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Authors
Torres-Cruz, Terry J
Tobias, Terri L Billingsley
Almatruk, Maryam
et al.

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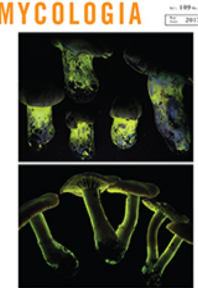

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Bifiguratus adelaidae, gen. et sp. nov., a new member of Mucoromycotina in endophytic and soil-dwelling habitats

Terry J. Torres-Cruz [a], Terri L. Billingsley Tobias[a], Maryam Almatruk[a], Cedar N. Hesse[b], Cheryl R. Kuske[b], Alessandro Desirò[c], Gian Maria Niccolò Benucci[c], Gregory Bonito[c], Jason E. Stajich [d], Christopher Dunlap[e], A. Elizabeth Arnold[f], and Andrea Porras-Alfaro [a]

[a]Department of Biological Sciences, Western Illinois University, Macomb, Illinois 61455; [b]Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545; [c]Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, Michigan 48824; [d]Department of Plant Pathology and Microbiology, University of California, Riverside, California 92521; [e]National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604; [f]School of Plant Sciences and Department of Ecology and Evolutionary Biology, The University of Arizona, Tucson, Arizona 85721

ABSTRACT
Illumina amplicon sequencing of soil in a temperate pine forest in the southeastern United States detected an abundant, nitrogen (N)-responsive fungal genotype of unknown phylogenetic affiliation. Two isolates with ribosomal sequences consistent with that genotype were subsequently obtained. Examination of records in GenBank revealed that a genetically similar fungus had been isolated previously as an endophyte of moss in a pine forest in the southwestern United States. The three isolates were characterized using morphological, genomic, and multilocus molecular data (18S, internal transcribed spacer [ITS], and 28S rRNA sequences). Phylogenetic and maximum likelihood phylogenomic reconstructions revealed that the taxon represents a novel lineage in Mucoromycotina, only preceded by *Calcarisporiella*, the earliest diverging lineage in the subphylum. Sequences for the novel taxon are frequently detected in environmental sequencing studies, and it is currently part of UNITE's dynamic list of most wanted fungi. The fungus is dimorphic, grows best at room temperature, and is associated with a wide variety of bacteria. Here, a new monotypic genus, *Bifiguratus*, is proposed, typified by *Bifiguratus adelaidae*.

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Bryophyte; dimorphic; endophyte; environmental sampling; Mucoromycota; Pinaceae; soil

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INTRODUCTION

Although fungal species are being described continuously, and have been for centuries, the majority of fungi remain undiscovered. Working estimates predict that 5.1 million species of fungi exist, yet only ~100,000 species have been described formally (Blackwell 2011). A recent study on the tree of life encompassing more than 1000 uncultivated organisms highlighted the large number of organisms for which cultured representatives are unknown (Hug et al. 2016). Indeed, new sequencing techniques frequently reveal large numbers of uncharacterized taxa that have not been described because of the lack of representative reference specimens in herbaria and/or culture collections (Martin et al. 2011). A major gap in our understanding of fungal diversity lies in determining the connections between fungi detected through next-generation sequencing or other culture-free methods and fungi already known to mycologists (Nilsson et al. 2016; Hesse et al. 2016). One approach is to use next-generation data sets to guide subsequent efforts to isolate target fungi from the same environments, thus providing biological material for further functional and molecular analyses (Hesse et al. 2016).

Many branches of the fungal tree of life are still poorly resolved and underrepresented (or lacking) in phylogenies. This is particularly true for lineages of early diverging terrestrial fungi (including those formerly classified as Zygomycota; Spatafora et al. 2016). Improved sampling of diversity in these groups is critical for illuminating key evolutionary innovations in the fungal kingdom and assessing the ecological functioning of novel taxa or those reported previously as nonculturable (or uncultured) (Grigoriev et al. 2011).

A previous investigation of the mycobiota associated with soil in a pine forest in North Carolina, USA (Hesse et al. 2016), detected a fungal taxon by 28S nuclear

CONTACT Andrea Porras-Alfaro a-porras-alfaro@wiu.edu
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ribosomal RNA (= 28S) sequencing. This fungal taxon was common in the soil and responded positively to forest nitrogen (N)-fertilization treatments (Weber et al. 2013; Hesse et al. 2016). Particle filtration of soils from the same site subsequently yielded two isolates (TLT265, YP243) representing the same 28S phylotype. When looking for similar sequences in the National Center for Biotechnology Information (NCBI), one other match (AZ0501) with >99% similarity for the internal transcribed spacer [ITS], 28S, and 18S regions was found. These three strains were compared and found to be similar in morphology and molecular characters. Here, we report and describe a novel taxon, *Bifiguratus adelaidae*, and place it as an early diverging lineage in Mucoromycotina through multilocus and phylogenomic analyses. Mucoid growth on the edges of the colonies suggested the presence of bacteria on the fungus, which was evaluated using culturing, microscopy, and next-generation sequencing techniques.

MATERIALS AND METHODS

Sample collection and site description.—As part of a previous study, soil cores were collected from a free air CO_2 enrichment (FACE) experiment located in a *Pinus taeda* plantation in the Blackwood Division of the Duke Forest, North Carolina, USA (35°58′N, 79°06′W; elevation 130 m). The FACE site was established in 1983 (Weber et al. 2013). In 2005, the CO_2 enrichment plots were quartered and annual N-fertilization experiments began in two randomly selected quadrants in the form of ammonium nitrate pellets ($11.2\,g\,N\,m^{-2}$). Soil sampling was designed to take into account the long-term field experiment and to minimally disturb soils in the plots. The sampling was performed within the fertilized and unfertilized quadrants of ring 5 (Weber et al. 2013). In October 2013, 10-cm soil cores were collected from one N-fertilized and one unfertilized quadrant within ambient CO_2 FACE plot 5. Immediately after collection, each core was sectioned into multiple vertical strata: Oi horizon (forest floor), Oa horizon (0–2 cm deep), upper A horizon (2–5 cm deep), and lower A horizon (5–10 cm deep). Each soil fraction was homogenized in an individual zip-lock bag. Soil samples were transported back to the laboratory and stored at 4 C until use.

Fungal isolation.—Fungi were isolated following two techniques, standard serial dilution methods for soil (O'Donovan et al. 2013) and a particle filtration-pasteurization method (Mueller et al. 2004). Detailed methods for isolation can be found in Hesse et al. (2016). Briefly, for the particle filtration method, seven sieves were used to separate different particle sizes (4000, 2000, 250, 500, 125, 63, and <63 μm). One gram of soil was loaded in the upper sieve (4000 μm). Particles were washed with 4–6 L of autoclaved sterile water for 10 min. The last rinse was 1 L of 70% ethanol to surface-sterilize the soil. The soil particles from each sieve were transferred to 9 mL of 0.2% water agar (used to suspend the particles). Dilutions of 10^{-1} and 10^{-2} were plated on peptone yeast glucose agar supplemented with tetracycline (50 mg/L) and streptomycin (50 mg/L). Cultures were incubated at 25 C for 15 d. Plates were checked regularly to obtain pure cultures.

Cultures and specimens from this study are preserved in the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Culture Collection (Peoria, Illinois), the herbarium of the University of Michigan (Ann Arbor, Michigan), the Robert L. Gilbertson Mycological Herbarium (Tucson, Arizona), and the Western Illinois University Fungarium (Macomb, Illinois).

Morphological characterization.—Mycelium from each isolate (TLT265, YP243, and AZ0501) was plated on seven types of media treated with tetracycline (50 mg/L) and streptomycin (50 mg/L). Hereafter, the use of antibiotics in media is represented with a plus sign (+). The seven media tested were 1.3% malt extract agar (MEA+) (Difco Laboratories, Sparks, Maryland), Sabouraud dextrose agar (SDA+) (Oxoid, Hampshire, England), Emerson yeast starch agar (EYSA+) (Difco Laboratories, Detroit, Michigan), Reasoner's 2A agar (R2A+) (Difco Laboratories, Sparks, Maryland), Czapek-Dox Agar (CDA+) (Sigma-Aldrich, St. Louis, Missouri), peptone yeast agar (PYG+) (Mueller et al. 2004), and potato dextrose agar (PDA+) (Oxoid). Media were prepared following the manufacturers' instructions. Cultures from each isolate were incubated separately at two temperatures, 25 and 35 C, for 30 d in darkness. The Elcometer 6210 RAL Chart K7-2014 was used as the color standard to describe the colonies. All cultures were produced from the same source culture for each strain.

Mycelial growth rates were measured on MEA+. Inoculum consisted of one 4 mm diam agar plug from the growing edge of a fungal colony on MEA+. Plates were incubated at five different temperatures at 10 C intervals from 5 to 45 C in the dark. Plates were examined every 5 d for 1 mo after cultures were established. Radial mycelial growth rates were calculated using arithmetic mean values per day by measuring

colony diameter (Aoki et al. 2013). Three replicates were measured for each isolate at each temperature.

The production of sporangiospores was assessed using corn meal agar (CMA+) (Sigma-Aldrich) supplemented with 50 mg/L chloramphenicol (Sigma-Aldrich). Cultures were incubated at 25 C and evaluated for 3 wk.

Microscopy.—Tissue of each isolate was collected from the growing edge of a 15-d-old colony on MEA+, SDA +, EYSA+, and PYG+ that had been incubated at 25 and 35 C. Hyphae were stained with Bluemount (Hardy Diagnostics, Santa Maria, California) and compared under the light microscope.

Tissue was fixed for scanning electron microscopy (SEM) by incubation for 24 h at 4 C in a solution of 25 mM phosphate buffer (Sigma-Aldrich) at pH 7 and 3% gluteraldehyde (Sigma-Aldrich). The fixative solution then was poured off, and a 1% osmium tetroxide solution (Electron Microscopy Sciences, Hatfield, Pennsylvania) was added to the tissue. After incubating at 4 C for 24 h, the fungal tissue was rinsed three times with 25 mM phosphate buffer (pH 7) and then soaked in the following ethanol series for 15 min for dehydration of the tissue (30%, 50%, 65%, 75%, 89%, 95%, 3× 100%) and kept overnight at 100%, with two more 100% soaks the next day. After fixation, the specimens were dried in a carbon dioxide critical point dryer (Tousimis, Rockville, Maryland) and immediately placed in a sputter coater for a 2-min coating of gold (Structure Probe, West Chester, Pennsylvania). The SEM images were obtained on a JEOL JSM-6010LA microscope (JEOL, Tokyo, Japan) at the National Center for Agricultural Utilization Research, Peoria, Illinois, USA. Typical operating conditions were an accelerating voltage of 10 kV and a spot size of 30.

DNA extraction, amplification, and sequencing.—DNA was extracted from the cultures obtained, and 28S nuc rRNA was amplified following the protocols described below. The sequences were compared against GenBank sequences (Altschul et al. 1997). The two cultures (TLT265 and YP243) matched the same phylotype as the one detected through culture-free sequencing (Hesse et al. 2016). The 28S sequences for TLT265 and YP243 were compared against GenBank sequences using BLASTn. One isolate with high similarity (>99%) from another study was identified (BLAST score 1125 for both isolates; e value = 0). This isolate (AZ0501, GenBank accession number HM123225) was collected in 2007 in a study of endophytic fungi in a montane forest in southeastern Arizona (U'Ren et al. 2010). AZ0501 was isolated from photosynthetic tissue of a bryophyte (*Leucobryum* sp., Dicranaceae; U'Ren et al. 2010). This strain was obtained from the Robert L. Gilbertson Mycological Herbarium at The University of Arizona (accession number MYCO-ARIZ-AZ0501) and characterized.

Genomic DNA was extracted from cultures of each isolate using the Wizard genomic DNA purification kit (Promega, Madison, Wisconsin). Polymerase chain reaction (PCR) was used to amplify the partial 18S nuc rRNA (=18S) with primers NS1 and NS4 (White et al. 1990), the partial 28S using primers LR0R and LR3 (Vilgalys and Hester 1990), and the ITS1-5.8S-ITS2 nuc rRNA (ITS barcode) using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Each 25 μL of PCR mixture contained 12.5 μL of PCR master mix (Promega), 3 μL of 1% bovine serum albumin (BSA), 1 μL of each primer (5 μM), 6.5 μL of nuclease-free water, and 1 μL of DNA. Water was used in place of template for negative controls. The following PCR conditions were used: 95 C for 5 min; 35 cycles of 94 C for 30 s, annealing at 50 C for 30 s, and extension at 72 C for 45 s; and a final extension at 72 C for 7 min. PCR products were checked using gel electrophoresis (1.2 % agarose in Tris-acetate-EDTA buffer). All samples were sequenced using the forward primer at Beckman Coulter Genomics (Danvers, Massachusetts). Sequences obtained were trimmed and edited using Sequencher (Gene Codes, Ann Arbor, Michigan). Similar sequences were identified by comparison with GenBank sequences via the Basic Local Alignment Search Tool (BLASTn option) (Altschul et al. 1997) and comparison with the UNITE database (Kõljalg et al. 2013). The ITS partial 28S sequence of AZ0501 had been submitted previously to GenBank (HM123225; U'Ren et al. 2010). The sequences obtained here were deposited at GenBank (ITS: KU702608, KU702388; 28S: KU702551, KU702228; 18S: KU702505, KX372676, KX372677). The percentage similarity over the shared length for each locus among the three isolates (estimate of evolutionary divergence between sequences) was determined by the number of nucleotide differences per site between sequences in MEGA7 (Kumar et al. 2016).

Phylogenetic analyses.—Fungal sequences were edited in Geneious 8.1.7 (http://www.geneious.com; Kearse et al. 2012) and used as queries to conduct BLAST searches for sequence similarity. Sequences were aligned with MUSCLE (Edgar 2004). The alignments were trimmed with GBlocks 0.91b (Castresana 2000) using the less stringent conditions and then manually edited. The 18S and 28S data sets were concatenated into a single data set of 1581 homologous

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nucleotide bases. Prior to phylogenetic inference, the best-fit models of nucleotide substitution were estimated using jModelTest 2.1.9 (Darriba et al. 2012), for 18S and 28S data separately. Bayesian inference on the phylogeny of these taxa was carried out using MrBayes 3.2.5 (Ronquist et al. 2012). The Markov chain Monte Carlo was run for 5 million generations under the TrN+I+G and TIM3+G nucleotide substitution models for 18S and 28S, respectively. Representatives of Mucoromycotina, Mortierellomycotina, and Glomeromycotina were selected to resolve the position of the novel fungal taxon within the recently erected phylum Mucoromycota (Spatafora et al. 2016). Alignments were submitted to TreeBASE (S19770).

Genome sequencing and phylogenomic analysis.—Isolate AZ0501 was grown in liquid yeast mold (YM) medium for approximately 3 wk. Hyphal tissue was filtered from the growth medium, and genomic DNA was extracted using the FastDNA SPIN Kit for soils (MP Biomedicals, Santa Ana, California). DNA was quantified using a QuBit 2.0 Fluorometer (Life Technologies, Waltham, Massachusetts). DNA was fragmented using a Covaris E220 focused-ultrasonicator (Woburn, Massachusetts) and fragment sizing assessed using Agilent 2100 BioAnalyzer (Santa Clara, California). Fragments were made blunt and adenylated at the 3' end to ligate Illumina sequencing adapters (San Diego, California). A unique index code was added during PCR amplification. Concentration was confirmed using KAPA Library quantification kit (Kapa Biosystems, Wilmington, Massachusetts) on an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, California). The AZ0501 Illumina library was sequenced using Illumina's MiSeq v2 500-cycle reagent kit (San Diego, California) as a spike-in with rRNA gene amplicon libraries on five separate Illumina MiSeq runs. On each run, this isolate constituted about 30% of the sequences. Sequences from individual MiSeq runs were combined into a single FASTA file and assembled de novo using the Velvet software package (Zerbino 2010) with a k-mer value of 61 bp. Genome annotation for developing genes for phylogenomic analyses was performed with MAKER (Holt and Yandell 2011) supported by ab initio gene prediction on the assembled contigs with Augustus (Stanke et al. 2004) using *Rhizopus delemar* parameters and self-trained GeneMarkHMM-ES (Ter-Hovhannisyan et al. 2008). Raw reads were deposited to the Sequence Read Archive (SRA) as Bioproject PRJNA368732 and Biosample SAMN06270744. Genome was deposited to GenBank (INSDC: MVBO00000000).

Phylogenomic analyses were performed with an approach previously applied for zygomycete genomes (Spatafora et al. 2016). The data and analysis pipeline are available at https://github.com/zygolife/Bifiguratus_Phylogenomics. Briefly, the approach used 434 protein coding genes that are typically single copy in most fungi. Each phylogenetic marker was summarized as a profile hidden Markov model (HMM) (HMMER3; Eddy 2011) from an alignment of the orthologs from several dozen diverse fungi. The phylogenomic pipeline searched each HMM against the protein set of a species with HMMSEARCH, and the best hit from each species was saved. A multiple sequence alignment (MSA) containing the orthologous copies from each species was constructed with HMMALIGN of these copies guided by the marker HMM. Each gene MSA was trimmed with trimAl (Capella-Gutiérrez et al. 2009), and the trimmed MSAs were concatenated into a single master alignment with a Perl script built with BioPerl (Stajich et al. 2002). Phylogenetic tree inference was performed with RAxML (8.2.8) using autoMRE and rapid boostrapping (-f a) (Stamatakis 2014).

Analysis of associated bacteria.—Mucoid growth was observed on the edges of cultures under some conditions, especially PYG+ at 35 C. Light microscopy and culturing were used to evaluate the presence of bacteria on these mucoid areas. Bacteria present on fungal tissue were streaked onto R2A, from the three isolates grown on the seven media described above. The cultures were incubated at 25 C for 7 d. We attempted to remove bacteria from the fungus using different antibiotics, media, and temperatures, with little success. However, their growth was especially limited on MEA+ at 25 C.

DNA was extracted and amplified from the bacterial cultures following the protocol used for fungal samples (above). DNA amplification, sequencing, and identification were performed as described above, except that we used universal primers for the 16S rRNA (= 16S) gene (8F and 1492R; Lane 1991) and an annealing temperature of 56 C. Sequencing was performed using primer 1409R. Further sequencing of the *gyrA* gene of *Bacillus* strains was performed with primers p-gyrA-F and p-gyrA-R (Roberts et al. 1994) for identification (Chun and Bae 2000). Sequences were deposited to GenBank (KX397340–KX397345 for 16S; KX670823–KX670827 for *gyrA*).

Next-generation sequencing was used with the purpose of confirming the presence of bacteria that may have evaded culturing. To assess dynamics of bacteria associated with this novel fungal taxon, isolates were grown on three media (PYG+, MEA+, and SDA+) at 25 and 35 C. Three replicates were evaluated, one for each isolate (TLT265, YP243, and

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AZ0501), for a total of 18 samples. Tissue was collected from all samples and sent to MRDNA Molecular Research LP (Shallowater, Texas) for DNA extraction and Roche 454 FLX titanium sequencing. The 16S universal bacterial primer 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) was used in a modified version of amplicon pyrosequencing (bTEFAP) (Dowd et al. 2008). A single-step 30-cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, California) was run under the following conditions: 94 C for 3 min; followed by 28 cycles of 94 C for 30 s, 53 C for 40 s, and 72 C for 1 min; with a final elongation step at 72 C for 5 min. Amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience, Beverly, Massachusetts). Samples were sequenced with Roche (San Jose, California) 454 FLX titanium instruments and reagents following the manufacturer's procedures. With each new box of DNA extraction reagents, two negative controls were run. No detectable amplification with universal 16S primers was detected on the controls.

Multiplex tags were used to parse the sequence library into 18 respective samples using the QIIME software package (Caporaso et al. 2010). The sequences obtained were denoised using AmpliconNoise (Quince et al. 2011), quality filtered and trimmed, and operational taxonomic units (OTUs) were clustered in USEARCH (Edgar 2010) using the UPARSE (Edgar 2013) algorithm considering a pairwise sequence identity percentage of 0.97. Chimera filtering was performed using UCHIME (Edgar et al. 2011) comparing with the RDP Gold database (http://drive5.com/usearch/manual/cmd_uchime_ref.html). Taxonomy was assigned using the RDP Classifier (Wang et al. 2007) against the Greengenes database (DeSantis et al. 2006) and corroborated with BLAST (Altschul et al. 1997) matches against GenBank sequences (Benson et al. 2011). Singletons and sequences assigned to mitochondria were removed. Raw reads were deposited to the Sequence Read Archive (SRA) as study accession SRP082174 (Bioproject PRJNA318907).

RESULTS

Two isolates of the focal fungus (YP243 and TLT265) were obtained using filtration-pasteurization methods from soil. Both isolates were obtained from the lower A horizon (5–10 cm deep) of a N-fertilized plot under ambient CO_2 in a pine plantation in North Carolina, USA. Isolates YP243 and TLT265 were obtained from the 63 μm and 2000 μm particle sizes, respectively, and grown at 25 C on PYG+ (Hesse et al. 2016). These isolates were evaluated with the only known, cultured closest relative in GenBank, isolate AZ0501, which was obtained from surface-sterilized photosynthetic tissue of a moss (U'Ren et al. 2010). Isolates YP243 and TLT265 share >99% similarity for the ITS, 18S, and 28S sequences with AZ0501 (SUPPLEMENTARY TABLE 1). Morphological characterization of the

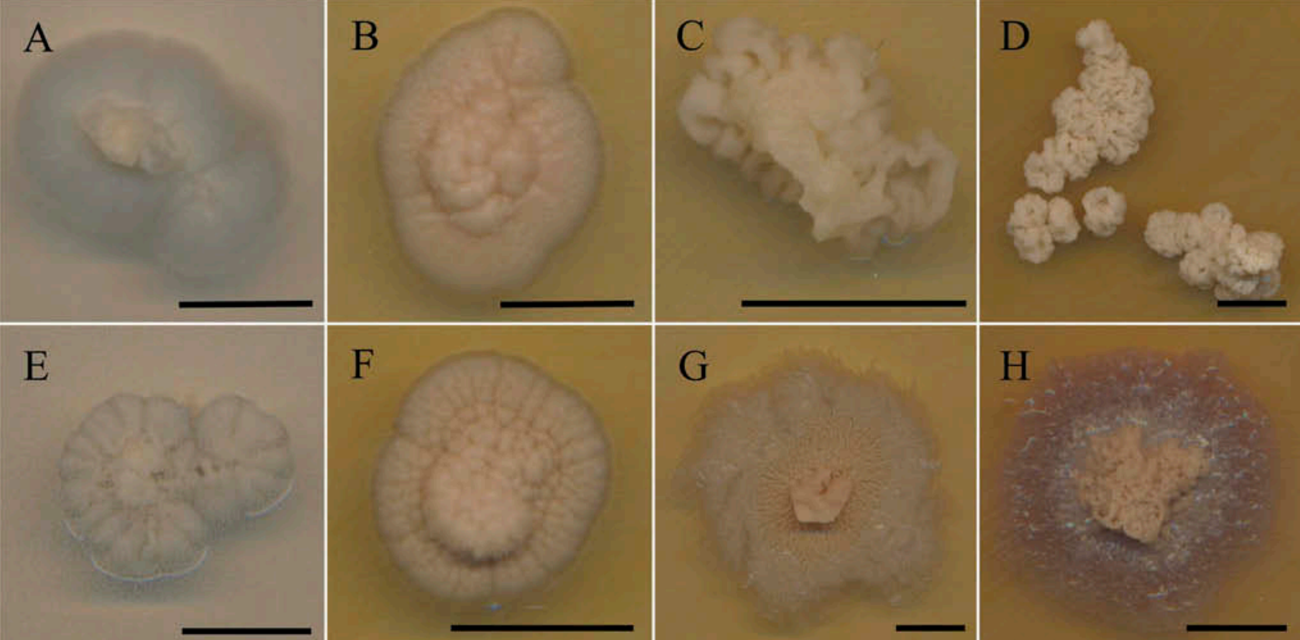

Figure 1. *Bifiguratus adelaidae* colony morphology on different media and temperatures. A. MEA+ at 25 C. B. SDA+ at 25 C. C. EYSA+ at 25 C. D. PYG+ at 25 C. E. MEA+ at 35 C. F. SDA+ at 35 C. G. EYSA+ at 35 C. H. PYG+ at 35 C. Each image represents a different isolate. All isolates (AZ0501, TLT265, YP243) had consistent growth characters on each medium. Bars = 10 mm.

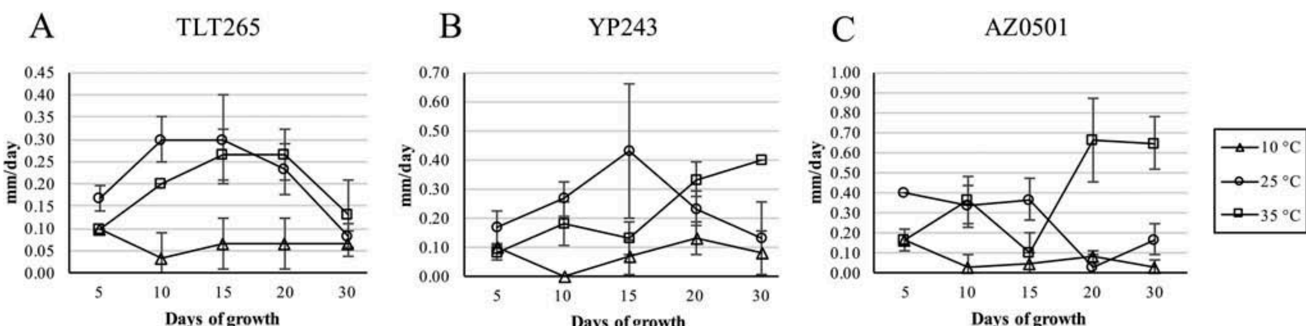

Figure 2. Radial growth rates of *B. adelaidae* per day on MEA+ under three different temperatures during 30 d. Vertical bars indicate standard deviation. A. Isolate TLT265. B. Isolate YP243. C. Isolate AZ0501. Δ = 10 C; ○ = 25 C; □ = 35 C.

Figure 3. Light microscopy of *B. adelaidae* isolate TLT265. A. Yeast cells. B. Putative intercalary chlamydospores (dark purple) in coenocytic hyphae. C. Sporangium. Bars = 25 µm.

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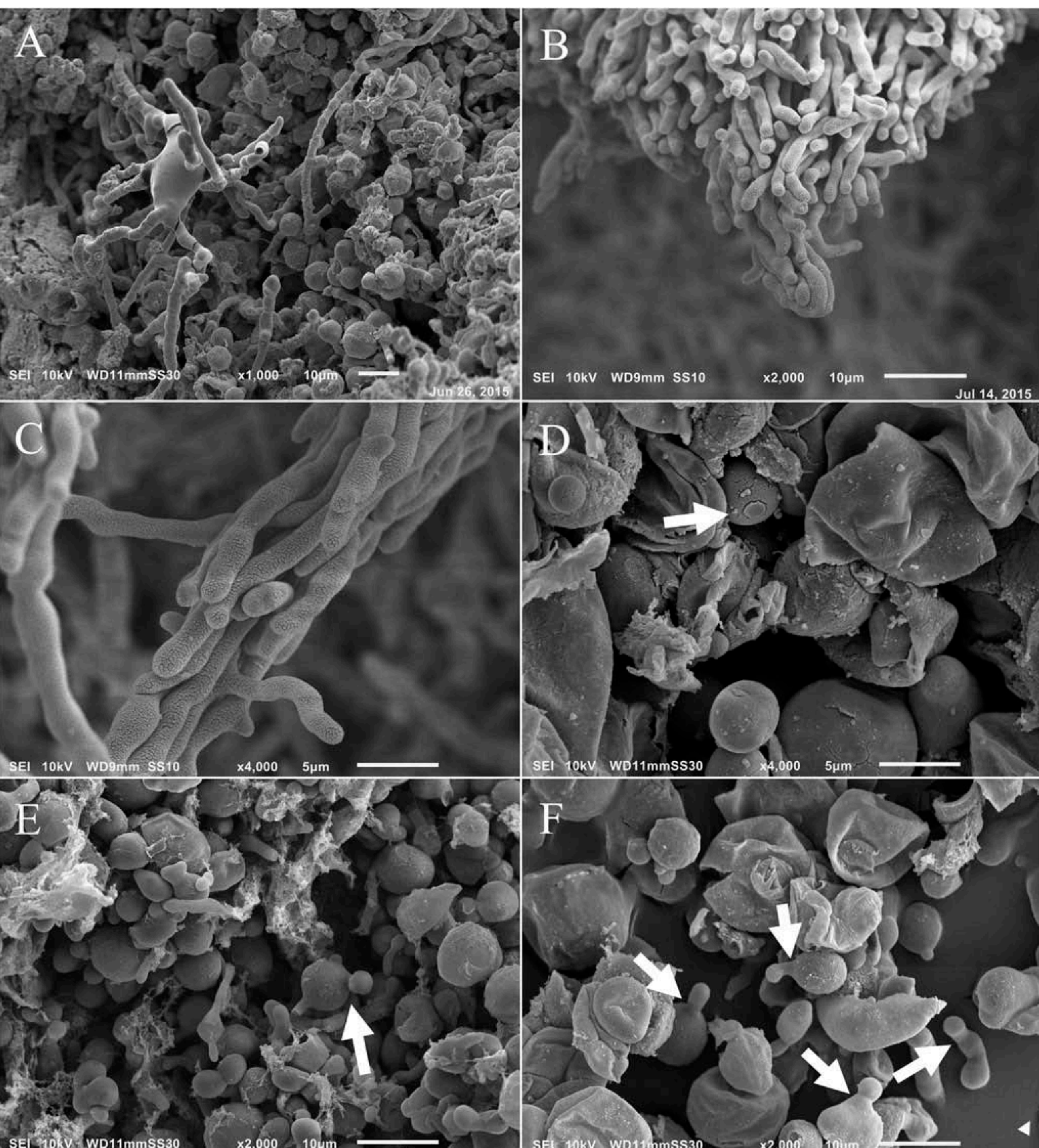

Figure 4. Scanning electron micrographs of *B. adelaidae* hyphal (A–C) and yeast (D–F) structures. A. Putative germinating chlamydospore with several germ tubes. B. Profuse vegetative hyphae. C. Hyphal wall surface ornamentations. D. Yeast bud scar (arrow). E. Budding of yeast cells (arrow). F. Germinating yeast cells, arrows point to germ tubes. Micrograph E is from YP243; all other images are from isolate TLT265.

isolates is provided in the taxonomic description, including growth on different media (FIGS. 1, 2) and microscopy (FIGS. 3, 4).

Phylogenetic analyses.—Given the lack of available data for genomes of fungi such as *Endogone* and *Sphaerocreas*, and because ITS sequences could not be aligned across this phylum, concatenated 18S and 28S analyses containing

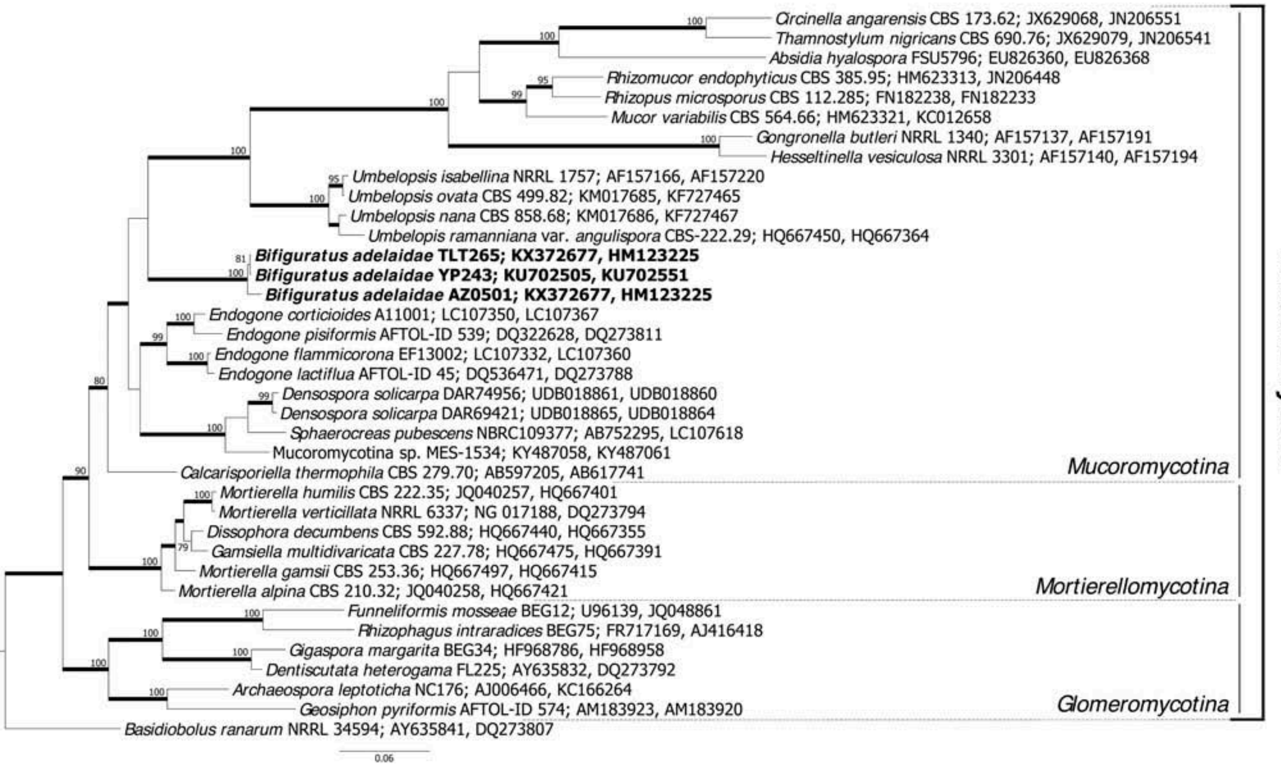

Figure 5. Phylogenetic placement of *Bifiguratus adelaidae* within Mucoromycota (Spatafora et al. 2016) based upon concatenated 18S and 28S rRNA gene sequence alignment. *Basidobolus ranarum* was used as outgroup. Branches with Bayesian posterior probabilities values >0.95 are thickened and ML bootstrap support values >70 are shown.

more complete taxon sampling were used to estimate the phylogenetic placement of these strains within Mucoromycotina. Isolates AZ0501, TLT265, and YP243 clustered together within a well-supported monophyletic clade that branched after early diverging lineages such us *Calcarisporiella* (Hirose et al. 2012), *Densospora* (McGee 1996), *Endogone* (Yamamoto et al. 2015), and *Sphaerocreas* (Hirose et al. 2014) and unidentified Mucoromycotina (Truong et al. 2017) (FIG. 5).

Genome sequencing and phylogenomic analysis.— Sequencing generated about 6.8 million high-quality reads, of which 5.6 million reads assembled into 568 scaffolds of at least 500 bp. The final assembly had an N_{50} of ~928 kbp, an overall genome assembly length of ~19.5 Mbp, and a GC content of 48.3%. The taxon represented by these three strains has 8876 predicted protein-coding genes. This is a nearly complete genome, but it lacks RNA transcriptome data to provide additional support for gene models. The assembly shows a high degree of completeness with a Benchmarking Universal Single-Copy Orthologs (BUSCO) score of 82.1% using Fungi ODB9 set. The genome is 86% complete. Out of the 290 BUSCO groups searched, we found 217 complete single-copy, 21 complete duplicated, 12 fragmented, and 40 missing BUSCO orthologs (Simão et al. 2015). These results are similar to other Mucoromycotina (TABLE 1). Indicative of a nearly complete genome assembly, we identified 237 of 248 superconserved core eukaryotic genes (CEGs). The CEGMA pipeline (Parra et al. 2007) suggests 95.6% genome completeness (SUPPLEMENTARY TABLE 2).

Table 1. Benchmarking Universal Single-Copy Orthologs (BUSCO) results for *Bifiguratus adelaidae* and other Mucoromycotina.

BUSCO	*Bifiguratus adelaidae*	*Phycomyces*	*Calcarisporiella*
Complete BUSCO orthologs	238	266	266
Complete and single-copy BUSCO orthologs	217	214	217
Complete and duplicated BUSCO orthologs	21	52	49
Fragmented BUSCO orthologs	12	13	13
Missing BUSCO orthologs	40	11	11
Total BUSCO groups searched	290	290	290

The maximum likelihood phylogenetic tree generated with RAxML was produced from a concatenated alignment of 434 protein-coding genes (165,307 residues across 10 taxa). The phylogenomic tree places the new taxon as one of the early diverging lineages in Mucoromycotina, only preceded by *Calcarisporiella*, the first diverging known lineage in the subphylum (FIG. 6).

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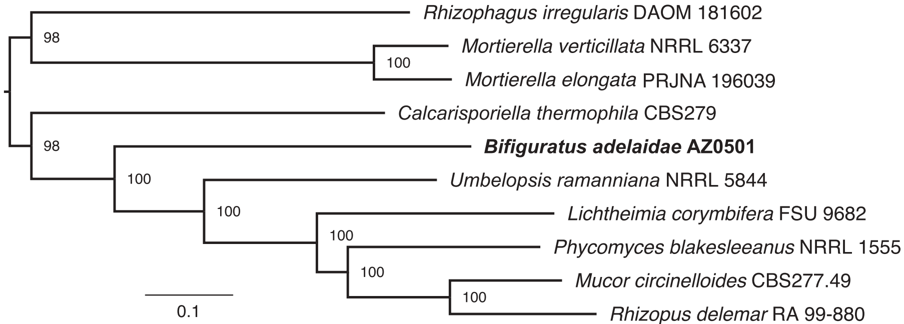

Figure 6. RAxML phylogenetic tree of Mucoromycota based on a concatenated alignment of 434 conserved orthologous proteins. *Bifiguratus adelaidae* isolate AZ0501 is in bold. Bootstrap values are shown on respective branches.

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Analysis of associated bacteria.—Two genera of bacteria were successfully isolated and identified based on 16S: *Bacillus licheniformis* (KX397340–KX397344) and *Stenotrophomonas* sp. (KX397345). Because of the limitations of the 16S rRNA gene in resolving members of the *Bacillus subtilis* complex, identities of the *Bacillus licheniformis* strains were confirmed using the *gyrA* gene (KX670823–KX670827) (SUPPLEMENTARY TABLE 3). Both bacteria were recovered from PYG+, SDA+, and CZP+ at 25 C. Although bacteria were not isolated from fungal isolate AZ0501, they were observed on this isolate using SEM (FIG. 7C, D) and were confirmed by direct sequencing (FIG. 7A).

Culture-independent methods based on 454-amplicon pyrosequencing were used to assess bacterial communities associated with the three isolates grown on different media at two temperatures (25 and 35 C). A majority of the sequences turned out to be fungal mitochondria amplicons, which were removed from the analysis. The remaining data show numerous bacterial taxa associated with these three fungal isolates (FIG. 7A). However, aside from *Bacillus, Methylobacterium* spp., and *Propionibacterium*, most bacterial taxa were in low read abundance (<10 sequences/OTU), and in 4 of the 18 treatments, no bacteria were detected (SUPPLEMENTARY TABLE 4). Venn diagrams (FIG. 7B) show that few bacterial OTUs are shared between isolates, with the majority of OTUs being distinct for each isolate. *Bacillus* and *Methylobacterium* were the dominant taxa associated with YP243 and TLT265; however, *Bacillus* was not detected from isolate AZ0501 in the 454-amplicon data set, nor when isolates were grown on MEA+ and SDA+ media (FIG. 7A, B). Isolates TLT265 and YP243 showed higher bacterial richness than isolate AZ0501. Variation in bacterial taxa and their relative abundance (in terms of sequence reads) was observed among temperature and medium treatments (FIG. 7A, B). A greater number of bacterial OTUs were detected from strains obtained from soil and those growing on MEA+ (FIG. 7B). Although a core set of bacterial OTUs are present at both temperatures (25 and 35 C), incubation temperature appeared to shift the bacterial communities in these isolates. In contrast, three *Methylobacterium* OTUs were consistently detected from all three isolates at 25 and 35 C on MEA+ and SDA+.

TAXONOMY

Bifiguratus T.J. Torres-Cruz & A. Porras-Alfaro, gen. nov. FIGS. 1, 3, 4

MycoBank MB818093

Typification: Bifiguratus adelaidae T.J. Torres-Cruz & A. Porras-Alfaro.

Etymology: From the Latin *"figuratus,"* in reference to the dimorphic characteristic of the type species.

Bifiguratus adelaidae T.J. Torres-Cruz & A. Porras-Alfaro, sp. nov. FIGS. 1, 3, 4

MycoBank MB818094

Typification: UNITED STATES. ARIZONA: Cochise County, Coronado National Forest, eastern Chiricahua Mountains, dried culture from an isolate from surface-sterilized photosynthetic tissue of *Leucobryum* sp., Arizona, USA, Jun 2007, *J.M. U'Ren, A.E. Arnold, et al.* (**holotype** MICH 134798). Ex-type culture MYCO-ARIZ-AZ0501 = NRRL 66559.

Etymology: In honor of Adelaida Chaverri Polini, a female Costa Rican scientist.

Full description: Cultures of the three strains grown on MEA+ at 25 C present a white (RAL 9003) smooth flat colony with entire margins (FIG. 1A). At 35 C, colonies are smaller than at 25 C, white (RAL 9003), and 1–2 mm raised with undulate margins and a rough rugose surface (FIG. 1E). On SDA+ at 25 C, colonies are cream (RAL 9001), rough, and 1–2 mm raised with entire margins (FIG. 1B), whereas at 35 C the colonies have the same characteristics but are more corrugated (FIG. 1F). Colony characteristics on CMA+ at 25 C are consistent with those observed on

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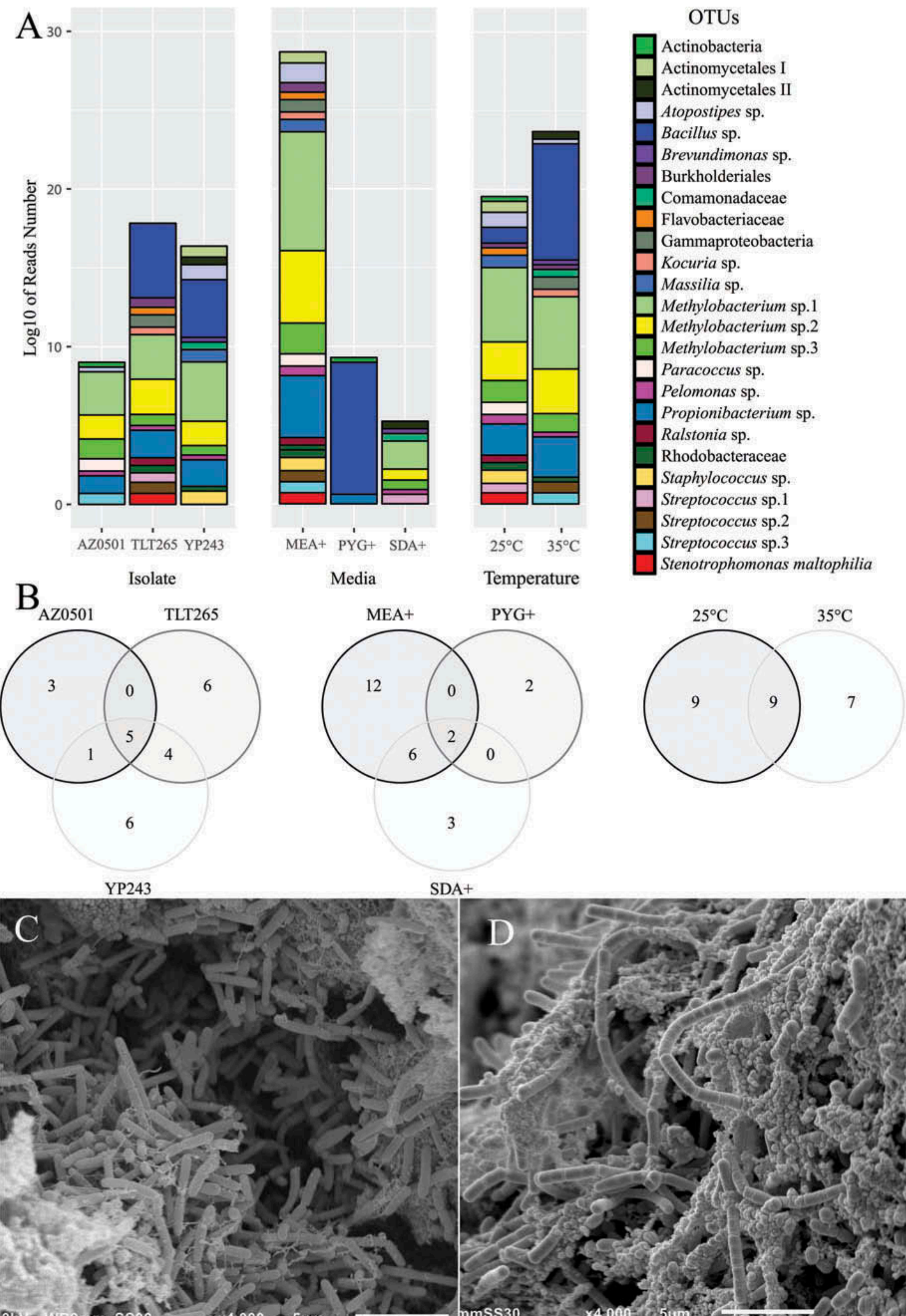

Figure 7. Bacteria associated with isolates of *B. adelaidae*. A. Sequence read abundance of the 25 bacterial operational taxonomic units (OTUs) detected through the 454-amplicon pyrosequencing of three isolates of *B. adelaidae* grown at different temperatures and on different medium types. OTU relative sequence abundance is expressed as $\log_{10}$ of read number. The plot was generated with the R package PHYLOSEQ (McMurdie and Holmes 2013). Bars with less than one read in a single sample group are not displayed in bar plots. B. Venn diagrams of the bacterial OTUs comparing diversity between isolates, media, and temperatures (left to right). C, D. Scanning electron micrographs of bacteria present on *B. adelaidae* isolate AZ0501. Bars = 5 µm.

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SDA+. On EYSA+ at 25 C, colonies are pure white (RAL 9010), rough; 2–3 mm high colonies have lobate margins (FIG. 1C). On this medium at 35 C, colonies are light ivory (RAL 1015) and flat but still wrinkled, with a filamentous margin (FIG. 1G) and a more mucoid texture is observed at the edges with a cream pigmentation (RAL 9001). On PYG+ at 25 C, colonies are highly corrugated (FIG. 1D), irregular, cream (RAL 9001), with undulated margins, and are 3–4 mm high. At 35 C on PYG+, colonies are cream (RAL 1001), corrugated, and raised, but the edges present a mucoid growth with undulated margins (FIG. 1H) that have a translucent beige-red color (RAL 3012). The presence of a mucoid texture at the edges of the fungus growing at 35 C on EYSA+ and PYG+ (FIG. 1G–H) is related to more abundant growth of bacteria in the cultures, with respect to MEA+ and PDA+. On PDA+, the fungus has the same morphological characteristics as those on PYG + at 25 C and the morphology remains the same at 35 C. None of the strains grew on R2A and CDA; fungus-associated bacteria colonized the plates instead. All cultures were produced from the same source culture for each strain, such that the bacteria appear to have been present in the source and became dominant on R2A and CDA media.

As cultures age (i.e., after more than 1 mo of incubation), their morphology changes. In some cases (e.g., SDA+), colonies become more mucoid and flat and are overcome by bacterial growth. However, in most cases (e.g., MEA+), cultures present more hyphal growth at the edges and colonies become darker, turning brown.

Colonies on MEA+ have radial mycelial growth rates of 0.4 mm/d at 25 C and 0.2 mm/d at 35 C in the dark, during the first 15 d of incubation. Growth rates at different incubation temperatures are shown in FIG. 2. The three isolates did not grow at 5 C or at 45 C. The isolates generally grew more rapidly at 25 C during the first 2 wk of incubation. After 15 days, the growth rate decreased at 25 C and increased at 35 C for YP243 and AZ0501.

The three strains possess coenocytic mycelium with hyphae ca. 2 μm diam (15 day old culture, MEA+, 25 C; FIGS. 3B and 4B). When observed by SEM, hyphal surfaces present rough texture with surface ornamentations (FIG. 4B–C). No sporangiospores were observed, although multiple attempts were made using PDA, CMA, and several temperatures. The fungus produces what appear to be intercalary and terminal chlamydospores 5–10 μm diam, which appear globose to subglobose (FIGS. 3B, 4A), but additional studies are necessary to confirm the function of these structures. SEM observations revealed the presence of yeast-like cells (FIG. 4D–F). Bud scars and budding yeasts were observed (FIG. 4D–E), and germ tubes were visualized on yeast-like cells (FIG. 4F). They were commonly observed when the fungus was cultivated on MEA+ (FIG. 4E) and EYSA+ (FIG. 4D, F) at 25 C. Thus, these isolates appear to be dimorphic, i.e., with both mycelial and yeast-like growth.

Other cultures examined: UNITED STATES. NORTH CAROLINA: Blackwood Division of the Duke Forest free air CO_2 enrichment (FACE) experiment (35°58′N, 79°06′ W; elevation 130 m), Oct 2013, *C.R. Kuske*, MICH 134800 (NRRL 66560 = WIU-APA-TLT265) and MICH 134801 (NRRL 66561 = WIU-APA-YP243).

Substrate: Because only three cultures are currently available for this fungus, we based analyses of substrates and potential hosts on analyses of available ITS rRNA data from BLASTn analysis. Two cultures (YP243 and TLT265) were recovered from soil, and apparently related fungi have been detected on several other soil samples using direct sequencing from environmental samples including temperate forest soils (HQ021892, DQ421188, DQ421187), truffle grounds soils (JF927042, JF927041), rice field soils (AB520601), and humus under *Pinus sylvestris* (GU559100).

Our previous report suggested that this species represented up to 9% of 454 sequences in individual samples at the Duke Forest (Hesse et al. 2016). It is rarely found in the forest floor and is more common on the deeper horizons (Hesse et al. 2016). Hesse et al. (2016) showed that the species increases in abundance with soil depth and reaches the highest abundance in a 5–10 cm depth. It responds to the combination of N fertilization and CO_2 by increasing in relative abundance at the 2–5 cm and 5–10 cm deep intervals (Hesse et al. 2016).

In addition to occurring as a culturable endophyte in *Leucobryum* (AZ0501; U'Ren et al. 2010), environmental data show that related strains have been detected as a root-associated fungus of the orchid *Cymbidium kanran* (KU141166; Hong et al. 2015) and sweet chestnut, *Castanea sativa* (EF040836; Peintner et al. 2007).

Distribution: This species is known from the Northern Hemisphere. In the United States, phylotypes consistent with *B. adelaidae* have been detected from the Duke Forest, North Carolina (YP243 and TLT265); Bartlett Experimental Forest, New Hampshire (HQ021892); Cedar Creek Ecosystem Science Reserve, Minnesota (DQ421188, DQ421187); and Coronado National Forest, Arizona (AZ0501). It has also been detected in Italy (JF927042, JF927041, EF040836), Japan (AB520601), Sweden (GU559100), and South Korea (KU141166).

DISCUSSION

Fungal isolates obtained from soil (TLT265, YP243) and as an endophyte (AZ0501) provided the

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opportunity for morphological, multilocus, and phylogenomic description of a new endophytic and soil-dwelling species of Mucoromycota with an apparent wide distribution across the Northern Hemisphere. This dimorphic species is affiliated with epihyphal bacteria and appears to have a wide host range spanning multiple lineages of plants, as well as subsurface soils in diverse forests. This fungus is part of UNITE's "most wanted" fungi as cluster code UCL7_006587, ranked number 1 at the phylum level based on number of studies and number 4 based on number of sequences (Nilsson et al. 2016). Description of this species exemplifies the potential to use environmental sequencing to guide taxonomic discovery. Its description and characterization represents a step towards bridging the gap between fungal taxonomy and molecular ecology.

Asexual reproduction among lineages formerly known as zygomycetes can be documented by the formation of chlamydospores, sporangiospores, yeast cells, or arthrospores (Fuller 1978; Benny et al. 2014). *Bifiguratus adelaidae* presents coenocytic hyphae, globose intercalary chlamydospores, and yeast-like cells. The presence of yeast-like cells in other Mucoromycotina taxa has been described (e.g., *Mucor*, *Benjaminiella*, and *Mycotypha*; Benny et al. 2014). After budding, scar tissue is left behind and the mother cell exhibits a crater-like ring of scar tissue (Chant and Pringle 1995; Powell et al. 2003), consistent with similar structures observed in *B. adelaidae* (FIG. 4D). Budding yeasts were also viewed (FIG. 4E). Chlamydospores form in swellings of coenocytic young hyphae (Benny et al. 2014). We observed what appears to be germinating chlamydospores (FIG. 4F), which also have been observed in other dimorphic fungi (e.g., Ascomycota such as *Candida albicans* and *Candida dubliniensis*). In those species, chlamydospores can generate daughter chlamydospores, bud to form daughter yeast cells, and germinate to produce pseudohyphae and hyphae (Citiulo et al. 2009). Chlamydospores have been reported in other Mucoromycotina fungi, including *Umbelopsis* (Wang et al. 2013), *Ambomucor* (Liu and Zheng 2015), and *Isomucor* (Ivanildo et al. 2012). Chlamydospore formation can be induced by bacteria associated with fungi, as seen in three Mucoromycotina species (*Phycomyces blakesleeanus*, *Mucor bacilliformis*, and *Mucor hiemalis*) where a lipopeptide produced by *Ralstonia solanacearum* induces the production of chlamydospores as a survival strategy for the fungus (Spraker et al. 2016). Perhaps the presence of bacteria on *B. adelaidae* could influence chlamydospore production (FIG. 3B), a topic that will require further study.

Phylogenetic inferences based on the concatenated partial 18S and 28S loci placed *B. adelaidae* as a novel lineage in Mucoromycota (FIG. 5). In the phylogenomic analysis (FIG. 6), the *B. adelaidae* branch diverges after *Calcarisporiella*, the earliest known branching Mucoralean fungus or sister to Mucoromycotina. It is distinct from taxa such as *Endogone*, *Sphaerocreas*, and *Umbelopsis*.

Fungi in Mucoromycotina and related lineages in Mucoromycota often associate with other organisms, including plants and bacteria (Benny et al. 2014; Bonfante and Desirò 2017). Glomeromycotina form arbuscular mycorrhizal associations, some species of Mortierellomycotina can occur with mosses and other plants (Melo et al. 2014), and diverse Mucoromycotina present associations with living plants. For example, some Mucoromycotina grow on liverworts in the thallus midrib and subterranean axes (Field et al. 2015a, 2015b). Species of *Umbelopsis* associate with roots of orchids (Yu et al. 2015), and some *Mucor* and *Umbelopsis* were isolated from woody roots of ponderosa pine and Douglas fir (Hoff et al. 2004). In this study, isolates of the new lineage *B. adelaidae* were obtained from soil and as a putative endophyte of *Leucobryum* sp. (U'Ren et al. 2010). Additional studies are needed to assess whether these fungi function in a symbiotic capacity with plants.

Many lineages of early diverging terrestrial fungi are often associated with bacteria. For example, gram-positive endobacteria related to the Mollicutes occur in cytoplasm of Glomeromycotina (Naumann et al. 2010; Toomer et al. 2015). Recent studies have provided evidence of the presence of bacteria in some Mucoromycotina, such as *Endogone*, where similar Mollicutes-related endobacteria have been detected (Desirò et al. 2015). Endobacteria occur in the cytoplasm of Mortierellomycotina, such as *Mortierella elongata* (Sato et al. 2010; Uehling et al. 2017) and *Rhizopus* (Partida-Martinez and Hertweck 2005). Based on SEM micrographs, we conclude that at least some of these bacteria are epihyphal in *B. adelaidae*, but additional testing is necessary to determine if any of the bacteria detected on this fungus are endobiotic. To our knowledge, this is the first report of *Bacillus licheniformis*, *Methylobacterium*, and *Stenotrophomonas* living in association with a fungus (but see the *Bacillus* clade identified by Hoffman and Arnold 2010).

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The type species described here was named in honor of Adelaida Chaverri Polini. Dr. Chaverri was a world-recognized tropical biologist and role model for women in Latin America in science, technology, engineering, and mathematics. Her major contributions were in the study and conservation of tropical montane forests and treeless alpine grasslands (*páramos*). As a biologist, she recognized the importance of mycorrhizal fungi and contributed to the description of a fungus in the Acaulosporaceae, a fungus in the same phylum as the species described here (Sieverding et al. 1988). She supported the creation of Costa Rica's National Park Service, providing the basis for extensive conservation of biological diversity. In 2013, she was included in the *Galería de la Mujer* (Women Gallery), one of the highest honors given to Costa Rican women for their dedication to defending and improving women human rights (Kappelle and Cleef 2004).

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ORCID

Terry J. Torres-Cruz http://orcid.org/0000-0002-9193-3249
Jason E. Stajich http://orcid.org/0000-0002-7591-0020
Andrea Porras-Alfaro http://orcid.org/0000-0002-9053-7973

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