
190 **across *Cryptococcus* and *Kwoniella***

191 Sexual cycles have been described for eight *Cryptococcus* species, seven of which are
192 primarily heterothallic (*C. neoformans*, *C. deneoformans*, *C. gattii*, *C. bacillisporus*, *C.*
193 *deuterogattii*, *C. amylolentus*, *C. floricola*), while one (*C. depauperatus*) is homothallic (**Fig 1A**)
194 [23, 46, 73-77]. In *Kwoniella*, sexual cycles are known for only two species (*K. mangrovensis* and
195 *K. heveanensis*), both heterothallic [44, 51, 65]. To identify sexual cycles in species where they
196 remain unknown, we attempted to induce mating and detect sexual structures in species for which
197 at least two strains of opposite mating types are available. Crosses were performed under
198 conditions known to stimulate mating in *Cryptococcus* [78] and examined for the presence of
199 hyphal growth or other sexual structures, including basidia and basidiospores.

200 In *C. decagattii*, sexual reproduction was confirmed under laboratory conditions upon
201 crossing strains 7685027 (*MAT*α) and CBS11687 (*MAT***a**), resulting in the formation of sexual
202 structures (**S2A Fig**). In contrast, crosses between the two *C. tetragattii* strains (IND107 and

203 CBS11718) did not produce observable sexual structures (**S2B Fig**). For *Cryptococcus* sp. 3,
204 sister to *C. depauperatus*, we observed key differences: while *C. depauperatus* is homothallic and
205 exhibits continuous hyphal growth throughout its lifecycle [23], *Cryptococcus* sp. 3 grew mostly as
206 yeast and did not produce sexual structures alone, suggesting it is unlikely to be homothallic (**S2C**
207 **Fig**). Finally, within clade D species, we tested *Cryptococcus* sp. 4, where two strains of opposite
208 mating types were available. Although some hyphal growth was observed, it was inconsistent, and
209 no discernible sexual structures were detected in the resulting mycelia even after prolonged
210 incubation (**S2D Fig**). Given the absence of confirmed sexual reproduction in most of the species
211 tested, we resorted to genomic data and BLAST-based searches to investigate *MAT* gene content
212 and organization. This approach enabled us to determine the likely breeding system (heterothallic
213 or homothallic) for species without defined sexual cycles and classify *MAT* loci configurations as
214 tetrapolar, pseudobipolar, or bipolar across the two genera. The results of these analyses are
215 summarized in **Fig 1B**.

216 Expanding on previous studies that identified the tetrapolar-to-bipolar transition in the
217 ancestor of the *Cryptococcus* lineage containing human pathogens (clade A) [34, 77], our analysis
218 confirmed that all species in this clade, including *C. decagattii* and *C. tetragattii*, have a bipolar
219 mating configuration. This analysis resolves gaps stemming from the previous lack of genome
220 assemblies for both mating types in these species. In contrast, most of the other non-pathogenic
221 *Cryptococcus* and *Kwoniella* species appeared to be heterothallic and tetrapolar, consistent with
222 the proposed ancestral state of basidiomycetes [9, 79, 80]. However, three exceptions were
223 identified: *Cryptococcus* sp. 3 (clade C), *K. europaea* (clade E), and *K. fici* (clade H) (**Figs 1B** and
224 **1E**). In *Cryptococcus* sp. 3 and *K. fici*, BLAST searches revealed that the key *MAT* genes (*HD* and
225 the pheromone receptor *STE3*) are close together on the same chromosome, a hallmark of bipolar
226 configurations (**Fig 1E**). In contrast, *K. europaea* exhibited a pseudobipolar configuration, with the
227 *HD* and *STE3* genes residing on the same chromosome but separated by ~8.95 Mb (**Fig 1E**).

These findings prompted us to precisely determine the composition of each *MAT* loci across species and retrace the chromosomal structural changes underlying the configurations found in all bipolar species.

Delineation of the *MAT* loci structure in *Cryptococcus* and *Kwoniella* species

The structure and gene composition of the *MAT* locus has been previously determined for several *Cryptococcus* species within clades A, B, and C [16, 23, 34, 35, 46, 77, 81]. In contrast, analyses of *Kwoniella* species have been limited to *K. mangrovensis* and *K. heveanensis*, relying on fosmid library data in the absence of genome sequencing and focusing on a single mating type, leaving the precise structure and full gene composition of the *MAT* loci unresolved [65, 66]. To overcome these limitations and expand the analysis to newly included species, we conducted synteny analyses complemented by gene genealogies to comprehensively characterize *MAT* loci across *Cryptococcus* and *Kwoniella*.

Our analyses revealed that the *HD* locus in tetrapolar *Cryptococcus* and *Kwoniella* species is a small chromosomal region containing only the *HD1*/*SXI1* and *HD2*/*SXI2* genes, which are divergently transcribed from a shared promoter region (**S3 Fig**). Similar to other basidiomycetes, differences between mating types are primarily confined to the N-terminal regions of the encoded proteins. This conserved two-gene structure suggests strong selection to maintain the *SXI1*-*SXI2* pair as a single inheritable unit. Notably, this conservation persists in *Kwoniella* species, even though the *HD* locus is predominantly located in subtelomeric regions, which are inherently more prone to variability and rearrangements (**S3J–S3O Fig**).

In contrast to the *HD* locus, the *P/R* locus is notably more variable, spans a larger chromosomal region, and exhibits substantial variation across species and between mating types within the same species (**Figs 2** and **S4**). The *P/R* locus is significantly more expanded in tetrapolar *Cryptococcus* species, averaging 91.7 kb compared to 29.3 kb in *Kwoniella* species

(**Figs 2, S4 Fig**, and **S2 Appendix**; $P < 0.0001$, Mann–Whitney U Test). Within *Cryptococcus*, the *P/R* locus ranges from 84.5 kb estimated in *Cryptococcus* sp. 5 (*P/R a2*) to 101.5 kb in *C. floricola* (*P/R a1*), while in *Kwoniella*, it spans from 24.3 kb in *K. newhampshirensis* (*P/R a1*) to 34.4 kb in *K. heveanensis* (*P/R a2*) (**S2 Appendix**).

Evolution of the *P/R* locus in *Kwoniella*

Gene content analysis of the *Kwoniella P/R* locus revealed 9 genes shared across all species (**Figs 1C** and **2C-D,** and **S3 Appendix**). These include a single pheromone gene (*MF*α or *MF***a**) containing the canonical CAAX motif at its C-terminus, a pheromone receptor (*STE3*α or *STE3***a**), and two additional genes (*STE20* and *STE12*) with established roles in mating-related functions and known to reside within *MAT* in *Cryptococcus* pathogens [34, 82, 83]. Other genes identified include *DBP10* and *REI1*, both implicated in ribosome biogenesis in *Saccharomyces cerevisiae* [84, 85]; *ATG7*, an autophagy-related gene [86]; a C2 domain-containing protein (*C2prot*), whose ortholog in *S. pombe* (Ync13) coordinates exocytosis, endocytosis, and cell-wall integrity during cytokinesis [87]; and *NDC80*, encoding a component of the NDC80 kinetochore complex, essential for chromosome segregation and spindle checkpoint activity [88]. In *K. heveanensis* and other species of clade G, the *P/R* locus has expanded to include two additional genes: *BPS3*, encoding a protein of unknown function with BTP/PZ and CAP-Gly domains (also present in the *MAT* locus of *Cryptococcus* pathogens), and *UPB6,* a predicted deubiquitinating enzyme associated with the 26S proteasome [89]. Of these genes, three (*DBP10*, *REI1* and *NDC80*) are predicted to be essential in *S. cerevisiae* and in *C. neoformans*, based on evidence from previous studies [84, 85, 88, 90, 91].

Within a species, the *P/R a2* allele is generally larger than the *a1* allele (**Figs 2C-D**, **S4 Fig** and **S2 Appendix**), primarily due to the presence of an additional gene of unknown function. This size difference is also accompanied by truncation of the *STE12* gene within the *a2* allele, with

fragments of *STE12* flanking the *STE3* gene on both sides in several species, such as in *K. mangrovensis* and *K. pini* (**Figs 2C** and **S4**). Similarly, fragments of the 3' end of *NDC80* and *BSP3* were detected at the right edge of the *P/R a2* allele in certain clade E/F and clade G species, respectively, in addition to apparently intact copies of these genes (**Figs 2C** and **S4**). These fragments seem to be remnants of structural rearrangements, potentially associated with duplication tracks at the inversion breakpoints. To elucidate the origin of these truncations and structural differences, we reconstructed the likely rearrangements leading to the extant *P/R* configuration in *Kwoniella*. In clades E/F, three distinct inversions are predicted to have shaped the locus: the first inversion involved *STE3* and *STE12*, truncating *STE12* in the *a2* allele; the second relocated *DBP10* and *REI1* from the edge of the locus to its center; and the third moved *NDC80* from the edge to the middle of the locus (**S5A Fig**). The first two inversions appear to be conserved in clade G, suggesting they are likely ancestral in *Kwoniella*. In contrast, two additional rearrangements relocating *BSP3-NDC80* and *UBP6* to the center of the locus (**S5B Fig**) seem to be clade G-specific events. Collectively, this data suggests that the *P/R a2* allele has experienced more modifications compared to the *P/R a1* allele.

The *P/R* locus in *K. ovata* and *K. endophytica* present further complexity in that it contains *STE3***a** and *MF***a** alleles alongside truncated versions of *STE3*α, with *K. ovata* additionally harboring a truncated *MF*α gene (**Fig 3A**). This unique configuration suggests that the extant *P/R* locus in these species may have arisen through intra-*P/R* recombination, resulting in a mosaic assembly of loci elements derived from both mating types. Supporting this hypothesis, synteny analysis showed that the left side of the *P/R* locus resembles that of other *P/R a2* strains, whereas the right side aligns more closely with the *P/R a1* allele (**Fig 3A**). Gene genealogies further revealed trans-specific polymorphism in several genes. While deep trans-specific polymorphism has been well-documented for *STE3*, *STE12*, and *MF* genes, which were ancestrally integrated into the *P/R* locus across *Cryptococcus* and *Kwoniella* [23, 46, 66, 77], a shallower trans-specific

303 polymorphism pattern was observed for *NDC80*, *BSP3*, *ATG7*, and *UBP6*. Specifically, *NDC80*
304 exhibited trans-specific polymorphism within clades E/F and within clade G (**Fig 3B**), while *ATG7*,
305 *BSP3*, and *UBP6* displayed this pattern exclusively within clade G (**S5C Fig**). Notably, the *NDC80*
306 allele at the right edge of the *P/R* locus in *K. ovata* and *K. endophytica* clustered more closely with
307 *a1* alleles from other species (**Fig 3B**), and a similar pattern was found in *K. dendrophila*, together
308 pointing to a shared evolutionary history where the extant *P/R* locus resulted from past intra-*P/R*
309 recombination. While this event may have initially produced strains with intercompatible
310 pheromone and receptor pairs, potentially enabling self-filamentous growth, subsequent
311 evolutionary pressures appear to have led to the differential loss of *STE3*α and *MF*α mating-type
312 alleles, ultimately eliminating self-filamentation. Consistent with this, solo cultivation of these
313 species in V8 pH 5 media did not induce hyphal growth, indicating that extant species are no
314 longer self-fertile and instead retain relics of the ancestral recombination event. Collectively, these
315 findings unveil a complex evolutionary history of the *P/R* locus in *Kwoniella*, marked by dynamic
316 structural rearrangements, recombination, and lineage-specific expansions.

317

318 **Evolution and expansion of the *P/R* locus in *Cryptococcus***

319 Compared to *Kwoniella*, the *P/R* locus in tetrapolar *Cryptococcus* species has undergone
320 significant expansion, averaging 95.4 kb in clade B and 86.9 kb in clade D, a difference that is not
321 statistically significant (P = 0.063, Mann–Whitney U Test; **Figs 2** and **S4**; **S2 Appendix**). It also
322 exhibits substantially more complex rearrangements between mating types, precluding the precise
323 reconstruction of the evolutionary events leading to the extant configurations (**Figs 2**, **S4B**, and
324 **S4E**). Another striking feature is the consistently lower GC content of the *P/R* locus relative to
325 genome-wide averages (**Figs 2**, **S4A** and **S4D**; **S4 Appendix**). For instance, in *C. amylolentus*
326 CBS6039 (clade B), the GC content of the *P/R* locus is 51.24%, compared to a genome-wide GC
327 content of 53.36% (*t*-test: t = 65.03, P < 0.0001; permutation test: P = 0.0288), with Z-scores

ranging from –1.38 to –4.00 across clade B strains. This trend is even more pronounced in clade D species, where the GC content difference can approach 10%. For example, in *Cryptococcus* sp. DSM108351, the *P/R* locus GC content is 49.17%, compared to a genome-wide average of 59.24% (*t*-test: $t = 199.19$, $P < 0.0001$; permutation test: $P = 0.002$), with Z-scores ranging from –6.25 to –7.22 across clade D strains. Further analysis of codon composition revealed a significant enrichment of AT-rich codons in the *P/R* locus compared to genome-wide sequences (**S4 Appendix**). For instance, in *Cryptococcus* sp. DSM108351, the *P/R* locus contains 13,429 AT-rich codons versus 13,390 GC-rich codons, whereas genome-wide sequences contain 2,877,796 AT-rich codons versus 3,475,722 GC-rich codons ($\chi^2 = 245.86$, $P < 0.0001$). Similar patterns were observed in clade C species, such as *C. amylolentus* CBS6039 ($\chi^2 = 21.22$, $P < 0.0001$; **S4 Appendix**). These findings suggest that the lower GC content of the *P/R* locus is likely driven by a bias towards AT-rich codons, potentially resulting from mutation accumulation associated with reduced or suppressed recombination in this region.

Despite these shared features, the *P/R* locus gene content shows striking variation between *Cryptococcus* species of clades B and D (**Fig 1C**). While both clades share a core set of 7 genes (*MF* with two to four copies, *STE3*, *STE12*, *BSP3*, *MYO2*, *STE20*, and *RPL39*; **S3 Appendix**), several genes within the *P/R* locus in clade B species are instead located in the right flanking region of the locus in clade D species (**Figs 2A-B**, **S4B**, and **S4E**) and similarly positioned outside, but proximal to, the *P/R* locus in *Kwoniella* species (**Figs 2C-D**, **S4H**, **S4K**, and **S4N**). Additionally, two genes (*ATG7* and *C2prot*) located within the *Kwoniella P/R* locus are also found within the *P/R* locus in two of the four clade D species, but absent from the locus of other tetrapolar *Cryptococcus* clades. Interestingly, although only 7 genes are shared between clades B and D, the *P/R* loci are similar in overall size. This similarity arises because clade D species harbor up to 15 genes (**Figs 1C**, **2B**, and **S4E**) that are absent from the *P/R* locus in other *Cryptococcus* or *Kwoniella* clades. Extending these comparisons across all tetrapolar *Cryptococcus* and *Kwoniella*

clades revealed that only four genes (*MF*, *STE3*, *STE20*, and *STE12*) are conserved within the *P/R* loci, likely reflecting the ancestral configuration (**Figs 1C-D**, **S3 Appendix**). These findings suggest that the ancestral *P/R* locus consisted of a very small set of genes and independently expanded in the different lineages by incorporating distinct gene sets through lineage-specific rearrangements.

A final notable distinction of the *P/R* locus in *Cryptococcus* is the presence of multiple pheromone genes per mating type [34, 46]. Unlike *Kwoniella*, which retains a single pheromone gene per mating type, all heterothallic *Cryptococcus* species, including the newly identified clade D species, exhibit multiple copies, with up to four pheromone genes per mating type. These pheromone genes are often arranged in divergent orientations (**Figs 2A-B**, **S4B**, and **S4E**), a configuration that may act as inverted repeats. Such an arrangement facilitates the formation of inversion loops during recombination, driving both historical and potentially ongoing structural rearrangements, and the expansion of the *P/R* locus in *Cryptococcus*.

MAT **locus evolution and structural dynamics in** ***Cryptococcus*** **pathogens**

The fusion of the *P/R* and *HD* loci into a single nonrecombining *MAT* locus represents a pivotal transition in the evolution of breeding systems in basidiomycetes, marking a shift from tetrapolar to bipolar systems. This transition has been extensively documented in the *Cryptococcus* lineage that includes all of the human pathogens (clade A). Building on previous work, we expanded the analysis of the *MAT* locus to all recognized species in this clade (**Figs 1**, **4**, **S6**, and **S7**; **S1 Appendix**). For *C. deneoformans*, the analysis included the well-studied congenic strains JEC21α and JEC20**a**, their progenitor strains NIH12α and NIH433**a**, and another *MAT***a** strain (NIH430) (**S6 Fig** and **S1 Appendix**). For *C. neoformans*, we examined multiple strains of both mating types across its four main lineages (VNI, VNII, VNBI, and VNBII), including eight newly sequenced genomes assembled telomere-to-telomere (T2T) (**Fig 4A** and **S1**

Appendix). This comprehensive dataset offered unprecedented resolution for investigating intra-specific structural dynamics of the *MAT* locus.

Synteny analysis across species revealed that the *MAT***a** allele is generally longer than the *MAT*α allele in both *C. neoformans* ($P < 0.0001$, Mann–Whitney U Test) and the *C. gattii* species complex ($P = 0.04142$, Mann–Whitney U Test; **Fig 4C and S2 Appendix**). A similar trend is observed in *C. deneoformans*, although limited sampling precluded statistical support. The *MAT***a** allele also exhibits greater structural diversity than the *MAT*α allele among closely related species, such as in the *C. gattii* complex (**S7 Fig**). This pattern extends to *C. neoformans*, where pronounced rearrangements, predominantly inversions, have occurred even among *MAT***a** strains of the same VN lineage (e.g., VNI strains Bt130 vs. IUM96-2828, or strain Bt206 compared to other VNBII strains; **Fig 4D**). Notably, the breakpoints for these rearrangements are often associated with the pheromone genes, supporting the hypothesis that multiple pheromone genes may act as mediators of structural changes. In contrast, the *MAT*α allele structure in both *C. neoformans* and the *C. gattii* species complex is more conserved, with only limited inversions involving the two pairs of pheromone genes flanking the *PRT1* and *ZNF1* genes (**Figs 4D** and **S7**). Interestingly, a comparison of the entire *MAT* chromosome across *C. neoformans* strains showed that most of the intraspecific structural variation is confined to the *MAT* locus, with no major structural changes detected outside this region, apart from differences within the centromeric regions, which are inherently more dynamic (**Fig 4B**).

Beyond these structural changes, species-specific gene losses (e.g., *NCP1* and *NCP2*, which are absent in most species) and TEs further drove structural differences in *MAT* allele configurations (**S7 Fig**). TE activity, in particular, may play a significant role as the *C. deneoformans MAT* locus has been estimated to contain over five times more TEs than the rest of the genome, excluding centromeric regions [16, 34, 92]. In addition to previously identified TE remnants, our analysis revealed novel transposon insertions. Specifically, two distinct KDZ

403 transposons, recently characterized in *C. neoformans* as large mobile elements (~11 kb) with
404 terminal inverted repeats (TIRs) and target site duplications (TSDs) [93], were detected within the
405 *MAT* locus in 5 of the 23 analyzed strains. *KDZx* was identified in strains 125.91, C45, PMHc1023,
406 and T4, while *KDZ1* was found in strain Ze90-1 (**Fig 4D)**. Together, these findings underscore the
407 dynamic evolution of the *MAT* locus in *Cryptococcus* pathogens, shaped by an interplay of
408 structural rearrangements, gene loss, and TE activity.

409 In *C. neoformans*, the VNI lineage is hypothesized to have initially diverged with only the
410 *MAT*α allele, potentially due to a bottleneck associated with a small founding population, and later
411 acquired the *MAT***a** allele via introgression through mating between a VNBI *MAT***a** strain and VNI
412 *MAT*α strain [94]. To date, only five VNI *MAT***a** strains have been identified [94-99], two of which
413 were fully sequenced (T2T) and annotated in this study (125.91 and Bt130), while a third strain
414 (IUM96-2828) was sequenced in a previous study [100] and annotated as part of this work.
415 However, synteny analysis revealed significant structural divergence between the *MAT***a** alleles of
416 VNBI strains (Ftc555-1 and Bt63) and these VNI strains (**Fig 4D**), raising questions about the
417 proposed introgression hypothesis.

418 To address this, we reanalyzed SNP data leveraging newly generated reference genome
419 sequences. Genome-wide SNP analysis, using the VNBI *MAT***a** strain Ftc555-1 as the reference,
420 revealed markedly lower SNP densities within the *MAT***a** locus in VNI strains compared to VNBII
421 strains (**Figs 5A** and **5B**). In contrast, across the rest of the genome, VNI *MAT***a** strains exhibited
422 higher divergence from the VNBI reference, while VNBII strains showed lower SNP densities (**Fig**
423 **5A** and **5B**). Interestingly, the typically high SNP densities between VNI and VNBI strains are
424 restored immediately downstream of the *MAT* locus and approximately five genes upstream,
425 consistent with previous reports of recombination hotspots flanking the *MAT* locus [101].
426 Phylogenetic analyses further supported these observations; VNBI strains clustered more closely
427 with VNBII strains in trees inferred from genome-wide and chr. 5-specific SNPs (**Figs 5C** and **5D**),

428 whereas those constructed with *MAT*-specific SNPs showed closer clustering of VNI and VNBI
429 *MAT***a** strains, supporting a more recent shared ancestry for the *MAT***a** allele (**Fig 5E**). Together,
430 these findings support and refine the introgression model illustrated in **Fig 5F**, suggesting that
431 lineage-specific rearrangements occurred following the introgression event to account for the
432 observed structural differences.

433

434 **Evidence for independent fusion of *P/R* and *HD* loci in *Cryptococcus* sp. 3**

435 Our initial screening of *MAT* gene content revealed that the *P/R* and *HD* loci-associated
436 genes are adjacent on the same chromosome in *Cryptococcus* sp. 3, indicating a fusion of these
437 two regions (**Fig 1E**). While only a single strain (CMW60451) is currently available for this species,
438 limiting precise determination of the *MAT* locus length, the minimum estimated size is ~90 kb
439 based on the distance between the two most distal pheromone genes (**Fig 6C**). If the boundaries
440 are extended to include additional genes known to be part of the *MAT* locus in pathogenic
441 *Cryptococcus* species, the *MAT* locus could span ~120 kb, from *RPL22* on the left to *STE12* on
442 the right (**Fig 6C**). Under this broader definition, a total of 15 genes are shared between
443 *Cryptococcus* sp. 3 and clade A species (**Figs 1C** and **6C,** and **S3 Appendix**), including for
444 example four *MF*α pheromone gene copies and three genes typically associated with the *HD* locus
445 (*RPL22*, *CAP1*, and *SPO14*). Additionally, two distinct regions within the *MAT* locus contain TEs:
446 one harboring a complete KDZ-like transposon and another with only remnants of this element
447 (**Fig 6C**).

448 Further analysis uncovered an unusual combination of *MAT* alleles. Unlike the bipolar
449 *Cryptococcus* pathogens, where *MAT*α strains carry *HD1* (*SXI1*) linked to *STE3*α and *MAT***a**
450 strains have *HD2* (*SXI2*) linked to *STE3***a**, *Cryptococcus* sp. 3 retains only *SXI2*, which is instead
451 linked to *STE3*α. Although this distinct configuration suggested an independent fusion event at the
452 origin of the *MAT* locus in *Cryptococcus* sp. 3, the uncertain phylogenetic placement of clade C

relative to clade A left open the possibility that the two clades share a more recent common ancestor. In this scenario, the fusion of the *P/R* and *HD* loci could have occurred in their shared ancestor rather than independently in both lineages.

To test this hypothesis, we analyzed the phylogenetic clustering patterns of genes within the predicted *MAT* region shared with pathogenic *Cryptococcus* species. Genes ancestrally recruited to the *P/R* locus, such as *STE3, STE12,* and *MYO2*, were excluded from the analysis because they exhibit deeper trans-specific polymorphism predating the divergence of *Cryptococcus* species [23, 46, 66, 77], making them less informative. Instead, we focused on the genealogies of four genes (*BSP3, PRT1, SPO14,* and *CAP1*), which are part of the *MAT* locus in pathogenic species and display mating-type-specific clustering within them [34]. The analysis revealed that *Cryptococcus* sp. 3 sequences do not group with either the **a** or α allele-specific clusters from pathogenic species; rather, their sequences are distinct and positioned outside these clusters, which is more consistent with an independent fusion event than one inherited from a common ancestor (**S8 Fig**).

Next, to investigate the chromosomal rearrangements underlying the linkage of *P/R* and *HD* loci in *Cryptococcus* sp. 3, synteny blocks were reconstructed with SynChro [102], employing *K*. *shandongensis* as reference. The choice of *K. shandongensis* was guided by several factors: (i) it is tetrapolar, with the *P/R* and *HD* loci located on separate chromosomes, representing the ancestral configuration (**Figs 1**, **S3N**, and **S4N**); (ii) *Kwoniella* species exhibit significantly fewer interchromosomal rearrangements compared to *Cryptococcus* species, likely due to their simpler and smaller centromeres, as well as a lower TE density, given that a higher TE density is a known factor promoting genomic rearrangements in *Cryptococcus* [48, 103-105]; and (iii) it retains a 14-chromosome karyotype, which is the predicted ancestral state for both genera [48] (**Figs 6** and **S9A**).

477 The nuclear genome of *Cryptococcus* sp. 3, fully assembled into 13 complete
478 chromosomes (**S1 Appendix**), represents a reduction from the ancestral 14-chromosome
479 karyotype. This reduction raised the possibility that the observed linkage of the *P/R* and *HD* loci
480 could be a result of this karyotype transition. Specifically, the fusion of chromosomes containing
481 the *P/R* and *HD* loci during chromosomal rearrangement could account for their linked
482 configuration in this species. To test this hypothesis, we examined which centromere was lost
483 during the karyotype transition. Synteny analysis and centromere mapping traced the missing
484 centromere to a region on chr. 3, corresponding to the *CEN4* region in *C. neoformans* H99 (**S9B**
485 **Fig**). This region no longer contains the LTR-rich sequences typically associated with
486 *Cryptococcus* centromeres (**S9B Fig**) [35, 48, 106, 107]. Additional comparisons with *K.*
487 *shandongensis* suggest that this centromere loss possibly resulted from an intercentromeric
488 recombination event leading to the inactivation and eventual loss of one of the resulting
489 centromeres (**S9B Fig**). Because the *MAT* locus in *Cryptococcus* sp. 3 resides on chr. 11 (**Fig 6A**),
490 which is unrelated to the chromosome affected by the centromere loss, this indicates that the
491 physical linkage of the *P/R* and *HD* loci (i.e., colocation on the same chromosome) in
492 *Cryptococcus* sp. 3 arose through a separate mechanism, independent of the reduction from 14
493 to 13 chromosomes.

494 To further investigate the mechanism underlying the tetrapolar-to-bipolar transition in
495 *Cryptococcus* sp. 3, we compared its *MAT*-containing chromosome to chromosomes harboring
496 the *P/R* and *HD* loci in *K. shandongensis*. Synteny analysis revealed significant alignment between
497 chr. 11 of *Cryptococcus* sp. 3 and large regions of chrs. 12 and 5 in *K. shandongensis* carrying
498 the *P/R* and *HD* loci, respectively (**Fig 7A**). Additionally, a small region derived from the *P/R*
499 chromosome is embedded towards the left end of chr. 11 within a region otherwise predicted to
500 have originated from the *HD* chromosome. This suggests that the physical linkage of the *P/R* and
501 *HD* loci in *Cryptococcus* sp. 3 likely began with a chromosomal translocation that brought the two

502 loci onto the same chromosome, initially separated by a considerable distance. A subsequent
503 inversion then relocated the *HD* locus closer to the *P/R* locus, resulting in their fusion (events 1-3
504 in **Fig 7A**). Notably, the centromere flanking regions of chr. 11 in *Cryptococcus* sp. 3 show
505 considerable synteny to the *CEN12*-flanking region in the *K. shandongensis P/R* chromosome.
506 This suggests that the two centromeres share a similar evolutionary origin and that the
507 translocation event did not directly involve centromeric recombination but rather occurred
508 elsewhere in the chromosomes (**Fig 7A**).

509

510 **Revisiting the model of *P/R* and *HD* loci linkage in *Cryptococcus* pathogens**

511 Based on these findings, which reflect a broader genomic analysis and species sampling
512 than any previous study, we revisited a previously proposed model for the physical linkage and
513 subsequent fusion of *P/R* and *HD* loci in *Cryptococcus* pathogens. This model, originally
514 formulated from comparisons between the tetrapolar species *C. amylolentus* (clade B) and the
515 bipolar pathogen *C. neoformans* (clade A), posited that the physical linkage was initiated by a
516 chromosomal translocation mediated by ectopic recombination between repetitive elements within
517 centromeric regions of *P/R*- and *HD*-containing chromosomes. This translocation would place the
518 two *MAT* loci on the same chromosome, initially separated by the centromere. Subsequent
519 rearrangements, such as inversions or transpositions, were hypothesized to bring the two loci into
520 closer proximity, ultimately leading to their fusion and establishing the contiguous *MAT* locus
521 characteristic of *Cryptococcus* pathogens [35, 36].

522 To reassess this hypothesis, we expanded our analysis to include multiple *Cryptococcus*
523 species spanning the four clades, focusing on synteny and chromosomal architecture. In *C.*
524 *neoformans*, the *MAT* locus resides on chr. 5. Our analysis revealed that its centromere (*CEN5*)
525 shares conserved synteny on both flanking regions with *CEN12* of *K. shandongensis*, as well as
526 with *CEN11* of *Cryptococcus* sp. 3 (clade C) and *CEN9* of *Cryptococcus* sp. DSM108351 (clade

527 D) (**Fig 6B**). These conserved synteny patterns indicate a shared evolutionary origin for these
528 centromeres, strongly suggesting that they were not directly involved in the translocation that
529 linked the *P/R* and *HD* loci in *C. neoformans*. In contrast, analysis of the *P/R* chromosome (chr.
530 10) in *C. amylolentus* (clade B) reveals that its centromere-flanking regions align with two distinct
531 chromosomes, indicative of intercentromeric recombination and pointing to a derived rather than
532 ancestral centromere structure. Comparison between *C. neoformans* and *K. shandongensis*
533 further suggests a model in which the physical linkage and subsequent fusion of the *P/R* and *HD*
534 loci in *C. neoformans* arose through a mechanism similar to that proposed for *Cryptococcus* sp.
535 3, involving a chromosomal translocation outside the centromeric region, followed by an inversion
536 event that brought the two loci into closer proximity (**Fig 7B**). In *C. neoformans*, however, the
537 inversion seems to have relocated the *P/R* locus closer to the *HD* locus near the left chromosomal
538 end (events 3 in **Fig 7B**). These broader analyses refine the earlier model and suggest that the
539 physical linkage of *P/R* and *HD* loci in *Cryptococcus* species likely arose through alternative
540 mechanisms, independent of centromeric recombination.

541

542 **Tetrapolar to bipolar and pseudobipolar transitions in *Kwoniella***

543 We recently reported that karyotype reduction within *Kwoniella* occurred both
544 progressively and independently over time, often accompanied by the formation of "giant"
545 chromosomes (up to 18 Mb) through repeated chromosome fusion events [48]. While such events
546 could potentially lead to physical linkage of *P/R* and *HD* loci, only two *Kwoniella* species harbor
547 both loci on the same chromosome (**Fig 1B**).

548 The first instance is in *K. europaea* (clade E), where the *HD* and *P/R* loci are located on
549 the same chromosome but remain far apart (~8.95 Mb) (**Figs 1E** and **S4G**), representing a
550 pseudobipolar configuration. This transition appears to have resulted from a previously
551 documented chromosomal translocation specific to *K. europaea* [48]. In this event, the progenitor

552 “giant” chr. 1 of clade E species underwent a translocation with chr. 2, giving rise to the extant
553 pseudobipolar arrangement. Given their physical distance, the two loci are still expected to
554 recombine.

555 The second instance is in *K. fici*, where the *HD* locus is located less than 13 kb away from
556 the *P/R* locus gene cluster, both localized near the terminal region of chr. 1 (**Fig 8D**). This
557 chromosome in *K. fici* is also a “giant” chromosome, likely formed independently through similar
558 chromosomal fusion events as those documented for *Kwoniella* clades E and G species [48].
559 Interestingly, following the divergence from *Kwoniella* clade I (*K. shandongensis/K.*
560 *newhampshirensis*), an ancestral fusion of *K. shandongensis* chrs. 11 and 12 resulted in the
561 emergence of the *P/R* chromosome, observed as a single chromosome in most *Kwoniella* species
562 (exemplified by chr. 2 in *Kwoniella* sp. DSM27419; **Fig 8A**) [48]. In *K. fici*, however, this ancestral
563 fusion product has fragmented further; a portion now resides at the terminal region of *K. fici* chr.
564 1, while another segment is located on chr. 4 (**Fig 8C**). Similarly, the majority of the *K.*
565 *shandongensis HD* (chr. 5), aligns with the opposite end of *K. fici* chr. 1, although a small portion
566 is present on chr. 4, adjacent to the *P/R*-derived region. This pattern suggests that translocations
567 likely contributed to the genesis of the *P/R* and *HD* loci fusion in *K. fici*, with blocks derived from
568 the ancestral *P/R* and *HD* chromosomes brought together during these events. Notably, only a
569 few key *HD*-associated genes (*SXI1*, *SXI2*, and *RPL22*) are positioned near the *P/R* locus gene
570 cluster at the right end of chr. 1 (**Fig 8D)**. This apparently bipolar arrangement retains both *HD*
571 genes (*SXI1* and *SXI2*), contrasting with the presence of only one *HD* gene in opposite mating
572 types of bipolar *Cryptococcus* species. Together, these observations suggest intricate
573 rearrangements leading to this unique derived configuration in *K. fici*.

574

575

576

577 **Analysis of sexual reproduction in *Kwoniella mangrovensis***

578 *Kwoniella mangrovensis* (clade G) is known for its ability to reproduce sexually in the lab
579 [51, 65] and characterized by having only three chromosomes. To better understand its
580 reproductive biology, we analyzed the outcomes of sexual reproduction, focusing on
581 recombination, ploidy variation, and potential self-fertility in the progeny.

582 The two originally *K. mangrovensis* sequenced strains, CBS8507 (*P/R a1*, *HD b1*) and
583 CBS8886 (*P/R a2*, *HD b1*), were found to share identical *HD b1* alleles, making them incompatible
584 for mating (**Fig 9A**). However, Guerreiro et al. [65] previously characterized a third strain,
585 CBS10435, as haploid and compatible with CBS8507. We Illumina-sequenced CBS10435 and
586 confirmed that this strain carries the *P/R a2* allele and a distinct *HD b2* allele, rendering it
587 compatible with CBS8507 (**Fig 9A**). Next, we performed crosses between CBS8507 and
588 CBS10435, which were able to mate on both CMA and V8 pH 5 media. These crosses produced
589 basidia with characteristic globose and lageniform morphologies, consistent with prior descriptions
590 of the species (**Fig 9B**) [51]. Progeny were recovered by dissecting eight clusters of cells
591 germinating from basidia along the hyphae (see **S5 Appendix** for details). A total of 51 F1 progeny
592 were obtained from mating patches from both media types. To ensure that we avoided analyzing
593 duplicated genotypes potentially resulting from asexual clonal propagation, we screened the
594 progeny for evidence of recombination applying a PCR-RFLP approach with two markers per
595 chromosome, positioned on either side of the centromere (**S5 Appendix**). Putative recombination
596 events (allele exchange) as well as heterozygosity at several loci were detected, and nine F1
597 progeny with distinct genotypes were selected for whole-genome sequencing. SNPs identified
598 between the parental strains served as markers to infer inheritance patterns associated with
599 recombination and loss of heterozygosity (LOH). Additionally, we combined read coverage
600 analysis with FACS to assess ploidy in these isolates.

601 Our results showed that the majority of F1 progeny were non-haploid (**Figs 9C-D**, **S10,**
602 **and S11**). Genotyping and FACS results were largely concordant; however, some discrepancies
603 (e.g., MP11 and MP18) suggested variability within cell populations, possibly stemming from
604 intrinsic genomic instability. Indeed, mapping of reads to a combined reference genome (CBS8507
605 and CBS10435) revealed uneven coverage for certain chromosomes. For example, chr. 2 of
606 CBS10435 exhibited low coverage in progeny MP59 (**Fig 9D**). Although CBS8507 and CBS10435
607 were previously reported as haploid [65], our FACS analysis revealed that CBS10435
608 predominantly consists of diploid cells, with a subpopulation of haploids (**Figs 9C** and **S11**). This
609 difference in ploidy between the parental strains likely explains the observed aneuploidy in the
610 progeny.

611 Analysis of the *MAT* gene content across the F1 progeny showed that some carried
612 compatible *P/R* and *HD* alleles (*a1a2b1b2*), potentially enabling self-fertility (**Fig 9C**). To
613 investigate this, we tested the F1 progeny under mating conditions and compared their
614 phenotypes to a cross of the parental strains (**Figs 9E-F**, and **S12**). On CMA and V8 pH 5 media,
615 progeny with fully compatible *MAT* alleles (MP16, MP18, MP54) produced hyphae and basidia
616 similar to the parental cross. Some hyphal growth was also observed in solo culture of strains
617 MP49, MP51 and MP59, exclusively on V8 pH 5, but the filaments were shorter and had no
618 structures resembling basidia (**S12 Fig**). These isolates showed a phenotype similar to previously
619 reported self-filamentous *K. mangrovensis* strains, which are characterized by highly branched,
620 irregular hyphae that lack visible clamp cells [65]. Taken together, our analyses reveal that sexual
621 reproduction in *K. mangrovensis* involves recombination and can generate non-haploid progeny
622 with the potential for self-fertility.

Discussion

Our broad comparative genomic analysis of *Cryptococcus* and *Kwoniella MAT* loci provided new insights into their evolution and structural dynamics. These include distinct expansions of the *P/R* locus and additional independent events of *HD-P/R* fusion, which are summarized in **Fig 10** as a visual roadmap of key transitions.

The archetypal basidiomycete *MAT* loci are thought to consist of a compact *P/R* region, with a single pheromone receptor and one pheromone gene, and an unlinked *HD* region containing two divergently transcribed homeodomain transcription factors (*HD1* and *HD2*). While this configuration is retained in some lineages, such as in *Malassezia* species [20], various studies have shown that in most other basidiomycetes, the *HD* locus remains largely conserved (except in some mushroom-forming species that have undergone segmental duplications of the *HD* genes; reviewed in [9, 28]), whereas the *P/R* locus has experienced lineage-specific expansions and structural rearrangements. For example, in *Ustilago maydis*, the size difference between the two allelic forms of the *P/R* locus (4.5 kb in the *a1* allele vs. 8 kb in the *a2* allele) results from the presence of two additional genes (*lga2* and *rga2*) in the *a2* allele that direct mitochondrial uniparental DNA inheritance (mito-UPI) [108, 109]. Similarly, several *Microbotryomycete*s (*Pucciniomycotina*) yeasts harbor substantially expanded *P/R* loci incorporating additional genes [26, 110, 111]. Likewise, tetrapolar *Cryptococcus* species exhibit an expanded *P/R* locus [35, 46, 77], and in *C. amylolentus* this region has also been implicated in mito-UPI [112].

Our analysis of the *P/R* locus across tetrapolar *Cryptococcus* and *Kwoniella* revealed distinct evolutionary trajectories, presumably originating from a shared ancestral locus containing four genes (*MF, STE3, STE12,* and *STE20*) (**Fig 10**, event 1). In *Kwoniella*, the *P/R* locus remains relatively compact across species, averaging 29.3 kb with a core set of nine conserved genes (**Fig 10**, event 2). Its evolution has involved (i) mating-type-specific changes, including the insertion of an additional gene of unknown function and *STE12* truncation in *a2* alleles; (ii) lineage-specific

648 small-scale expansions through the incorporation of two additional genes (*BSP3* and *UBP6*) via
649 inversions; and (iii) intra-*P/R* locus recombination in *K. ovata*, *K. endophytica*, and *K. dendrophila*,
650 generating a mosaic structure. The latter suggests that recombination suppression may not be
651 fully established, though whether such recombination events are rare or recurrent remains unclear.

652 In contrast, the *P/R* locus in tetrapolar *Cryptococcus* species has undergone significant
653 expansion, averaging 95.4 kb in clade B and 86.9 kb in clade D. Despite their similar sizes,
654 substantial differences in gene content suggest that these expansions occurred independently
655 after the divergence of the two lineages from their most recent common ancestor (**Fig 10**, event
656 5). A defining feature of *Cryptococcus P/R* loci is the duplication of pheromone (*MF*) genes, with
657 each mating type harboring multiple copies, typically arranged in divergent orientations. This
658 pattern is also observed in expanded *P/R* loci of *Microbotryomycetes* yeasts [26, 110, 111]. These
659 duplications may have evolved to strengthen mating partner recognition, suggesting that high
660 pheromone production is an important feature of the mating process in these species. However,
661 they also introduce structural instability by facilitating the formation of inverted repeats, which can
662 promote inversion loops and allele-specific structural rearrangements, potentially extending
663 regions of recombination suppression between *P/R* alleles.

664 One way to mitigate structural instability would be to cluster pheromone gene pairs closer
665 together, thereby restricting the extent of rearrangements. Consistent with this, we observed
666 reduced structural variation in *MAT*α strains of *C. neoformans* and *C. gattii* species complex,
667 where the two pheromone gene pairs are positioned in close proximity, separated by only two
668 genes (*ZNF1* and *PRT1*). In contrast, *MAT***a** strains exhibit a more dispersed arrangement of
669 pheromone genes, which appears to contribute to greater structural diversity across strains,
670 including the VNI-specific rearrangements that arose following introgression of the *MAT***a** allele
671 from the VNBI lineage. Once established, the presence of multiple pheromone gene copies may
672 be maintained as they could serve as templates for intrallelic repair via gene conversion, a non-

673 reciprocal exchange that can occur during meiosis, primarily between homologous sequences but
674 also at lower frequencies between sister chromatids, or within duplicated regions on a single
675 chromosome during mitosis [113, 114]. If confirmed, this mechanism would resemble the role of
676 palindromic sequences in preserving gene integrity on the human Y chromosome [115-117].

677 A striking feature of the *P/R* locus in tetrapolar *Cryptococcus* species is its consistently
678 lower GC content compared to genome-wide averages, particularly in clades B and D, where it is
679 significantly enriched for AT-rich codons. One likely explanation is that recombination suppression
680 in this region has allowed AT-biased mutational pressure to accumulate over time, a pattern
681 observed in other fungi, including another *Tremellomycetes* lineage, *Trichosporonales*, where *MAT*
682 loci also exhibit reduced GC content [18]. Additionally, this AT enrichment may reflect relaxed
683 selective pressure on codon usage, particularly if *P/R* locus genes are expressed primarily under
684 specific mating conditions rather than constitutively. Indeed, genes with stage-specific expression
685 may experience weaker selection for translational efficiency, leading to reduced codon
686 optimization, as observed in *Arabidopsis thaliana*, where genes with restricted tissue-specific
687 expression tend to show lower codon adaptation [118]. The *frq* gene in *Neurospora crassa*
688 provides another example of how codon usage bias can influence regulatory processes:
689 optimization of *frq* codons disrupts circadian clock function, highlighting how non-optimal codon
690 usage can be functionally relevant [119]. These findings raise the possibility that codon usage
691 within the *P/R* locus may be shaped not only by recombination suppression and mutational biases
692 but also by selective constraints related to mating-type regulation and expression timing.

693 Another contributing factor could be the reduction of GC-biased gene conversion (gBGC),
694 which in many species counteracts AT-biased mutations in recombining regions [120-122]. If
695 suppressed recombination also limits gBGC in the *P/R* locus, this could further contribute to
696 localized GC depletion. Interestingly, the absence of a similar GC drop in the *MAT* locus of
697 *Cryptococcus* pathogens, despite the presence of recombination suppression, suggests that the

genomic GC background plays a role. In high-GC genomes (e.g., clades B and D), reduced recombination may lead to a more pronounced AT accumulation, whereas in *Cryptococcus* pathogens, which have a lower genomic GC content overall [48], the absence of such a shift suggests their genomes may already be at equilibrium under mutation pressure. Further investigation of codon adaptation indices, genome-wide relative synonymous codon usage (gw-RSCU), recombination rates, and expression profiles across diverse *Cryptococcus* species will be essential for disentangling the relative contributions of mutation bias, selection, and recombination dynamics in shaping the evolution of *P/R* loci regions.

Beyond the previously documented tetrapolar-to-bipolar transition in pathogenic *Cryptococcus* lineages, we identified three additional transitions: two resulting in a bipolar configuration, in *Cryptococcus* sp. 3 and *K. fici* (events 6 and 4 in **Fig 10**, respectively), and one leading to a pseudobipolar arrangement in *K. europaea* (**Fig 10**, event 3). Our findings suggest that the transition to bipolarity in *Cryptococcus* sp. 3 likely occurred independently of that observed in *Cryptococcus* pathogens (Clade A). First, we confirmed that the fusion of *P/R* and *HD* loci is unrelated to reduction from 14 to 13 chromosomes in this species and instead was initiated by a chromosomal translocation followed by an inversion event. Second, the *MAT* locus in *Cryptococcus* sp. 3 retains only *SXI2* (*HD2*), which is linked to the *STE3*α allele, differing from *Cryptococcus* pathogens, where *SXI2* is associated with *STE3***a**. Third, phylogenetic analyses revealed that many genes within the *MAT* locus in *Cryptococcus* sp. 3 do not group with **a** or α allele-specific clusters from pathogenic species. If the *MAT* locus fusion predated the divergence of these clades, one would expect to see trans-specific polymorphism, with alleles of the same mating type clustering across species. However, this pattern is not observed, which is more consistent with an independent fusion event in *Cryptococcus* sp. 3 rather than inheritance from a common ancestor with Clade A. Nevertheless, the absence of additional strains for this species,

722 limits our ability to fully delineate the *MAT* locus boundaries and confirm whether the opposite *MAT*
723 allele carries the expected *SXI1/HD1–STE3***a** combination.

724 Furthermore, it remains uncertain whether the initial translocation event that brought the
725 *P/R* and *HD* loci onto the same chromosome occurred independently in *Cryptococcus* sp. 3 or in
726 a common ancestor of clades A and C. This uncertainty stems from two main factors. First, the
727 phylogenetic relationships among clades A (bipolar), B (tetrapolar), and C (which includes
728 *Cryptococcus* sp. 3) remain unresolved. Second, although our synteny analyses indicate that the
729 translocation breakpoints at the origin of the physical linkage of the *MAT* loci appear to differ
730 between *Cryptococcus* sp. 3 and pathogenic species (which would favor the possibility of
731 independent translocation events), these differences might also result from distinct chromosomal
732 rearrangements that accumulated along the two lineages. Further sampling and additional
733 comparisons will be required to conclusively resolve these relationships and gain deeper insight
734 into the broader implications of these transitions.

735 The composition of *MAT* loci can provide insight into both past and more recent transition
736 events. For example, *K. fici* also underwent a transition to fused *MAT* loci, yet both *SXI1* (*HD1*)
737 and *SXI2* (*HD2*) have been retained. This could suggest that the fusion event is relatively recent,
738 and that insufficient time has passed for one *HD* gene to decay in each mating type, as observed
739 in *Cryptococcus* pathogens, where remnants of *SXI2* persist next to *SXI1* in the *MAT*α alleles of
740 some species [123]. However, retaining both *HD* genes in each allele is the most common
741 organization in other bipolar basidiomycetes with fused *MAT* loci, including several *Microbotryum*
742 [27], *Sporisorium* [30], and *Ustilago* species [31, 124, 125]. As in these fungi, this configuration
743 may be functionally advantageous in *K. fici*, potentially reflecting differences in mating strategies
744 or genomic constraints. For instance, if both *HD* genes have additional roles beyond mating that
745 do not require heterodimerization, their retention could be selectively maintained, although this
746 possibility remains largely unexplored in basidiomycetes.

747 In contrast, beyond *Cryptococcus* pathogens and *Cryptococcus* sp. 3 identified here, the
748 only other documented case of a bipolar system with fused *MAT* loci containing a single *HD* gene
749 per mating type occurs in the *Trichosporonales* [18]. A few other known cases of *HD* gene loss in
750 heterothallic bipolar species, albeit distinct, include: (i) *Microbotryum violaceum* on *Silene*
751 *caroliniana*, where *HD1* is lost in *a2* strains, while *a1* retains both *HD* genes [27]; (ii) *Microbotryum*
752 *superbum*, where loss of *HD* function in mating compatibility may have relaxed constraints on *HD2*
753 maintenance in *a1* strains, leading to its disruption, while *a2* strains retains both *HD* genes [126];
754 and (iii) xerotolerant *Wallemia* species, where *HD1* is retained in *MAT a1*, while the opposite
755 mating type appears to lack any *HD* gene, potentially indicating another case of *HD* loss-of-
756 function in mating-type determination [127]. Because *HD* compatibility is no longer an independent
757 determinant of mating success in bipolar species, maintaining both *HD* genes in each *MAT* allele
758 may no longer be necessary. Thus, their loss could result from genomic streamlining, eliminating
759 redundancy once *MAT* loci became fused and recombination was suppressed.

760 The tetrapolar-to-bipolar transition in *Cryptococcus* pathogens has been hypothesized to
761 result from a chromosomal translocation mediated by ectopic recombination between repetitive
762 elements in the centromeric regions of the *P/R*- and *HD*-containing chromosomes [35]. This model
763 had support from several observations: (i) *Cryptococcus* centromeres are enriched with species-
764 specific long-terminal-repeat retrotransposons (LTRs) from the Ty3/*Gypsy* and Ty1/*copia* families
765 shared among different centromeres, potentially facilitating recombination [23, 46, 48, 106, 107];
766 (ii) chromosomal arm exchanges have been identified during the divergence of *Cryptococcus*
767 lineages from their common ancestor [35, 105]; and (iii) the repair of experimentally induced
768 double-strand breaks at centromere-specific transposons in *C. neoformans* frequently results in
769 chromosomal translocations [105].

770 Our expanded comparative analysis, however, indicates that the centromere of the *C.*
771 *neoformans MAT* chromosome, along with extended flanking regions, is conserved across most

Cryptococcus species and in *K. shandongensis*, suggesting an ancestral organization. In contrast, in *C. amylolentus* this region aligns to two distinct chromosomes, implying that intercentromeric recombination occurred as a derived rather than ancestral feature. Therefore, these findings provide an alternative perspective to previous interpretations that proposed intercentromeric recombination as the primary event initiating the tetrapolar-to-bipolar transition in *Cryptococcus* pathogens.

Our analyses suggest that translocations outside centromeric regions may have played a more prominent role in repositioning the *P/R* and *HD* loci onto the same chromosome (**Fig 7**). Such non-centromeric translocations appear to underlie the transition to bipolarity in several *Ustilaginales* species, including *Sporisorium scitamineum* [30] and in the common ancestor of *Ustilago hordei*, *U. nuda*, and *U. bromivora* [31]. Non-centromeric translocations events are also readily apparent across other genomic regions when comparing genomes of different *Cryptococcus* species [48] and even among strains within the same species, as observed in *C. neoformans* [100, 128]. Whether such translocations are influenced by TEs has not yet been systematically analyzed in *Cryptococcus*; however, studies in other model organisms, including *Saccharomyces cerevisiae*, have shown that translocations can occur via homologous recombination between retrotransposons on different chromosomes [129]. Given the abundance of TEs in *Cryptococcus* genomes [48], a similar mechanism could have contributed to the chromosomal relocation of *MAT* loci and, ultimately, to the tetrapolar-to-bipolar transition.

Mating-type chromosomes with genetically linked *P/R-HD* loci can also arise from the fusion of entire ancestral *P/R* and *HD* chromosomes, as reported in *Microbotryum violaceum* on *Silene paradoxa* [27]. In *Kwoniella*, repeated and independent chromosome fusions have dramatically reduced chromosome numbers, with some extant species retaining only 3 (clade E) or 5 (clades G and H) chromosomes, descending from a 14-chromosome ancestral karyotype [48]. Yet, aside from the bipolar arrangement in *K. fici* (clade H) and the pseudobipolar

797 arrangement in *K. europaea* (clade E), no other instances of *P/R-HD* linkage were detected in
798 *Kwoniella*. This observation reinforces the view that while chromosomal fusions and translocations
799 provide the mechanistic basis for tetrapolar-to-bipolar transitions, they do not inherently drive shifts
800 in breeding systems. Instead, whether a species remains tetrapolar or transitions to bipolarity likely
801 depends on other related factors such as mating ecology (e.g., the degree of outcrossing vs.
802 selfing/inbreeding), selection for mating efficiency, and long-term genomic stability.

803 The colonization of new habitats, including host-jumps, can lead to significantly altered
804 dispersal patterns and changes in mating success, which may drive genetic rearrangements and
805 facilitate adaptation to new niches. While sexuality is very frequent in some fungal groups (e.g.,
806 mushroom-forming basidiomycetes), asexual reproduction is widespread across the fungal
807 kingdom, and approximately 20% of known fungal species lack documented evidence of sexual
808 cycles [130, 131]. This is particularly evident in many fungal pathogens of animals, where sexual
809 reproduction is rarely observed or remains entirely unknown [132, 133].

810 Among the species analyzed in this study, sexual cycles are documented in only 8 of 17
811 *Cryptococcus* species and 2 of 18 *Kwoniella* species. Research on these fungi is significantly
812 hindered by the difficulty of culturing them, resulting in many species being known from only a
813 single isolate [47]. Unlike many mushroom-forming basidiomycetes, most *Tremellomycetes* are
814 parasites of lichens and other fungi, producing inconspicuous fruiting bodies or reproductive
815 structures embedded within host tissues. This makes direct observation of their sexual
816 reproduction in the field and the isolation of compatible partners for experimental studies
817 particularly challenging. While genomic analysis of *MAT* loci content and organization allows for
818 inferences about potential breeding systems (heterothallism vs. homothallism), experimental
819 mating assays remain the most definitive approach for confirming sexual reproduction and viable
820 progeny production. Expanding sampling efforts and discovering new species in their natural
821 environments are crucial for uncovering the full diversity of *Tremellomycetes*, gaining deeper

822 insights into their reproductive strategies, and understanding their ecological roles and
823 evolutionary trajectories [47, 49].

824 Our experimental analysis confirmed a sexual cycle in *Cryptococcus decagattii*,
825 demonstrating its ability to undergo mating and sporulation. However, we were unable to induce
826 sexual reproduction in several other lineages, including *C. tetragattii* and *Cryptococcus* sp. 4. The
827 failure to observe mating under laboratory conditions may stem from suboptimal culture conditions
828 rather than an inherent inability to reproduce sexually, underscoring the need to identify
829 appropriate mating conditions and compatible partners. Notably, *Cryptococcus* sp. 3 is closely
830 related to *C. depauperatus*, a homothallic species, yet it does not undergo sexual reproduction
831 alone, suggesting that it is not homothallic. This is consistent with its genomic composition, which
832 indicates a heterothallic, bipolar *MAT* configuration.

833 One possible approach to overcome these challenges and induce the sexual state is the
834 targeted deletion of *CRG1*, a regulator of G protein signaling that negatively regulates Gα proteins
835 activated through pheromone receptors, thereby downregulating signaling from a pheromone-
836 bound *STE3* receptor [134, 135]. *C. neoformans*, *crg1*Δ mutants are hypersensitive to mating
837 signals, exhibit hyperfilamentation, and show enhanced mating efficiency, particularly under
838 conditions that would otherwise be non-permissive for sexual reproduction [135, 136]. Given that
839 *CRG1* is conserved across *Cryptococcus* and *Kwoniella*, its deletion in cryptic lineages with
840 compatible mating-type strains could facilitate mating and reveal latent sexual cycles, contingent
841 on establishing these species as genetically tractable.

842 In *K. mangrovensis*, progeny analysis from a mating cross revealed significant ploidy
843 variation, with a high incidence of aneuploid strains. The primary factor contributing to this
844 outcome appears to be the difference in ploidy between the parental strains: while CBS8507 is
845 haploid, FACS analysis indicated that CBS10435 predominantly consists of diploid cells. As
846 observed in other fungi, haploid-diploid mating can result in chromosome mis-segregation and

847 aneuploid progeny due to improper chromosome pairing and segregation during meiosis. This
848 effect may be further exacerbated in *K. mangrovensis* by its highly reduced karyotype (3 chrs.),
849 where errors in the segregation of its disproportionately large chromosome may have an outsized
850 impact on ploidy balance. Despite this, genomic comparisons of F1 isolates with parental strains
851 revealed patterns consistent with recombination, with chromosome segments mapping to different
852 parents, supporting the occurrence of meiotic exchange. Additionally, loss of heterozygosity (LOH)
853 was observed in some regions. While ploidy estimates from genome-wide read depth analysis
854 and FACS were generally concordant, some discrepancies suggest either intrinsic genomic
855 instability or methodological differences in DNA content estimation, potentially stemming from
856 differences in cell wall composition, nuclear DNA organization, or cell cycle stage, which could
857 affect staining efficiency and fluorescence intensity in FACS-based ploidy assessment.

858 Interestingly, some of the *K. mangrovensis* F1 progeny, by virtue of their diploid/aneuploid
859 status, carried compatible *P/R* and *HD* alleles (*a1a2b1b2*), suggesting the potential for self-fertility.
860 Under mating conditions these strains formed hyphae and basidia similar to those produced by
861 the parental cross, with some even exhibiting more extensive filamentation. Further investigation
862 of the *K. mangrovensis* life cycle, including controlled crosses with haploid strains, synchronized
863 cultures, and genomic analyses of progeny, will be necessary to clarify the extent to which
864 aneuploidy results from chromosomal instability and is tolerated versus differences in parental
865 ploidy during mating.

866 Overall, our findings highlight both the challenges and opportunities in understanding the
867 reproductive biology and evolutionary trajectories of *Cryptococcus* and *Kwoniella*. Comparative
868 genomic analyses revealed distinct evolutionary paths shaping *MAT* loci architecture, including
869 independent *P/R* locus expansions, diverse modes of *HD-P/R* fusion, and convergent transitions
870 in mating systems. Additional analysis of sexual cycles will require expanded strain sampling,
871 optimized mating conditions, and genetic approaches such as *CRG1* deletion to uncover latent

reproductive potential. Beyond laboratory investigations, assessing sexual recombination in natural populations and exploring the hidden diversity of *Cryptococcus* species — particularly in their likely African origin [49] — will be crucial for understanding their speciation, ecology, and adaptation, as well as the evolutionary forces shaping *MAT* locus evolution and reproductive transitions, with possible implications for pathogenic emergence.

Materials and methods

Strains and media

Strains studied in this work were routinely grown on YPD medium (10 g/L yeast extract, 20 g/L Bacto Peptone, 20 g/L dextrose, and 20 g/L agar) unless indicated otherwise. *C. neoformans* and *C. deneoformans* strains were incubated at 30°C, while other *Cryptococcus* and *Kwoniella* species strains were grown at room temperature (20-23°C). A complete list of strains used in this study is provided in **S1 Appendix**. Strain NCYC1536 was obtained from the National Collection of Yeast Cultures (Norwich, UK), and strain CMW60451 was isolated in October 2022, from a bark beetle (*Lanurgus* sp.) infesting twigs of *Widdringtonia cedarbergensis* in the Cederberg Mountains, South Africa.

Genomic DNA extraction

High-molecular weight (HMW) DNA was extracted with a cetyltrimethylammonium bromide (CTAB) extraction as previously described [105], minimizing sharing stress during sample preparation. DNA quality was evaluated by determining the A260/A280 and A260/A230 ratios on NanoDrop spectrophotometer (Thermo). Integrity and fragment size were analyzed using clamped homogeneous electric field (CHEF) electrophoresis, and gDNA for short-read whole-genome sequencing (Illumina) was extracted with a phenol:chloroform-based protocol, both as previously

896 described [48]. DNA concentration for all samples was measured with Qubit dsDNA Assay Kits
897 (Invitrogen) on the Qubit fluorometer.

898

899 **Genome sequencing**

900 Whole-genome sequencing was performed with PacBio, Nanopore, and Illumina
901 technologies. For *K. heveanensis* BCC8398 and *K. mangrovensis* CBS10435, Illumina
902 sequencing at the Broad Institute Genomics Platform utilized “fragment” and “jumping” libraries,
903 constructed and sequenced as previously specified [48]. All other Illumina sequencing was
904 conducted at the Duke University Sequencing and Genomic Technologies (DUSGT) Core, with
905 libraries prepared with Kapa HyperPlus library kit and sequenced as paired-end 2 x 150 bp reads
906 on various Illumina platforms. For PacBio sequencing, 15- to 20-kb insertion-size libraries were
907 prepared and run on a PacBio RS II or Sequel (2.0 chemistry) system at the DUSGT. Nanopore
908 sequencing was carried out in-house. Single-strain libraries were prepared with either the SQK-
909 LSK108 or SQK-LSK110 kit, whereas up to three DNA samples were barcoded with the SQK-
910 LSK109 and EXP-NBD103/EXP-NBD104, or SQK-NBD114.24 kits. Libraries were prepared
911 according to the manufacturer’s protocols, either individually or pooled, and sequenced on R9 flow
912 cells (FLO-MN106) or R10 flow cells (FLO-MIN114) for 48-72 hours at default voltage on a MinION
913 Mk1B or MinION Mk1C system. The MinION software version available at the time of each run
914 was applied. Further details on specific genome sequencing platforms, basecalling, and
915 demultiplexing are provided in **S1 Appendix**.

916

917 **Genome assembly**

918 Complete genomes were assembled with Canu [137] using Nanopore or PacBio
919 sequencing data and default parameters. The accuracy of the assemblies was improved through

initial error correction with Medaka (https://github.com/nanoporetech/medaka) for Nanopore-based assemblies, followed by up to five rounds of iterative polishing with Pilon v1.22 [138] (`--fix all`) for all assemblies, using Illumina reads aligned to the first pass-polished assembly with BWA-MEM v0.7.17-r1188 [139]. Contigs containing only rDNA sequences, detected by Barrnap (https://github.com/tseemann/barrnap) (`--kingdom euk`), or those classified as mitochondrial DNA, were excluded from the final nuclear genome assembly. To confirm assembly completeness and evaluate telomeric regions, Nanopore/PacBio and Illumina reads were realigned to the Canu-corrected assembly using minimap2 v2.9-r720 [140] and BWA-MEM, respectively, and read coverage profiles were examined in the Integrative Genomics Viewer (IGV) [141]. Draft genome assemblies for *K. europaea* PYCC6162 and *K. botswanensis* CBS12717 were generated with SPAdes v3.15.3 using default settings, while the assembly for *K. heveanensis* BCC8398 and *K. mangrovensis* CBS10435 was constructed with Allpaths [142]. Genome assemblies and raw sequencing data have been deposited in DDBJ/EMBL/GenBank under the BioProject numbers listed in **S1 Appendix**, which also provides specific details on sequencing platforms, basecalling, demultiplexing, and assembly parameters for each genome.

Gene prediction and annotation

Gene models were predicted on repeat-masked assemblies using either BRAKER2 v2.1.5 [143] or Funannotate v1.8.9 (https://github.com/nextgenusfs/funannotate), following previously described methodologies [20, 23, 48]. Manual inspection and correction were performed exclusively for the mating-type gene models, including the prediction and addition of short mating pheromone precursor genes across all species. Functional annotation was integrated into the final gene models using the Funannotate `"annotate"` module, which incorporated data for PFAM and InterPro domains, Gene Ontology (GO) terms, fungal transcription factors, Cluster of Orthologous Genes (COGs), secondary metabolites, Carbohydrate-Active Enzymes (CAZYmes), secreted

proteins, proteases (MEROPS), and Benchmarking Universal Single-Copy Orthologs (BUSCO) groups. InterPro domain data, COG annotations, and secondary metabolite predictions were obtained by InterProScan v5.55-88.0, eggNOGmapper v.2.1.7 (eggNOG DB version: 5.0.2) [144], and AntiSMASH v6.1.0 [145], before being passed to Funannotate `annotate` with the options `--iprscan, --eggnog`, and `--antismash`. Specific parameters are provided in **S1 Appendix**.

Ortholog identification and sequence alignment

To construct the phylogenomic data matrix, single-copy orthologs (SC-OGs) were identified across *Cryptococcus* and *Kwoniella* species, as well as three outgroup species (*Tremella mesenterica* ATCC28783, GCA_004117975.1; *Saitozyma podzolica* DSM27192, GCA_003942215.1; and *Bullera alba* JCM2954, GCA_001600095.1) using OrthoFinder v3.0.1b1 with options: `-M msa -S diamond_ultra_sens -I 1.5 -M msa -A mafft -T fasttree -t 48 -a 6`. A total of 3,086 SC-OGs shared among all species were identified. The amino acid sequences of these SC-OGs were individually aligned with MAFFT v7.310 [146] using `--localpair --maxiterate 1000` and trimmed with TrimAl v1.4.rev22 [147] using the options `-gappyout -keepheader`. The same approach was applied to construct the phylogenomic data matrix for *C. neoformans* strains representing different VN groups, along with three outgroup strains of *C. deneoformans*. This analysis identified 5,439 SC-OGs shared across all strains, which were subsequently aligned and trimmed as described above.

Species phylogeny and estimation of topological support

The updated phylogeny of *Cryptococcus* and *Kwoniella* species (**Figs 1A** and **S1**) was inferred using two complementary strategies: (i) concatenation-based partitioned maximum likelihood (ML) phylogeny reconstruction in IQ-TREE v2.1.6 [148], and (ii) gene-based

coalescence analysis in ASTRAL v5.7.8 [149]. For the concatenation approach, individual amino acid alignments for 3,086 single-copy orthologs (SC-OGs) were combined into a partitioned supermatrix (52 taxa, with 3,086 partitions and 1,686,691 sites) using the *"-p"* option in IQ-TREE. The edge-linked proportional partition model [150] was applied to account for differences in evolutionary rates across partitions, and the best-fit substitution model for each partition was identified with ModelFinder [151] based on the Bayesian information criterion. The ML tree was inferred with the parameters *--seed 54321 -m MFP -msub nuclear -B 1000 -alrt 1000 -T 14*, incorporating 1,000 ultrafast bootstrap (UFboot) replicates [152] and Shimodaira–Hasegawa approximate likelihood ratio tests (SH-aLRT) for branch support assessment. For the coalescence-based approach, ML gene trees were independently constructed for each SC-OG alignment using IQ-TREE's *"-S"* option, which performs both model selection and tree inference for individual alignments. The resulting trees were then input to ASTRAL to reconstruct a species phylogeny under default settings. Quartet support values were computed with the *-t 2* option, providing quartet support metrics for the primary topology (q1) and alternative topologies (q2, q3), and local posterior probability (LPP) support. Genealogical concordance and topological support for branches in the concatenated ML tree were evaluated using the gene concordance factor (gCF) and site concordance factor (sCF) metrics as implemented in IQ-TREE. This analysis utilized both the best-scoring concatenated ML tree (concat.treefile) and the set of gene trees (loci.treefile), with options *-t –gcf -p --scf 100*. The concatenation-based approach was also employed to resolve relationships within *C. neoformans* strains representing different VN groups (**Fig 4A**). Phylogenetic trees were visualized and annotated with iTOL v7.

Gene genealogies

Amino acid sequences of genes of interest were extracted from the corresponding OGs identified by OrthoFinder. These sequences were manually inspected and reannotated as needed.

994 Curated protein sequences were aligned and trimmed as above, and ML phylogenies were
995 reconstructed and visualized as above. Detailed model parameters are provided in the tree files
996 accessible at https://doi.org/10.5281/zenodo.14851287.

997

998 **Synteny analyses, *MAT* loci delineation, and centromere identification**

999 Conserved synteny blocks in pairwise genome comparisons were determined using
1000 SynChro [102], with the delta parameter set to 3 to ensure high stringency. Comparisons shown
1001 in **Figs 6A**, **8A** and **S9A** employed the *K. shandongensis* genome assembly as the reference.
1002 Detailed linear synteny plots comparing chromosomes and specific genomic regions, including
1003 the *MAT* loci, were generated with EasyFig v2.2.2 [153] using BLASTN. Centromeres positions
1004 were determined by in silico analysis as previously described [48], combining the detection of
1005 centromere-associated LTR elements and synteny analysis. Centromere lengths were estimated
1006 as the intergenic regions between flanking centromeric genes (**S1 Appendix**). *MAT* loci were
1007 initially identified using BLAST searches, with *C. neoformans*-derived *MAT* genes and their
1008 flanking proteins as queries. The *HD* loci were defined as the regions spanning the *HD1* and *HD2*
1009 genes. The *P/R* loci were delineated based on structural comparisons between mating types. For
1010 species with available strains of opposite mating types, the *P/R* locus was defined as the region
1011 where synteny between the mating types is disrupted, with the boundaries corresponding to the
1012 restoration of synteny. For species with only a single strain analyzed, the *P/R* locus length was
1013 inferred based on the distance between *P/R*-flanking genes, as determined from comparisons
1014 involving opposite mating types. Statistical analyses of *MAT* loci lengths across species, clades,
1015 or mating-types were conducted with Python3 with Pandas, Seaborn, Matplotlib, and SciPy
1016 libraries. Differences between the two groups were assessed using the two-sided Mann–Whitney
1017 U test. Scripts and raw data are available at https://doi.org/10.5281/zenodo.14851287. To

enhance figure clarity and readability, plots were refined by adjusting color schemes, modifying labels, and manually adding additional features using Adobe Illustrator.

Analysis of gene content in *MAT* loci, their frequency across species, and gene essentiality classification

A curated gene presence/absence matrix was constructed following the delineation of the *MAT* loci across species. Genes were categorized as “present” (coded as “1”), "absent" (coded as “0”), “unclear” (coded as “?”), or “pseudogene” (coded as “pseudo”). Unclear cases indicated instances where gene presence could not be conclusively determined due to incomplete or uncertain *MAT* locus boundaries. For frequency calculations, pseudogenes were treated as present, while unclear cases were treated as absent. The presence of either *SXI1* or *SXI2* was scored as present and combined for frequency quantification. The matrix also included information on gene essentiality, exclusively inferred from studies on *C. neoformans* H99 (i.e., gene essentiality in other species was extrapolated solely from H99-based predictions). Essentiality classifications were based on experimentally validated studies [34, 154, 155] and predictions from a recent high-throughput transposon mutagenesis and sequencing (TN-seq) study (https://bbillmyre.shinyapps.io/Crypto_TN_seq_viewer/) [91]. Genes without orthologs in H99 were categorized as “unknown” due to the absence of data for classification. A custom R script (`0_calculate_gene_presence_frequency.R`) was developed to analyze and plot gene presence frequencies, sorted by frequency. The presence/absence matrix was also visualized as a heatmap using a custom R script (`1_plot_gene_matrix_based_on_frequency.R`), with genes ordered by their frequency across strains. While *SXI1* and *SXI2* were shown as separate columns in the heatmap, their ranking reflected their combined presence frequency. These analyses and visualizations were conducted in R using the dplyr, ggplot2, ComplexHeatmap, and circlize packages. Additionally, gene presence/absence across clades was analyzed using a custom

Python script (*2_compare_MAT_gene_content_across_clades.py*) that calculates clade-specific and pairwise gene presence, identify genes shared across all specified clades, and highlight genes unique to individual clades. Scripts are available at https://doi.org/10.5281/zenodo.14851287, and the gene presence/absence input matrices are provided in **S2 and S3 Appendices**.

GC content and codon composition analysis

Genome-wide GC content and deviations from the mean GC% were analyzed using a custom Python script (*0_gc_content_analysis_and_plots.py*), employing a non-overlapping sliding window approach (1 kb for whole-genome analyses, 0.5 kb for zoomed-in views). Regions of interest (e.g., *P/R* loci) were highlighted using BED file coordinates. The resulting plots were further refined in Adobe Illustrator for producing **Fig 2**. To assess whether the *P/R* locus exhibited lower GC content than genome-wide averages, we used another custom Python script (*1b_gc_content_analysis.py*) that calculated GC content for the *P/R* locus and compared it to 100 randomly sampled regions of the same size, either genome-wide or on the same chromosome. A one-sample *t*-test assessed whether the *P/R* locus GC content differed significantly from the sampled mean, and a permutation test (10,000 iterations) generated a null distribution to calculate *P*-values and Z-scores. The analysis was automated using the shell script (*1a_run_script_vs_wg.sh*), consolidating results into a summary table (**S4 Appendix**). Codon usage analysis was conducted with a third custom Python script (*3_codon_usage_analysis.py*), which compared codon frequencies in the *P/R* locus and genome-wide coding sequences. Codons were classified as AT- or GC-rich, and their counts were compared between the two regions. Chi-square tests ($P < 0.05$) evaluated: (i) differences in codon usage (64 codons) and (ii) AT/GC composition differences. For codon usage, the null hypothesis assumed the codon distributions in the *P/R* locus matched genome-wide distributions. Scripts are raw data are

accessible at https://doi.org/10.5281/zenodo.14851287 and raw data is also summarized in **S4 Appendix**.

Variant analysis and SNP distribution in *C. neoformans MAT*a strains

Multi-genome variant analysis was performed with the Snippy pipeline v4.6.0 (https://github.com/tseemann/snippy) using the newly assembled genome of *C. neoformans* Ftc555-1 (VNBI) as the reference, and the following parameters: `--cpus 10, --unmapped, --mincov 10, and --minfrac 0.9`. Paired-end reads were obtained from the NCBI SRA database and are provided in **S1 Appendix**. Three datasets were independently used: (i) the entire genome, (ii) only the *MAT* locus region (chr_5:153,773-277,230), and (iii) only chr. 5. The *MAT* locus and chr. 5 were extracted from the reference genome to generate region-specific GenBank files, which were used as inputs for Snippy to identify SNPs and construct region-specific phylogenies. Visualization of SNV distributions was based on the whole-genome dataset and included both chromosome-wide and zoomed-in views of specific regions. A custom Python script was used to process merged SNV datasets, compute total SNP counts for each strain, and generate visualizations with gradient color-coding for SNP density and annotated regions of interest. Final composite figures were refined using Adobe Illustrator. Core SNP alignments (core.aln) from each dataset were used for phylogenetic reconstruction with IQ-TREE2, incorporating 10,000 UFboot replicates and SH-aLRT tests for branch support. Scripts for SNV analysis and visualization are available at https://doi.org/10.5281/zenodo.14851287.

Chromosome composition analysis of *K. mangrovensis* progeny

To characterize the chromosome composition of *K. mangrovensis* progeny, a combined nuclear reference genome was constructed using the genome assemblies of the two parental

1092 strains, CBS8507 and CBS10435. For CBS8507, the original assembly consisted of four contigs,
1093 two of which represented the same chromosome but were fragmented at the rDNA array. To
1094 streamline downstream analyses and facilitate data interpretation, these two contigs were merged
1095 into a single contig. A small gap (5 Ns) was introduced to connect the contigs in the correct
1096 orientation, aligning the rDNA genes consistently in the same direction and resulting in a finalized
1097 3-chromosome assembly. For CBS10435, the draft assembly, which comprised 37 contigs, was
1098 reordered and reoriented to match the 3-chromosome assembly of CBS8507. This was achieved
1099 using the D-GENIES tool [156], which employs minimap2 for genome alignment. Raw Illumina
1100 paired-end reads from selected *K. mangrovensis* progeny, along with the combined reference
1101 genome, were subsequently processed through the sppIDer pipeline [157]. The sppIDer workflow
1102 sequentially mapped the Illumina short reads to the combined reference genome, applied stringent
1103 quality filtering (MQ > 3), and generated depth-of-coverage plots. Chromosome number and ploidy
1104 for each progeny were estimated by integrating the depth-of-coverage data from sppIDer with flow
1105 cytometry results.

1106

1107 **Mating assays and phenotyping on mating inducing media**

1108 Mating assays using *K. mangrovensis* strains CBS8507 and CBS10435 were performed
1109 as previously described [65, 66]. Equal amounts of cells from each strain were mixed on V8 agar
1110 (10 g/L yeast extract, 20 g/L Bacto Peptone, 20 g/L dextrose, 20 g/L agar; pH 5) or corn meal agar
1111 (CMA; 15 g/L corn meal agar, 5 g/L agar) and incubated in the dark at room temperature
1112 (approximately 20-23°C) for up to one month. Plates were regularly monitored for the development
1113 of mating structures. Random progeny (F1) was recovered by microdissecting individual cells from
1114 cell clusters [78] embedded in or on the surface of the mating media (V8 and CMA) at the edges
1115 of mating patches. A total of 51 progeny were collected from dissection plates, grown axenically
1116 on YPD plates, and stored as glycerol stocks at -80 °C. Selected F1 progeny (MP11, MP15, MP16,

1117 MP18, MP49, MP50, MP51, MP54 and MP59) were assessed for hyphal formation and mating
1118 structures on V8 pH5 and CMA media, compared to the parental cross (CBS8507 x CBS10432)
1119 and the solo culture of each parental strain. Assays were repeated 3 separate times to ensure
1120 consistent phenotypic observations.

1121 Potentially compatible strain pairs from *Cryptococcus* species lacking documented sexual
1122 reproduction (IND107 x CBS11718; CBS11687 x 7685027; and DSM108351 x NCYC1536) were
1123 tested for mating ability under conditions known to induce mating in *C. neoformans* and *C.*
1124 *deneoformans* (V8 media, in the dark, and at room temperature) for up to a month, with regular
1125 monitoring [78]. Solo cultures of each strain were also evaluated for their capacity to undergo
1126 sexual reproduction without a compatible partner. Similar experiments were performed for strains
1127 of newly described species lacking known compatible partners. All assays were repeated three
1128 times to confirm phenotype consistency.

1129

1130 **Analysis of *K. mangrovensis* progeny by PCR-restriction fragment length polymorphism**
1131 **(RFLP) analysis**

1132 *Kwoniella mangrovensis* has only three chromosomes. To analyze the F1 progeny from
1133 the cross between strains CBS8507 and CBS10432, six primer pairs (one per chromosomal arm)
1134 were selected for the initial screening. PCR primers were manually designed to produce a distinct
1135 pattern between the two parental alleles after digestion of the PCR products with specific
1136 restriction enzymes. As a positive control for heterozygosity, PCR products from a 1:1 DNA mixture
1137 of both parental strains were amplified for each primer pair, followed by digestion with the
1138 corresponding restriction enzyme. The results of this analysis and primer sequences are provided
1139 in **S5 Appendix**.

1140

1141 **Microscopy**

1142 The edge of yeast colonies and mating patches were examined and photographed to
1143 assess hyphal growth, basidia, and spores. Imaging was performed with a Zeiss Axio Scope.A1
1144 microscope equipped with an Axiocam Color camera, using the ZEN Lite V3.4 software.

1145

1146 **Fluorescence-activated cell-sorting (FACS)**

1147 Fluorescence-activated cell sorting (FACS) was performed to determine the ploidy of *K.*
1148 *mangrovensis* strains CBS8507, CBS10432, and nine progeny strains from their cross, as
1149 previously described [158]. Briefly, strains were grown overnight at room temperature (21-23°C)
1150 on YPD medium, harvested, and washed with PBS. Cells were fixed using 2 ml of 70% ethanol at
1151 4°C overnight. Fixed cell pellets were washed with 1 ml of NS buffer (10 mM Tris-HCl pH 7.2, 250
1152 mM sucrose, 1 mM EDTA pH 8.0, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.4 mM
1153 phenylmethylsulfonyl fluoride, and 7 mM β-mercaptoethanol) and then stained with 5 µl of
1154 propidium iodide (0.5 mg/ml) in 180 µl NS buffer with 20 µl of RNase (10 mg/ml) at 4°C overnight.
1155 Lastly, 50 µl of stained cells were diluted in 2 ml of 50 mM Tris-HCl pH 8.0 and sonicated for 1 min
1156 before analysis at the Duke Cancer Institute Flow Cytometry Shared Resource. Data were
1157 collected from 10,000 cells using the FL1 channel on a Becton-Dickinson FACScan and analyzed
1158 with FlowJo software. Strains JEC21 and XL143 were respectively used as haploid and diploid
1159 controls [159].

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Figures

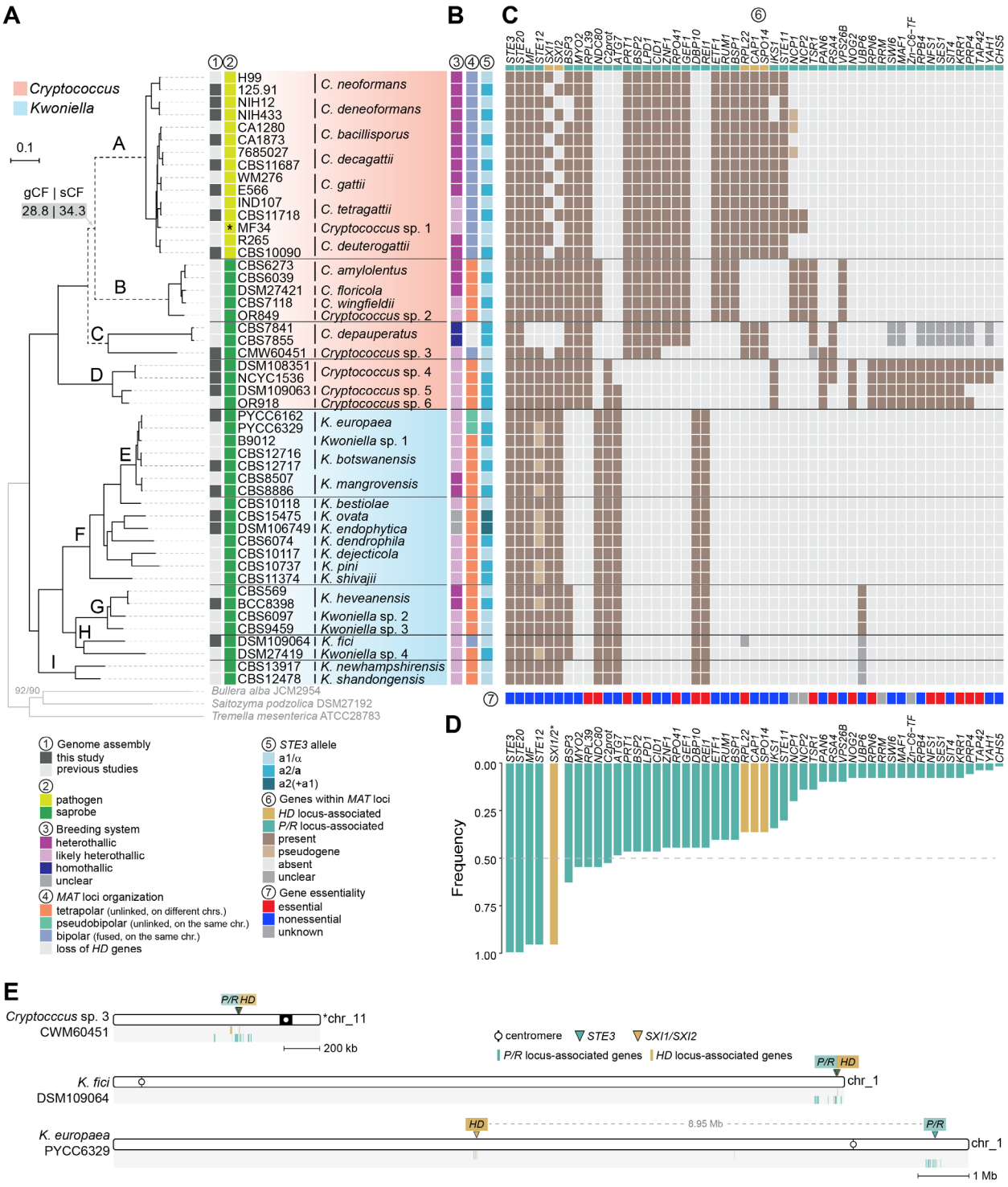

A
B
C
D
E
Cryptococcus
Kwoniella
0.1
gCF | sCF
28.8 | 34.3
92/90
Bullera alba JCM2954
Saitozyma podzolica DSM27192
Tremella mesenterica ATCC28783
C. neoformans
C. deneoformans
C. bacillisporus
C. decagattii
C. gattii
C. tetragattii
Cryptococcus sp. 1
C. deuterogattii
C. amylolentus
C. floricola
C. wingfieldii
Cryptococcus sp. 2
C. depauperatus
Cryptococcus sp. 3
Cryptococcus sp. 4
Cryptococcus sp. 5
Cryptococcus sp. 6
K. europaea
Kwoniella sp. 1
K. botswanensis
K. mangrovensis
K. bestiolae
K. ovata
K. endophytica
K. dendrophila
K. dejecticola
K. pini
K. shivajii
K. heveanensis
Kwoniella sp. 2
Kwoniella sp. 3
K. fici
Kwoniella sp. 4
K. newhampshirensis
K. shandongensis
Genome assembly
this study
previous studies
pathogen
saprobe
Breeding system
heterothallic
likely heterothallic
homothallic
unclear
MAT loci organization
tetrapolar (unlinked, on different chrs.)
pseudobipolar (unlinked, on the same chr.)
bipolar (fused, on the same chr.)
loss of HD genes
STE3 allele
a1/α
a2/a
a2(+a1)
Genes within MAT loci
HD locus-associated
P/R locus-associated
present
pseudogene
absent
unclear
Gene essentiality
essential
nonessential
unknown
Frequency
0.00
0.25
0.50
0.75
1.00
Cryptocccus sp. 3
CWM60451
*chr_11
200 kb
centromere
STE3
SXI1/SXI2
P/R locus-associated genes
HD locus-associated genes
K. fici
DSM109064
chr_1
K. europaea
PYCC6329
8.95 Mb
1 Mb

Fig 1. Phylogeny, breeding systems, and *MAT* loci organization in *Cryptococcus* and *Kwoniella*. (A) Phylogenetic tree of strains analyzed in this study, inferred by maximum likelihood analysis of a concatenated data matrix comprising protein alignments of 3,086 single-copy genes. Branch lengths are shown as the number of substitutions per site (scale bar). All branches are supported >95% based on SH-aLTR and UFboot tests, unless otherwise indicated. Gene concordance factor (gCF) and site concordance factor (sCF) were assessed to evaluate genealogical concordance across branches, revealing an ambiguous placement of clades A, B and C (dashed branches; see S1 Fig for details). An asterisk (*) denotes that VGV has not yet been associated with human infections. **(B)** Predicted breeding systems, *MAT* locus organization, and mating-type identity of the *P/R* locus. Breeding systems are classified as heterothallic or homothallic for species with defined sexual cycles, and as likely heterothallic for species inferred to exhibit heterothallism based on *MAT* gene content. **(C)** Heatmap of gene presence/absence and pseudogene annotations within the inferred *MAT* loci across species. Genes are ordered by their frequency of presence, with the most conserved genes on the left. Genes marked as "unclear" indicate instances where there is uncertainty regarding their presence within the *MAT* loci because these regions could not be precisely determined (see text for details). *BSP3* and *IKS1*, which were evicted from the *MAT* locus in *C. deneoformans* [34], are listed as absent in this species. Gene essentiality, as predicted or experimentally validated in strain H99 in prior studies rather than direct assessments in each species, is displayed at the bottom. **(D)** Gene presence frequency plot summarizing panel B. Pseudogenes were treated as present for frequency calculations, while unclear cases were treated as absent. The identification of either *SXI1* or *SXI2* was scored as "present" and combined for frequency quantification. **(E)** *MAT* loci locations in *Cryptococcus* sp. CWM60451, *K. fici*, and *K. europaea*, illustrating bipolar and pseudobipolar arrangements, respectively. Genes typically found either within or near the *HD* and *P/R* loci in tetrapolar species are designated as *HD*- and *P/R*-associated genes and their respective chromosomal locations are indicated by gold or teal vertical bars. The data underlying this Figure can be found in **S1 Appendix** and at https://doi.org/10.5281/zenodo.14851287.

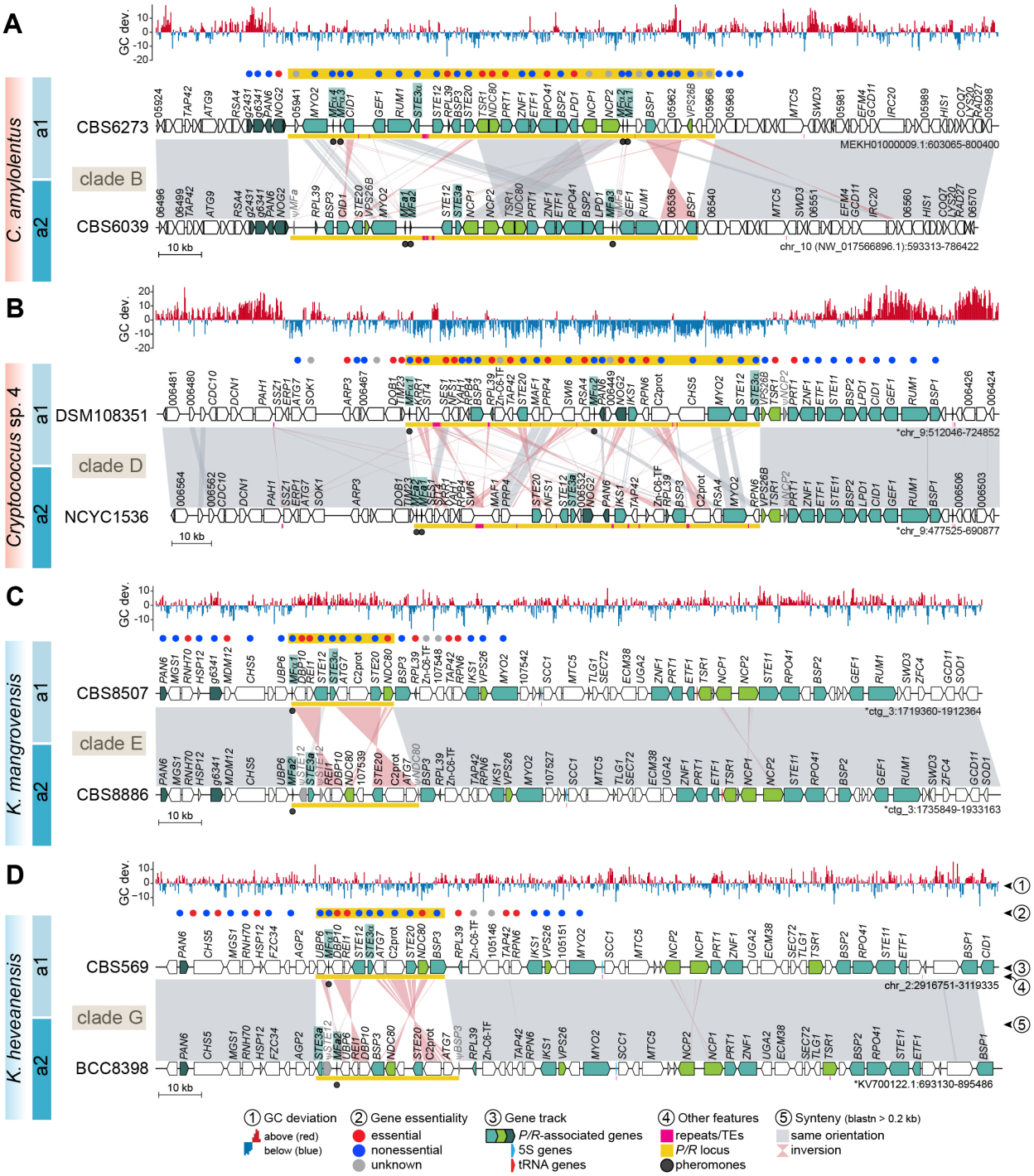


Fig 2. Synteny analysis of the *P/R* locus in tetrapolar *Cryptococcus* and *Kwoniella* species. (A-B) Detailed views of the *P/R* locus of representative *Cryptococcus* species from clades B and D. In both clades, the *P/R* locus exhibits lower GC content compared to other genomic regions. In clade D species, the predicted *P/R* locus contains only a subset of the genes found within the *P/R* locus of clade B species or the *MAT* locus of pathogenic *Cryptococcus* species. The additional genes in clade B or pathogenic species are instead located in the immediate downstream region, suggesting independent expansion of this genomic segment in clade D species. **(C-D)** *P/R* locus region in representative *Kwoniella* species of clades E and G.

In *a2* strains, the *STE12* gene appears truncated. Remnants of the *NDC80* gene (in clade E/F species) or the *BSP3* gene (in clade G species) are observed at the right edge of the *P/R a2* allele, likely resulting from inversion events. In all panels, *P/R*-associated genes are colored teal if their orthologs in *Cryptococcus* pathogens are located within the *MAT* locus, with darker teal indicating genes positioned in the flanking regions, and bright green for genes present within the *P/R* locus of *C. amylolentus*, but absent in most *Cryptococcus* pathogens. The *P/R* allele of each strain (*a1* or *a2*) is indicated on the left. Chromosomes inverted relative to their original assembly orientations are marked with asterisks. GC content is depicted as the deviation from the genome average, calculated in 0.5 kb non-overlapping windows. Gene essentiality within and in the immediate vicinity of the *P/R* locus is inferred from predictions or experimental validations in *C. neoformans* H99 rather than direct assessments in these species. Other features are annotated as shown in the key. See S4Fig for additional comparisons. The data underlying this Figure can be found at https://doi.org/10.5281/zenodo.14851287.

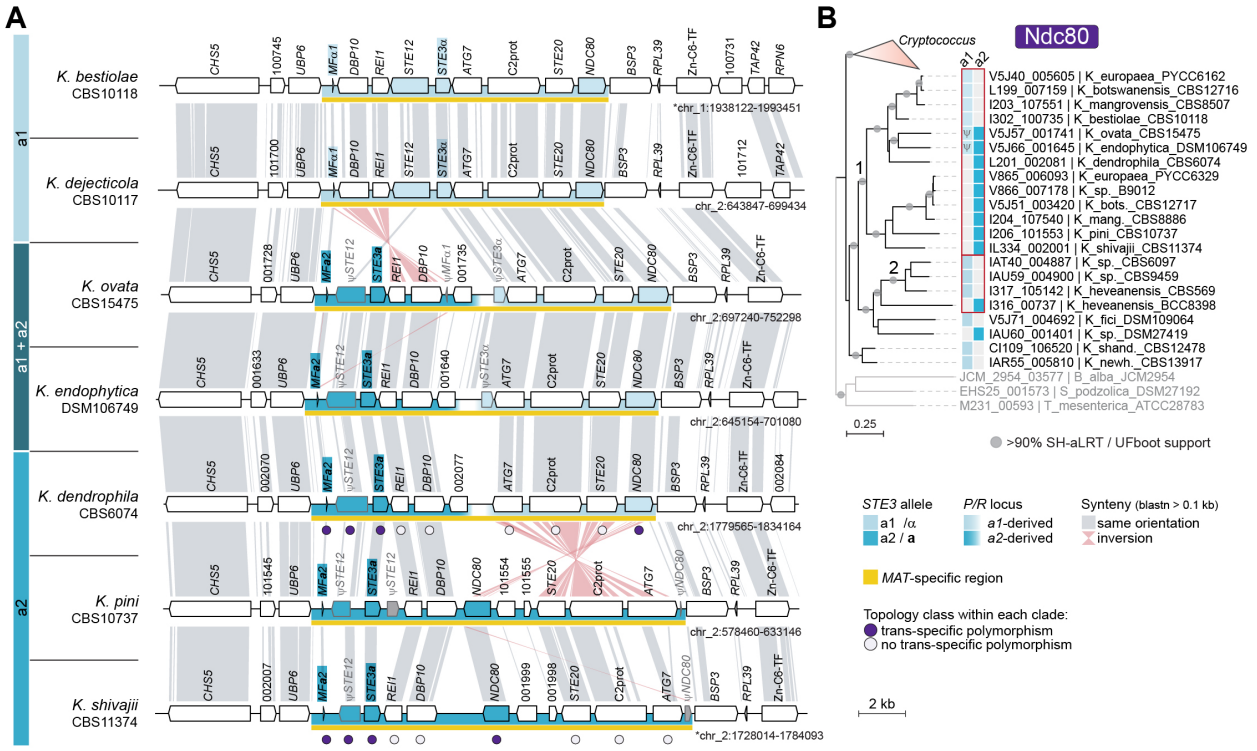


Fig 3. The *P/R* locus in *K. ovata*, *K. endophytica*, and *K. dendrophila* likely arose through intra-*P/R* recombination. **(A)** Synteny analysis comparing *P/R a1* and *P/R a2* alleles in select *Kwoniella* species from clade F. The *P/R* loci of *K. ovata* and *K. endophytica* contain *STE3***a** and *MF***a** alleles, along with truncated (ψ) versions of *STE3*α. Additionally, *K. ovata* harbors a truncated *MF*α gene. Synteny analysis indicates that the left side of the *P/R* locus in *K. ovata*, *K. endophytica*, and *K. dendrophila* is structurally more similar to *P/R a2* alleles of *K. pini* and *K. shivajii*, while the right side aligns more closely with the *P/R a1* alleles of *K. bestiolae* and *K. dejecticola*. **(B)** Gene genealogy of the *NDC80* gene inferred with IQ-TREE2 (model JTT+G4) showing trans-specific polymorphism across clade E/F species (labeled as 1) and within clade G species (labeled as 2). The *NDC80* alleles of *K. ovata*, *K. endophytica*, and *K. dendrophila* cluster more closely with *a1* alleles than with other *a2* alleles. The data underlying this Figure can be found at https://doi.org/10.5281/zenodo.14851287.

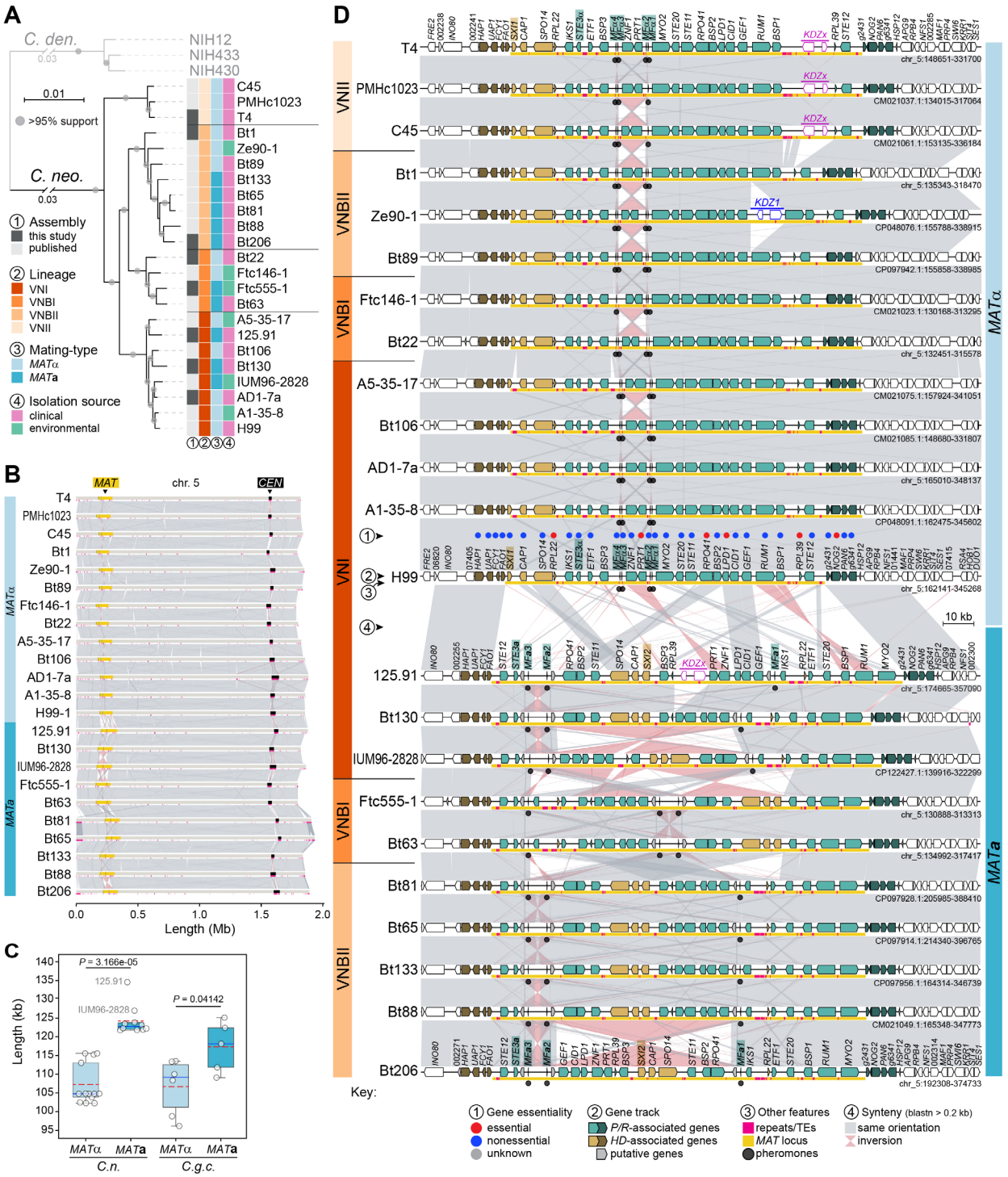


Fig 4. Synteny analysis of the mating-type locus (*MAT*α and *MAT*a) across different *Cryptococcus neoformans* lineages. (A) Phylogenetic tree of representative *C. neoformans* strains from different VN lineages, inferred using maximum likelihood analysis of a concatenated matrix of protein alignments from 5,439 single-copy genes. Branch support is based on SH-aLTR and UFboot tests. Mating types were determined based on the allelic version of the pheromone receptor gene (*STE3*). **(B)** Synteny analysis of chr. 5, where the *MAT* locus resides, showing significant conservation across strains, except within the *MAT*

and centromere regions, which exhibit rearrangements. Strains are organized by mating type and lineage, as in panel D. **(C)** Box plot comparing *MAT* locus size between α and **a** strains of *C. neoformans* (*C.n.*) and *C. gattii* species complex (*C.g.c.*). The red dashed line, blue line, and boxes denote the mean value, median value, and interquartile range, respectively. Outliers are labeled with the corresponding strain name. Statistical significance (*P*-values) was determined by the Mann–Whitney U test. **(D)** Detailed synteny of the *MAT* locus, highlighting strain- and mating-type-specific rearrangements. The *MAT***a** configuration is more variable both across and within VN lineages, existing in three distinct configurations, whereas the *MAT*α structure is more conserved, with limited rearrangements between ancestrally linked homeodomain (*HD*) and pheromone receptor (*P/R*) genes. Observed rearrangements appear to result from inversions mediated by identical copies of pheromone genes. KDZ transposons are detected within the *MAT* locus of certain strains. Genes typically associated with the *P/R* and *HD* loci in tetrapolar species are colored green and gold, respectively, with darker shades denoting genes flanking the *MAT* locus in *Cryptococcus* pathogens. Gene essentiality within the *MAT* locus, as predicted or experimentally validated in strain H99, is indicated by color-coded circles as given in the key. The data underlying this Figure can be found at https://doi.org/10.5281/zenodo.14851287.

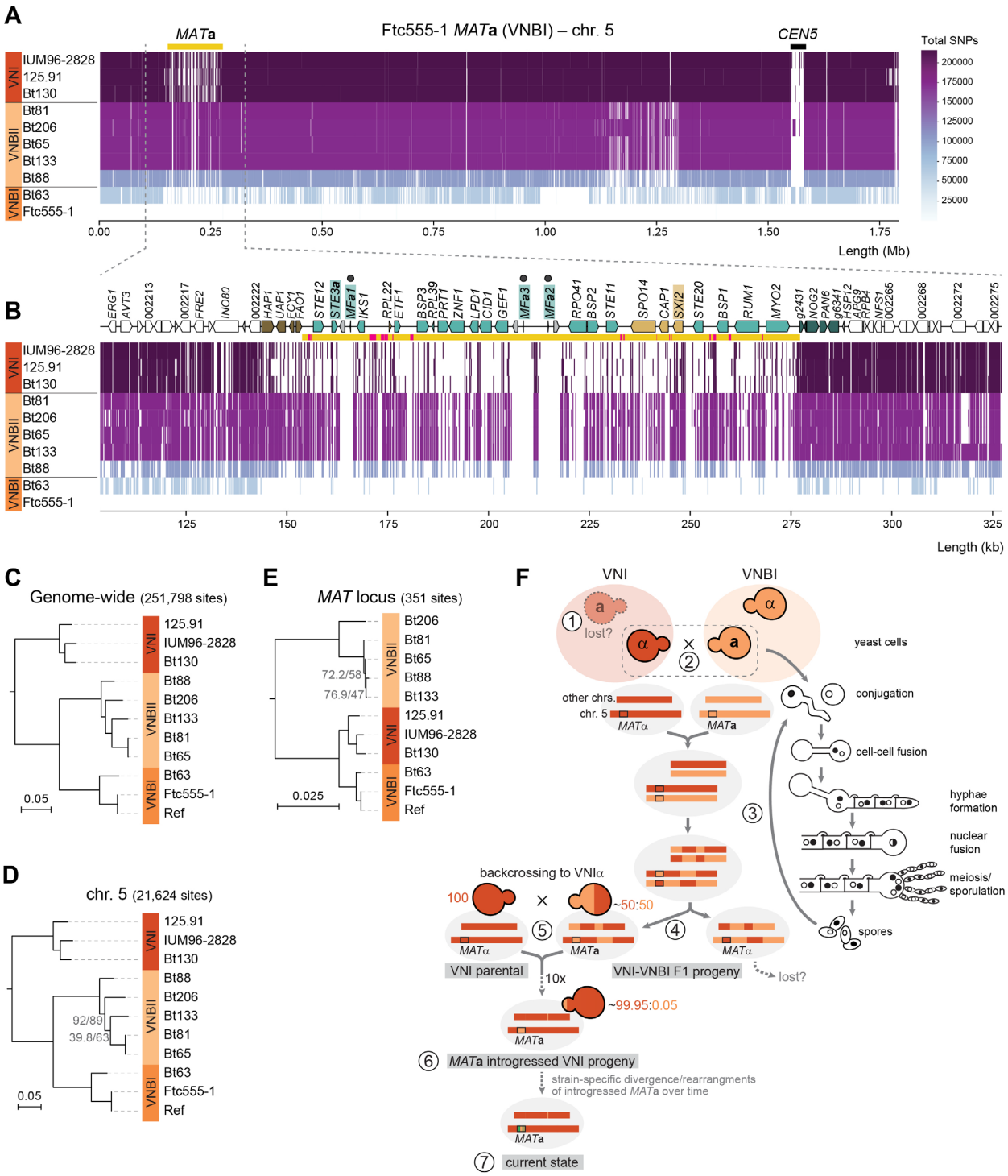


Fig 5. SNP distribution, phylogenetic analysis, and revised introgression model of the *MAT*a allele from VNBI into VNI. (A) Genome-wide distribution of SNPs across chromosome 5 in *C. neoformans MAT***a** strains from different VN lineages relative to the *C. neoformans* Ftc555-1 VNBI genome reference. Empty spaces in the plots represent either sites identical to the reference genome (monomorphic sites) or regions excluded due to low coverage or repetitive/duplicated sequences. Strains on the y-axis are ordered by the total number of SNPs across the genome, with a color gradient (right) indicating SNP counts: darker colors

represent higher SNP densities relative to the reference (strains positioned at the top), while lighter colors indicate fewer SNPs (strains at the bottom). Reads from the reference strain were also mapped as a control. The x-axis shows genomic coordinates. **(B)** Zoomed-in view of the *MAT* locus region plus 50 kb flanking regions, with genes and other features represented as in Fig 4. Notably, VNI strains exhibit a markedly lower number of SNPs at the *MAT***a** locus relative to the VNBI reference, compared to VNBII strains. **(C)** Midpoint-rooted tree based on genome-wide SNPs across all chromosomes, illustrating the evolutionary relationships of the strains. **(D)** Midpoint-rooted tree constructed using SNPs from chromosome 5 only, showing similar relationships to the genome-wide analysis. **(E)** Midpoint-rooted tree based on SNPs restricted to the *MAT* locus, highlighting a closer relationship between VNI and VNBI strains. In all trees, branches are shown as the number of substitutions per site (scale bars) and have >95% support based on SH-aLTR and UFboot tests, unless otherwise indicated. "Ref" represents the reference genome assembly employed for read mapping and is thus identical to Ftc555-1 reads, serving as a control to validate the SNP calling pipeline **(F)** Proposed model for the introgression of the VNBI *MAT***a** allele into VNI, shown alongside the key stages of the *C. neoformans* sexual cycle (right). Step 1: possible loss of the *MAT***a** allele in the VNI lineage, likely due to a bottleneck; step 2: mating and cell-cell fusion between a VNBI (*MAT***a**) and a VNI (*MAT*α) strain; step 3: formation of dikaryotic hyphae, nuclear fusion, meiosis, recombination, and sporulation; step 4: some of the resulting F1 progeny emerged with a ~50:50 VNBI:VNI genome composition, retaining the VNBI *MAT***a** locus; step 5: repeated backcrossing of the *MAT***a** F1 progeny to VNI *MAT*α strains progressively purging VNBI genomic regions while retaining the introgressed *MAT***a** allele; step 6: after ~10 backcrosses, the genome became ~99.95% VNI, with only the VNBI *MAT***a** locus and possibly a small fraction (~0.05%) of the VNBI genome remaining; step 7: over time, the introgressed VNBI *MAT***a** locus diverged and underwent rearrangements within the VNI background, reflecting strain-specific changes. The data underlying this Figure can be found at https://doi.org/10.5281/zenodo.14851287.

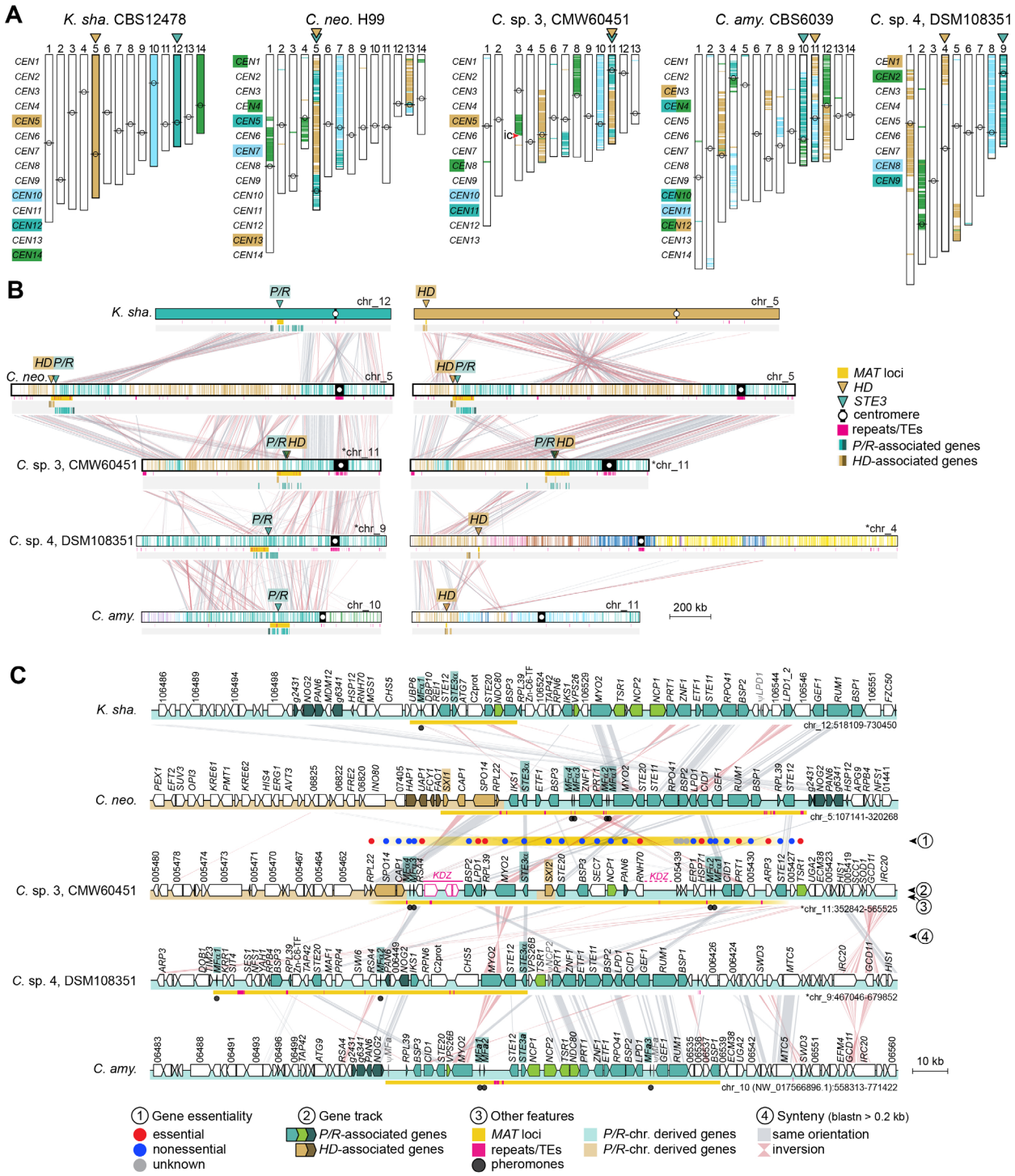


Fig 6. Transition to linked *P/R-HD* loci in *Cryptococcus* sp. CMW60451 and *Cryptococcus* pathogens. (A) The karyotype of *K. shandongensis* (with 14 chrs.) served as the reference for reconstructing synteny blocks in pairwise comparisons. For simplicity of visualization, only synteny blocks corresponding to *P/R*- and *HD*-containing chromosomes (chrs. 12 and 5, colored teal and gold, respectively) were plotted in representative *Cryptococcus* species of clades A (*C. neo.* H99), B (*C. amy.* CBS6039), C (*C.* sp. CMW60451), and D (*C. sp.* DSM108351). Besides the *MAT*-containing chromosomes, synteny blocks of

1862 two additional chromosomes in *K. shandongensis* (chrs. 10 and 14), which correspond to a large portion of
1863 *P/R* and *HD* chromosomes in *C. amylolentus* were also plotted (color-coded light blue and green,
1864 respectively). Other synteny blocks can be visualized in **S9A Fig**). A red arrowhead pinpoints the predicted
1865 location of an inactivated centromere (ic) in *Cryptococcus* sp. CMW60451. **(B)** Linear chromosome plots
1866 depicting gene synteny conservation across species with zoomed-in views depicted in **(C)**.

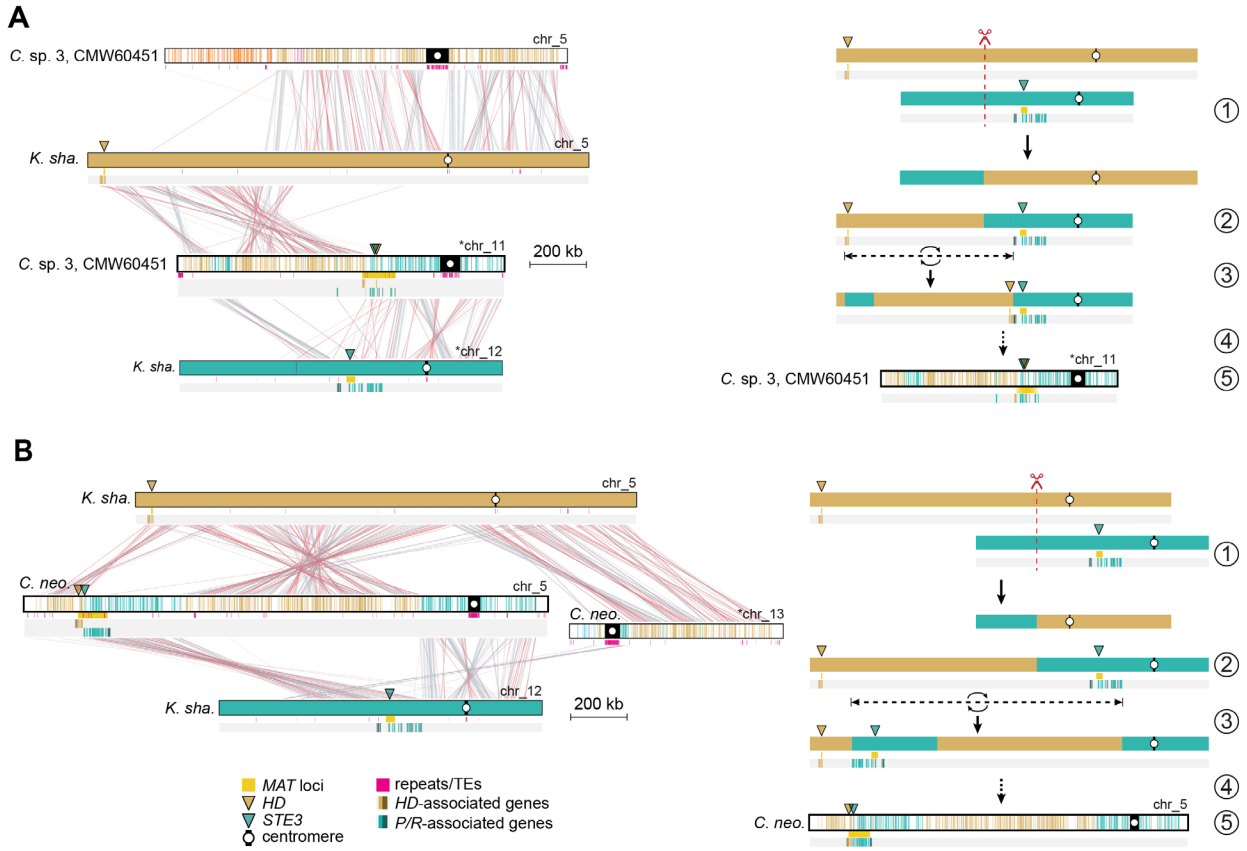


Fig 7. Hypothesized mechanisms underlying the tetrapolar-to-bipolar transitions in *Cryptococcus* sp. 3 and in *Cryptococcus* pathogens. Comparative synteny analysis between *K. shandongensis* and **(A)** *Cryptococcus* sp. 3 or **(B)** *C. neoformans*, illustrating the inferred sequence of chromosomal rearrangements leading to this transition. In both panels, the left side displays pairwise alignments, with syntenic regions connected by red (collinear) and gray (inverted) links, while the right side depicts a stepwise model of the proposed structural changes. The transition likely began with (1) an initial chromosomal break and reciprocal translocation (red dashed line), leading to (2) the repositioning of the *P/R* locus onto the same chromosome as the *HD* locus. (3) In *Cryptococcus* sp. 3, a subsequent inversion relocated the *HD* locus closer to the presumed ancestral position of the *P/R* locus, whereas in *Cryptococcus* pathogens, an inversion instead shifted the *P/R* locus toward the original location of the *HD* locus. (4) Additional structural modifications further refined the newly linked configuration, ultimately resulting in (5) the extant *MAT* locus organization observed in each species. These models suggest that despite independent evolutionary trajectories, both lineages underwent convergent genomic rearrangements that facilitated the transition to bipolar mating systems.

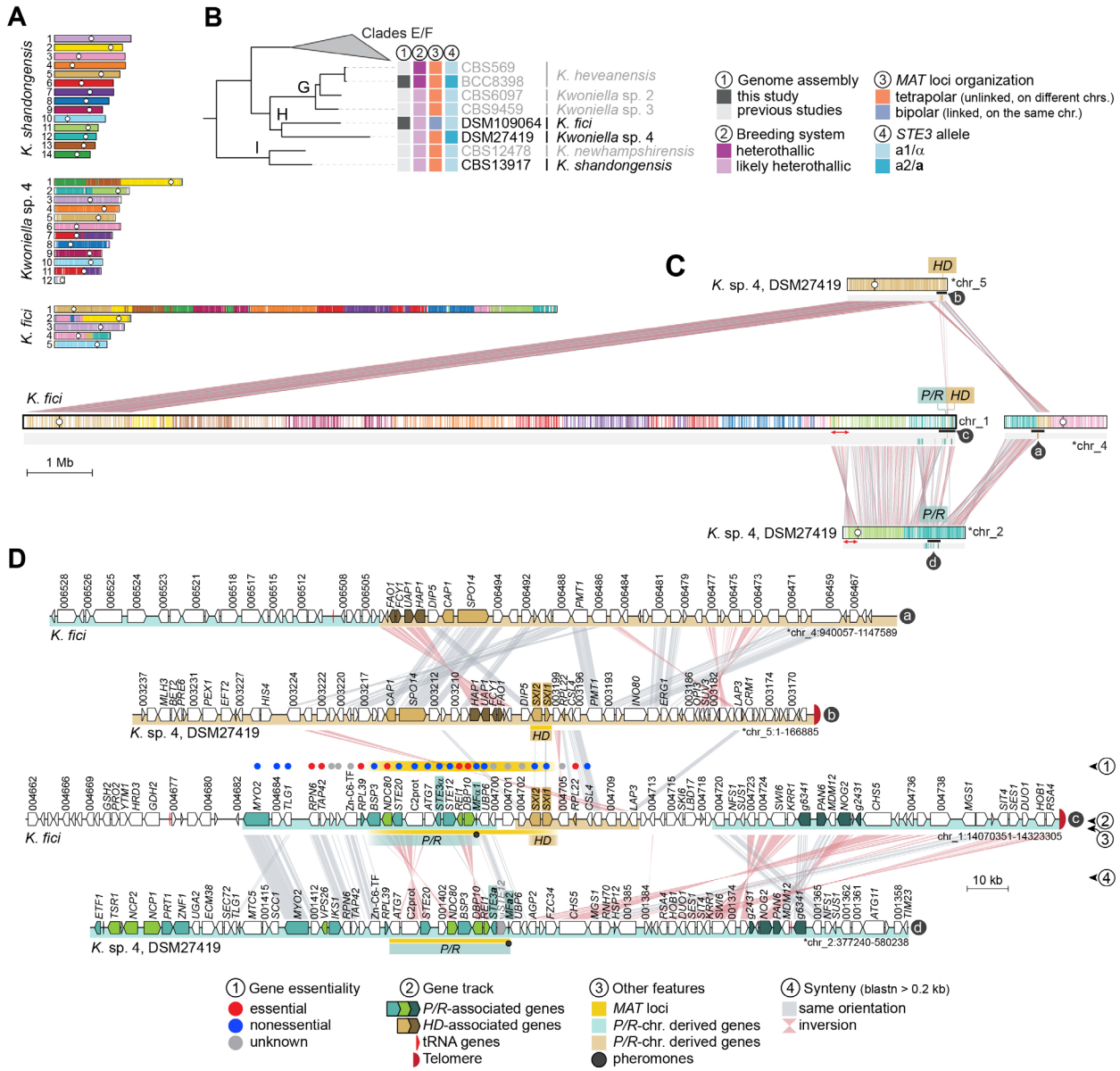
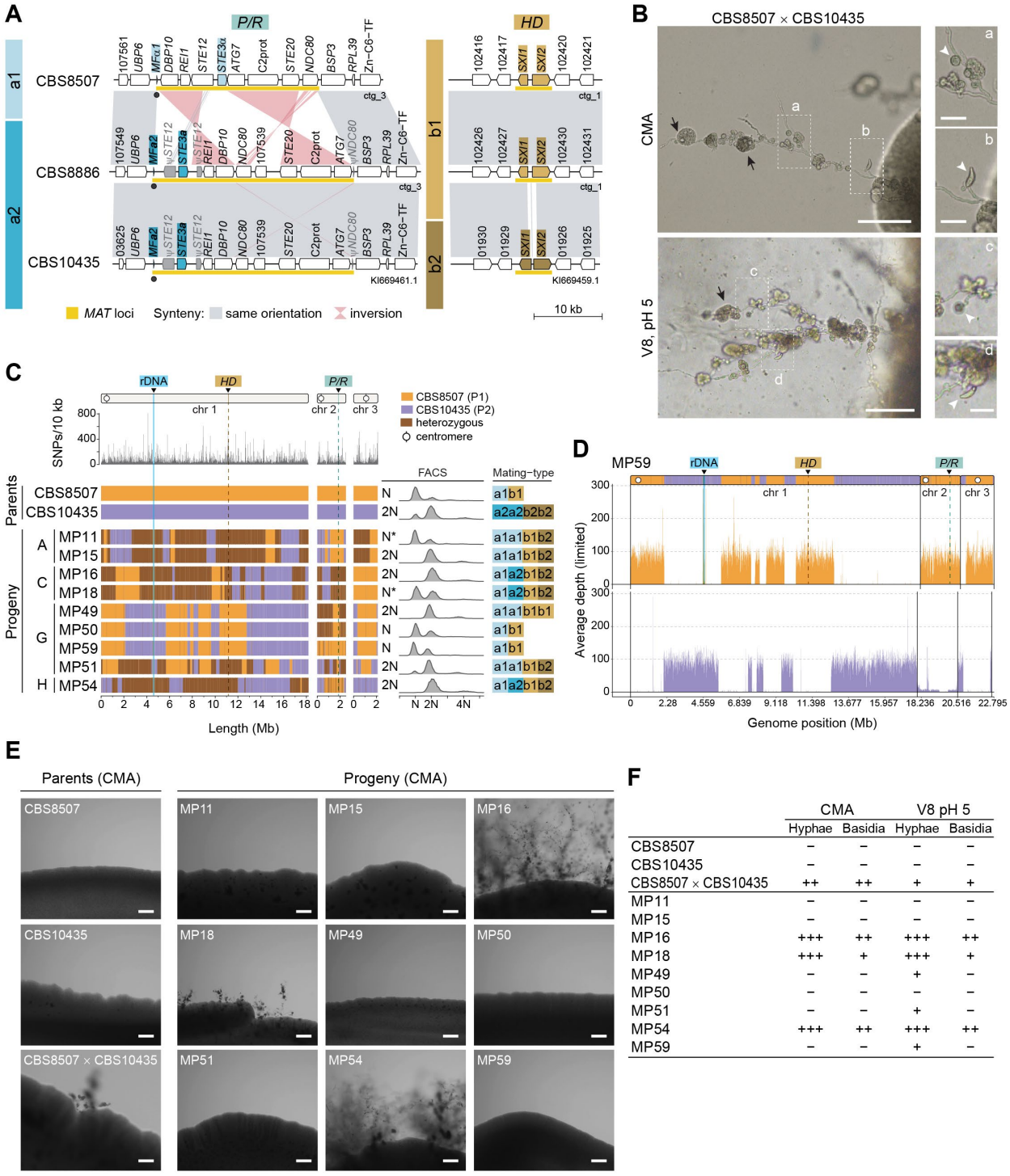


Fig 8. Linkage of *P/R* and *HD* loci in *Kwoniella fici* and *MAT* locus structure (A) The karyotype of *K. shandongensis* (with 14 chrs.) served as a reference for reconstructing synteny blocks in pairwise comparisons with *Kwoniella* sp. 4 and *K. fici*. **(B)** Phylogenetic relationships among selected *Kwoniella* species, highlighting their *MAT* locus organization and inferred breeding systems. **(C)** Synteny comparison between *K. fici* and its closest relative *Kwoniella* sp. 4, illustrating chromosomal rearrangements underlying the linkage of *P/R* and *HD* loci in *K. fici*. A red double-headed arrow marks that the centromere-proximal regions of chr. 2 in *Kwoniella* sp. 4 correspond to a region near the fusion point on the *K. fici* "giant" chromosome (chr. 1), whereas the telomere-proximal regions of *Kwoniella* sp. 4 chr. 2 align with more internalized regions. This suggests that a large pericentric inversion targeting the centromere-adjacent region is associated with this fusion event, as previously reported for other *Kwoniella* species [48]. **(D)** Detailed gene-level organization of the *MAT* locus in *K. fici* compared to *Kwoniella* sp. 4. Each track (labeled a–d in dark circles) corresponds to a specific region marked by a black bar in panel C. Chromosomes inverted relative to their original assembly orientations are marked with asterisks.



	CMA		V8 pH 5	
	Hyphae	Basidia	Hyphae	Basidia
CBS8507	–	–	–	–
CBS10435	–	–	–	–
CBS8507 × CBS10435	++	++	+	+
MP11	–	–	–	–
MP15	–	–	–	–
MP16	+++	++	+++	++
MP18	+++	+	+++	+
MP49	–	–	+	–
MP50	–	–	–	–
MP51	–	–	+	–
MP54	+++	++	+++	++
MP59	–	–	+	–

Fig 9. Sexual reproduction and recombination in *Kwoniella mangrovensis*. (A) Synteny analysis of the *P/R* and *HD* loci among three *K. mangrovensis* strains of different mating types (CBS8507, *a1b1*; CBS8886, *a2b1*; and CBS10435, *a2b2*). Note the pronounced divergence in the N-terminal domains of both *HD1* (*SXI1*) and *HD2* (*SXI2*) gene products, which are known to mediate mating-type specificity in other basidiomycetes. **(B)** Micrographs showing hyphal filaments extending from colony peripheries in a sexual cross between CBS8507 and CBS10435 after 2 weeks of incubation on CMA and V8 pH 5 media, in the

dark, at room temperature. Insets depict two types of basidia: globose (a–c) and lageniform (b–d). Black arrows indicate clusters of cells emerging near basidia (either at the surface or embedded), scored as potential meiotic progeny (basidiospores). Single cells from these clusters were isolated using a micromanipulator, cultured into colonies, and genotyped by PCR-RFLP (see S5 Appendix). Putative recombinants were further analyzed using Illumina sequencing. Scale bars = 100 μm (25 μm in insets). **(C)** Genotypic analysis of selected meiotic progeny. SNP density between the parental strains was calculated as the number of SNPs per 10 kb (top). The genotypes of 9 segregants, derived from four distinct cell clusters, were inferred from SNP data and are depicted as follows: orange for regions inherited from CBS8507, purple for regions inherited CBS10435, and brown for heterozygous regions (i.e. inherited from both parents). Instances of recombination or loss of heterozygosity (LOH) are detected by changes in genotype along the chromosomes. Discrepancies in ploidy, as inferred from FACS and sequencing read coverage, suggest potential genomic instability (marked by asterisks). Mating-type identity was inferred through sequencing, coverage analysis, and FACS. **(D)** Sequencing coverage plot for progeny MP59 with color-coded contributions from each parent. Haplotypes blocks inferred from SNP data are overlaid for each chromosome for comparison (additional progeny data is presented in S8 Fig). **(E)** Self-filamentation phenotype of *K. mangrovensis* progeny. Parental strains CBS8507 and CBS10435 (grown individually and in co-culture) and their recovered progeny were cultivated on CMA at room temperature. Self-filamentation was assessed after 2 weeks of incubation. While neither parental strain exhibited self-filamentation in solo culture, their co-culture produced hyphal filaments and basidia. Progeny MP16, MP18, and MP54 also exhibited robust self-filamentation on both CMA and V8 pH 5 (see S9Fig), whereas progeny MP49, MP51, and MP59 displayed weaker filamentous growth, detectable only on V8 pH 5 (S9 Fig). Scale bars = 200 μm. **(F)** Summary of the growth phenotype of the recovered progeny. The production of mycelium and basidia was classified as: extensive (+++), when observed across the entire periphery of the mating patch; moderate (++), when restricted to specific areas of the mating patch; poor (+), when limited to a single spot of the matching patch and slower to develop; and negative (–), when no filamentous growth was observed. Results represent observations from two independent tests.

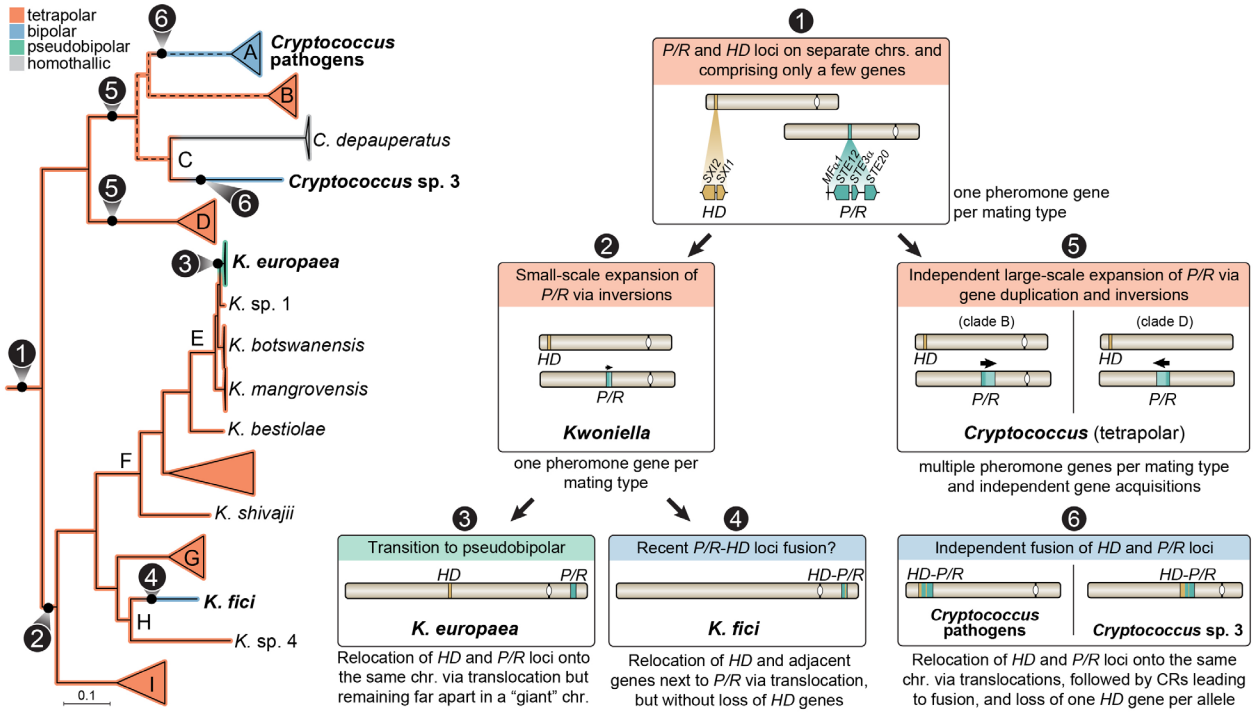


Fig 10. Summary of the evolutionary transitions in *MAT* locus organization across *Kwoniella* and *Cryptococcus* species. Schematic representation of key chromosomal rearrangements and evolutionary events underlying transitions from tetrapolar to bipolar and pseudobipolar mating configurations. The phylogenetic tree highlights inferred changes in *MAT* locus structure, with color-coded branches representing both extant and reconstructed *MAT* configurations. Dashed lines indicate unresolved phylogenetic relationships among clades A, B, and C. Insets summarize distinct evolutionary stages, including the ancestral organization of *P/R* and *HD* loci (1), small- and large-scale expansions of the *P/R* locus in *Kwoniella* and *Cryptococcus* (2, 5), relocation of *P/R* and *HD* loci onto the same chromosome leading to pseudobipolar or fused *MAT* configurations in *Kwoniella* (3, 4), and independent *HD-P/R* fusion events in *Cryptococcus* pathogens and *Cryptococcus* sp. 3 (6). These transitions highlight the diverse pathways by which chromosomal rearrangements have shaped *MAT* locus evolution in these fungal lineages.