Novel taxa of thermally dimorphic systemic pathogens in the *Ajellomycetaceae* (*Onygenales*)

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Summary

Recent discoveries of novel systemic fungal pathogens with thermally dimorphic yeast-like phases have challenged the current taxonomy of the *Ajellomycetaceae*, a family currently comprising the genera *Blastomyces, Emmonsia, Emmonsiellopsis, Helicocarpus, Histoplasma, Lacazia* and *Paracoccidioides*. Our morphological, phylogenetic and phylogenomic analyses demonstrated species relationships and their specific phenotypes, clarified generic boundaries and provided the first annotated genome assemblies to support the description of two new species. A new genus, *Emergomyces,* accommodates *Emmonsia pasteuriana* as type species, and the new species *Emergomyces africanus,* the etiological agent of case series of disseminated infections in South Africa. Both species produce small yeast cells that bud at a narrow base at 37 °C and lack

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Conflict of interest
None to declare.

adiaspores classically associated with the genus *Emmonsia*. Another novel dimorphic pathogen, producing broad-based budding cells at 37 °C and occurring outside North America, proved to belong to the genus *Blastomyces*, and is described as *Blastomyces percursus*.

Keywords

Blastomyces; *Emergomyces*; *Emmonsia*; *Ajellomycetaceae*; phylogeny; genomics

Introduction

Recent discoveries of novel systemic human pathogens with a thermally-dimorphic pathogenic phase that consist of budding yeast cells have challenged the current taxonomy of the family *Ajellomycetaceae* (order *Onygenales*)[1]. For nearly 100 years only four genera of classical systemic pathogens, each containing just one or two species, were recognized in the order *Onygenales*, i.e. *Coccidioides*, *Blastomyces*, *Histoplasma* and *Paracoccidioides*. All these fungi reside in their filamentous forms in soil or guano, and upon inhalation by the host they morphologically shift to an invasive spherule (*Coccidioides*) or a yeast-like form in the host's pulmonary system. While phylogenetic analyses have placed the genus *Coccidioides* in the family *Onygenaceae*, *Blastomyces, Histoplasma* and *Paracoccidioides* proved to be members of the family *Ajellomycetaceae*[2].

Another documented genus within the *Ajellomycetaceae* is *Emmonsia*, until recently known mainly for species that cause pulmonary infections in small mammals. Until the description of *Emmonsia pasteuriana* in 1998, the genus *Emmonsia* contained two species: the genetically homogeneous *Emmonsia crescens* and a more diverse species, *Emmonsia parva* [3]. These are the etiological agents of adiaspiromycosis, a pulmonary disease of terrestrial mammals and occasionally of humans[4,5]. They differ from classical dimorphic pathogenic fungi by their pathogenic phase consisting of large, thick-walled adiaspores instead of budding yeast cells. *Emmonsia crescens* has adiaspores often over 100 μm in diameter and a maximum growth temperature of 37 °C, while the adiaspores of *Emmonsia parva* are mostly 15–25 μm in diameter and the fungus grows up to 40 °C. Multilocus phylogenetic analysis suggested these species to be less closely related than anticipated. *Emmonsia parva* clustered with *Blastomyces dermatitidis / B. gilchristii*, while *Emmonsia crescens* took a rather isolated position[1]. Recent phylogenomic analysis supported *Emmonsia crescens* as a sister group to the clade including *Histoplasma*, *Emmonsia parva* and *B. dermatitidis / B. gilchristii*[6]. Taken together, the genetic evidence suggests that, in spite of striking morphological, ecological, and pathophysiological similarities, etiological agents of adiaspiromycosis are polyphyletic.

Since the 1970's, novel pathogens have emerged with phylogenetic, morphological and clinical similarities to known members of the *Ajellomycetaceae.* Schwartz et al.[1] summarized reports of numerous additional human cases due to novel species in the family *Ajellomycetaceae*, most of which remained undescribed. Recently, Wang et al.[7] reported on another novel species from China. Several of these novel taxa are opportunistic pathogens of immunocompromised hosts, primarily persons infected with HIV[1]. An important emerging

species associated with disease in advanced HIV infection was found in South Africa with at least 56 cases reported since being correctly identified in 2008[8,9]. The agent causing this mycosis was closely related to *Emmonsia pasteuriana*, which is also known to infect patients with AIDS and in patients with other immune disorders[10–12].

The present work studies the relationships of unclassified isolates from clinical sources with existing *Emmonsia* and *Blastomyces* species and other members of the *Ajellomycetaceae* by means of comprehensive morphological and phylogenetic analyses involving both multilocus and whole genome sequencing. We describe the new genus *Emergomyces* to include *Emmonsia pasteuriana* as type species (*Emergomyces pasteurianus* comb. nov.) and *Emergomyces africanus* sp. nov. for a fungus formerly mentioned by Schwartz et al. as '*Emmonsia* sp. 5'[1]. Another dimorphic human pathogen is described as *Blastomyces percursus* sp. nov. (formerly named '*Emmonsia* sp. 3'[1]). We completed the first annotated genome assemblies for these novel species, which may guide the development of new diagnostics.

Materials and methods

Strains and phenotypes

Reference strains were taken from the collection of the Centraalbureau voor Schimmelcultures (CBS) of CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, the University of Alberta Microfungus Collection and Herbarium (UAMH), Devonian Botanic Garden, Edmonton, Canada (now UAMH Centre for Global Microfungal Biodiversity, Toronto, Canada) and the National Collection of Pathogenic Fungi (NCPF), Mycology Reference Laboratory, Bristol, U.K., supplemented by kind donations of individual researchers. Twenty-four strains were selected for detailed morphological and molecular study (Table 1). These were part of a larger dataset comprising 109 strains (Table S1) used in multilocus analyses. Reference strains belonging to *Coccidioides, Paracoccidioides*, *Blastomyces* and *Histoplasma* as well as the novel taxa described here were handled in biosafety level 3 (BSL-3) laboratories; *Emmonsia crescens* and *Emmonsia parva* were handled at BSL-2. Strains were cultured on 2 % Malt Extract Agar (MEA, Oxoid) plates using inoculum from lyophilized, cryo-preserved or fresh mycelium. Cultures were incubated for 28 days at a temperature of 24 °C.

Microscopic observations were done with slide cultures using MEA, as an optimal medium for conidium formation. Agar blocks of ~0.5 cm^2 were placed on agar plates and inoculated at the four sides. The block was subsequently covered with a sterile cover slip (~2 cm^2). Plates were incubated at 24 °C for 7, 14, 21 and 28 days in a closed plastic box with sterile gauze soaked with 5 ml sterile water to avoid drying of the culture. Slides were made by Shear's mounting medium without pigments. Micrographs were taken using a Nikon Eclipse 80i microscope and DS Camera Head DS-Fi1/DS-5 m/DS-2Mv/DS-2MBW using NIS-Element freeware package (Nikon Europe, Badhoevedorp, The Netherlands). Dimensions were determined with the Nikon Eclipse 80i measurement module and the mean and standard deviation were calculated from measurements of 50 conidia.

Cardinal temperatures were determined on MEA at 5, 15, 21, 24, 27, 30, 33, 36, 37, 40 and 42 °C. Growth rates were determined in triplicate after 4 wk incubation. Thermal dimorphism was evaluated by incubation on MEA and Brain Hearth Infusion agar (BHI, BD Difco) for one to four weeks, using temperature switch from 24 °C to 37 °C as sole stimulus for transition[13].

DNA extraction, PCR and sequencing

Fungal material was harvested for DNA extraction using MasterPure™ Yeast DNA Purification Kit from Epicentre (Madison, WI, U.S.A.). Five gene regions were amplified. The first two loci were ITS and LSU of the rDNA operon[14]. The universal fungal locus ITS1-5.8-ITS2 of the rDNA was amplified with primers ITS5[15] and ITS4 operated under standard PCR conditions[14]. Partial LSU of the rDNA operon was amplified using LR0R and LR5 primer set [16] under the same PCR conditions but with cycle extension of 90 seconds. Partial β-tubulin (*TUB2*) covering the variable 5′-end containing four small introns, was amplified with TUB2Fd and TUB4Fd primer set[17], partial gene encoding elongation factor 3 (*TEF3*) with Al50+51_EF3_2900_F and Al50+51_EF3_3300_R primer set, and 60S ribosomal protein L10 (*RP 60S L1*) with AlGr52_412-433_F1 and Algr52_1102_1084_R1 primer set[18]. Primers and PCR protocols were designed and tested for the development of potential secondary fungal barcodes[18]. PCR products were visualized on 1 % agarose gels. Positive PCR products were sequenced in cycle-sequencing reaction using ABI big dye terminator v.3.1 with a modified manufacturer's protocol)[18]. Following the cycle-sequencing reaction, a capillary electrophoresis system (Life Technologies 3730XL DNA analyser) was used for performing bidirectional sequencing. Sequences obtained were manually edited and consensus sequences stored in a Biolomics database[19].

Sequence alignment and phylogenetic analysis

Obtained sequences were aligned with MAFFT v. 6.850b with default settings except for the 'genafpair' option[20]. Data sets for the five loci were assembled in a single multilocus dataset using Sequence Matrix software [21]. For both the ITS and multilocus datasets, a maximum likelihood phylogeny was inferred using RAxML v.8.0.0 employing GTRCAT model and 1,000 bootstrap replicates[22]. Bootstrap branch support above 70 % was considered as significant. Multiple sequences of species and genera outside the focus of our analysis were collapsed or represented by one or two strains. The interspecific variation (%) was estimated and included in Table S2. The multilocus data set was additionally analyzed by Markov chain Monte Carlo (MCMC) algorithm with MrBayes v. 3.2.6[23] on the CIPRES portal (http://www.phylo.org) with four simultaneous runs for 10 million generations, with a sampling frequency of 1000 trees. A burn-in tree sample of 25 % was discarded. Bayesian posterior probabilities from 50 % majority-rule consensus trees with a probability value higher than 0.90 were considered as significant.

Genome sequencing and *de novo* assembly

Three strains were selected for genome sequencing, including *Ea. pasteuriana* CBS 101426, and BP222 and CBS 136260 initially mentioned as *Emmonsia* sp.3 and *Emmonsia* sp.5 respectively[1] (Table 1). Genomic DNA of strain BP222, isolated from a brain abscess in an immunocompetent person in South Africa[24] was extracted and a library with insert sizes

ranging from 500 to 1500 bp was sequenced on the Illumina MiSeq platform to obtain paired-end reads of 300 bp. Strain CBS 136260, isolated from a skin biopsy in an HIV-infected patient[9] was sequenced using IonTorrent, generating unpaired reads from 8 to 361 bp. For strain CBS 101426 (= UAMH 9510 = NCPF 4236) of *Ea. pasteuriana*, isolated from cutaneous lesions in an Italian woman with advanced HIV infection[10,25] 100 ng of genomic DNA was sheared to approximately 250 bp for library construction, using a Covaris LE instrument and prepared for sequencing as previously described[26]. A library with 180-base inserts was constructed and sequenced on an Illumina HiSeq 2000 platform to generate 101 bp paired-end reads, producing average genome coverage of 191X.

Both Illumina and IonTorrent reads of BP222 and CBS 136260 were assembled using the SPAdes assembler v3.1.1[27]. Next, Pilon v1.16[28] was used to correct the best assembly from each species, resolving single nucleotide errors (SNPs), artifactual indels, and local mis-assemblies, as previously described for *Paracoccidioides* species[29]. The 101-bp Illumina reads of *Ea. pasteuriana* were assembled using ALLPATHS-LG[30] with default parameters. All three *de novo* assemblies were evaluated using GAEMR package (http://www.broadinstitute.org/software/gaemr/), which revealed no aberrant regions of coverage, GC content or unexpected sequence similarity suggestive of contamination. Scaffolds representing the mitochondrial genome were separated out from the nuclear assembly.

Gene prediction and annotation

Genes were predicted and annotated by combining calls from multiple methods to obtain the best consensus model for a given locus. These included *ab initio* predictions (GlimmerHMM, Augustus, Snap, GeneMark-ES), homologous inference (Genewise, TBlastN), and gene model consolidation programs (EvidenceModeler)[31]. For protein coding-gene name assignment we combined HMMER PFAM/TIGRFAM, Swissprot and Kegg products. Kinannote was used to annotate protein kinases[32]. To evaluate the completeness of predicted gene sets, the representation of highly conserved genes in a wide range of eukaryotic taxa (core eukaryotic genes; CEGs) were analyzed using CEGMA genes [33] with the CoreAlyze tool (http://sourceforge.net/projects/corealyze/).

Identification of orthologs and phylogenomic analysis

To examine the phylogenetic relationship of novel sequenced species relative to other dimorphic fungi, single copy orthologs of species from the family *Ajellomycetaceae* were determined and clustered using OrthoMCL (version 1.4)[34] with a Markov inflation index of 1.5 and a maximum e-value of 1e-5. A total of 19 genomes from the *Onygenales* order and three *Aspergillus* genomes were chosen for comparative analyses. These additionally include the following genomes: four *Blastomyces* SLH 14081 (ACBU00000000), ATCC 26199 (AEII00000000), ATCC 18188 (ADMK00000000), ER-3 (ACBT00000000), *Emmonsia* species UAMH 139 (LDEV00000000) and UAMH 3008 (LCZI00000000), *Histoplasma capsulatum* WU24 (AAJI01000000), *H. capsulatum* G186AR (ABBS01000000), *Paracoccidioides lutzii* Pb01 (ABKH02000000), *P. brasiliensis* Pb03 (ABHV02000000), *P. brasiliensis* Pb18 (ABKI02000000), *Coccidioides immitis* RS (AAEC00000000), *C. posadasii* CBS 113859 = Silveira (ABAI02000000), *Uncinocarpus reesii* UAMH 1704 (AAIW00000000), *Microsporum gypseum* CBS 118893 (ABQE00000000), *Trichophyton*

rubrum CBS 118892 (ACPH01000000), *Aspergillus nidulans* FGSC A4 (AACD00000000), *A. flavus* NRRL3357 (AAIH00000000), *A. fumigatus* Af293 (AAHF01000000). To estimate the species phylogeny, orthologs present in a single copy (1:1) in all of 22 genomes were identified. Multiple protein sequence alignment was performed for each single-copy ortholog cluster using MUSCLE to generate sequence alignments of the same length. Then, the cluster multiple alignments were concatenated, and a phylogeny was estimated using RAxML v7.7.8 [22] with model PROTCATWAG with a total of 1,000 bootstrap replicates.

Data availability statement

The assemblies and annotations of the described species genomes were deposited at DDBJ/ENA/GenBank under the following accession numbers: strain BP222 (PRJNA284520), strain CBS 101426 (PRJNA234734), and strain CBS 136260 (PRJNA284519).

Results

Multilocus phylogeny

To examine the phylogenetic relationships of the novel species, we used a panel of 107 strains from the family *Ajellomycetaceae* and multilocus phylogenetic analysis of concatenated sequences of ITS, LSU, *TUB2, TEF3* and *RP 60S L1*. Using *Coccidioides* species (fam. *Onygenaceae*) as outgroup, five monophyletic clades were clearly recognizable and highly supported within the family *Ajellomycetaceae*, representing the new genus, the systemic pathogens in the genera *Paracoccidioides, Histoplasma* and *Blastomyces*, and the recently established environmental genus *Emmonsiellopsis*[35] (Fig. 1). Within the *Ajellomycetaceae*, the genus *Emmonsiellopsis* is located in an ancestral basal position, followed by *Paracoccidioides*. Bootstrap support (BS) and posterior probabilities (PP) of the genera *Emmonsiellopsis* and *Paracoccidioides* were both high (BS/PP 100/1).

Two clades (green and grey boxes; Fig. 1) were analyzed in detail. The upper clade (green box) contains *Blastomyces* in its current sense and in total is interpreted to represent the genus *Blastomyces*. The *Blastomyces* clade has five monophyletic subgroups, all supported by highest BS/PP values of 100/1 (black dots). The uppermost clade contains the etiological agents of blastomycosis in North America, *B. dermatitidis* and *B. gilchristii*, which could not be separated with the chosen set of loci. The closest clade to *B. dermatitidis / B. gilchristii* is indicated as *Blastomyces* sp. 1 and includes the strain CBS 139874 (= UAMH 3398) originating from Canada, which was reported as an unusual case of blastomycosis[36]. The nearest clade to the group *B. dermatitidis / B. gilchristii / Blastomyces* sp. 1 (BS/PP 76/0.99) includes strains of a novel species causing systemic mycosis in healthy human hosts, described here as *Blastomyces percursus*. Two of the three *B. percursus* strains, BP222 and NCPF 4091 originating from South Africa are grouped together (BS/PP 86/1). A third strain, CBS 139878 = UAMH 7425 originating from Israel, is slightly divergent. Two further clades (BS/PP 91/1, 100/1) include strains previously described as *Emmonsia parva*. The clade denoted as *Blastomyces* sp. 2 includes the genome-sequenced strain CBS 139879 = UAMH 139[6] isolated by W. L. Jellison from a weasel in the U.S.A. This is clearly separated from the clade including another classical *Ea. parva* strain, CBS 139881 (= UAMH 130) isolated

by C.W. Emmons, and confirms its basal position and the generic distinctions among *Ea. parva* strains noted previously[3]. The emmonsia-like strains producing small adiaspores phylogenetically cluster in *Blastomyces*, while *Ea. crescens* remains outside *Blastomyces* (Fig. 1).

The lower clade in Fig. 1 (grey box) is clearly separated from *Blastomyces* and *Histoplasma*, and has a large diversity of isolates, including emerging dimorphic species. The entire clade is judged to represent a novel genus, which is described as *Emergomyces*. The clade is monophyletic with BS/PP 99/1 support and includes three well-defined subclades. The monophyletic *Es. pasteurianus* cluster (BS/PP 100/1) comprises the type strain CBS 101426 = UAMH 9510 and two additional strains. A second large monophyletic cluster (BS/PP 100/1) comprises seven strains obtained from HIV-infected individuals with disseminated mycoses in South Africa[9]. The new taxon *Emergomyces africanus* has the genome-sequenced strain CBS 136260 as type. The group is separated from *Es. pasteurianus* by a single strain from a human infection in Germany, CBS 102456 (= UAMH 10427), which is significantly different from *Es. africanus* (e.g. by 19/508 ITS alignment difference). Thus it is denoted as *Emergomyces* sp. 6 (formerly *Emmonsia* sp. 6 in Schwartz et al.[1]). A sister-clade to the *Es. pasteurianus*-group comprises the recently described species *Es. orientalis*[7] and another undescribed taxon, *Emergomyces* sp. 2 (as *Emmonsia* sp. 2 in Schwartz et al.[1]) represented by two strains from Canada. All species of the *Emergomyces* clade produce small yeast-like cells when grown at 37 °C *in vivo* and *in vitro*, in contrast to the large yeast cells of *B. dermatitidis* and the large, thick-walled adiaspores of *Ea. crescens* which are difficult to obtain in culture.

Annotated genome assemblies of *Emergomyces pasteurianus, Es. africanus* and *Blastomyces percursus*

To better characterize the relationships of novel species and to provide reference genomes, we sequenced and assembled the genomes of the type or representative strains for each new species, *B. percursus* strain BP222, *Es. africanus* strain CBS 136260, and *Es. pasteurianus* strain CBS 101426. The species were sequenced using different technologies (Illumina MiSeq, IonTorrent and Illumina HiSeq 2000, respectively) and *de novo* assembled. The genome assembly sizes of these three species were 32.3 Mb in *B. percursus*, 29.7 Mb in *Es. africanus*, and 32.4 Mb in *Es. pasteurianus* (Table 2, Fig. 2A). These assembly sizes are similar to those of other species within the *Ajellomycetaceae* including *Ea. parva, Ea. crescens, H. capsulatum* and *P. brasiliensis*[6,29,37], which have an average of 30 Mb. Further, the genomes of these species are not as expanded as are the genomes of the closely related species *B. dermatitidis* and *B. gilchristii*, which are 66.6 Mb and 75.4 Mb, respectively[6]. This includes the closely related strain BP222 representative of *B. percursus*, suggesting that the remarkable genome expansion in *B. dermatitidis* and *B. gilchristii* occurred recently.

The total number of predicted genes in *B. percursus, Es. africanus* and *Es. pasteurianus* was similar to that found in other *Ajellomycetaceae*, as well as in distantly-related dimorphic fungal pathogens in the family *Onygenaceae*, such as *Coccidioides* (Fig. 2). Numbers of predicted genes for *B. percursus, Es. africanus* and *Es. pasteurianus* were 10,293, 8,769 and 8,950, respectively (Table 2, Fig. 2A). Despite the smaller contig size in two assemblies

(Table 2), we found high representation of core eukaryotic genes in all genomes, providing evidence that their gene sets are nearly complete; *Es. africanus* included 88 % of core eukaryotic genes, while *B. percursus* and *Es. pasteurianus* gene sets included 96–98 % (Fig. 1B). Based on their completeness, the *B. percursus*, *Es. africanus* and *Es. pasteurianus* reference genome assemblies can define a wide set of genes that is shared across the dimorphic pathogenic fungi. We classified these references according to the mating type locus. *Es. africanus* and *Es. pasteurianus* contained mating type alpha (*MAT1-1*; locus ID ACJ72_07256 and AI78_01298, respectively), while *B. percursus* contained the mating type HMG (*MAT1-2*; locus ID ACJ73_00817).

Phylogenomics of *Es. pasteurianus, Es. africanus* and *B. percursus*

To compare gene content and conservation, we identified orthologous gene clusters in the three genomes sequenced here, *Onygenales* genomes of other dimorphic pathogens (*Blastomyces*, *Histoplasma, Paracoccidioides*, and *Coccidioides*) and two dimorphic non-human pathogenic species, *Ea. parva* and *Ea. crescens*, the etiological agents of adiaspiromycosis in small mammals. As outgroups, three *Aspergillus* genomes were also included. Using 2,851 single copy core genes present in all strains, we estimated a strongly supported phylogeny of these organisms using RAxML (Fig. 3). *Blastomyces percursus* clustered with the primary pathogen *B. dermatitidis / B. gilchristii* (100 % bootstrap support). *Blastomyces* sp. 2 (strain UAMH 139; formerly *Ea. parva*) was also closely related, branching earlier as a sister species within the *Blastomyces* clade (Fig. 3; green box). *Es. africanus* and *Es. pasteurianus* clustered in a single, strongly supported (100 % bootstrap replicates) clade as sister species, and this clade was sister to *Ea. crescens* (Fig 3; grey box). The *Ea. crescens – Emergomyces* clade is a sister group of the clade including *Histoplasma* and *Blastomyces*, with *Paracoccidioides* in a basal position.

Multilocus and genome analyses demonstrated that (i) one group of novel dimorphic species (*Emergomyces*; grey box) formed a single, derived clade within the *Ajellomycetaceae* distinct from the classic dimorphic pathogens and classic *Emmonsia* species; (ii) a second group (*B. percursus*; within green box) clustered as a separate clade within the genus *Blastomyces* as close relative to the dimorphic pathogens *B. dermatitidis / B. gilchristii* and (iii) the etiological agents of adiaspiromycosis (*Ea. parva* and *Ea. crescens*) in the current sense are polyphyletic with *Ea. parva* strains having small adiaspores grouping with *Blastomyces* and *Ea. crescens* having large adiaspores occupying a unique position.

Members of the *Emergomyces* clade produce filamentous phases similar to those of other members of the *Ajellomycetaceae*, that is, solitary, single-celled conidia being formed on short narrow pedicels on swollen conidiogenous cells at 24 °C (Figs 4, 5). Their responses at 37 °C generate the most significant differences from the *Blastomyces* clade. Species of the *Emergomyces* clade (grey boxes in Figs 1, 3) produce small budding yeast cells (<5 μm) with a narrow base; budding in *Es. pasteurianus* may be uni-, bi- or multipolar. In contrast, members of the *Blastomyces* clade (green boxes in Figs 1, 3) typically produce larger yeast cells (>5 μm) with budding at a broad base in the case of *B. dermatitidis, B. gilchristii,* and *B. percursus.*

Emergomyces Dukik, Sigler & de Hoog, **gen. nov.** – MycoBank MB 818569

Etymology: referring to the newly emerged group of species during the last few decades.

Colonies on MEA at room temperature white to beige, slow growing, filamentous. Hyphae hyaline, thin-walled. Conidiophores are short, branched, arising at right angles from hyphae, slightly swollen at the top, sometimes with short, secondary conidiophores. Conidia solitary, one-celled, often subspherical, produced on narrow pedicels on swollen conidiophores or sessile, or alongside hyphae. At 37 °C small yeast cells having narrow buds typically formed but larger cells with broader based buds sometimes present.

Type species: Emmonsia pasteuriana Drouhet *et al.*[10].

Differential diagnosis: The genus is distinguished from *Emmonsia* and *Blastomyces* species by small yeast cells budding at narrow bases at 37 °C. It is clearly segregated from remaining genera of *Ajellomycetaceae* by multilocus sequence data. Strains attributed to the genus have been isolated from humans and cause primarily disseminated disease frequently characterized by cutaneous lesions.

Emergomyces pasteurianus (Drouhet, Guého & Gori) Dukik, Sigler & de Hoog, **comb. nov.** – MycoBank MB 818570

Basionym: Emmonsia pasteuriana Drouhet, Guého & Gori, *in* Drouhet, Guého, Gori, Huerre, Provost, Borgers & Dupont – *J. Mycol. Méd.* 8: 90, 1998[10].

Colonies at 24 °C on MEA whitish, composed of hyaline hyphae. Conidiophores short, slender, unbranched, arising at right angles from narrow hyphae and slightly swollen at the tip; bearing 1–3 (up to 8) conidia on short thin pedicels or sometimes sessile alongside hyphae. Conidia subhyaline, slightly verruculose, thin-walled, one-celled, subspherical, 2–3 × 3–4 μm. At 37 °C budding cells present which are ellipsoidal, 2–4 μm in length, budding at a narrow base, in addition to broad-based budding cells.

Physiology: minimum growth temperature 6 °C, optimum 24 °C, reaching 35 mm diam, maximum 40 °C.

Type: CBS 101426 = UAMH 9510 = IP 2310.95 = NCPF 4236, isolated from cutaneous lesion of 40-year-old female with advanced HIV disease and a history of injection drug use with disseminated fungal infection, Italy, 1984, reported in 1998[25].

Differential diagnosis: This species is characterized by formation of small, ellipsoidal yeast-like cells (2–4 μm) at 37 °C, showing narrow-based mostly unipolar budding, with rare bi- or multipolar scars, intermingled with broad-based budding cells. *In vivo*, along with small thin-walled yeast cells, few larger thick-walled yeast cells of 8–10 μm were observed[10].

Emergomyces africanus Dukik, Kenyon, Govender, Schwartz & de Hoog, **sp. nov.** – Fig. 4, MycoBank MB 818571

= *Emmonsia* sp., Kenyon et al. – *N. Engl. J. Med.* 369: 1416. 2013[9].

= *Emmonsia* sp. 5, Schwartz et al. – *PLoS Pathog.* 11: e1005198 p. 2. 2015[1].

Etymology: referring to the species causing outbreaks in South Africa.

Holotype: New Somerset Hospital, Cape Town, South Africa, specimen of culture CBS 136260 (preserved in metabolically inactive condition in liquid nitrogen) from skin biopsy of an HIV-infected male, collected by N.P. Govender, 11 June 2010.

Colonies on MEA at 24 °C, 4 wk moist, circular, flat or slightly raised towards the center, reaching 21 mm diam, often with central hyphal tufts but otherwise lacking aerial mycelium. The firm hyphal mat is almost concolorous with agar. Colony reverse warm-buff in the centre, light buff around, radially sulcate. Mycelium delicate, hyphae 1.4–2.5 μm in diam, hyaline, septate, branched, with few spirally twisted hyphae. Conidiophores mostly one-celled, solitary, arising at right angles from vegetative hyphae, 0.6–1.5 μm in diam, with a septum at the base and mostly swollen at the tip; usually forming short, secondary conidiophores. Conidia emerging from swollen tips on narrow pedicels, each forming a terminal conidium, establishing a grouping or "floret" of 4 to 8 conidia. Conidia solitary, occasionally in chains of two or four, subspherical, slightly shortened along the vertical axis, 1.2–3.2 × 1.7–3.8 μm (2.2 ± 0.5 × 2.7 ± 0.5, n = 45), smooth-walled to finely roughened; rhexolytic, sometimes adherent to the conidiophore; rarely sessile.

Colonies on MEA at 37 °C, 4 wk smooth, glistening, cream-coloured to greyish-brown, reaching 5 mm diam. Yeast cells abundant, ovoidal to subspherical, 1.7–5.3 × 0.9–2.2 μm (2.9 ± 0.73 × 1.6 ± 0.31, n = 45), mostly single, occasionally multiple. Budding unilateral from a narrow base. Some swollen and short hyphae also present

Physiology: minimum growth temperature 6 °C, optimum 24–27 °C reaching 21 mm diam, maximum 40 °C.

Differential diagnosis: Es. africanus is differentiated by having small, ovoidal to subspherical yeast cells below 5 × 3 μm at 37 °C, budding at narrow bases at the poles. At 24 °C, conidia are borne in a complex cluster or "floret" of four to eight conidia formed individually at the ends of slender stalks.

Blastomyces percursus Dukik, Muñoz, Sigler & de Hoog, **sp. nov.** – Fig. 5, MycoBank MB 817662

= *Emmonsia* sp. 3, Schwartz et al. – *PLoS Pathog*. 11: e1005198 p. 2. 2015 [1].

Etymology: referring to the ability of the fungus to infect multiple sites of human patients.

Holotype: Israel, specimen of culture CBS 139878 (preserved in metabolically inactive condition in liquid nitrogen) from granulomatous lesion on lip of otherwise healthy patient with disseminated infection, isolated by I. Polachek, November 1993; living strain CBS 139878 = Kemna 408–93 = UAMH 7425 = UAMH 7426.

Colonies on MEA 24 °C, 4 wk flat, reaching 42–43 mm diam, with a loose, whitish felt of aerial mycelium and often with central hyphal tufts. Margin flat, with reptant hyphae. Colony reverse pale buff, warm-buff at the centre, radially sulcate from the centre. Exudate absent. Hyphae 1.1–2.8 μm in diam, hyaline, septate, irregularly branched, locally swollen,

with some spirally twisted hyphae. Conidiophores solitary, arising at right angles from vegetative hyphae, mostly swollen around the middle or near the end, 1.6–4.1 (2.2 ± 0.59) μm wide, with a septum at the base and directly below the conidium, sometimes bearing 2–4 secondary conidiophores; solitary conidia produced on short and narrow pedicels of < 1 μm long. Conidia holothallic, subspherical, 1.5–4.4 × 1.7–4.6 μm (2.7 ± 0.6 × 2.6 ± 0.5, n = 45), smooth to slightly roughened, rhexolytic, sometimes adherent to the conidiophore. Chlamydospore-like cells occasionally present on short lateral branches, having thickened cell walls and often a median septum.

Colonies on MEA at 37 °C, 4 wk smooth, shiny, cream to greyish-brown, reaching about 6 mm diam. Commonly short and swollen hyphal elements present with shorter intercalary cells and disarticulating into smaller fragments, intermingled with large yeast-like cells with uni- or bipolar budding at a broad base, 5.2–12.2 × 2.4–6.5 μm (8.1 ± 1.7× 4.8 ± 0.90, n = 45).

Physiology: minimum growth temperature 9–15 °C, optimum 27 °C reaching 48 mm, maximum 40 °C.

Differential diagnosis: This species differs in having more elaborate conidiophores bearing conidia on stalks, in contrast to the simple conidiogenous cells bearing single conidia that are typical of *B. dermatitidis / B. gilchristii*. Yeast cells produced at 37 °C are subspherical, over 5 μm long, and bud at a broad base as in *B. dermatitidis*.

Discussion

Recent reports document the emergence of infections in humans caused by new types of systemic thermo-dimorphic fungi over the past few decades[1,9,12,24,25,38–40]. Initially, these fungi were considered emmonsia-like, based on the microscopic morphology of conidia, which cluster in florets on conidiophores[1]. However, their yeast-like appearance in tissue and in culture at 37 °C differed from classical species of *Emmonsia*. The disease caused by *Emmonsia* species is adiaspiromycosis, a pulmonary disease occurring in rodents and other small burrowing animals in which the fungus resides as spherical structures in the lung known as adiaspores which enlarge but do not multiply, in contrast to the endosporulating spherules of *Coccidioides* species[5]. Human infections are uncommon[4,5]. The discovery of adiaspiromycosis occurred in 1942 when Emmons and Ashburn[41] observed a fungus producing spherule-like structures up to 20 μm in the lungs of rodents trapped in Arizona, U.S.A. The fungus was described originally as *Haplosporangium parvum*, but was reclassified as the type species of a new genus *Emmonsia* as *Emmonsia parva*. The second described species, *Ea. crescens* differed in producing giant adiaspores in tissue up to 200 μm in diameter, which could be reproduced *in vitro* at 37 °C [5,42]. Peterson and Sigler[3] found that *Ea. parva*, the type species of *Emmonsia*, is phylogenetically closely related to *B. dermatitidis*. This was confirmed in our multilocus and phylogenomic data. With the type species of the genus *Emmonsia* clustering in *Blastomyces*, the former genus becomes a synonym of *Blastomyces*. Peterson and Sigler[3] showed *Ea. crescens* to belong to a single, rather invariable clade and our data indicate that the species is separated from *Blastomyces* by *Histoplasma*. Its exact phylogenetic position and its relationship to other taxa within the

Ajellomycetaceae remain unresolved. *Ea. crescens* is one of only three taxa within the *Ajellomycetaceae* including *B. dermatitidis* and *H. capsulatum* that are heterothallic and have proven ajellomyces-like sexual stages[43].

The first human-associated species classified in *Emmonsia* was *Ea. pasteuriana*[10] but it was fundamentally different particularly from *Ea. crescens* in the type of infection caused (disseminated cutaneous mycosis rather than limited pulmonary disease); in production of yeast-like cells rather than adiaspores in tissue; and in its occurrence in humans, *Emergomyces pasteurianus* was first isolated in 1994 from an Italian woman with advanced HIV-infection presenting with disseminated skin lesions in which small budding yeast cells of 2–4 μm were observed in tissue and in culture at 37 °C [10,25]. Additional reports can be attributed to *Es. pasteurianus.* A case of disseminated infection occurred in Spain in an HIV-infected person who was also a liver transplant recipient and presented with pulmonary and skin lesions[44]. Malik et al.[11] reported an *Es. pasteurianus* disseminated infection in an Indian woman with advanced HIV who presented with multiple non-tender skin lesions and pulmonary disease. Two further cases involved disseminated skin infection in non-HIV infected patients from Guangzhou, China; one occurred in a renal transplant recipient[45] and another in a male receiving high dose corticosteroids[46]. Additionally, we report that *Es. pasteurianus* has been isolated from a patient in South Africa (Fig. 1, Table 1). The clinical, morphological and phylogenetic analyses described here determined that *Ea. pasteuriana* and several other emmonsia-like fungi with yeast stages formed a single, derived clade in the *Ajellomycetaceae*, described here as *Emergomyces* with type species *Es. pasteurianus*. The genus is supported by BS/PP 99/1 and contains several subclades (Fig. 1).

The emergence of *Es. africanus* as the cause of disease among HIV-infected persons from South Africa and the Kingdom of Lesotho has been remarkable with respect to the numbers of cases reported[1,8,9,39]. Thirteen cases were discovered during an initial surveillance program in South Africa using broad-range fungal PCR assay of all deep fungal infection clinical isolates[9]; the number of reported cases was soon expanded to 52 [8]. Fifty-one patients had advanced HIV disease, and the other was a renal transplant recipient. Ninety-five percent of patients had widespread skin lesions, which were protean and often misdiagnosed. Isolates were primarily cultured from skin and bone-marrow biopsies or blood culture. Another species, *Es. orientalis* has been described separately for a strain from Beijing, China causing disseminated infection in an individual with diabetes[7]. Other fungi appear to warrant placement in *Emergomyces.* These include a strain recovered from the lung tissue of a male with rheumatoid arthritis treated with low doses of corticosteroids in Germany[38] (Fig. 1 as *Emergomyces* sp. 6). Molecular analysis of two isolates from immunocompromised patients in Canada[40] placed them as sister clade to *Es. orientalis* (Fig. 1 as *Emergomyces* sp. 2). Taken together, these cases underline the potential of *Emergomyces* species as new cosmopolitan opportunistic pathogens in the immunocompromised host. Most persons infected with *Emergomyces* species have impaired cellular immunity. Some cases now attributed to *Es. africanus* infection were misclassified because they were incorrectly diagnosed by histopathological examination as *H. capsulatum*[1,9]. Based on the sequencing data, none of the *Histoplasma* strains retained in the CBS collection represents any of the novel dimorphic fungi described here. The

environmental reservoir is unproven although early evidence has implicated soil (I. Schwartz, unpublished data). Infection of animals has not been reported.

Strains identified as *Blastomyces percursus* came from immunocompetent and immunocompromised hosts. The type strain (CBS 139878 = UAMH 7425) was found to cause granulomatous oral lesions in an immunocompetent patient in Israel[47]. Molecular analyses by Peterson & Sigler[3] and Schwartz et al.[1] placed this isolate in the *Blastomyces* clade. Our analysis of this strain and two additional isolates confirmed this, and showed a clear separation between this clade as compared with *B. dermatitidis* / *B. gilchristii*. Two other strains are from cases in South Africa[9,24]. The first case was isolated from ulcerated skin of an HIV-infected person in Johannesburg in 1986 and it was originally identified as *B. dermatitidis* (NCPF 4091). The second strain (BP222) came from a 52-year-old previously healthy male with a cerebellar abscess. The diagnosis was based on a brain tissue biopsy which showed budding yeast cells suggestive of *B. dermatitidis*. However, sequencing of the ITS locus showed partial alignment with the ITS sequences of the South African emmonsia-like strains. The patient received amphotericin B followed by oral itraconazole therapy with good clinical response.

The polyphyletic nature of the analyzed dimorphic human pathogens and the etiological agents of adiaspiromycosis (*Ea. parva* and *Ea. crescens*) which are separated from each other by species with other types of pathogenic phases suggest that members of the *Ajellomycetaceae* have undergone multiple evolutionary transitions allowing infection of humans and other mammals. In addition, it shows how, in spite of phylogenetic transitions, they have retained their mesophilic morphology including sporulation with solitary, slightly to moderately rough-walled conidia; *Histoplasma* is exceptional in its production of conidia of two sizes, the larger being coarsely ornamented (tuberculate). Major differences between species and genera (*Blastomyces, Emergomyces, Histoplasma, Paracoccidioides*) are in their invasive forms, whereby *Histoplasma* is again exceptional in its intracellular growth of small yeast cells in host macrophages. It may be noted that the biological coherence of taxa in *Ajellomycetaceae* is not only underlined by monomorphic filamentous stages occurring throughout the family, but also by *Ajellomyces* teleomorphs having elaborate morphology of gymnothecia, asci and ascospores. From a point of view of ambient morphology at room temperature, sexual as well as clonal, all members of *Ajellomycetaceae* show a number of highly conserved traits, which likely are linked to their alternating life cycle with mammal hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

Ea   Emmonsia

Es   Emergomyces

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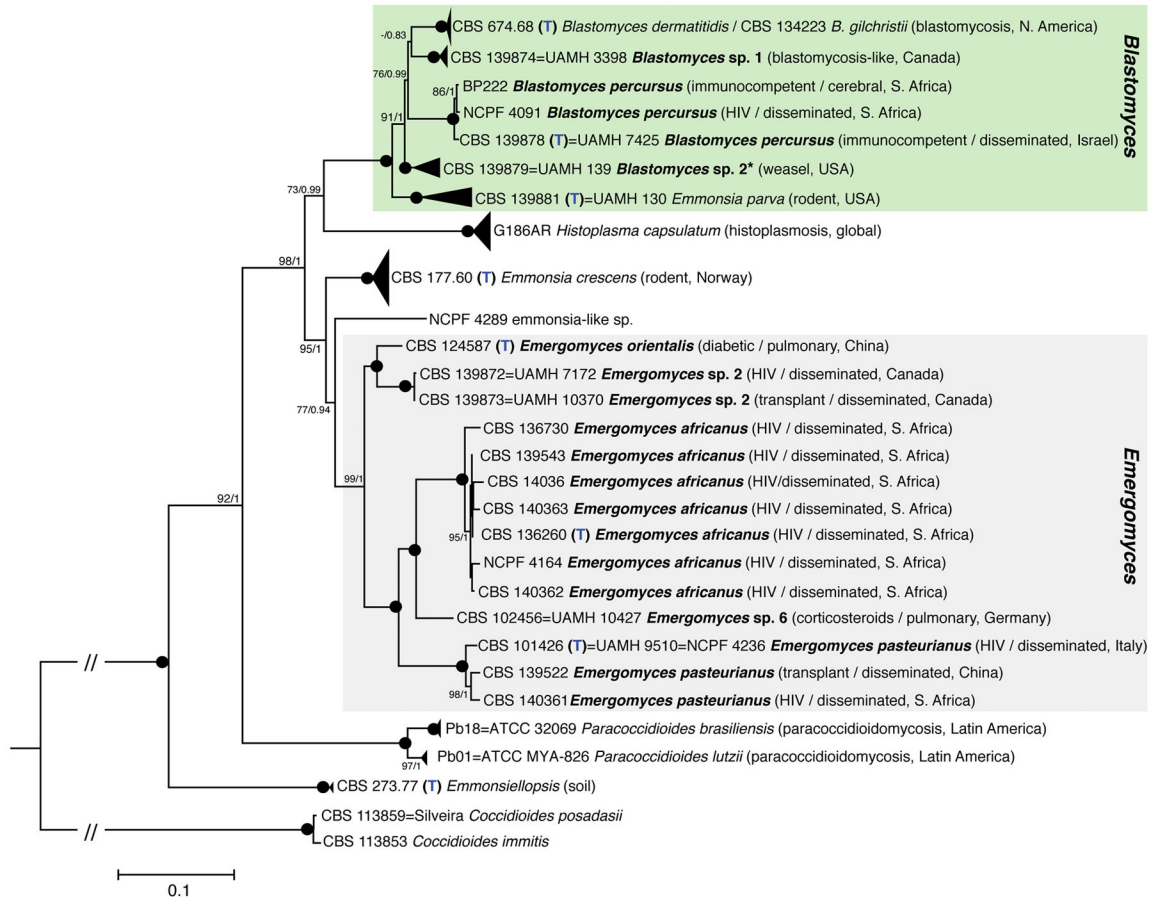
Fig. 1.

RAxML tree constructed from concatenated dataset including five regions: ITS, LSU, *TUB*, *TEF3* and *RP 60S L1*. The genera and species that are subject of this analysis are shown in bold font and represented by all analyzed strains (Table 1). Multiple sequences of other species/genera are collapsed (represented by one strain that is: type or neotype, genome sequenced or key strain from yet undescribed species). Bootstrap support (BS) from RAxML > 70% (left) and Bayesian posterior probability (PP) >0.80 are given at the nodes. Fully supported branches (100/1) are depicted as black circles. (*) Formerly *Emmonsia parva.* (T) – Type strain.
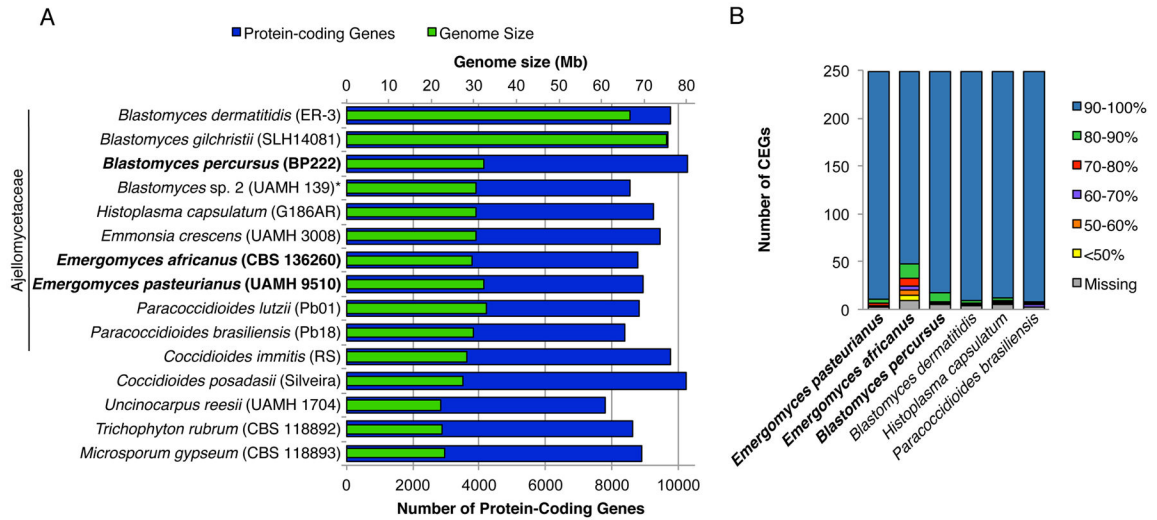
Fig. 2.

(A) Genome sizes in megabases (Mb) and total number of protein-coding genes of the sequenced and assembled strains of *Emergomyces africanus*, *Es. pasteurianus* and *Blastomyces percursus*, and other previously sequenced and assembled species from the *Ajellomycetaceae*. * formerly *Emmonsia parva* **(B)** Conservation of core eukaryotic genes (CEGs) across *Es. africanus*, *Es. pasteurianus*, *B. percursus* and other compared genomes.
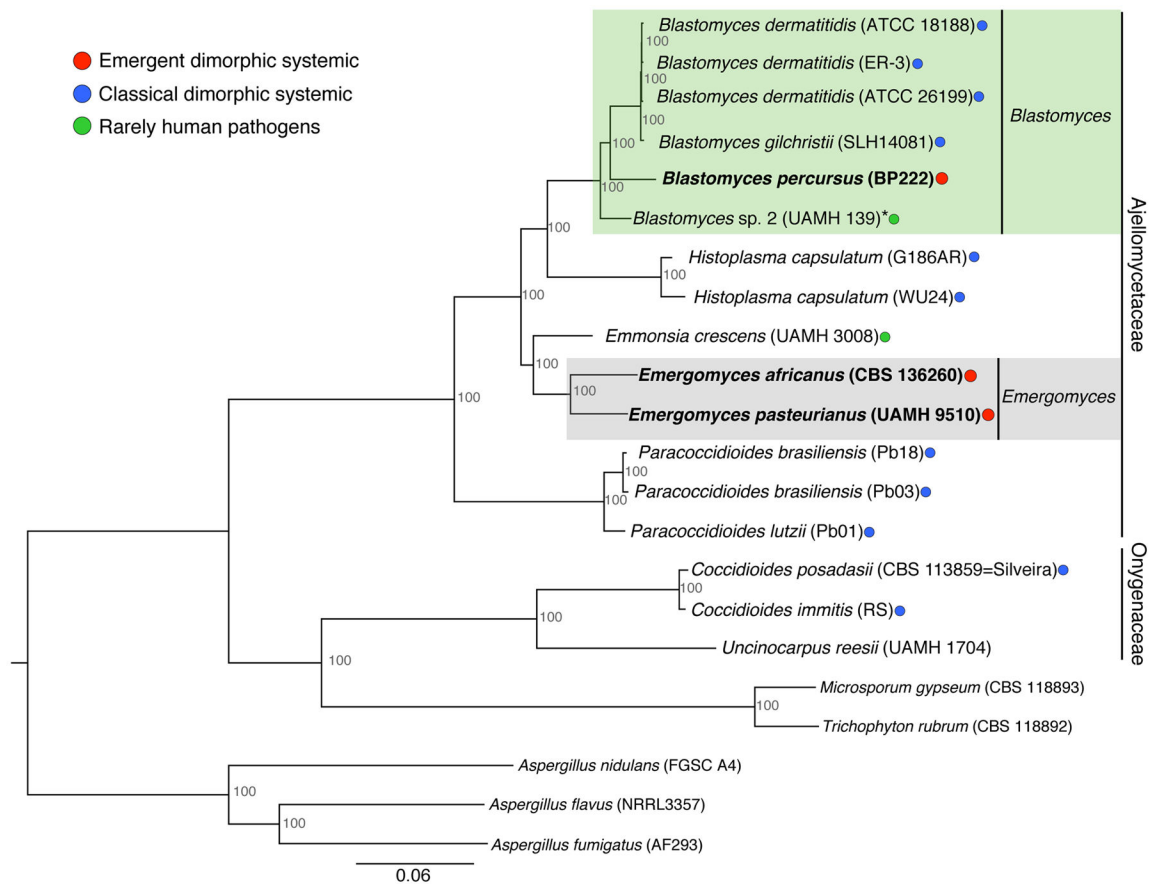
Fig. 3.

Phylogeny of *Emergomyces africanus*, *Es. pasteurianus* and *B. percursus*. Maximum likelihood tree inferred from concatenated protein alignments of 2,851 core genes based on 1,000 replicates; all nodes were supported by 100% of bootstrap replicates. Novel emergent species are highlighted with red color circles.
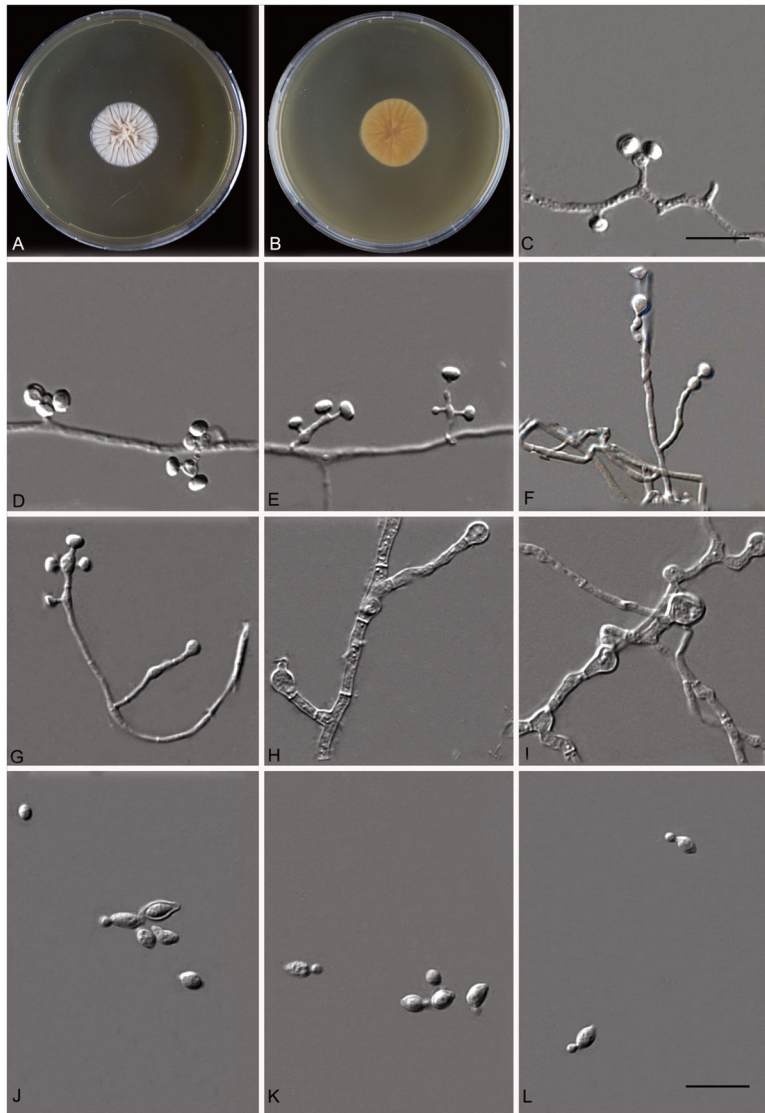
Fig. 4.
Emergomyces africanus sp. nov., CBS 126360 (type strain). **(A, B)** Colony on MEA after 4 weeks at 24 °C, obverse and reverse. **(C–I)** Conidia of mycelial phase. **(J–L)** Small yeast cells at 37 °C. Scale bars = 10 μm.
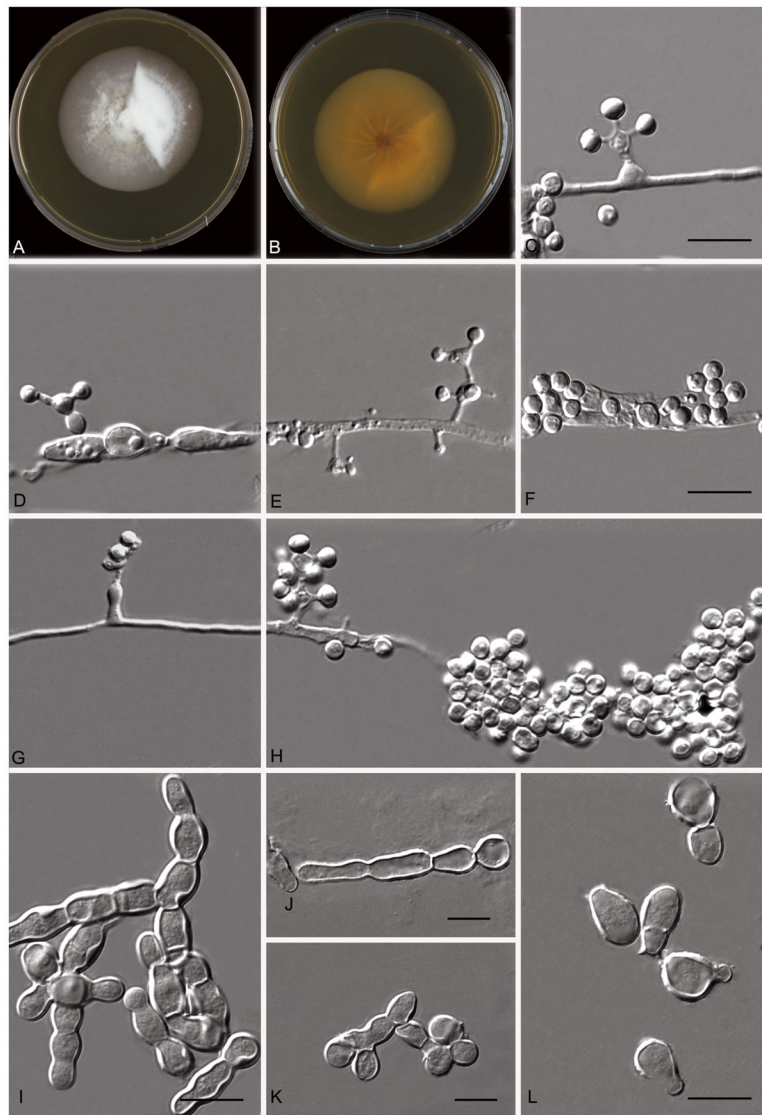
Fig. 5.
Blastomyces percursus sp. nov., CBS 139878 (type strain). **(A, B)** Colony on MEA after 4 weeks at 24 °C, obverse and reverse. **(C–H)** Conidia of mycelial phase. **(I–K)** Hyphal elements in transition to yeast-like growth at 37 °C. **(L)** Large yeast with unipolar or bipolar budding at a broad base at 37 °C. Scale bars = 10 μm.

Table 1

Strains used in this analysis and shown in phylogenetic trees. Only the sequences from the taxa described in this paper were submitted to GenBank.

Taxon name	CBS accession	Other collections	Source	Country	References	GenBank Nr. (ITS, LSU, *TUB2, TEF3, RP60SL1*)	GenBank genome Nr.
B. dermatitidis	CBS 674.68 (T)	ATCC 18188; B788(a); IHEM 3783; UAMH 3539	human, blastomycosis	USA	6		ADMK00000000
B. dermatitidis		ER-3 (ATCC MYA 2586)	soil under woodpile	USA	6		ACBT00000000
B. gilchristii	CBS 134223 (T)	SLH14081	human, blastomycosis	Canada	6		ACBU00000000
B. percursus	CBS 139878 (T)	UAMH 7425	human, immunocompetent, mucocutaneous	Israel	43, 47	KY195964, KY195971, KY195936, KY195942, KY195949	
B. percursus		BP222	human, immunocompetent, brain abscess / pneumonia	South Africa	24		LGTZ00000000
B. percursus		NCPF 4091	human, disseminated / cutaneous	South Africa	9	KY195963, KY195972, KY195940, KY195943, –	
Blastomyces sp. 1	CBS 139874	UAMH 3398	human, alcoholic / diabetes, meningo-encephalitis	Canada	1,36		
Blastomyces sp. 2 *	CBS 139879	UAMH 139	weasel	USA	3, 6		LDEV00000000
Ea. parva	CBS 139881	UAMH 130	rodent lung	USA	3		
Es. africanus	CBS 136260 (T)		human, HIV, disseminated	South Africa	9		LGUA00000000
Es. africanus	CBS 136730		human, HIV, disseminated	South Africa	9	KY195959, KT155137, KT155489, KT156143, –	
Es. africanus	CBS 140362		human, HIV, disseminated	South Africa	9	KY195961, KY195968, KY195939, –, KY195952	
Es. africanus	CBS 140363		human, HIV, disseminated	South Africa	9	KY195958, KY195965, –, –, KY195954	
Es. africanus	NCPF 4164		human, HIV, disseminated	South Africa	9	KY195960, –, KY195941,	

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Taxon name	CBS accession	Other collections	Source	Country	References	Genbank Nr. (ITS, LSU, TUB2, TEF3, RP60SL1)	GenBank genome Nr.
Es africanus	CBS 140360		human, HIV, disseminated	South Africa	9	KY195957, KY195967, KY195937, –, KY195955	
Es africanus	CBS 139543		human, HIV, disseminated	South Africa	9	KY195956, KY195966, KY195935, KY195944, KY195953	
Ea. crescens	CBS 177.60 (T)	ATCC 13704; UAMH 3008	rodent, lung	Norway	42		LCZI00000000
Es. pasteurianus	CBS 101426 (T)	IP 2310.95: UAMH 9510: NCPF 4236	human, HIV, disseminated	Italy	10		LGRN00000000
Es. pasteurianus	CBS 140361		human	South Africa	This study	KY195962, KY195969, KY195938, KY195946, KY195950	
Es. pasteurianus	CBS 139522	F003	human, renal transplant, disseminated	China	45	KT155632, KT155632, KY195934, KY195947, KY195951	
Es. orientalis	CBS 124587		human, diabetes, disseminated	China	7		
Emergomyces sp. 2°	CBS 139872	UAMH 7172	human, HIV, disseminated	Canada	3		
Emergomyces sp. 2°	CBS 139873	UAMH 10370	human, transplant, disseminated	Canada	40		
Emergomyces sp. 6†	CBS 102456	UAMH 10427	human, rheumatic / corticosteroids, pulmonary	Germany	38		

* Formerly Emmonsia parva.

° Formerly *Emmonsia* sp. 2 in Schwartz et al. *PLoS Pathog.* 11: e1005198 p. 2. 2015.

† Formerly *Emmonsia* sp. 6 in Schwartz et al. *PLoS Pathog.* 11: e1005198 p. 2. 2015.

Abbreviations used: B = *Blastomyces*, Ea = *Emmonsia*, Es = *Emergomyces*, T = type strain, CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; NCPF = National Collection of Pathogenic Fungi, Bristol, England; UAMH = Centre For Global Microfungal Biodiversity

Table 2

Assembly and annotation statistics for *Emergomyces pasteurianus*, *Es. africanus*, *Blastomyces percursus*. For comparison, GC-content %, genome size and number of genes were included for reference genomes of *B. dermatitidis* (ER-3), and *Blastomyces* sp. 2 (UAMH 139).

Genus	Emergomyces		Blastomyces		
Species	**Es. africanus**	**Es. pasteurianus**	**B. percursus**	**B. dermatitidis**	**B. sp. 2***
Strain	CBS 136260	UAHM 9510	BP222	ER-3	UAMH 139
Scaffolds	4,444	1,643	3,868	25	2,682
Scaffold N50 (kb)	13.8	45.9	12.8	5,550	31.17
Scaffold N90 (kb)	2.5	8.8	4.0	2,312	3.4
Max Scaffold (kb)	79.7	267.9	71.4	10,302.04	265.3
Assembly GC	43.5	44.5	47.3	37.1	44.7
Total Length (Mb)	29.7	32.4	32.3	66.6	30.3
Coding-protein genes	8,769	8,950	10,293	9,755	8,563

*
Formerly *Emmonsia parva*.