Supplementary Appendix

Human blastomycosis in South Africa caused by *Blastomyces percursus* and *Blastomyces africanus* sp. nov., 1967 – 2014

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Materials and methods

Morphological description

The archived isolates had been stored in water at room temperature. All isolates were sub-cultured onto Sabouraud agar or potato dextrose agar (PDA) (Diagnostic Media Products [DMP], Sandringham, South Africa) and incubated at 25ºC and 30 ºC for up to 4 weeks for the mycelial phase. Isolates were transferred onto brain heart infusion (BHI) agar with or without 5% sheep blood (DMP) and incubated for 1-3 weeks at 37ºC and 40ºC for conversion to the yeast phase. The diameter of the mould-phase colonies was measured in millimetres each week using a ruler. The morphology of both phases was observed using standard light microscopic examination of lacto phenol cotton blue preparations and differential interference contrast (Nomarski) microscopic examination of unstained preparations, using an Olympus BX53 with a Nikon camera and calibrated Eclipse software. Mycelial growth was also inoculated onto a urea agar slope (DMP) and incubated at 37ºC for 6 days. Yeast and mycelial isolates were examined using

light microscopy and electron microscopy. Descriptive terminology used follows that of Jiang *et al*. (2018) [1].

Electron microscopy (EM)

Yeast and mycelial cultures were fixed by flooding with 2.5% glutaraldehyde in 0.2M phosphate buffer, pH 7.1, for several hours. Routine EM processing was as follows: pieces of agar on which the fungus was growing, were cut out and rinsed in buffer, post-fixed for 2 hours in 1% osmium tetroxide, buffer-rinsed and dehydrated in an ethanol series [2]. For transmission EM, specimens were then infiltrated with a low viscosity epoxy resin, polymerized, sectioned at 70 nm on a Leica EM UC6 ultracut microtome, and double-stained with uranyl acetate and lead citrate. For scanning EM, dehydrated specimens were placed in hexamethyldisilazane, air dried overnight, mounted on stubs and coated with carbon. Specimens were viewed on a Zeiss Crossbeam 540 scanning electron microscope at 1kV, or at 80kV on a FEI BioSpirit Twin TEM fitted with an Olympus Quemesa CCD camera and OSIS calibrated software.

Antifungal susceptibility testing

The inoculum was prepared from fresh cultures and the turbidity adjusted using a turbidometer to the equivalent of a McFarland 1 standard (2.5 X 10^5) for the yeast phase and a McFarland 2 standard (5.0 X 10^5) for the mycelial phase [3]. Customised, round-bottomed frozen 96-well micro titre plates, containing two-fold dilution ranges of itraconazole, voriconazole, posaconazole, fluconazole, flucytosine, anidulafungin, caspofungin and micafungin, were immediately inoculated (TREK Diagnostic Systems, Inc., Cleveland, Ohio, USA). BMD MIC endpoints were read at 50% inhibition for fluconazole, voriconazole, posaconazole, itraconazole, flucytosine, caspofungin, anidulafungin and micafungin. Etest MICs for amphotericin B,

voriconazole, itraconazole and posaconazole were determined using Roswell Park Memorial Institute (RPMI) 1640 plates containing 2% glucose (DMP) according to the manufacturer's recommendations. Etest MIC endpoints were read as follows: 80% inhibition for voriconazole, posaconazole, itraconazole (i.e. micro-colonies within the elliptical zone of inhibition were ignored) and 100% for amphotericin B. All plates were incubated at 35ºC and read by 3 independent observers at 6 days. Quantitative colony counts were performed to assess the purity and accuracy of the final inoculum: the suspension was homogenized and 20 µℓ was spread over the surface of a Sabouraud agar plate and incubated at 35ºC until the number of viable colonies could be counted.

<u>DNA extraction, PCR amplification and sequencing</u>

Fungal DNA was extracted from 20 yeast-phase isolates using the Zymo ZR fungal/bacterial DNA MiniPrep Kit (Zymo Research Corp, USA) according to the manufacturer's instructions. PCR and sequencing of 5 genes (internal transcribed spacer [*ITS*], large subunit [*LSU*], actin, β-tubulin and intein *PRP8*) was performed as described by Kenyon *et al*. (2013) [4]. Briefly, each reaction contained 2.5 µl of 1X buffer (Life Technologies), 1.6 µl magnesium chloride (1.6 mM; Life Technologies), 0.25 µl deoxynucleotide triphosphate mix (0.1 mM; Life Technologies), 1 µl forward primer and 1 µl reverse primer (0.5 µM, Inqaba Biotechnical Industries, Pretoria, South Africa), 0.2 µl (1.0 U, HotStart Taq polymerase), 2 µl (genomic DNA) and 16.45 µl distilled water in a 25 µl reaction. DNA was denatured at 96°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, with a final extension step of 7 min at 72°C. The samples were sequenced in a 3130 Sequencer (Applied Biosystems, Life Technologies Corporation, USA) using the same primers. Sequences were subjected to BLAST analyses in GenBank (www.ncbi.nlm.nih.gov) for identification. Sequences were then submitted to GenBank

(Accesion ITS: MH571851, MH571852, MH571853, MH571854, MH571855, MH571856, MH571857, MH571858, MH571859, MH571860, MH571861, MH571862, MH606201, MH606202, MH606203, MH606204, MH606205, MH606206, MH606207, MH606208; LSU: MH644816, MH644817, MH644818, MH644819, MH644820, MH644821, MH644822, MH644823, MH644824, MH644825, MH644826, MH644836, MH644837, MH644838, MH644839, MH644840, MH644841, MH644842, MH644843).

MLST phylogenetic analysis

Sequences of *Emergomyces africanus, Emergomyces pasteurianus, Histoplasma capsulatum*, *Paracoccidioides lutzii, Paracoccidioides brasilliensis, B. dermatitidis*, *B. gilchristii*, *B. parvus* and *B. silverae* were obtained from Genbank and were added for phylogenetic analysis. All DNA sequences were aligned with MAFFT version 7 as described by Kenyon *et al*. (2013) [4]. The datasets for the five genes and the six MLST loci were analysed with the following settings: Q-INS-I, scoring matrix= 1PAM/K=2, Gap opening penalty=1.53, offset value=0. Threshold score=39{E=8.4e-11. Sequences were trimmed with BioEdit version seven and also checked manually [5, 6]. On MEGA we used Models to select the best DNA Models and the models for each gene region were as follows: *ITS*=TN93+I, *LSU*=JC, intein *PRP8*= K2+G, beta tubulin= K2+G and actin gene [7].

Phylogenetic analysis using whole-genome single nucleotide polymorphisms

Reads were aligned to the *Blastomyces percursus* assembly strain BP222 (GenBank accession GCA_003206225.1_ASM320622v1) using BWA-MEM version 0.7.12 [8, 9]. Variants were then identified using GATK version 3·4 [10]. Briefly, indels were locally realigned, haplotypeCaller was invoked in GVCF mode with ploidy = 1, and genotypeGVCFs was used to predict variants in

each strain. All VCFs were then combined and sites were filtered using variantFiltration with QD < 2.0, FS > 60.0, and MQ < 40.0. Individual genotypes were then filtered if the minimum genotype quality < 50, percent alternate allele < 0.8, or depth < 10. Reads from *B. dermatitidis* strain ER3 and *B. parvus* strain UAMH 130 were also included and variants were called for all samples as described above. For phylogenetic analysis, the 1,712,033 sites with an unambiguous SNP in at least one isolate and with ambiguity in at most 10% of isolates were concatenated; insertions or deletions at these sites were treated as ambiguous to maintain the alignment. Maximum likelihood phylogenies were constructed using RAxML version 8.2.4 using the GTRCAT nucleotide substitution model and bootstrap analysis based on 1,000 replicates [9].

Results

Examination of cultured isolates of *Blastomyces percursus* and *Blastomyces emzantsi*

Two distinguishable morphological groups were identified based on examination of cultures grown on Sabouraud agar (at 25°C) and brain heart infusion agar (at 37°C). Twelve isolates (group 1) resembled *B. percursus* morphologically (Figure S1). The remaining 8 isolates more closely resembled *B. parvus*: at 25°C, colonies were white and peripherally floccose, becoming wrinkled and slightly raised in the centre. Microscopically, at 25°C, both groups had septate hyphae with conidiophores arising at right angles to the hyphae or terminally, with unicellular globose-to-subspherical conidia borne singly or in clusters on small secondary conidiophores. All isolates demonstrated cellular enlargement, swelling and giant cell formation, with an increase in temperature between 25°C and 37°C, as well as the presence of thick-walled, chlamydospore-like cells at saprobic and intermediate (30°C) temperatures. As all group 1 isolates conformed to previous descriptions of *B. percursus*; they will not be described further [1, 11].

Description of *Blastomyces emzantsi*

On Sabouraud agar at 25°C, colonies were white and granular (powdery, wrinkled), with aerial hyphal tufts on centrally-raised areas, and with distinct though narrow, floccose margins. The reverse side was white-cream. Colonies grew to between 15 – 18 mm in diameter after one week of incubation, 50 – 55 mm after three weeks. On BHI agar at 37°C, colonies were beige, butyrous and cerebriform, occasionally retaining some white, aerial hyphae. The reverse was tan in colour, and colonies reached a diameter of 10 mm after 3 weeks of incubation. The saprobic phase consisted of slender, hyaline, septate hyphae [(0.9)–1.3-2.8–(4.2) µm in diameter; n = 50], with limited branching, and occasional helical hyphal gyres. Abundant spherical or subspherical conidia [(1.9)-3.4-(3.8) µm; n=50] were borne singly: either terminally; on short pedicles laterally; or on longer primary or secondary conidiophores. In the latter, up to 4 conidia were positioned on each terminally-inflated, primary conidiophore. It was common for conidia to be arranged in opposite pairs along the hyphae. Conidia were variously ornamented, from smooth (younger spores) to tuberculate (mature spores). During conidiogenesis, there were also distinctive clavate, complanate cells terminally/extending at right angles from the hyphae, many of which developed into conidiophores by septation (maybe more than once) and rounding of the terminal portion, although most of the other lateral conidiophores were slender and more delicate. Clavate cells were noted which were similar *Emmonsia sola* illustrated in Jiang *et al*. (2018) [1]. Laterally-borne conidia and clavate cells were less obviously septate, than terminally-positioned structures. As cultures age, intra/endohyphal growth occurred, and there was a tendency for hyphal filaments to adhere, forming rope-like bundles. With an increase in temperature, there was generalised swelling and vacuolation of all cells, lipid droplets accumulated intracellularly, lateral conidiophores became ampulliform, and doliform giant cells developed from the enlarged cells of fragmenting hyphal filaments. There was incomplete transition both *in vitro* and *in vivo*, so at

37°C, hyphae, fragmented enlarged hyphal elements, adiaspore-like cells that budded multilaterally [9.0–10.2 x 13.8-20.9 µm (n=50), infrequently as large as 22.3-24.3 x 26.4-34.2 µm (n = 4)], and yeast-like budding cells were infrequently observed [5.4-8.5 x 9.6-14.3 µm (n = 50)] on culture.

Figure S2 Phylogenetic analysis of *Blastomyces percursus* and *Blastomyces emzantsi*

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Figure S1: Microscopy of *Blastomyces percursus* (group 1 isolates), providing corroborative and supplementary information to the two published descriptions of this species [1, 11]. (a – i) cultures on Sabouraud agar after 3 weeks, 25°C; (j - n) cultures on Sabouraud agar after 3 weeks, 30°C; (a) floccose colony with a felt of white, aerial, hyphal tufts; (b) conidia generally solitary, and may be subtended by either narrow or ampulliform primary conidiophores; (c) an inflated primary conidiophore with 3 delicate secondary conidiophores; (d) laterally-borne, solitary conidia may have stout or slender conidiophores, and may also extend from pyramidal, chlamydospore-like, intercalary cells (arrow); (e) tangential section through part of an intercalary, thick-walled cell; (f) abundant conidia production; (g) section through hyphal cells and two conidia (arrows); (h) variable verrucose conidia ornamentation; (i) low-power, scanning micrograph illustrating aerial hyphal tufts; (j) increase in temperature induces cellular swelling, most obviously of terminal cells; (k) cell enlargement with increased temperature is not limited to conidia and terminal cells, as this section through an enlarged, thin-walled, intercalary cell illustrates; (l) conidia swell and become filled with oil droplets. Note the wall of an empty enlarged cell (arrow); (m) budding of cells into hyphal lumina was common, particularly at intermediate temperatures; (n) sections through hyphal cells revealed the multiple endohyphal/intrahyphal arrangement. Scale bars: a, b, c, e, m, n = 1 µm; d, g, k = 2 µm; f, j, l = 4 µm; h = 0.5 µm; i = 200 µm.

Figure S2: Maximum likelihood tree inferred from *ITS*, *LSU*, intein *PRP8*, beta tubulin and actin genes alignment of *ITS2*-*LSU*-*PRP8*-β-tubulin-actin based on 1000 replicates. Sequences of *Paracoccidioides lutzii*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Emergomyces africanus* were obtained from Kenyon *et al*. (2013) and *Blastomyces silverae, Blastomyces helicus, Blastomyces parvus* and *Emmonsia sola* were

obtained from Jiang *et al.* (2018). Phylogenetic analysis of each gene: *ITS*, *LSU*, intein *PRP8*, beta tubulin and actin gene sequences revealed 2 distinct groups. **A-E:** these groups corresponded to the recently-named *B. percursus* (group 1 strains comprising 12 isolates with only 7 isolates with detailed clinical information) and a separate cluster, here as designated *B. emzantsi* (group 2 comprising of 8 archived isolates with only 3 isolates with clinical information). Both groups were closely related to, yet separate from, *B. dermatitidis, B. gilchristii, B. parvus* and *B. silverae.* Only *LSU* could not separate *B. parvus* and, *B. silverae* from *B. emzantsi*.