Molecular and Genetic Evidence for a Tetrapolar Mating System in the Basidiomycetous Yeast Kwoniella mangrovensis and Two Novel Sibling Species

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Kwoniella mangrovensis has been described as a sexual species with a bipolar mating system. Phylogenetic analysis of multiple genes places this species together with Kwoniella heveanensis in the Kwoniella clade, a sister clade to that containing two pathogenic species of global importance, Cryptococcus neoformans and Cryptococcus gattii, within the Tremellales. Recent studies defining the mating type loci (MAT) of species in these clades showed that, with the exception of C. neoformans and C. gattii, which are bipolar with a single biallelic multigene MAT locus, several other species feature a tetrapolar mating system with two unlinked loci (homeodomain [HD] and pheromone/receptor [P/R] loci). We characterized several strains from the original study describing K. mangrovensis; two MAT regions were amplified and sequenced: the STE20 gene (P/R locus) and the divergently transcribed SXI1 and SXI2 genes (HD locus). We identified five different mating types with different STE20/SXI allele combinations that together with results of mating experiments demonstrate that K. mangrovensis is not bipolar but instead has a tetrapolar mating system. Sequence and gene analysis for a 43-kb segment of the K. mangrovensis type strain MAT locus revealed remarkable synteny with the homologous K. heveanensis MAT P/R region, providing new insights into slower evolution of MAT loci in the Kwoniella compared to the Cryptococcus clade of the Tremellales. The study of additional isolates from plant substrates in Europe and Botswana using a combination of multilocus sequencing with MAT gene analysis revealed two novel sibling species that we name Kwoniella europaea and Kwoniella botswanensis and which appear to also have tetrapolar mating systems.

Ungular mating-type loci (MAT) are specialized regions of the genome that determine sexual identity of haploid cells and progression through the sexual cycle (1). There is considerable interest in the genetic characterization of MAT loci due to their central role in fungal life cycles, their connection to lifestyle and virulence (viz., in human or plant-pathogenic taxa), and the impact of sexual recombination on population genetics and speciation. The discovery of remarkable convergence in the structure of sex-determining genomic regions from studies in animals, plants, and fungi illuminates the forces that shape the evolutionary trajectories of MAT loci or sex chromosomes in eukaryotes (2).

In basidiomyctetes, two major types of MAT loci have so far been recognized (3). The tetrapolar mating system of the corn smut Ustilago maydis or the mushroom Coprinopsis cinerea is governed by two small (<10-kb) unlinked loci: one encodes members of the homeodomain (HD) family of transcription factors, which heterodimerize upon mating to generate an active transcription regulator (HD locus), and the other encodes lipopeptide pheromone precursors and 7-transmembrane pheromone receptors that mediate intercellular signaling (P/R locus). Alleles at both loci must differ for mating to occur, and in many cases each locus is multiallelic; hence, numerous mating types exist in certain tetrapolar species. On the other hand, in the bipolar system of the human pathogen Cryptococcus neoformans or the barley smut Ustilago hordei, the two MAT loci found in the tetrapolar species are linked and form a single, large (>100-kb) multigene locus rich in repetitive elements and containing several additional genes, either related or unrelated to mating. In the bipolar species, there are normally only two possible versions for the content of the single MAT locus, and hence only two mating types exist. Recent studies have suggested that the basidiomycete bipolar mating systems are evolutionarily derived from tetrapolar ancestral states (4–6).

A comparative study of the MAT structure of C. neoformans and sibling taxa in the pathogenic species complex, including the analysis of gene order, phylogeny, and synonymous substitution rates, led to the proposal of an evolutionary model that posits a series of gene acquisitions, chromosomal translocations, and recombination events leading to expansion of the MAT-specific regions and linkage of the two loci of a postulated tetrapolar ancestor to form the large derived locus of the bipolar Cryptococcus species (5). The expansion of this nonrecombining sex-determining genomic region mirrors the models proposed for the genesis of sex chromosomes in multicellular eukaryotes (2). This study has since been expanded to include several other closely aligned sap-

Received 9 March 2013    Accepted 15 March 2013
Published ahead of print 22 March 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00065-13.
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doi:10.1128/EC.00065-13
robic species in the order Tremellales (class Tremellomycetes, subphylum Agaricomycotina), namely, Cryptococcus amylolentus, Cryptococcus heveanensis, Filobasidiella depauperata, and Tsuchiyaec wingfieldii (7–10). The latter studies disclosed sexual states in two species previously classified as asexual, viz. C. amylolentus and C. heveanensis (the telemorphs were named Filobasidiella amylolenta and Kwoniella heveanensis) (8, 11) and further revealed that these heterothallic species, as well as the more distantly related dimorphic jelly fungus Tremella mesenterica, all have tetrapolar mating systems with unlinked P/R and HD loci, thus providing additional support for the tetrapolar-to-bipolar evolutionary model (5, 12). The P/R locus was found to be biallelic, whereas the HD locus is multiallelic. This comparative genomics approach has also shown some conservation of gene content within MAT gene clusters. For example, the P/R locus region generally includes, besides the genes encoding pheromones (MF) and pheromone receptors (STE3), genes encoding elements of the pheromone-activated signaling cascade (STE11, STE1, STE2, and STE20), as well as other genes that may be unrelated to mating (e.g., BSP3, LPD1, RPO41). The HD locus consists of two divergently transcribed HD genes, homologous to the single SXI1 or SXI2 genes found in either of the C. neoformans mating types, and the flanking MAT-specific or nonspecific regions include additional genes common to most of the studied species (e.g., RPL22, SPO14). Information on the genes contained in those loci may be found in previous publications (3, 5, 8, 9, 12).

Kwoniella mangrovensis (the original spelling, mangroviensis, was considered an orthographical error) was described as a sexual species with a bipolar mating system based on the study of several isolates from mangrove habitats in the Bahamas and Florida Everglades (13). On the basis of phylogenetic analysis of multiple genes (14), this species belongs, together with Kwoniella (Cryptococcus) heveanensis, to the Kwoniella clade, a sister clade to that which contains the pathogenic Cryptococcus neoformans and Cryptococcus gattii, as well as C. amylolentus (Filobasidiella clade, which we henceforth rename as the Cryptococcus clade for reasons that will be explained in Discussion). In view of the results of previous studies that explored the MAT locus structure in those yeasts, it seemed essential to examine this genomic region in strains from the original description of K. mangroviensis to verify its mating system and to seek additional insights on the evolution of MAT in the Tremellales. Statzell-Tallman et al. (13) included in their study two cork isolates from Europe which had divergent internal transcribed spacer (ITS) sequences but were considered conspecific with the mangrove strains. We expanded our study to include additional isolates from different substrates and geographic locations, identified as K. mangroviensis on the basis of large subunit (LSU) rRNA gene sequences, and applied a multilocus sequencing approach to reassess species limits and disclose putative cryptic species. We also determined sequences of putative MAT genes from the same strains to elucidate the mating system and to gain additional evidence for defining species boundaries.

In this study, we provide evidence for a tetrapolar mating system in K. mangroviensis and insight into the MAT locus structure of this species, which revealed remarkable similarities to the more closely related saprobic yeasts in the Tremellales. We performed phylogenetic analyses of MAT genes and of housekeeping genes in a multilocus approach for an enlarged group of strains of K. mangroviensis and closely related species, which revealed two hitherto unrecognized taxa comprising strains from plant-related sub-

strates in Europe and Africa. The results obtained clearly illustrate the power of the combined analysis of housekeeping genes and MAT genes in resolving species boundaries and providing insights into the mating systems of species whose sexual status may or may not be known.

MATERIALS AND METHODS

Yeasts strains and media. The yeast cultures used in this study are listed in Table S1 in the supplemental material. Strains were obtained from the following culture collections: Portuguese Yeast Culture Collection, Portugal (PYCC); Agricultural Research Service Culture Collection, United States (NRRL); The Spanish Type Culture Collection, Spain (CECT); Centraalbureau voor Schimmelcultures, The Netherlands (CBS); Culture Collection of Industrial Microorganisms, Slovenia (ZIM); (Agro) Industrial Fungi & Yeasts Collection, Belgium (MUCL). All isolates were grown and maintained on MYP medium at 25°C and 4°C, respectively. MYP medium contained 0.7% (wt/vol) malt extract, 0.25% (wt/vol) Soytone, 0.05% (wt/vol) yeast extract, and 1.5% agar.

Mating experiments. To test for sexual compatibility, isolates were grown on MYP agar plates at 25°C for 3 days. Pairs of cell suspensions were then inoculated and mixed together on plates containing CMA, YCB, or MEA medium at 25°C and examined with a phase-contrast microscope (Zeiss, Germany) for the presence of filaments and sexual structures, up to 4 weeks of incubation. CMA medium contained 1.5% (wt/vol) corn meal agar (Difco) and 0.5% (wt/vol) agar. YCB medium contained 1.17% (wt/vol) yeast carbon base (Difco) and 2% agar. MEA medium contained 2.5% (wt/vol) malt extract (Difco) and 2% (wt/vol) agar.

Fluorescence microscopy and staining. Cells and mycelial structures from positive matings were stained with Calcofluor white (0.05% solution; BD) and Sytox green (5 mM; Invitrogen) to detect the cell wall and nuclei, respectively, using the protocols described by Metin et al. (9).

FACS analysis. The fluorescence-activated cell-sorting (FACS) protocol was modified from Tanaka et al. (15). Cells were grown overnight at 25°C in yeast extract–peptone–dextrose (YPD) broth, collected by centrifugation, and washed with 1 × phosphate-buffered saline (PBS). Cells were then fixed in 1 ml of 70% ethanol overnight at 4°C with mild agitation. Upon centrifugation, cells were resuspended and washed with 1 ml of NS buffer (10 mM Tris-HCl [pH 7.2], 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.55 mM phenylmethylsulfonyl fluoride, 0.1 mM ZnCl₂, 0.049% 2-mercaptoethanol). Cells were then resuspended in 180 µl NS buffer with 14 ml RNase A (15 mg/µl), and 6 ml of propidium iodide (1.0 µg/µl; CALBIOCHEM) and incubated in the dark for 2 to 4 h at room temperature. After incubation, 50 µl of the cells were mixed with 500 ml of Tris-PI mix (482 µl 1 M Tris [pH 7.5] and 18 µl propidium iodide, 1 µg/µl). Flow cytometry was performed on 10,000 cells with a slow laser scan, on the FL1 channel with a Becton, Dickinson FACSScan. Selected haploid and diploid C. neoformans reference strains were used as controls for ploidy determination.

Physiological characterization. Physiological tests were performed on sterile 96-well microplates (NunclonΔ Surface, Denmark) according to Kurtzman et al. (16) and read with a StatFax 2100 microplate reader (Awareness Technology Inc., United States) using absorbance measures at 630 nm. Media were prepared according to a protocol available at the CBS website (http://www.cbs.knaw.nl/collections/DefaultInfo.aspx?page = YeastMethods). The inoculum was grown overnight on liquid MYP medium at 25°C, in a Certomat U orbital shaker (Sartorius, Germany) at 48 rpm, and diluted in sterile distilled water for microplate inoculation. Each well contained 200 µl of medium and 5 µl of diluted inoculum (initial absorbance of 0.04). After inoculation, the microplates were sealed with a sealing pellicle (NunclonΔ Surface, Denmark) and were incubated at 25°C in a Denley WellWarm 1 (Denley) microplate incubator with shaking, up to 3 weeks. Each strain was tested in independent duplicates. The ability to grow at 30°C, 35°C, and 37°C was determined by inoculating MYP slants with each culture and incubating in water baths at the appropriate temperature for up to 4 days.

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DNA extraction. Genomic DNA for MAT gene amplification and sequencing and for MLST was obtained using a simplified phenol-chloroform extraction method followed cell disruption using glass beads. Genomic DNA for fosmid library preparation was obtained using the cetyltrimethylammonium bromide (CTAB) extraction method described by Metin et al. (9).

Degenerate PCR. Identification of potential MAT genes in K. maragroensis relied on the design of degenerate primers for moderately conserved MAT genes in C. neoformans and relatives. Sequences of homologs of several Cryptococcus MAT genes, CAP1, LPD1, RPL22, RPO41, STE3, STE12, and STE20 (pheromone cluster) and SX11 and SX12 (HD cluster), from C. neoformans, C. gattii, C. amylolentus, K. (C.) heveanensis, and T. wingfieldii were aligned with BioEdit 7.0.9.0 software (17), using the built-in ClustalW version 1.4 (18) with default parameters. The newly designed primers were tested for PCR amplification and sequencing of putative MAT gene fragments with K. maragroensis opposite mating type strains CBS 8507 (=K01) and CBS 10435 (=K02). The newly obtained sequences were checked for mating-type-specific polymorphisms, which are expected only for genes within MAT. Those sequences were also used to design new, less, or nondegenerate primers, specific for K. maragroensis, namely, for STE20 and SX11/SX12. PCR products with the expected size were gel extracted or directly purified using the QiAquick gel extraction kit (Qiagen) or Illustra GFX PCR DNA and the gel band purification kit (GE Healthcare), according to the manufacturer’s instructions. In some cases, an intermediate cloning step was necessary prior to sequencing and used the TOPO TA cloning kit (Invitrogen), as described by Metin et al. (9). Primers and PCR amplification conditions used in this study are presented as supplemental material (see Text S1 in the supplemental material). Primers were synthesized by Integrated DNA Technologies (United States) or STABVida (Portugal). To generate fosmid libraries from K. maragroensis strains CBS 8507 (K01) and CBS 10435 (K02), using the procedure described by Findley et al. (8). Approximately 1,500 fosmid clones were picked into 96-well plates and transferred to 384-well plates for long-term storage at −80°C. The 384-well plates were replicated onto high-density filters for hybridizations using MAT gene probes prepared from the amplicons obtained using degenerate PCR, as described by Metin et al. (9). Positive fosmids were confirmed using colony PCR with specific primers for K. maragroensis.

Fosmid library preparation and probing. We employed the Copy Control Fosmid Library production kit (Epipcentric, Madison, WI) to generate fosmid libraries from K. maragroensis strains CBS 8507 (K01) and CBS 10435 (K02), using the procedure described by Findley et al. (8). Approximately 1,500 fosmid clones were picked into 96-well plates and transferred to 384-well plates for long-term storage at −80°C. The 384-well plates were replicated onto high-density filters for hybridizations using MAT gene probes prepared from the amplicons obtained using degenerate PCR, as described by Metin et al. (9). Positive fosmids were confirmed using colony PCR with specific primers for K. maragroensis.

MLST analysis. Multilocus sequence typing (MLST) of selected strains of K. maragroensis was based on the following nuclear loci: D1/D2 domains of the LSU rRNA gene (LSU), ITS1-5.8S rRNA gene-ITS2 (ITS), a fragment of the gene encoding the largest subunit of RNA polymerase II (RPB1), a fragment of the gene encoding the second-largest subunit of RNA polymerase II (RPB2), a fragment of the gene encoding translation elongation factor 1 alpha (TEFI), and a fragment of the gene encoding a licensing factor required for DNA replication initiation and cell proliferation (MCM7). The first five loci have been previously used as phylogenetic markers for fungi (e.g., by the Assembling the Fungal Tree of Life [AFTOL] consortium [19]), and MCM7 was proposed more recently for the same purpose by Schmitt et al. (20). The RPB1 and TEF1 gene fragments contained two and one intron, respectively, whereas RPB2 and MCM7 did not include introns. Specific or degenerate primers were ordered or designed as needed and used to amplify and sequence the six loci. MLST primer information and PCR conditions are available in Text S1 in the supplemental material.

DNA sequencing. Purified PCR amplification products were sequenced by STABVida (Portugal). Primers used for PCR amplification were also used for sequencing, except for ITS and LSU, in which case internal primers ITS1 (21) and NL4 (22), respectively, were used, and for RPB2 only the reverse primer was used for sequencing, yielding a <700-bp sequence. Fosmid DNA was extracted with Jet Starr, the novel plasmid purification system (Genomed), according to the manufacturer’s instructions. Fosmid DNA was sequenced and assembled by STABVida (Portugal), using next-generation sequencing technology.

Bioinformatic and phylogenetic analyses. Annotation of the different genomic loci was done manually by comparing the obtained sequences with the annotated sequences of C. neoformans var. neoformans JEC21 and/or C. heveanensis CBS 569. The comparison was done by pairwise alignment (BioEdit 7.0.9.0) and using BLASTn, BLASTp, BLASTx, or tBLASTx (2.2.26 version) (23) searches. Multiple sequence alignments were performed with the genomic sequences using either MUSCLE (24) or the ClustalW built-in version of MEGA version 5.05 (25) with the default parameters. Independent alignments and phylogenetic analyses were performed for each locus. For MLST, independent alignments were concatenated manually for each strain. Phylogenetic relationships were inferred by the maximum likelihood (ML) method based on the general time reversible (GTR) model (26) from 1,000 bootstrap replicates, using gamma distributed of 5 discrete gamma categories, all sites of the data set, all the codon positions and noncoding sites, and nearest-neighbor interchange (NNI) as the ML heuristic method in MEGA version 5.05. To detect recombination in the MLST data set, we used the D statistics test of Bruen et al. (27) implemented in the SplitsTree software (28). For phylogenetic analysis of MAT genes, the neighbor-joining method based on the Kimura 2-parameter model or the ML method as detailed above were employed, also using MEGA version 5.05. Sequences from GenBank were used when available. Accession numbers for sequences used for MLST and MLST analyses are listed in Table S1 in the supplemental material. Sequences from C. neoformans var. neoformans JEC20 (MATa) and JEC21 (MATa), C. neoformans var. grubii 125.91 (MATa) and H99 (MATa), C. gattii E566 (MATa) and WM276 (MATa), and C. heveanensis CBS 569 (A1B1) and BCC 8398 (A2B2) were all retrieved either from GenBank or DOE Joint Genome Institute (JGI), while all sequences from Tremella mesenterica ATCC 24923 were retrieved from JGI, and therefore no accession number is provided. Upon annotation of the fosmid insert sequence, synteny analysis was based on comparison of gene order and orientation in the homologous region of closely aligned species.

Nucleotide sequence accession numbers. Obtained DNA sequences of MLST loci and MAT genes were submitted to EMBL under the accession numbers given in Table S1 in the supplemental material. Sequences of the STE3/STE12 gene fragments were submitted to EMBL under the following accession numbers: HF564892 (strain K06), HF564893 (K27), HF564894 (K10), HF564895 (K25) and HF564896 (K29). The annotated sequence of the P/R locus fragment of strain K01 (fosmid insert) was submitted to EMBL under accession number HE997060.

RESULTS

Mating experiments. We performed crosses between the K. maragroensis strains listed in Table S1 in the supplemental material in several possible combinations, using strains K01, K02, and K06 as the main mating type testers. In some cases, dikaryotic hyphae with basidia in clusters as described by Statzell-Tallman et al. (13) were formed, and those crosses were considered fertile (Fig. 1A). Nuclear staining confirmed the presence of dikaryotic hyphae, young dikaryotic basidia (Fig. 1B), and mature basidia with four mononucleated compartments (data not shown). Strains K04, K05, and K06 were self-filamentous, but filaments produced in monoculture were highly branched, irregular in shape, and lacked clamp connections (data not shown). The observed mating pattern (Fig. 1A) is only partially concordant with the mating type status designated for each strain by Statzell-Tallman et al. (13) and is not compatible with a bipolar system as originally proposed. For example, K02 (originally designated MAT A) crossed with K01 and K06 (originally designated MAT alpha) but not with K04 or K05, and the latter crossed with K06 but not K01. If K04 or K05 were MAT A strains, they should mate with K01 and K06. The mating type status of each strain revealed by the MAT gene strat-
egy (see below) demonstrated a tetrapolar mating system that is compatible with the observed results. No fertile crosses were observed with strains K09, K10, K11, K14, K16, K25, K26, K28, and K29 in any of the tested combinations: all nine strains were crossed with K01, K02, and K06; K10, K11, K14, and K16, and K09, K25, K28, and K29, were crossed in all combinations; K10 and K14 were crossed with K09, K25, K28, and K29. Filamentous structures consisting mainly of branched pseudohyphae were produced by strain K22 in monoculture.

**Phylogeny and species limits of *K. mangrovensis***. A multilocus sequence typing (MLST) approach was applied to a set of 14 strains previously identified as *K. mangrovensis* on the basis of the sequences of the D1/D2 domains of the LSU rRNA gene (*LSU*). The set of strains comprised five strains included in the original study of Statzell-Tallman et al. (13), four seawater isolates from the Bahamas (K01, K02, K04, K06) and one cork isolate (CECT 11955 = K25), and nine additional strains: one clinical isolate from the United States (K09), four isolates from Mopane trees in Botswana, one seawater isolate from the British Virgin Islands (CBS 11279 = K27), one isolate from grape juice in Switzerland (CBS 7868 = K28), one isolate from grape berries in Slovenia (ZIM 605 = K29) obtained by Cadež et al. (29), and one isolate from a tree hollow in Brazil (K30) (see Table S1 in the supplemental material). The isolates from the Florida Everglades and the beach isolate reported by Statzell-Tallman et al. (13) were no longer viable (J. W. Fell, personal communication) and could thus not be studied. FACS analyses showed that strains K01, K02, K04, K06, K09, K10, K14, K17, K21, K25, K28, K29, and K30 are all haploid (data not shown). Preliminary results of PCR fingerprinting with all the strains previously identified as *K. mangrovensis* listed in Table S1 in the supplemental material (data not shown) demonstrated that some strains were indistinguishable based on fingerprints and therefore closely related (e.g., K04 and K05, K10 and K11, K25 and K26). We thus selected genetically distinct strains or those from different samples or geographic origins for the MLST analysis. We also included in the MLST analysis the taxa studied by Findley et al. (14) but added two recently described species in the *Kwoniella* clade: *Cryptococcus pinus* (30), including the type strain as well as a recent isolate from insect larvae in Belgium (MUCL 53261; see Table S1 in the supplemental material), and *Cryptococcus shivajii* (31). *Tremella mesenterica* served as an outgroup for the analysis. We performed a preliminary phylogenetic analysis of *LSU* and *ITS* with all sequences available in GenBank and found three other recently described species—*Cryptococcus cuniculi*, *Cryptococcus tronadorensis*, and *Kwoniella shandongensis*—to be loosely linked to the *Cryptococcus* and *Kwoniella* clades (see Fig. S1 in the supplemental material). However, since they formed a weakly supported lineage separate from the two main clades, we have not included them in the MLST analysis.

The following six loci were used for the MLST analysis: *LSU*, *ITS*, *RPB1*, *RPB2*, *TEF1*, and *MCM7*. We were able to amplify and sequence most of the loci for all taxa; accession numbers of the obtained sequences are given in Table S1 in the supplemental material. However, we were unable to amplify *MCM7* from *K. kevicanensis*, *F. depauperata*, or *T. wingfieldi*. Firstly, we performed phylogenetic analyses, using maximum likelihood (ML), of the individual loci (see Fig. S2 to S4 in the supplemental material). We assessed congruence of the different trees by checking possible conflicts in nodes with >70% bootstrap support as proposed by

![FIG 1 Mating in *K. mangrovensis*. (A) Results of mating experiments with *K. mangrovensis* strains: Y, formation of dikaryotic hyphae and basidia; N, no filamentous structures observed; empty cells, not determined; *, self-filamentous (see the text); M.T., molecular mating type designation according to MAT gene analyses. (B) Micrographs of mating between strains K01 and K02. Dikaryotic hyphae with clamp connections and young basidia; pairs of nuclei (arrows) are seen in the hyphae and basidia. Scale bar represents 10 μm. Upper panel, phase contrast image; central panel, fluorescence image showing cell walls and septa stained with Calcofluor white; lower panel, fluorescence image showing cell walls, septa, and nuclei stained with Calcofluor white and Sytox green.](ec.asm.org/749)
Hillis and Bull (32). We found only limited conflict in the nodes connecting *Cryptococcus bestiolae*, *Cryptococcus dejecticola*, and *Bullera dendrophila* in the TEF1 and MCM7 trees (see Fig. S4A and B in the supplemental material). Phylogenetic analyses of the concatenated sequences of five loci (Fig. 2) or of six loci (excluding the three above-mentioned species; see Fig. S5 in the supplemental material) generated trees in which most branches had strong bootstrap support and confirmed the two main lineages recognized by Findley et al. (14): the *Cryptococcus* clade, which comprises the pathogenic species complex, as well as *C. amylolentus*, *T. wingfieldii*, and *F. depauperata*; and the *Kwoniella* clade, which comprises *K. mangrovensis*, the remaining *Cryptococcus* species (including the two strains of *C. pinus* and *C. shivajii*), and *Bullera dendrophila*. *Cryptococcus bestiolae* was frequently the *K. mangrovensis* closest relative (except in the TEF1 tree; see Fig. S4A), and *K. heveanensis* appeared to be basal to the *Kwoniella* clade, but with moderate bootstrap support. Moreover, in phylogenetic trees of individual loci, namely, LSU, ITS, and TEF1 (see Fig. S2A and B)

FIG 2 Phylogenetic relationships among *K. mangrovensis* and selected members of the Tremellales based on the analysis of concatenated sequences of five genomic loci (LSU, ITS, RPB1, RPB2, and TEF1). Analysis performed with 3,457 positions in the final data set. The tree was constructed using the maximum likelihood method implemented in the MEGA 5.05 software. Numbers on branches are bootstrap values (>50) from 1,000 replicates. *T. mesenterica* was used as the outgroup in the analysis. T, type strain. GenBank/EMBL accession numbers of the sequences used in the analysis are listed in Table S1 in the supplemental material; additional sequences were retrieved from JGI.
and S4A), *K. heveanensis* was not or only weakly connected to the *Kwoniella* clade.

The phylogenetic analysis of the combined MLST loci (Fig. 2) further revealed significant genetic heterogeneity among *K. mangrovensis* strains that clustered into three strongly supported and closely related lineages: one includes the type strain (K01) as well as K02, K04, K06, and K27; another includes K10, K14, K17, and K22; and the third includes K25, K28, and K29. The three lineages are also apparent when the *Kwoniella* botswanensis MAT locus was included in the analysis (see Fig. S5 in the supplemental material). Moreover, those three lineages are genetically distinct in each of the six loci, except LSU (see Fig. S2A in the supplemental material), and the strains of each lineage clustered consistently on each tree (see Fig. S2 to S4), thus suggesting an absence of genetic exchange between the three lineages. We have applied the *Φ*₂, statistical test (27) to the 6-gene data set of the 12 *K. mangrovensis* strains mentioned above and found no significant evidence for recombination. However, inclusion of strains K09 and K30 in the same test resulted in a significant probability for recombination. The latter two strains occupied inconsistent or intermediate positions on the trees (Fig. 2; see also Fig. S2 to S4) and will be further discussed below. Nucleotide differences in the coding regions of the four protein-coding genes between strains of the three above-mentioned lineages were almost always synonymous, and in RPB1 and TEF1, most of the nucleotide differences were located in the introns (data not shown). The phylogenetic trees depicted in Fig. 2 and Fig. S2 to S5 suggest that *K. mangrovensis* should be confined to the strongly supported clade represented by the five strains isolated from seawater in tropical mangrove regions of the Atlantic, while the other two sister lineages, represented by the three European isolates and the four Botswana isolates, respectively, correspond to two additional sibling species, for which the names *Kwoniella europaea* and *Kwoniella botswanensis* are proposed (Fig. 2). This proposal is supported by the results of the mating experiments, because fertile crosses were observed only among the strains of *K. mangrovensis* but not between strains of *K. mangrovensis* and any of the two novel taxa. Crosses among strains of *K. europaea* or *K. botswanensis* were, however, infertile, and thus we are unable to confirm their conspecificity based only on mating assays. In spite of the apparent absence of sexual reproduction in either *K. europaea* or *K. botswanensis*, their phylogenetic placement in the *Kwoniella* clade (Fig. 2) argues for their inclusion in the genus *Kwoniella* in view of the recent implementation of the “one fungus, one name” rule in fungal nomenclature (33). Based on physiological test results, the three species are similar (see Table S2). However, *K. mangrovensis* can be distinguished from *K. europaea* and *K. botswanensis* by the inability of the former to grow at 35°C and to assimilate L-tartaric acid. *K. europaea* and *K. botswanensis* differ only in the assimilation of alpha-methyl-glucoside (fast in the latter and delayed in the former) and L-tartaric acid.

Evolutionary distances between the three sibling *Kwoniella* species calculated on the basis of the observed nucleotide differences in the six MLST loci are presented in Fig. 3 (distance bars). The distances between *K. mangrovensis* and *K. europaea* or *K. botswanensis* were much higher than the distance between *C. amylolentus* and *T. wingfieldii* and slightly higher than the distance between *C. neoformans var. neoformans* and *C. neoformans* var. *grubii*, which supports a separate taxonomic status for the former species. As for *K. europaea* and *K. botswanensis*, the calculated distance between them is lower than the distances of any of the two to *K. mangrovensis* and slightly higher than the distance between *C. amylolentus* and *T. wingfieldii*. In the absence of additional data, a varietal status could be assigned to the two sister taxa. However, the observed congruence of MLST gene genealogies and MAT gene data (see below) support separation of *K. europaea* and *K. botswanensis* at the species level. Formal descriptions of the newly proposed species are presented in the Taxonomy section below.

The two remaining strains (K09 and K30) formed a separate

![FIG 3 Jukes-Cantor distances based on concatenated sequences of five loci. Distances were computed among or between the following taxa: *K. botswanensis* (Kb), *K. mangrovensis* (Km), *K. europaea* (Ke), *C. pinus* (Cp), *C. amylolentus* (Ca), *T. wingfieldii* (Tw), *C. neoformans* var. *neoformans* (Cnn), *C. neoformans* var. *grubii* (Cng), and *C. gattii* (Cg). Values indicated above each bar represent the average distances; vertical range lines represent extreme values when different strains of the same taxon were compared.](image-url)
branch that is positioned next to *K. europaea* and *K. botswanensis* in the combined MLST trees (Fig. 2; see also Fig. S5 in the supplemental material). Strain K30 had unique alleles for all MLST loci, except for *RBP2*, which had an identical sequence to that of K09; however, K09 shared the alleles of *LSU*, *ITS*, and *MCM7* with *K. europaea*. FAC5 analyses demonstrated that both strains are haploid (data not shown). We have thus refrained from assigning a definite taxonomic status to either of these two anomalous strains until additional strains and respective genetic data are available to resolve if they are interspecies hybrids or novel taxa. As for the two strains of *C. pinus* studied and in spite of their different origins (see Table S1 in the supplemental material), they had only a few differences in the sequences of three of the six MLST loci and were thus considered conspecific (Fig. 2 and 3; see also Fig. S2 to S5 in the supplemental material).

**Identification of MAT genes in *K. mangrovensis* and sibling species.** Our degenerate PCR-based strategy enabled us to amplify fragments of genes with homologs in the *C. neoformans* MAT locus from two compatible mating strains of *K. mangrovensis*, K01 and K02, for the following loci: *LPD1*, *RPO41*, *STE20*, and *SXI*. In the latter case, we actually amplified a fragment containing the 5′ regions of the *SXI1* and *SXI2* genes, as well as the intergenic spacer (this fragment will be henceforth designated *SXI*), because each primer was anchored in the conserved homeodomain region of each gene. The orientation of the single primer combination that produced the expected amplicon indicates that *SXI1* and *SXI2* are adjacent and divergently transcribed in *K. mangrovensis*, as found previously in the tremellaceous tetrapolar species *C. amylolentus*, *K. heveanensis*, and *T. mesenterica* (8, 9). In the case of *STE20*, we amplified two fragments of the gene: one containing the kinase domain, toward the 3′ end of the gene (~450 bp), and the other spanning the Cdc42/Rac interactive binding (CRIB) domain, upstream of the kinase domain and containing one intron (~1,000 bp). Possible linkage to MAT was verified by comparing the sequences of each gene fragment from the two compatible strains of *K. mangrovensis*, because only genes inside MAT are expected to exhibit marked mating-type-specific polymorphisms. Significantly different alleles were found for *STE20* and *SXI* (e.g., 89% sequence similarity for the *STE20* kinase 450-bp fragment; 74% similarity for the *SXI2* 673-bp fragment) but not for *LPD1* or *RPO41* (99% sequence similarity for the *LPD1* 388-bp fragment; 99.7% sequence similarity for the *RPO41* 909-bp fragment). In view of previous results on MAT structure of closely related species in the *Cryptococcus* and *Kwoniella* clades (12) and the observed mating-type-specific polymorphisms of *STE20* and *SXI*, we predict that the former is located in the P/R region of MAT and the latter in the HD region. To confirm MAT specificity of those genes and to verify the mating system, we amplified and sequenced the *STE20* (CRIB-containing region) and *SXI* gene fragments from the full set of strains previously identified as *K. mangrovensis* (see Table S1). We were also able to amplify and sequence the same loci for other species in the *Kwoniella* clade, *B. dendrophila*, *C. bestioleae*, *C. dejecticola*, *C. pinus*, and *C. shinivajii* (for the latter two species and *B. dendrophila*, we could not amplify *SXI*). Accession numbers of the obtained sequences are given in Table S1 in the supplemental material. Phylogenetic analyses of the sequences of each locus are presented in Fig. 4 (*STE20*) and 5 (*SXI*). We were able to identify two *STE20* alleles in *K. mangrovensis* (KmA1 in strains K01, K03, K06, K07, and K27; and KmA2 in strains K02, K04, K05, and K08), two additional alleles in *K. botswanensis* (KbA1 in strains K10, K11, and K22; and KbA2 in strains K14, K16, K17, K20, K23, and K24), and two more alleles in *K. europaea* (KeA1 in strains K25, K26, and K29; and KeA2 in strain K28) (see Table S1; Fig. 4). Strains K09 and K30 each had unique *STE20* alleles. We also obtained an *STE20* sequence from the mating type A2 strain of *K. heveanensis* and found that it is mating type specific (67% similarity to the sequence of the A1 allele), in contrast with the findings of Metin et al. (9), who predicted that *STE20* was not located within MAT.

Observed nucleotide differences in *STE20* sequences between mating types of the same species were much lower in the three sibling *Kwoniella* species (ranging from 34 to 69) than in *K. heveanensis* (327) or the pathogenic *Cryptococcus* species (403 to 412). Nonetheless, nucleotide differences located in the coding sequence in the *Kwoniella* species were not always synonymous and led to 5 to 7 amino acid substitutions in the translated sequences between mating types (data not shown). The fact that mating type specificity of *STE20* is less pronounced in the three *Kwoniella* species is also apparent in the phylogenetic tree depicted in Fig. 4. In fact, the topology of the *Kwoniella* clade is more similar to the species phylogeny as obtained from the MLST analysis (Fig. 2), whereas among the species of the *Cryptococcus* clade, *STE20* follows a mating-type-specific phylogeny. Interestingly, whereas both *STE20* alleles of *K. mangrovensis* formed a single, well-supported clade, those of *K. botswanensis* and *K. europaea* seemed to follow a mating-type-specific phylogeny, with the KbA1 allele clustering with the KeA1 allele and vice versa (Fig. 4).

For the *SXI* region, we identified four alleles in *K. mangrovensis* (KmB1 in strains K01, K03, K04, K05, and K08; KmB2 in strain K02; KmB3 in strains K06 and K07; and KmB4 in strain K27), a single allele in *K. botswanensis* (KbB1), and three different alleles in *K. europaea* (KeB1 in strains K25 and K26; KeB2 in strain K28; and KeB3 in strain K29) (Fig. 5; see also Table S1 in the supplemental material). The finding of distinct alleles of the two putative MAT genes (*STE20* and *SXI*) in *K. mangrovensis*, *K. botswanensis*, and *K. europaea* provides further key support for their separation at the species level. For the two anomalous strains, K30 had a unique *SXI* allele, and K09 surprisingly shared one of the *SXI* alleles (KeB1) of *K. europaea* (Fig. 5), which hints to the possibility of this strain being a hybrid. The topology of the *SXI* tree (Fig. 5) reflects the high level of polymorphism of the 5′ regions of the *SXI* genes as observed in previous studies of related species (8, 9) that classified these genes as mating type unique, in contrast to the mating-type-specific or species-specific phylogenies displayed by other MAT genes.

Assuming that *STE20* belongs to the P/R locus, the latter appears to be biallelic in each sibling species, whereas the HD locus defined by the *SXI* alleles appears to be multiallelic, at least for *K. mangrovensis* and *K. europaea*. These results are compatible with the hypothesis that the three sibling *Kwoniella* species are tetrapolar. This hypothesis is further corroborated by the fact that the alleles at both loci are not linked, as they are found in different combinations in the different strains of each species (Fig. 4 and 5; see also Table S1 in the supplemental material). We have in fact identified five different mating types with different *STE20/SXI* allele combinations in *K. mangrovensis*, three different mating types in *K. europaea*, and two in *K. botswanensis* (see Table S1). The molecular mating types are fully concordant with the results of mating experiments for *K. mangrovensis* (Fig. 1A; see also Table S1). On the other hand, in *K. europaea*, we would expect fertile
matings between strains K25 and K28 or between K28 and K29. However, we did not observe any mating reactions with all four K. europaea strains. This result could be due to the fact that the tested strains are sterile or to nonoptimal mating conditions, but we were not able to confirm any of these hypotheses. Finally, in K. botswanensis, one likely explanation for the absence of observed sexual reproduction is that all studied strains have the same SXI allele (KbB1).

We tried to design degenerate primers to amplify also the STE3 and STE12 genes, which belong to the P/R locus in all heterothallic species of the Tremellales studied to date (12). This approach proved difficult due to the high sequence variability of those genes across species. However, we observed that in most taxa for which MAT structure has been elucidated, the two genes are almost always adjacent, although their relative orientation varies across different species. We designed one primer in the conserved domains of each gene and ordered also the reverse complement of each primer so as to perform PCR amplifications using all four possible primer combinations (see Text S1 in the supplemental material). We were able to obtain STE3 and STE12 gene fragments with only one primer combination from all KmA1 strains of K. mangrovensis tested but not from any of the KmA2 strains. The relative orientation of the successful primer pair suggests that the STE3 and STE12 genes are adjacent and divergently transcribed, at least in KmA1 strains of K. mangrovensis. The STE3/STE12 gene fragments had identical sequences in the KmA1 strains analyzed (K01, K06, K27). We were also able to amplify and sequence with the same primer pair STE3/STE12 gene fragments from two KeA1 strains of K. europaea (K25, K29) and one KbA1 strain (K10) of K. botswanensis (see Fig. S6 in the supplemental material) but not from the KeA2 or KbA2 strains. These results constitute further evidence that the STE20, STE3, and STE12 genes identified define the alleles of the P/R locus in the three sibling species.

Genetic structure of the P/R locus of K. mangrovensis. To further define the P/R locus, fosmid libraries were screened with

FIG 4 Phylogenetic tree based on STE20 coding sequences (CRIB-containing gene fragment) of K. mangrovensis and selected members of the Cryptococcus and Kwoniella clades. Analysis performed with 853 positions in the final data set. The trees were constructed using the maximum likelihood method implemented in the software MEGA 5.05. Numbers on branches are bootstrap values (>50) from 1000 replicates. T. mesenterica was used as the outgroup in this analysis. T, type strain. GenBank/EMBL accession numbers of the sequences used in the analysis are listed in Table S1 in the supplemental material.
MAT gene probes produced based on the PCR amplicons of LPD1, RPO41, STE20 (kinase fragment), and SXI of strain K01 (mating type A1B1) and on the STE20 kinase gene fragment of strain K02 (A2B2). Positive fosmids in hybridization experiments were confirmed by colony PCR using specific primers. We were able to confirm positive fosmids of strain K01 containing LPD1 (two fosmids), RPO41 (five fosmids), and STE20 (one fosmid: 2K2) genes but not SXI. For strain K02, we were able to confirm only positive fosmids for LPD1 (one fosmid) and RPO41 (two fosmids). Because none of the fosmids were positive for more than one probe and the LPD1 and RPO41 genes appeared not to be mating type specific, we focused on sequencing the 2K2 fosmid of K01.

The fosmid insert was found to be 43 kb long, and annotation of the sequence obtained enabled identification of 12 genes with homologs in the C. neoformans genome (Fig. 6A). Besides STE20, three other genes present in the P/R loci of other species in the Tremellales, STE3, STE12, and a putative pheromone precursor gene (MFA1), were found in the vicinity, which suggests that in fact the sequenced fosmid contains or is part of the P/R locus of K. mangrovensis. The annotation results also confirmed that the STE3 and STE12 genes are adjacent and divergently transcribed and are in the close vicinity of the STE20 gene. Synteny analysis with the homologous regions in C. neoformans, K. heveanensis, and T. mesenterica is presented in Fig. 6A. Some MAT genes are common to all species so far examined in the Tremellales (BSP3, MFA1, STE3, STE12, STE20; highlighted in black in Fig. 6A) and may thus have been present in the ancestral MAT locus, while others are common only to K. heveanensis and/or T. mesenterica (CNB00600, CNB00610, CND05260, CNE02670, CNF01610, CNG04540, and CNI00160; highlighted in yellow in Fig. 6A) and could thus have been more recently acquired into MAT in specific lineages. An almost complete synteny is observed between K. mangrovensis and the mating type A1B1 strain of K. heveanensis, the only exception being the pheromone precursor gene, which is inverted. This finding was unexpected because K. mangrovensis and K. heveanensis are not sister taxa (Fig. 2A) and previous comparisons of MAT structure had shown low levels of synteny, with several rearrangements, translocations, and inversions, even between closely related taxa (e.g., C. amylolentus and T. wingfieldii [8] or C. neoformans var. neoformans and C. neoformans var. grubii [5]). CNF01610 (chitin synthase gene) has a homolog in T. mesenterica, not far from CNG04540 but apparently not in K. heveanensis (Fig. 6A); however, due to the observed synteny between K. mangrovensis and K. heveanensis and the fact that CNF01610 is...
positioned next to CNG04540 in the former species, it may well also be present at the same position in *K. heveanensis*.

The putative pheromone gene of *K. mangrovensis* was identified by the characteristic C-terminal motif of lipopeptide pheromones in the deduced protein sequence, CAAX, where A is an aliphatic amino acid (34). It encodes a 39-amino-acid-long pheromone precursor, which showed slightly higher identity (64%) with the MFA1 homolog of *K. heveanensis* than with the MFA2 homolog (48%), and the *K. mangrovensis* gene was thus designated MFA1. Comparison with pheromone precursors of other species in the Tremellales is presented in Fig. 6B. The pheromone precursor of the *K. mangrovensis* mating type A1 strain appears to be more similar to the homologs of the alpha mating types of the pathogenic *Cryptococcus* species.

Phylogenetic analyses were conducted with the putative P/R region genes identified in *K. mangrovensis* and their homologs in *K. heveanensis*, *C. amylolentus*, *T. wingfieldii*, *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, *C. gattii*, and *T. mesenterica* (see Fig. S7 in the supplemental material). Previous analyses (5, 7–9) had shown that the different genes displayed species-specific, mating-type-specific, or mixed phylogenies, thus reflecting their evolutionary trajectories within MAT loci. For the genes common only to the MAT-linked regions of *K. mangrovensis*, *K. heveanensis*, and *T. mesenterica* (CNB00600, CNB00610, CND05260, CNE02670, CNF01610, CNG04540, and CN100160), the limited number of available sequences, often only for one mating type of each species, did not allow us to draw any definitive conclusions on their evolutionary trajectories (see Fig. S7). However, the observed phylogenies of the genes common to species of the two clades, for which alleles for different mating types of the same species were often available, were either mating type specific (MF, STE3, STE12) or mixed (BSP3, STE20), as demonstrated in Fig. 7 for selected genes. Based on the topology of the trees of the former three genes, it is apparent that those genes in *K. mangrovensis* K01 strain are closer to their homologs in *K. heveanensis* mating type A1 strain and in the alpha mating type strains of *C. neoformans* and *C. gattii*, which clustered on a branch together with those of *T. mesenterica*. On the other hand, and as observed by Metin et al. (9), the same genes of mating type A2 of *K. heveanensis* are more closely related to their counterparts in “a” mating type strains of *C. neoformans* and *C. gattii*, on a second branch of the same trees that also contains the *C. amylolentus* mating type A1 strain and T.
This observation suggests a common origin of the alleles on each branch of the trees depicted in Fig. 7 and that these genes were likely to be already present in the ancestral MAT locus of this group of tremellaceous yeasts. BSP3 (Fig. 7) and STE20 (Fig. 4; see also Fig. S7) displayed variable degrees of mating type specificity in the different species, and the observed topologies were in part more similar to the true species phylogeny (Fig. 2).

**TAXONOMY**

*Kwoniella botswanensis*. Á. Fonseca, D. Springer, et J. Heitman. MycoBank accession number MB 802261. Etymology: the specific epithet *botswanensis* (N.L. fem. adj.; pertaining to Botswana) refers to the geographical origin of strains that represent the species. Standard description: the species belongs to the *Kwoniella* clade (sensu Findley et al. [14]) in the Tremellales. *K. botswanensis* is possibly heterothallic with a tetrapolar mating system, but sexual structures have not been observed. After 1 week at 25°C, colonies on MYP agar are circular, white, ridged, glistening, and raised. The texture is butyrous, and the margins are entire. After 3 days at 25°C in YM broth, the cells are ellipsoid to globose, 4.9 to 7.1 μm, and they may be single or with one attached polar bud. Physiological characteristics are listed in Table S2 in the supplemental material. Unambiguous identification and phylogenetic placement is based on DNA sequences of the following nuclear loci: ITS (HF545756), RPB1 (HF545793), RPB2 (HF545813), TEF1 (HF545827), and MCM7 (HF545819). Strain DS258 was designated as the type strain and was isolated by Laura Rusche from the bark of a Mopane tree (*Colophospermum mopane*) in the Okavango Delta, Botswana. This strain has been deposited in the Portuguese Yeast Culture Collection, Portugal, as PYCC 6332 and in the Centraalbureau voor Schimmelcultures, The Netherlands, as CBS 12716. Other strains belonging to this species include DS282 (PYCC 6333, CBS 12717), DS333 (PYCC 6335), and DS729 (PYCC 6334), all isolated from the bark or trunks of Mopane trees in Botswana.

*Kwoniella europaea*. Á. Fonseca et M. Guerreiro. Mycobank accession number MB 802262. Etymology: the specific epithet *europaea* (L. fem. adj.; pertaining to Europe) refers to the geographical origin of strains that represent the species. Standard description: the species belongs to the *Kwoniella* clade (sensu Findley et al. [14]) in the Tremellales. The species is apparently heterothallic with a tetrapolar mating system, but sexual structures have not been observed. After 1 week at 25°C, colonies on MYP agar are circular, white, smooth, glistening, and raised. The texture is butyrous, and the margins are entire. After 3 days at 25°C in YM broth, the cells are ovoid, ellipsoid to globose, 4.5 to 8.0 x 3.5 to 5.1 μm, and may be single or with one attached polar bud. Physiological characteristics are listed in Table S2 in the supplemental material.
material. Unambiguous identification and phylogenetic placement are based on DNA sequences of the following nuclear loci: ITS (HE984339), RPB1 (HE997004), RPB2 (HE996978), TEF1 (HE997051), and MCM7 (HE996990). Strain CECT 11955 was designated as the type strain and was isolated by Carmela Beloch from cork oak (Quercus suber) bark in Badajoz, Spain (35). This strain has been deposited in the Portuguese Yeast Culture Collection, Portugal, as PYCC 6162 and in the Centraalbureau voor Schimmelcultures, The Netherlands, as CBS 12714. Other strains belonging to this species include CBS 7868 from grape juice in Switzerland and ZIM 605 (PYCC 6207) from grape berries in Slovenia (29).

DISCUSSION

Genetic heterogeneity in *K. mangrovensis* and unveiling of two novel sibling species. In their original description of *K. mangrovensis*, Statzell- Tallman et al. (13) studied 30 seawater isolates, mostly from mangrove habitats, 19 from the Bahamas and 11 from the Florida Everglades, as well as two cork isolates from Spain and one beach isolate from Florida. Crossing experiments revealed only seven fertile strains among the Bahamas isolates, one designated MAT A (strain K02) and the other six designated MAT alpha (which included K01, K03, and K06). The authors proposed a bipolar mating system for *K. mangrovensis* and deemed the non-mating strains to be sterile. They also determined LSU and ITS sequences for representative strains and found little genetic variability, except for one cork isolate and the beach isolate, which had one nucleotide substitution in LSU and 6 to 7 substitutions in ITS, compared to the mangrove strains. These differences were not considered sufficient by Statzell-Tallman et al. (13) to warrant separate taxonomic status for those strains without further mating and genetic studies.

Multilocus sequence typing using partial sequences of selected housekeeping genes has been used successfully for delineating species in different yeasts (36). In the present study, we have complemented the MLST approach with the analysis of MAT genes due to the central role played by MAT loci in regulating sexual reproduction and thus in determining species boundaries (1, 3). The multilocus approach involved five *K. mangrovensis* strains (four from seawater and one from cork) from the original study (13), as well as one additional isolate from the British Virgin Islands, one clinical isolate from the United States, four isolates from Mopane trees in Botswana, one isolate from a tree hollow in Brazil, and two isolates from grapes in Europe (see Table S1 in the supplemental material). The results showed that the tropical seawater isolates, which included the type strain of *K. mangrovensis*, formed a monophyletic lineage, while the four Botswana isolates and the three European strains formed two additional sister lineages (Fig. 2; see also Fig. S5 in the supplemental material). The consistent clustering of the strains on individual gene trees (see Fig. S2 to S4 in the supplemental material), the apparent absence of recombination between strains of each lineage, and the observed evolutionary distances between strains of the three sibling lineages (Fig. 3) suggested a genetic separation between them that led us to propose two novel species represented by the Botswana and European isolates, *K. botswanensis* and *K. europaea*. This proposal is further supported by the sequence data of the two putative MAT gene fragments, STE20 and SX1, obtained for the same strains, because distinct alleles of the two genes were found for strains of the three sibling species (Fig. 3 and 4). The geographic origin and source of the strains belonging to *K. mangrovensis* (water samples from mangrove habitats in the tropical western shores of the North Atlantic Ocean), *K. botswanensis* (Mopane trees in Botswana), or *K. europaea* (plant-related substrates in Europe); the observed physiological differences; as well as the results of mating experiments are in line with the separation of the three sibling taxa at the species level. It is interesting to note that the novel *Kwoniella* species appear to be associated with plant surfaces (albeit in very different ecosystems), and although the strains of *K. mangrovensis* were isolated from seawater, Statzell-Tallman et al. (13) speculated that they could have actually originated from the mangrove trees of the surrounding coastal areas. The remaining two strains (K09, the only clinical isolate; and K30, isolated from a tree hollow in Brazil) were placed separately from the three *Kwoniella* species on the MLST and MAT gene trees (Fig. 2, 4, and 5) and could represent additional sibling taxa. However, due to the inconsistent clustering on individual gene trees (see Fig. S2 to S4) and the limited number of strains, their taxonomic status is best considered unresolved at present. Moreover, K09 shared some alleles with *K. europaea* (LSU, ITS, MCM7, SX1) and could thus be an interspecies hybrid.

Taxonomic status of taxa in the *Cryptococcus* and *Kwoniella* clades. We included in our multilocus study several taxa in the *Cryptococcus* and *Kwoniella* clades, and the phylogenetic analyses of the combined data set (Fig. 2; see also Fig. S5 in the supplemental material) have confirmed that *B. dendrophila*, *C. bestioloae*, *C. dejecticola*, *C. pinus*, and *C. shivajii* all belong, together with *K. mangrovensis*, *K. botswanensis*, and *K. europaea*, to the *Kwoniella* clade. In view of the recent implementation of the “one fungus, one name” rule to fungal nomenclature (33), the above-mentioned *Bullera* species and four *Cryptococcus* species should all be transferred to the genus *Kwoniella*, but we refrain from formalizing this proposal here since a taxonomic revision of the genus is beyond the scope of the present paper. The phylogenetic position of *K. heveanensis* was not consistent in the individual gene trees (see Fig. S2 to S4 in the supplemental material), but the five-gene ML tree had this taxon at a basal position in the *Kwoniella* clade (Fig. 2) with 89% bootstrap support, thus justifying its inclusion in the genus, in accordance with the findings of Findley et al. (14). In the *Cryptococcus* clade, a single genus name should also be adopted, which should be *Cryptococcus* given that *C. neoformans* is the type species of the genus and has precedence over *Filobasidiella*. *Tsuchiyaea wingfieldii* was considered by Fonseca et al. (37) to be a synonym of *C. amyloleptus*. However, the MAT gene analyses of Findley et al. (8) led the authors to propose that the two taxa should be kept separate at the species level. Our multilocus and MAT gene analyses support the hypothesis of the latter authors even though the evolutionary distance between the two taxa is less than half the value observed for the pair *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* (Fig. 3). The ensuing transfer of *T. wingfieldii* to the genus *Cryptococcus* as *C. wingfieldii* should, however, await the study of more strains (only a single isolate is currently known) to obtain crucial supporting evidence.

Mating system of *K. mangrovensis* and sibling species. In contrast to the original assumption of a bipolar mating system (13), our results have shown that *K. mangrovensis* instead has a tetrapolar system with two unlinked loci: a P/R locus defined by at least the STE20 gene (see below) and an HD locus defined by two divergently transcribed SX1 genes (Fig. 3 and 4). Several lines of evidence support this conclusion. First, we found two alleles for
STE20 and four alleles for SXI among nine strains of *K. mangrovensis*. Moreover, the association of the different alleles at both loci was found to vary in the different strains, defining five different mating types of eight possible combinations (Fig. 3 and 4; see also Table S1 in the supplemental material). Based on the results of previous studies on the MAT loci of closely aligned taxa in the Cryptococcus and Kwoniella clades for which tetrapolar systems have been demonstrated (8, 9), *K. mangrovensis* STE20 defines a biallelic P/R locus and SXI defines a multialelic HD locus. Additionally, the adjacent position of the two divergently transcribed SXI1 and SXI2 homologs in *K. mangrovensis*, demonstrated by the fact that we amplified fragments of those two genes with a specific pair of primers anchored in the homeosomal regions of each gene, has been found only in tetrapolar species (*C. amylolentus, K. heveanensis*, and *T. mesenterica*), whereas in the bipolar pathogenic Cryptococcus species, only one gene is present in the MAT locus of each mating type (5). The results of mating experiments (Fig. 1) were concordant with the molecular mating types determined for the different strains and provide additional evidence supporting our hypothesis. The same line of reasoning is applicable to *K. botswanensis* and *K. europaea*, and the results obtained for the STE20 and SXI genes (Fig. 3 and 4) suggest that a tetrapolar mating system is also operating in these species. However, mating data were not conclusive. The possibility that *K. mangrovensis* would have a tetrapolar mating system had been suggested by Metin et al. (9) based on their study of the MAT locus of *K. heveanensis*, for which a tetrapolar system was demonstrated.

**Evolution of MAT loci in the Tremellales.** The order Tremellales comprises a large number of yeast producing taxa distributed into several lineages, as deduced from phylogenetic analyses of different regions of the rDNA cistron (38, 39). The large majority of species is asexual and classified in genera such as *Bullera, Cryptococcus, Dioszegia*, or *Fellomyces*, some of which are clearly polyphyletic (38). However, it is possible and even likely that many of these species actually correspond to haploid states of unrecognized heterothallic sexual taxa, as has been found for several species whose sexual states were uncovered upon mating of compatible strains of taxa previously regarded as purely asexual, e.g., *C. amylolentus* (8), *C. neoformans* (40), *K. heveanensis* (9), and *K. mangrovensis* (13). The order Tremellales contains in fact several sexual taxa classified in the genera Auriculibuller, Bulleromyces, Fibulobasidium, and Tremella, among others, but only limited evidence is available on the respective mating systems (38). Some species are assumed to be bipolar (e.g., *Auriculibuller fuscus, Bulleromyces albus*) and others tetrapolar (e.g., *Tremella spp.*) (38), but molecular evidence is lacking for most. In fact, information on the genetic structure of MAT loci of members of the Tremellales is available only for some species in the Cryptococcus and Kwoniella clades and for *T. mesenterica* (12).

In the present study, we have identified tetrapolar mating systems in at least two of the three sibling taxa in the *Kwoniella* clade, *K. mangrovensis* and *K. europaea*. Our findings raise to five or six the number of taxa in the Tremellales with tetrapolar systems that have been confirmed molecularly, thus lending further support to the hypothesis that this mating system was present in the common ancestor of the Cryptococcus and Kwoniella clades and that the bipolar system of the pathogenic *Cryptococcus* species arose more recently in the common ancestor of the latter species cluster (5, 8, 9, 12). It will be of considerable interest to explore the mating systems of members of other clades in the Tremellales, namely, those that contain putative bipolar species, to ascertain if the latter mating system has arisen independently more than once in different lineages.

Annotation of the sequence of a fosmid insert containing the STE20 gene from a mating type A1 strain of *K. mangrovensis* (strain K01) enabled us to determine the extended gene structure of the genomic region containing the putative P/R locus (Fig. 6A). Because we were not able to determine the structure of the corresponding region in a mating type A2 strain, we cannot as yet define with certainty the boundaries of the P/R locus in *K. mangrovensis*. However, because we confirmed the mating type specificity of STE20 and the MF and STE3 genes are also likely mating type specific, we predict that the P/R locus could span at least the 21-kb region between STE20 and MF, comprising a total of eight genes, which include CNB00600, CNB00610, CND05260, CN100160, and STE12. To verify this hypothesis, we need to sequence the corresponding region in a mating type A2 strain. As mentioned above, the most remarkable feature of the characterized region in *K. mangrovensis* (mating type A1B1) was the almost perfect synteny with the homologous region of *K. heveanensis* (mating type A1B1). Previous studies on the MAT loci of different taxa in the Tremellales had shown that these genomic regions are very plastic, with frequent gene inversions and translocations, not only between closely related taxa but also between complementary mating types of the same species (5, 8, 9). Our finding of conserved gene order and orientation (Fig. 6A) between two species that are not even very close relatives (Fig. 2) is therefore unprecedented in this group of yeasts. We predict that the P/R locus in *K. heveanensis* should span at least the same eight genes, because we confirmed the mating type specificity of STE20 in this species. Metin et al. (9) were able to determine only a 5-gene fragment of the P/R locus in a complementary mating type A2 strain of *K. heveanensis* and found that gene order is apparently not maintained between the mating types. We therefore predict that the P/R loci of mating type A1 strains and those of mating type A2 of *K. mangrovensis* and *K. heveanensis* form two diverged syntenic groups. A somewhat parallel situation was observed in the distantly related red yeasts of the Sporidiobolales (class Microbotryomycetes, subphylum Pucciniomycotina) for which genomic regions around the P/R locus exhibited extensive rearrangements between complementary mating types of each species, while synteny was well conserved across species when the homologous regions of the same mating type were compared (41). A similar conclusion was reached by Kellner et al. (6) when comparing the triallelic P/R loci of different smut species in the Ustilaginomycetes. These findings suggest that the MAT gene structure of each allele of the P/R loci in different species was fixed long before speciation occurred. In the Tremellales, this is not apparently the case in the Cryptococcus clade, where MAT restructuring appeared to be concomitant with speciation, but our limited evidence suggests synteny may have been conserved in the evolution of MAT in the *Kwoniella* clade. Determination of the genetic structure of the P/R locus in a mating type A2 strain of *K. mangrovensis* and in other species in the *Kwoniella* clade will be necessary to test our hypothesis. Our phylogenetic analyses of genes in the P/R locus (Fig. 7) further suggest that STE3 and STE12 should be ancient MAT genes in the Tremellales due to the apparent trans-specific polymorphisms deduced from the topology of the corresponding phylogenetic trees. However, the STE20 gene may have been recruited more recently into the MAT locus in some members of the *Kwoniella* clade (namely in *K. man-
grovensis) compared to the taxae in the Cryptococcus clade for which the association of STE20 with MAT appears to be more ancient in view of the observed mating-type-specific phylogeny (see Fig. 4). An alternative hypothesis is that STE20 is an ancient MAT gene in the Tremellales, but its evolutionary trajectory may have been recently reset via gene conversion in some Kwoniella species.

Possible future strategies to gain further insight into the MAT loci of K. mangrovensis include (i) the reprofiling of the mating type A2 strain fosmid library with additional MAT gene probes, (ii) a PCR-based approach to amplify and sequence MAT genes in the P/R locus of the mating type A2 strain based on the genes already identified in the homologous regions of the mating type A1 strain and of the complementary mating strains of K. heveanensis, or (iii) whole-genome sequence analysis of the mating type A1 and A2 strains of K. mangrovensis.

The present study has demonstrated that exploration of the MAT loci structure in different saprobic taxa in the Tremellales is revealing new insights on the origin and evolution of these key genomic regions in a very diverse but still lesser-known group of basidiomycetous yeasts. Ongoing and future genome sequencing projects will contribute to provide additional insights on this central and exciting aspect of fungal biology.

ACKNOWLEDGMENTS

Work carried out at CREM was partly supported by Fundação para a Ciência e a Tecnologia (Portugal) (FCT), project PTDC/BIA-MIC/110351/2009. Work at Duke was supported by NIH/NIAID R37 grant AI99115 and R01 grant AI50113 to J.H.

The authors wish to thank Beatriz Cabaleiro (Laboratory of Virology, University of the Balearic Islands), Agnieszka Cˇadež (ZIM culture collection), Kennio Ferreira-Paim (Universidade Federal do Triângulo Mineiro, Brazil), and Shawn Lockhart (CDC) for providing some of the isolates used in this study.

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