1   **Short title:** *Bifiguratus adelaidae*, a new member of Mucoromycotina

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

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26   *Abstract*

27   Illumina amplicon sequencing of soil in a temperate pine forest in the southeastern United States

28   detected an abundant, N-responsive fungal genotype of unknown phylogenetic affiliation. Two

29   isolates with ribosomal sequences consistent with that genotype were subsequently obtained.

30   Examination of records in GenBank revealed that a genetically similar fungus had been isolated

31   previously as an endophyte of moss in a pine forest in the southwestern USA. The three isolates

32   were characterized using morphological, genomic, and multi-locus molecular data (18S, ITS, and

33   28S rRNA sequences). Phylogenetic and maximum likelihood phylogenomic reconstructions

34   revealed that the taxon represents a novel lineage in Mucoromycotina, only preceded by

35   *Calcarisporiella*, the earliest diverging lineage in the subphylum. Sequences for the novel taxon

36   are frequently detected in environmental sequencing studies and it is currently part of UNITE's

37   dynamic list of most wanted fungi. The fungus is dimorphic, grows best at room temperature,

38   and is associated with a wide variety of bacteria. Here a new monotypic genus, *Bifiguratus*, is

39   proposed, typified by *Bifiguratus adelaidae*.

40   **KEY WORDS:** Bryophyte, dimorphic, endophyte, environmental sampling, Mucoromycota,

41   Pinaceae, soil.

42   **INTRODUCTION**

43   Although fungal species are being described continuously, and have been for centuries,

44   the majority of fungi remain undiscovered. Working estimates predict that 5.1 million species of

45   fungi exist, yet only ~100,000 species have been described formally (Blackwell 2011). A recent

46   study on the tree of life encompassing more than 1000 uncultivated organisms highlighted the

47   large number of organisms for which cultured representatives are unknown (Hug et al. 2016).

48   Indeed, new sequencing techniques frequently reveal large numbers of uncharacterized taxa that

49   have not been described due to the lack of representative reference specimens in herbaria and/or

50   culture collections (Martin et al. 2011). A major gap in our understanding of fungal diversity lies

51   in determining the connections between fungi detected through next-generation sequencing or

52   other culture-free methods, and fungi already known to mycologists (Nilsson et al. 2016; Hesse

53   et al. 2016). One approach is to use next-generation data sets to guide subsequent efforts to

54   isolate target fungi from the same environments, thus providing biological material for further

55   functional and molecular analyses (Hesse et al. 2016).

56   Many branches of the fungal tree of life are still poorly resolved and underrepresented (or

57   lacking) in phylogenies. This is particularly true in lineages of early diverging terrestrial fungi

58   (including those formerly classified as Zygomycota; Spatafora et al. 2016). Improved sampling

59   of diversity in these groups is critical for illuminating key evolutionary innovations in the fungal

60   kingdom and assessing the ecological functioning of novel taxa or those reported previously as

61   non-culturable (or uncultured) (Grigoriev et al. 2011).

62   A previous investigation of the mycobiota associated with soil in a pine forest in North

63   Carolina, USA (Hesse et al. 2016) detected a fungal taxon by 28S nrRNA amplicon sequencing.

64   This fungal taxon was common in the soil and responded positively to forest N fertilization

65   treatments (Weber et al. 2013; Hesse et al. 2016). Particle filtration of soils from the same site

66   subsequently yielded two isolates (TLT265, YP243) representing the same 28S nrRNA

67   phylotype. When looking for similar sequences in the National Center for Biotechnology

68   Information (NCBI), one other match (AZ0501) with >99% similarity for the ITS, 28S, and 18S

69   regions was found. These three strains were compared and found to be similar in morphology

70   and molecular characters. Here we report and describe a novel taxon, *Bifiguratus adelaidae*, and

71   place it as an early diverging lineage in Mucoromycotina through multi-locus and phylogenomic

72   analyses. The presence of mucoid growth on the edges of the colonies suggested the presence of

73   bacteria on the fungus, which was evaluated using culturing, microscopy, and next generation

74   sequencing techniques.

75   **MATERIALS AND METHODS**

76   *Sample collection and site description —*

77   As part of a previous study, soil cores were collected from a free air CO_2 enrichment

78   (FACE) experiment located in a *Pinus taeda* plantation in the Blackwood Division of the Duke

79   Forest, North Carolina, USA (35°58'N, 79°06'W; elevation 130 m). The FACE site was

80   established in 1983 (Weber et al. 2013). In 2005, the CO_2 enrichment plots were quartered and

81   annual N-fertilization experiments began in two randomly selected quadrants in the form of

82   ammonium nitrate pellets (11.2 g N m^{-2}). Soil sampling was designed to take into account the

83   long-term field experiment and to minimally disturb soils in the plots. The sampling was

84   performed within the fertilized and unfertilized quadrants of ring 5 (Weber et al. 2013). In

85   October 2013, 10 cm soil cores were collected from one N-fertilized and one unfertilized

86   quadrant within ambient CO_2 FACE plot 5. Immediately after collection, each core was

87   sectioned into multiple vertical strata: Oi horizon (forest floor), Oa horizon (0–2 cm deep), upper

88   A horizon (2–5 cm deep), and lower A horizon (5–10 cm deep). Each soil fraction was

89   homogenized in an individual zip-lock bag. Soil samples were transported back to the laboratory

90   and stored at 4 °C until use.

91   *Fungal isolation —*

92   Fungi were isolated following two techniques, standard serial dilution methods for soil

93   (Gupta et al. 2012) and a particle-pasteurization filtration method (Mueller et al. 2004). Detailed

94   methods for isolation can be found in Hesse et al. (2016). Briefly, for the particle filtration

95   method, seven sieves were used to separate different particle sizes (4000, 2000, 250, 500, 125,

96   63, and < 63 μm). One gram of soil was loaded in the upper sieve (4000 μm). Particles were

97   washed with 4–6 liters of autoclaved sterile water for 10 minutes. The last rinse was 1 liter of

98   70% ethanol to surface-sterilize the soil. The soil particles from each sieve were transferred to 9

99   ml of 0.2% water agar (used to suspend the particles). Dilutions of 10^{-1} and 10^{-2} were plated on

100 peptone-yeast glucose agar supplemented with tetracycline (50 mg/L) and streptomycin (50

101 mg/L). Cultures were incubated at 25 ºC for 15 days. Plates were checked regularly to obtain

102 pure cultures.

103   Cultures and specimens from this study are preserved in the USDA-ARS Culture

104 Collection (Peoria, Illinois), the herbarium of the University of Michigan (Ann Arbor,

105 Michigan), the Robert L. Gilbertson Mycological Herbarium (Tucson, Arizona), and the Western

106 Illinois University Fungarium (Macomb, Illinois).

107 *Morphological characterization —*

108   Mycelium from each isolate (TLT265, YP243, and AZ0501) was plated on seven types

109 of media treated with tetracycline (50 mg/L) and streptomycin (50 mg/L). Hereafter, the use of

110 antibiotics in media is represented with a plus sign "+". The seven media tested were 1.3% malt

111 extract agar (MEA+) (Difco Laboratories, Sparks, Maryland), Sabouraud dextrose agar (SDA+)

112 (Oxoid LTD, Hampshire, England), Emerson yeast agar (EYSA+) (Difco Laboratories, Detroit,

113 Michigan), Reasoner's 2A agar (R2A+) (Difco Laboratories, Sparks, Maryland), Czapek-Dox

114 Agar (CDA+) (Sigma-Aldrich, St. Louis, Missouri), peptone yeast agar (PYG+) (Mueller et al.

115 2004), and potato dextrose agar (PDA+) (Oxoid LTD, Hampshire, England). Media were

116 prepared per manufacturers' instructions. Cultures from each isolate were incubated separately at

117 two temperatures, 25 ºC and 35 ºC, for 30 days in darkness. The Elcometer 6210 RAL Chart K7–

118   2014 was used as the color standard to describe the colonies. All cultures were produced from

119   the same source culture for each strain.

120   Mycelial growth rates were measured on MEA+. Inoculum consisted of one 4 mm

121   diameter agar plug from the growing edge of a fungal colony on MEA+. Plates were incubated at

122   five different temperatures at 10 ºC intervals from 5–45 ºC in the dark. Plates were examined

123   every five days for a month after cultures were established. Radial mycelial growth rates were

124   calculated using arithmetic mean values per day by measuring colony diameter (Aoki et al.

125   2013). Three replicates were measured for each isolate at each temperature.

126   The production of sporangiopores was assessed using corn meal agar (CMA+) (Sigma-

127   Aldrich, St. Louis, Missouri) supplemented with 50 mg/L chloramphenicol (Sigma-Aldrich, St.

128   Louis, Missouri). Cultures were incubated at 25 ºC and evaluated for 3 weeks.

129   *Microscopy —*

130   Tissue of each isolate was collected from the growing edge of a 15-day old colony on

131   MEA+, SDA+, EYSA+, and PYG+ that had been incubated at 25 ºC and 35 ºC. Hyphae were

132   stained with Bluemount (Hardy Diagnostics, Santa Maria, California) and compared under the

133   light microscope.

134   Tissue was fixed for scanning electron microscopy (SEM) by incubation for 24 hours at

135   4°C in a fixative solution of 25 mM phosphate buffer (Sigma-Aldrich, St. Louis, Missouri) with

136   a pH 7 and 3% gluteraldehyde (Sigma-Aldrich, St. Louis, Missouri). The fixative solution then

137   was poured off and a 1% osmium tetroxide solution (Electron Microscopy Sciences, Hatfield,

138   Pennsylvania) was added to the tissue. After incubating at 4 °C for 24 hours, the fungal tissue

139   was rinsed three times with 25 mM phosphate buffer (pH 7) and then soaked in the following

140   ethanol series for 15 minutes for dehydration of the tissue (30%, 50%, 65%, 75%, 89%, 95%,

141   3X-100%) and kept overnight at 100%, with two more 100% soaks the next day. After fixation,

142   the specimens were dried in a carbon dioxide critical point dryer (Tousimis, Rockville,

143   Maryland) and immediately placed in a sputter coater for a 2-minute coating of gold (Structure

144   Probe, Inc., West Chester, Pennsylvania). The SEM images were obtained on a JEOL JSM–

145   6010LA (JEOL Ltd. Tokyo, Japan) at the National Center for Agricultural Utilization Research,

146   Peoria, Illinois, USA. Typical operating conditions were an accelerating voltage of 10kV and a

147   spot size of 30.

148   *DNA extraction, amplification, and sequencing* —

149   DNA was extracted from the cultures obtained and 28S nuclear ribosomal RNA (nrRNA)

150   was amplified following the protocols described below. The sequences were compared against

151   GenBank (Altschul et al. 1997). The two cultures (TLT265 and YP243) matched the same

152   phylotype as the one detected through culture-free sequencing (Hesse et al. 2016). The 28S

153   nrRNA sequences for TLT265 and YP243 were compared against GenBank using BLASTn. One

154   isolate with high similarity (>99%) from another study was identified (1125 BLAST score for

155   both isolates; e value= 0). This isolate (AZ0501, accession number HM123225) was collected in

156   2007 in a study of endophytic fungi in a montane forest in southeastern Arizona (U'Ren et al.

157   2010). AZ0501 was isolated from photosynthetic tissue of a bryophyte (*Leucobryum* sp.,

158   Dicranaceae; U'Ren et al. 2010). This strain was obtained from the Robert L. Gilbertson

159   Mycological Herbarium at The University of Arizona (accession number MYCO-ARIZ-

160   AZ0501) and characterized.

161   Genomic DNA was extracted from cultures of each isolate using the Wizard genomic

162   DNA purification kit (Promega, Madison, Wisconsin). PCR was used to amplify the partial 18S

163   nrRNA with primers NS1 and NS4 (White et al. 1990), the partial 28S nrRNA using primers

164   LR0R and LR3 (Vilgalys and Hester 1990) and the ITS1-5.8S-ITS2 nrRNA (ITS barcode) using

165   primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Each 25 μl of PCR

166   mixture contained 12.5 μl of PCR master mix (Promega, Madison, Wisconsin), 3 μl of 1%

167   bovine serum albumin (BSA), 1 μl of each primer (5 μM), 6.5 μl of nuclease-free water and 1 μl

168   of DNA. Water was used in place of template for negative controls. The following PCR

169   conditions were used: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s,

170   and extension at 72 °C for 45 s; and a final extension at 72 °C for 7 min. PCR products were

171   checked using gel electrophoresis (1.2 % agarose in Tris-acetate-EDTA buffer). All samples

172   were sequenced using the forward primer at Beckman Coulter Genomics (Danvers,

173   Massachusetts). Sequences obtained were trimmed and edited using Sequencher (Gene Codes,

174   Ann Arbor, Michigan). Similar sequences were identified by comparison with GenBank via the

175   Basic Local Alignment Search Tool (BLASTn option) (Altschul et al. 1997) and comparison

176   with the UNITE database (Kõljalg et al. 2013). The ITS-partial 28S nrRNA sequence of AZ0501

177   had been submitted previously to GenBank (HM123225; U'Ren et al. 2010). The sequences

178   obtained here were deposited at GenBank (ITS rDNA, KU702608, KU702388; 28S rDNA,

179   KU702551, KU702228; 18S rDNA, KU702505, KX372676, KX372677). The percentage

180   similarity over the shared length for each locus among the three isolates (estimate of

181   evolutionary divergence between sequences) was determined by the number of nucleotide

182   differences per site between sequences in MEGA7 (Kumar et al. 2016).

183   *Phylogenetic analyses* —

184   Fungal sequences were edited in Geneious v.8.1.7 (http://www.geneious.com, Kearse et

185   al. 2012), and used as queries to conduct BLAST searches for sequence similarity. Sequences

186   were aligned with MUSCLE (Edgar et al. 2004). The alignments were trimmed with GBlocks

187   v.0.91b (Castresana 2000) using the less stringent conditions and then manually edited. The 18S

188   and 28S rDNA datasets were concatenated into a single dataset of 1581 homologous nucleotide

189   bases. Prior to phylogenetic inference, the best-fit models of nucleotide substitution were

190   estimated using jModelTest v.2.1.9 (Darriba et al. 2012), for 18S and 28S rDNA data separately.

191   Bayesian inference on the phylogeny of these taxa was carried out using MrBayes v.3.2.5

192   (Ronquist et al. 2012). The Markov chain Monte Carlo was run for 5 million generations under

193   the TrN+I+G and TIM3+G nucleotide substitution models for 18S and 28S, respectively.

194   Representatives of Mucoromycotina, Mortierellomycotina, and Glomeromycotina were selected

195   to resolve the position of the novel fungal taxon within the recently erected phylum

196   Mucoromycota (Spatafora et al. 2016). Alignments were submitted to TreeBASE (S19770).

197   *Genome sequencing and phylogenomic analysis* —

198   Isolate AZ0501 was grown in liquid yeast mold (YM) medium for approximately three

199   weeks. Hyphal tissue was filtered from the growth medium and genomic DNA was extracted

200   using the FastDNA SPIN Kit for soils (MP Biomedicals, Santa Ana, California). DNA was

201   quantified using a QuBit. DNA was fragmented using a Covaris E220 and fragment sizing

202   assessed using Agilent's BioAnalyzer. Fragments were made blunt and adenylated at the 3' end

203   to ligate Illumina sequencing adapters. A unique index code was added during PCR

204   amplification. Concentration was confirmed using KAPA's Library quantification kit on an ABI

205   7500 Real Time machine. The AZ0501 Illumina library was sequenced using Illumina's v2 500-

206   cycle reagent kit as a spike-in with rRNA gene amplicon libraries on five separate Illumina

207   MiSeq runs. On each run this isolate comprised about 30% of the sequences. Sequences from

208   individual MiSeq runs were combined into a single FASTA file and assembled *de novo* using the

209   Velvet software package (Zerbino 2010) with a k-mer value of 61 bp. Genome annotation for

210 developing genes for phylogenomic analyses was performed with MAKER (Holt and Yandell

211 2011) supported by *ab initio* gene prediction on the assembled contigs with Augustus (Stanke et

212 al. 2004) using *Rhizopus delemar* parameters and self-trained GeneMarkHMM-ES (Ter-

213 Hovhannisyan et al. 2008). Raw reads were deposited to the Sequence Read Archive (SRA) as

214 Bioproject PRJNA368732 and Biosample SAMN06270744.

215   Phylogenomic analyses were performed with an approach previously applied for

216 zygomycete genomes (Spatafora et al. in 2016). The data and analysis pipeline are available at

217 https://github.com/zygolife/Bifiguratus_Phylogenomics. Briefly, the approach uses 434 protein

218 coding genes that are typically single-copy in most fungi. Each phylogenetic marker is

219 summarized as a profile Hidden Markov Model (HMM) (HMMER3, Eddy 2011) from an

220 alignment of the orthologs from several dozen diverse fungi. The phylogenomic pipeline

221 searches each HMM against the protein set of a species with HMMSEARCH and the best hit from

222 each species is saved. A multiple sequence alignment (MSA) containing the orthologous copies

223 from each species is constructed with HMMALIGN of these copies guided by the marker HMM.

224 Each gene MSA is trimmed with TRIMAL (Capella-Gutiérrez et al. 2009) and the trimmed MSAs

225 are concatenated into a single master alignment with a Perl script built with BioPerl (Stajich et

226 al. 2002). Phylogenetic tree inference was performed with RAXML (v8.2.8) using autoMRE and

227 rapid boostrapping (-f a) (Stamatakis 2014).

228 *Analysis of associated bacteria —*

229   Mucoid growth was observed on the edges of cultures under some conditions, especially

230 PYG+ at 35 ºC. Light microscopy and culturing were used to evaluate the presence of bacteria

231 on these mucoid areas. Bacteria present on fungal tissue were streaked onto R2A, from the three

232 isolates grown on the seven media described above. The cultures were incubated at 25 ºC for 7

233   days. We attempted to remove bacteria from the fungus using different antibiotics, media and

234   temperatures with little success. However, their growth was especially limited on MEA+ at 25

235   ºC.

236   DNA was extracted and amplified from the bacterial cultures following the protocol used

237   for fungal samples (above). DNA amplification, sequencing and identification were performed as

238   described above, except that we used universal primers for the 16S rRNA (8F and 1492R; Lane

239   1991) and an annealing temperature of 56 °C. Sequencing was performed using primer 1409R.

240   Further sequencing of the *gyrA* gene of *Bacillus* strains was performed with primers p-gyrA-F

241   and p-gyrA-R (Roberts et al. 1994) for identification (Chun and Bae 2000). Sequences were

242   deposited to GenBank (KX397340–KX397345 for 16S; KX670823–KX670827 for gyrA).

243   Next generation sequencing was used with the purpose of confirming the presence of

244   bacteria that may have evaded culturing. To assess dynamics of bacteria associated with this

245   novel fungal taxon, isolates were grown on three media (PYG+, MEA+, and SDA+) at 25 °C and

246   35 °C. Three replicates were evaluated, one for each isolate (TLT265, YP243, and AZ0501), for

247   a total of 18 samples. Tissue was collected from all samples and sent to MRDNA Molecular

248   Research LP (Shallowater, Texas) for DNA extraction and Roche 454 FLX titanium sequencing.

249   The 16S rRNA gene universal bacterial primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3')

250   was used in a modified version of amplicon pyrosequencing (bTEFAP®) (Dowd et al. 2008). A

251   single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, California)

252   was run under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for

253   30 seconds; 53 °C for 40 seconds and 72 °C for 1 minute; with a final elongation step at 72 °C

254   for 5 minutes. Amplicon products from different samples were mixed in equal concentrations

255   and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, Beverly,

256   Massachusetts). Samples were sequenced with Roche 454 FLX titanium instruments and

257   reagents following the manufacturer's procedures. With each new box of DNA extraction

258   reagents two negative controls were run. No detectable amplification with universal 16S primers

259   was detected on the controls.

260   Multiplex tags were used to parse the sequence library into 18 respective samples using

261   the QIIME software package (Caporaso et al. 2010). The sequences obtained were denoised using

262   AmpliconNoise (Quince et al. 2011), quality filtered and trimmed, and operational taxonomic

263   units (OTUs) were clustered in USEARCH (Edgar 2010) using the UPARSE (Edgar 2013) algorithm

264   considering a pairwise sequence identity percentage of 0.97. Chimera filtering was performed

265   using UCHIME (Edgar et al. 2011) comparing to the RDP Gold database

266   (http://drive5.com/usearch/manual/cmd_uchime_ref.html). Taxonomy was assigned using the

267   RDP Classifier (Wang et al. 2007) against the Greengenes database (DeSantis et al. 2006) and

268   corroborated with BLAST (Altschul et al. 1997) matches against GenBank (Benson et al. 2011).

269   Singletons and sequences assigned to mitochondria were removed. Raw reads were deposited to

270   the Sequence Read Archive (SRA) as study accession SRP082174 (Bioproject PRJNA318907).

271   **RESULTS**

272   Two isolates of the focal fungus (YP243 and TLT265) were obtained using filtration-

273   pasteurization methods from soil. Both isolates were obtained from the lower A horizon (5–10

274   cm deep) of a N-fertilized plot under ambient CO_2 in a pine plantation in North Carolina, USA.

275   Isolates YP243 and TLT265 were obtained from the 63 mm and 2000 mm particle size,

276   respectively, and grown at 25 ºC on PYG+ (Hesse et al. 2016). These isolates were evaluated

277   with the only known, cultured closest relative in GenBank, isolate AZ0501, which was obtained

278   from surface-sterilized photosynthetic tissue of a moss (U'Ren et al. 2010). Isolates YP243 and

279   TLT265 share >99% similarity for the ITS, 18S and 28S genes sequences with AZ0501 (TABLE

280   S1). Morphological characterization of the isolates is provided in the taxonomic description,

281   including growth on different media (FIGS. 1–2) and microscopy (FIGS. 3–4).

282   *Phylogenetic analyses* —

283   Given the lack of available data for genomes of fungi such as *Endogone* and

284   *Sphaerocreas*, and because ITS nrRNA sequences could not be aligned across this phylum,

285   concatenated 18S and 28S nrRNA analyses containing more complete taxon sampling were used

286   to estimate the phylogenetic placement of these strains within Mucoromycotina. Isolates

287   AZ0501, TLT265 and YP243 clustered together within a well-supported monophyletic clade that

288   branched after early-diverging lineages such us *Calcarisporiella* (Hirose et al. 2012),

289   *Densospora* (McGee 1996), *Endogone* (Yamamoto et al. 2015) and *Sphaerocreas* (Hirose et al.

290   2014) and unidentified Mucoromycotina (Truong et al. 2017) (FIG. 5).

291   *Genome sequencing and phylogenomic analysis* —

292   Sequencing generated a total of approx. 6.8M high quality reads, of which 5.6M reads

293   assembled into 568 scaffolds of at least 500bp. The final assembly had an N50 of ~928kbp, an

294   overall genome assembly length of ~19.5 Mbp, and a GC content of 48.3 %. The taxon

295   represented by these three strains has 8876 predicted protein-coding genes. This is a nearly

296   complete genome, but lacks RNA transcriptome data to provide additional support for gene

297   models. The assembly shows a high degree of completeness with a Benchmarking Universal

298   Single-Copy Orthologs (BUSCO) score of 82.1% using Fungi ODB9 set. The genome is 86%

299   complete. Out of the 290 BUSCO groups searched, we found 217 Complete Single-Copy, 21

300   Complete Duplicated, 12 Fragmented, and 40 missing BUSCO orthologs (Simão et al. 2015).

301   These results are similar to other Mucoromycotina (TABLE 1). Indicative of a nearly complete

302   genome assembly, we identified 237 of 248 super-conserved Core Eukaryotic Genes (CEGs).

303   The CEGMA pipeline (Parra et al. 2007) suggests 95.6% genome completeness (TABLE S2).

304   The Maximum Likelihood phylogenetic tree generated with RAXML was produced from a

305   concatenated alignment of 434 protein-coding genes (165,307 residues across ten taxa). The

306   phylogenomic tree places the new taxon as one of the early diverging lineages in

307   Mucoromycotina, only preceded by *Calcarisporiella*, the first diverging known lineage in the

308   subphylum (FIG. 6).

309   *Analysis of associated bacteria —*

310   Two genera of bacteria were successfully isolated and identified based on 16S rRNA

311   data: *Bacillus licheniformis* (KX397340–KX397344) and *Stenotrophomonas* sp. (KX397345).

312   Due to the limitations of the 16S rRNA gene in resolving members of the *Bacillus subtilis*

313   complex, identities of the *Bacillus licheniformis* strains were confirmed using the gyrA gene

314   (KX670823–KX670827) (TABLE S3). Both bacteria were recovered from PYG+, SDA+, and

315   CZP+ at 25 ºC. Although bacteria were not isolated from fungal isolate AZ0501, they were

316   observed on this isolate using SEM (FIG. 7C, 7D), and were confirmed by direct sequencing

317   (FIG. 7A).

318   Culture-independent methods based on 454 amplicon pyrosequencing were used to assess

319   bacterial communities associated with the three isolates grown on different media at two

320   temperatures (25 ºC and 35 ºC). A majority of the sequences turned out to be fungal

321   mitochondria amplicons, which were removed from the analysis. The remaining data show

322   numerous bacterial taxa associated with these three fungal isolates (Fig. 7A). However, aside

323   from *Bacillus, Methylobacterium* spp. and *Propionibacterium* most bacterial taxa were in low

324   read abundance (<10 sequences / OTU), and in four of the 18 treatments no bacteria were

325   detected (TABLE S4). Venn diagrams (Fig. 7B) show that few bacterial OTUs are shared between

326   isolates, with the majority of OTUs being distinct for each isolate. *Bacillus* and

327   *Methylobacterium* were the dominant taxa associated with YP243 and TLT265, however

328   *Bacillus* was not detected from isolate AZ0501 in the 454 dataset, nor when isolates were grown

329   on MEA+ and SDA+ media (FIG. 7A, 7B). Isolates TLT265 and YP243 show a higher bacterial

330   richness with respect to isolate AZ0501. Variation in bacterial taxa and their relative abundance

331   (in terms of sequence reads) was observed among temperature and media treatments (FIG. 7A,

332   7B). A greater number of bacterial OTUs were detected from strains obtained from soil and those

333   growing on MEA+ (FIG. 7B). Although a core set of bacterial OTUs are present at both

334   temperatures (25 ºC and 35 ºC), incubation temperature appeared to shift the bacterial

335   communities in these isolates. In contrast, three *Methylobacterium* OTUs were consistently

336   detected from all three isolates at 25 ºC and 35 ºC on MEA+ and SDA+.

337   **TAXONOMY**

338   **Bifiguratus** T. J. Torres-Cruz & A. Porras-Alfaro, gen. nov.

339   MycoBank MB 818093

340   FIGS. 1, 3–4.

341   *Typification*: *Bifiguratus adelaidae* T. J. Torres-Cruz and A. Porras-Alfaro.

342   *Etymology*: from the Latin "*figuratus*", in reference to the dimorphic characteristic of the

343   type species.

344   **Bifiguratus adelaidae** T. J. Torres-Cruz & A. Porras-Alfaro, sp. nov.

345   MycoBank MB 818094

346   FIGS. 1, 3–4.

347   *Typification:* UNITED STATES: Arizona, Cochise County, Coronado National Forest,

348 eastern Chiricahua Mountains, dried culture from an isolate from surface-sterilized

349 photosynthetic tissue of *Leucobryum* sp., Arizona, USA, Jun 2007, *J. M. U'Ren, A. E. Arnold, et*

350 *al.* (**holotype** MICH 134798). Ex-type culture MYCO–ARIZ–AZ0501 = NRRL 66559.

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

352 *Full description —*

353 Cultures of the three strains grown on MEA+ at 25 ºC present a white (RAL 9003)

354 smooth flat colony with entire margins (FIG. 1A). At 35 ºC colonies are smaller than at 25 ºC,

355 white (RAL 9003), and 1–2 mm raised with undulate margins and a rough rugose surface (FIG.

356 1E). On SDA+ at 25 ºC colonies are cream (RAL 9001), rough, and 1–2 mm raised with entire

357 margins (FIG. 1B), while at 35 ºC the colonies have the same characteristics but are more

358 corrugated (FIG. 1F). Colony characteristics on CMA+ at 25 ºC are consistent with those

359 observed on SDA+. On EYSA+ at 25 ºC colonies are pure white (RAL 9010), rough, 2–3 mm

360 high colonies have lobate margins (FIG. 1C). On this medium at 35 ºC, colonies are light ivory

361 (RAL 1015) and flat but still wrinkled, with a filamentous margin (FIG. 1G) and a more mucoid

362 texture is observed at the edges with a cream pigmentation (RAL 9001). On PYG+ at 25 ºC

363 colonies are highly corrugated (FIG. 1D), irregular, cream (RAL 9001), with undulated margins,

364 and are 3–4 mm high. At 35 ºC on PYG+ colonies are cream (RAL 1001), corrugated, and

365 raised, but the edges present a mucoid growth with undulated margins (FIG. 1H) that have a

366 translucent beige-red color (RAL 3012). The presence of a mucoid texture at the edges of the

367 fungus growing at 35 ºC on EYSA+ and PYG+ (FIGS. 1G–H) is related to more abundant growth

368 of bacteria in the cultures, with respect to MEA+ and PDA+. On PDA+ the fungus has the same

369 morphological characteristics as those on PYG+ at 25 ºC, and the morphology remains the same

370 at 35 ºC. None of the strains grew on R2A and CDA; fungal-associated bacteria colonized the

371   plates instead. All cultures were produced from the same source culture for each strain, such that

372   the bacteria appear to have been present in the source and became dominant on R2A and CDA

373   media.

374   As cultures aged (i.e., after more than one month of incubation) the morphology of the

375   cultures changed. In some cases (e.g., SDA+), colonies became more mucoid and flat, and were

376   overcome by bacterial growth. However, in most cases (e.g. MEA+), cultures presented more

377   hyphal growth at the edges and colonies became darker, turning brown.

378   Colonies on MEA+ have radial mycelial growth rates of 0.4 mm/d at 25 ºC and 0.2 mm/d

379   at 35 ºC in the dark, during the first 15 days of incubation. Growth rates at different incubation

380   temperatures are shown in FIG. 2. The three isolates did not grow at 5 ºC or at 45 ºC. The isolates

381   generally grew more rapidly at 25 ºC during the first two weeks of incubation. After 15 days of

382   growth, the growth rate decreased at 25 ºC and increased at 35 ºC for YP243 and AZ0501.

383   The three strains possess coenocytic mycelium with hyphae ca. 2 μm in diameter (15-day

384   old culture, MEA+, 25 ºC; FIGS. 3B and 4B). When observed by SEM, hyphal surfaces present

385   rough texture with surface ornamentations (FIG. 4B–C). No sporangiospores were observed,

386   although multiple attempts were made using PDA, CMA, and several temperatures. The fungus

387   produces what appear to be intercalary and terminal chlamydospores 5–10 μm in size, which

388   appear globose to sub-globose (FIGS. 3B, 4A), but additional studies are necessary to confirm the

389   nature of these structures. SEM observations revealed the presence of yeast-form cells (FIGS.

390   4D–F). Bud scars and budding yeasts were observed (FIGS. 4D–E), and germ tubes were

391   visualized on yeast-form cells (FIG. 4F). They were commonly observed when the fungus was

392   cultivated on MEA+ (FIG. 4E) and EYSA+ (FIGS. 4D, F) at 25 ºC. Thus these isolates appear to

393   be dimorphic.

394   *Other cultures examined*: UNITED STATES, North Carolina, Blackwood Division of the

395   Duke Forest free air CO_2 enrichment (FACE) experiment (35°58'N, 79°06'W; elevation 130 m),

396   Oct 2013, *C. R. Kuske,* MICH 134800 (NRRL 66560 = WIU–APA–TLT265) and MICH 134801

397   (NRRL 66561 = WIU– APA–YP243).

398   *Substrate —*

399   Because only three cultures are currently available for this fungus, we based analyses of

400   substrates and potential hosts on analyses of available ITS rRNA data from BLASTn analysis.

401   Two cultures (YP243 and TLT265) were recovered from soil, and apparently related fungi have

402   been detected on several other soil samples using direct sequencing from environmental samples

403   including temperate forest soils (HQ021892, DQ421188, DQ421187), truffle grounds soils

404   (JF927042, JF927041), rice field soils (AB520601), and humus under *Pinus sylvestris*

405   (GU559100). See 'distribution' section for geographic range information.

406   Our previous report suggested that this species represented up to 9% of the total 454

407   sequences in individual samples at the Duke Forest (Hesse et al.2016). It is rarely found in the

408   forest floor as is more common on the deeper horizons (Hesse et al. 2016). Hesse et al. (2016)

409   showed that the species increases in abundance with soil depth and reaches the highest

410   abundance in a 5–10 cm depth. It responds to the combination of N fertilization and CO_2 by

411   increasing in relative abundance at the 2–5 cm and 5–10 cm deep intervals (Hesse et al. 2016).

412   In addition to occurring as a culturable endophyte in *Leucobryum* (AZ0501, U'Ren et al.

413   2010), environmental data show that related strains have been detected as a root associated

414   fungus of the orchid *Cymbidium kanran* (KU141166, Hong et al. 2015) and sweet chestnut,

415   *Castanea sativa* (EF040836, Peintner et al. 2007).

416   *Distribution —*

417   This taxon is known from the Northern Hemisphere. In the United States, phylotypes

418   consistent with *B. adelaidae* have been detected from the Duke Forest, North Carolina (YP243

419   and TLT265), Bartlett Experimental Forest, New Hampshire (HQ021892), Cedar Creek

420   Ecosystem Science Reserve, Minnesota (DQ421188, DQ421187), and Coronado National

421   Forest, Arizona (AZ0501). It has also been detected in Italy (JF927042, JF927041, EF040836),

422   Japan (AB520601), Sweden (GU559100), and South Korea (KU141166).

423   **DISCUSSION**

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

425   provided the opportunity for morphological, multi-locus and phylogenomic description of a new

426   endophytic and soil-dwelling species of Mucoromycota with an apparent wide distribution across

427   the Northern Hemisphere. This dimorphic species is affiliated with epihyphal bacteria and

428   appears to have a wide host range spanning multiple lineages of plants, as well as subsurface

429   soils in diverse forests. This fungus is part of UNITE's "most wanted" fungi as cluster code

430   UCL7_006587, ranked #1 at the phylum level based on number of studies and #4 based on

431   number of sequences (Nilsson et al. 2016). Description of this species exemplifies the potential

432   to use environmental sequencing to guide taxonomic discovery. Its description and

433   characterization represents an effort to help bridge the gap between fungal taxonomy and

434   molecular ecology.

435   Asexual reproduction among lineages formerly known as zygomycetes can be achieved

436   by the formation of chlamydospores, sporangiospores, yeast cells, or arthrospores (Fuller 1978;

437   Benny et al. 2014). *Bifiguratus adelaidae* presents coenocytic hyphae, globose intercalary

438   chlamydospores, and yeast cells. The presence of yeast cells in other Mucoromycotina taxa has

439   been described (e.g., *Mucor*, *Benjaminiella* and *Mycotypha*; Benny et al. 2014). After budding,

440   scar tissue is left behind and the mother cell will exhibit a crater-like ring of scar tissue (Chant

441   and Pringle 1995; Powell et al. 2003), consistent with those observed in *B. adelaidae* (FIG. 4D).

442   Budding yeasts were also viewed (FIG. 4E). Chlamydospores form in swellings of coenocytic

443   young hyphae (Benny et al. 2014). We observed what appears to be germinating chlamydospores

444   (FIG. 4F), which also have been observed in other dimorphic fungi (e.g., Ascomycota such as

445   *Candida albicans* and *Candida dubliniensis*). In those species, chlamydospores can generate

446   daughter chlamydospores, bud to form daughter yeast cells, and germinate to produce

447   pseudohyphae and hyphae (Citiulo et al. 2009). Chlamydospores have been reported in other

448   Mucoromycotina fungi, including *Umbelopsis* (Wang et al. 2013), *Ambomucor* (Liu and Zheng

449   2015), and *Isomucor* (Ivanildo et al. 2012). Chlamydospore formation can be induced by bacteria

450   associated with fungi, as seen in three Mucoromycotina species (*Phycomyces blakesleeanus*,

451   *Mucor bacilliformis* and *Mucor hiemalis*) where a lipopeptide produced by *Ralstonia*

452   *solanacearum* induces the production of chlamydospores as a survival technique for the fungus

453   (Spraker et al. 2016). It is possible that the presence of bacteria on *B. adelaidae* could influence

454   chlamydospore production (FIG. 3B), a topic that will require further study.

455   Phylogenetic inferences based on the concatenated partial 18S and 28S rRNA loci placed

456   *B. adelaidae* as a novel taxon in Mucoromycota (FIG. 5). The *B. adelaidae* branch diverges after

457   *Calcarisporiella*, the earliest known branching Mucoralean fungus or sister to Mucoromycotina.

458   It is distinct from taxa such as *Endogone, Sphaerocreas,* and *Umbelopsis* (FIG. 6).

459   Fungi in Mucoromycotina and related lineages in Mucoromycota often associate with

460   other organisms, including plants and bacteria (Benny et al. 2014, Bonfante and Desirò 2017).

461   Glomeromycotina form arbuscular mycorrhizal associations, some species of

462   Mortierellomycotina can associate with mosses and other plants (Melo et al. 2014), and diverse

463 Mucoromycotina present associations with living plants. For example, some Mucoromycotina

464 associate with liverworts in the thallus midrib and subterranean axes (Field et al. 2015a; Field et

465 al. 2015b). Species of *Umbelopsis* associate with roots of orchids (Yu et al. 2015), and some

466 *Mucor* and *Umbelopsis* have been isolated from woody roots of ponderosa pine and Douglas fir

467 (Hoff et al. 2004). In this study, isolates of the new lineage *B. adelaidae* were obtained from soil

468 and as a putative endophyte of *Leucobryum* sp. (U'Ren et al. 2010). Additional studies are

469 needed to assess whether these fungi function in a symbiotic capacity with plants.

470   Many lineages of early diverging terrestrial fungi often associate with bacteria. For

471 example, Gram-positive endobacteria related to the Mollicutes occur in cytoplasm of

472 Glomeromycotina (Naumann et al. 2010; Toomer et al. 2015). Recent studies have provided

473 evidence of the presence of bacteria in some Mucoromycotina, such as *Endogone,* where similar

474 Mollicutes-related endobacteria have been detected (Desirò et al. 2015). Endobacteria occur in

475 the cytoplasm of Mortierellomycotina, like *Mortierella elongata* (Sato et al. 2010; Uehling et al.

476 2017) and *Rhizopus* (Partida-Martinez and Hertweck 2005). Based on SEM micrographs we

477 conclude that at least some of these bacteria are epihyphal in *B. adelaidae*, but additional testing

478 is necessary to determine if part of the taxa detected on this fungus are endobiotic. To our

479 knowledge this is the first report of *Bacillus licheniformis*, *Methylobacterium* and

480 *Stenotrophomonas* living in association with a fungus (but see the *Bacillus* clade identified by

481 Hoffman and Arnold 2010).

482 **ACKNOWLEDGMENTS**

501 The type species described here was named in honor of Adelaida Chaverri Polini. Dr.

502 Chaverri was a world-recognized tropical biologist and role model for women in Latin America

503 in science, technology, engineering, and mathematics. Her major contributions were in the study

504 and conservation of tropical montane forests and treeless alpine grasslands (*páramos*). As a

505 biologist, she recognized the importance of mycorrhizal fungi and contributed to the description

506 of a fungus in the Acaulosporaceae, a fungus in the same phylum as the species described here

507 (Sieverding et al. 1988). She supported the creation of Costa Rica's National Park Service,

508 providing the basis for extensive conservation of biological diversity. In 2013, she was included

509   in the *Galería de la Mujer* (Women Gallery), one of the highest honors given to Costa Rican

510   women for their dedication to defending and improving women human rights (Kappelle and

511   Cleef 2004).

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726

727   **LEGENDS**

728   Fɪɢ. 1. *Bifiguratus adelaidae* colony morphology on different media and temperatures. A. MEA+

729   at 25 ºC. B. SDA+ at 25 ºC. C. EYSA+ at 25 ºC. D. PYG+ at 25 ºC. E. MEA+ at 35 ºC. F. SDA+

730   at 35 ºC. G. EYSA+ at 35 ºC. H. PYG+ at 35 ºC. Bars= 10 mm. Images presented belong to

731   different isolates. All isolates (AZ0501, TLT265, YP243) showed consistent growth in each

732   media.

733   FIG. 2. Radial growth rates of *B. adelaidae* per day on MEA+ under three different temperatures

734   during 30 days. Vertical bars indicate standard deviation. A. Isolate TLT265. B. Isolate YP243.

735   C. Isolate AZ0501, triangle = 10 ºC circle = 25 ºC box= 35 ºC.

736   FIG. 3. Light microscopy of *B. adelaidae* isolate TLT265. A. Yeast cells. B. Putative intercalary

737   chlamydospores (dark purple) in coenocytic hyphae. C. Sporangium. Bars= 25 μm.

738   FIG. 4. Scanning electron micographs of *B. adelaidae* hyphal (A–C) and yeast (D–F) structures.

739   A. Putative germinating chlamydospore with several germ tubes. B. Profuse vegetative hyphae

740   C. Hyphal wall surface ornamentations. D. Yeast bud scar (arrow). E. Budding of yeast cells

741   (arrow). F. Germinating yeast cells, arrows point to germ tubes. Micrograph E is from YP243; all

742   other images are from isolate TLT265.

743   FIG. 5. Phylogenetic placement of *Bifiguratus adelaidae* within Mucoromycota (Spatafora et al.

744   2016) based upon concatenated 18S and 28S rRNA gene sequence alignment. *Basidobolus*

745   *ranarum* was used as outgroup. Branches with Bayesian posterior probabilities values >0.95 are

746   thickened and ML bootstrap support values >70 are shown.

747   FIG. 6. RAxML phylogenetic tree of Mucoromycota based on a concatenated alignment of 434

748   conserved orthologous proteins. *Bifiguratus adelaidae* isolate AZ0501 is in bold. Bootstrap

749   values are shown on respective branches.

750   FIG. 7. Bacteria associated with isolates of *B. adelaidae.* A. Sequence read abundance of the 25

751   bacterial operational taxonomic units (OTUs) detected through the 454 pyrosequencing of three

752   isolates of *B. adelaidae* grown at different temperatures and on different media types. OTU

753   relative sequence abundance is expressed as log10 of read number. The plot was generated with

754   the R package "phyloseq" (McMurdie and Holmes 2013). Bars with less than one read in a

755   single samples group are not displayed in bar plots. B. Venn diagrams of the bacterial OTUs

756   comparing diversity between isolates, media, and temperature (left to right). C, D. Scanning

757   electron micrographs of bacteria present on *B. adelaidae* isolate AZ0501. Bars= 5 μm.

758   **TABLES**

759   TABLE 1. Benchmarking universal single-copy orthologs (BUSCO) results for *Bifiguratus*

760   *adelaidae* and other Mucoromycotina.

761   **SUPPLEMENTARY MATERIAL**

762   TABLE S1. Estimates of evolutionary divergence between sequences of ITS, 28S, and 18S

763   nrRNA for isolates AZ0501, TLT265, and YP243 of *Bifiguratus adelaidae.*

764   TABLE S2. Statistics of the completeness of the *Bifiguratus adelaidae* genome using CEGMA

765   based on 248 CEGs

766   TABLE S3. Isolation of bacteria associated with *Bifiguratus adelaidae.*

767   TABLE S4. Bacterial operational taxonomic units (OTUs) detected by 454 pyrosequencing on

768   isolates of *Bifiguratus adelaidae* under different media and temperature treatments.
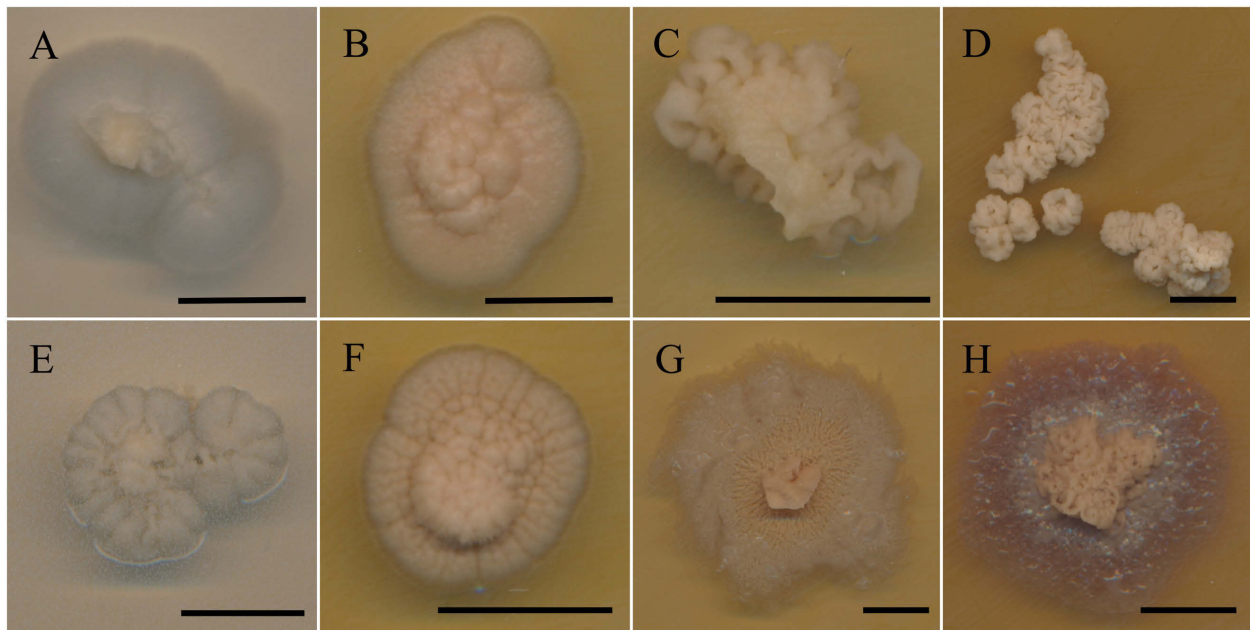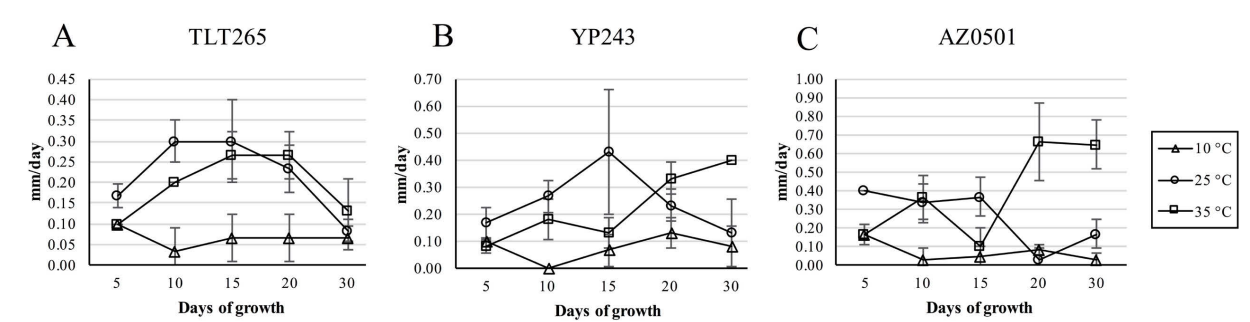
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Circinella angarensis CBS 173.62; JX629068, JN206551
Thamnostylum nigricans CBS 690.76; JX629079, JN206541
Absidia hyalospora FSU5796; EU826360, EU826368
Rhizomucor endophyticus CBS 385.95; HM623313, JN206448
Rhizopus microsporus CBS 112.285; FN182238, FN182233
Mucor variabilis CBS 564.66; HM623321, KC012658
Gongronella butleri NRRL 1340; AF157137, AF157191
Hesseltinella vesiculosa NRRL 3301; AF157140, AF157194
Umbelopsis isabellina NRRL 1757; AF157166, AF157220
Umbelopsis ovata CBS 499.82; KM017685, KF727465
Umbelopsis nana CBS 858.68; KM017686, KF727467
Umbelopis ramanniana var. *angulispora* CBS-222.29; HQ667450, HQ667364
Bifiguratus adelaidae TLT265; KX372677, HM123225
Bifiguratus adelaidae YP243; KU702505, KU702551
Bifiguratus adelaidae AZ0501; KX372677, HM123225
Endogone corticioides A11001; LC107350, LC107367
Endogone pisiformis AFTOL-ID 539; DQ322628, DQ273811
Endogone flammicorona EF13002; LC107332, LC107360
Endogone lactiflua AFTOL-ID 45; DQ536471, DQ273788
Densospora solicarpa DAR74956; UDB018861, UDB018860
Densospora solicarpa DAR69421; UDB018865, UDB018864
Sphaerocreas pubescens NBRC109377; AB752295, LC107618
Mucoromycotina sp. MES-1534; KY487058, KY487061
Calcarisporiella thermophila CBS 279.70; AB597205, AB617741
Mortierella humilis CBS 222.35; JQ040257, HQ667401
Mortierella verticillata NRRL 6337; NG 017188, DQ273794
Dissophora decumbens CBS 592.88; HQ667440, HQ667355
Gamsiella multidivaricata CBS 227.78; HQ667475, HQ667391
Mortierella gamsii CBS 253.36; HQ667497, HQ667415
Mortierella alpina CBS 210.32; JQ040258, HQ667421
Funneliformis mosseae BEG12; U96139, JQ048861
Rhizophagus intraradices BEG75; FR717169, AJ416418
Gigaspora margarita BEG34; HF968786, HF968958
Dentiscutata heterogama FL225; AY635832, DQ273792
Archaeospora leptoticha NC176; AJ006466, KC166264
Geosiphon pyriformis AFTOL-ID 574; AM183923, AM183920
Basidiobolus ranarum NRRL 34594; AY635841, DQ273807

Mucoromycotina
Mortierellomycotina
Glomeromycotina
Mucoromycota

0.06

FIGURE 6

Rhizophagus irregularis DAOM 181602
Mortierella elongata PRJNA196039
Mortierella verticillata NRRL 6337
Calcarisporiella thermophila CBS279.70
Bifiguratus adelaidae isolate AZ0501
Umbelopsis ramanniana NRRL 5844
Lichtheimia corymbifera FSU 9682
Phycomyces blakesleeanus NRRL 1555
Mucor circinelloides CBS277.49
Rhizopus delemar RA 99-880

0.1

FIGURE 7

A

Log10 of Reads Number

Isolate: AZ0501, TLT265, YP243
Media: MEA+, PYG+, SDA+
Temperature: 25°C, 35°C

OTUs
- Actinobacteria
- Actinomycetales I
- Actinomycetales II
- *Atopostipes* sp.
- *Bacillus* sp.
- *Brevundimonas* sp.
- Burkholderiales
- Comamonadaceae
- Flavobacteriaceae
- Gammaproteobacteria
- *Kocuria* sp.
- *Massilia* sp.
- *Methylobacterium* sp.1
- *Methylobacterium* sp.2
- *Methylobacterium* sp.3
- *Paracoccus* sp.
- *Pelomonas* sp.
- *Propionibacterium* sp.
- *Ralstonia* sp.
- Rhodobacteraceae
- *Staphylococcus* sp.
- *Streptococcus* sp.1
- *Streptococcus* sp.2
- *Streptococcus* sp.3
- *Stenotrophomonas maltophilia*

B

AZ0501 / TLT265 / YP243: 3, 0, 6, 1, 5, 4, 6

MEA+ / PYG+ / SDA+: 12, 0, 2, 6, 2, 0, 3

25°C / 35°C: 9, 9, 7

C

0kV WD9mm SS30   x4,000   5µm

D

mmSS30   x4,000   5µm