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Mooraboolomyces wintlei gen. & sp. nov. from Victoria, Australia

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Abstract

A new genus and species in the Mucoromycota, *Mooraboolomyces wintlei*, was isolated into culture from leaf litter from a national park in Victoria, Australia, and is described. The strain reproduced asexually to form sporangia and sporangiospores, with no production of zygospores, and sequencing of its genome and analysis supported a heterothallic mode of reproduction. Phylogenetic analyses using five DNA regions placed the strain within the Mucorales and Mucoraceae, clearly distinct from previously described genera. This represents the second genus in this phylum that has been identified from Australia and that is currently unique to this country.

Key words: biodiversity, heterothallism, Mucorales, *sex* locus, zygomycete

Introduction

Relative to the Dikarya fungi the numbers of genera and species of the basal fungal lineages are low. In 2019, just 338 species were estimated to have been described from the Mucoromycota phylum, which is one of the better researched early lineages (Voigt *et al.* 2021), yet nonetheless this is a small number in the context of the most recent estimates of 2.5 million species of fungi globally (Niskanen *et al.* 2023). Although limited in numbers, several groups across the world are exploring Mucoromycota diversity, especially species in order Mucorales, by the isolation of new species into culture, such as recently illustrated with 58 species described from China (Zhao *et al.* 2023). The basal lineages remain a fascinating area for research, in part due to their important roles in human activities and as fundamental to understanding the evolutionary trajectories within the Opisthokonts as some lineages have maintained genes that were lost in the Dikarya (Merényi *et al.* 2023, Galindo *et al.* 2021).

Furthermore, because the spores produced by species in the Mucoromycota are generally not optimized for aerial dispersal (with notable exceptions), some of the early-diverging species may have limited movement. Thus, it is possible that analysis of the biogeography of these species may reveal greater levels of endemism compared to more easily dispersed fungi in the Dikarya. However, our currently poor understanding of microfungal diversity, particularly in the Mucoromycota, is an impediment in testing such an hypothesis.

The traditional classification of species within the Mucoromycota that was based mainly on morphological features led to several proposed affiliations between taxa. Phylogenetic analyses based on DNA sequences subsequently highlighted, and in cases resolved, some of these ambiguous relationships (Hoffmann *et al.* 2013, Voigt & Wöstemeyer 2001). Thus, use of DNA barcodes has become a standard method to distinguish new taxa in this phylum.

In this study, a microfungus representing a new genus was identified from Victoria, Australia, and described as within the Mucorales, Mucoromycota.

Material and methods

Leaf litter was collected from the Brisbane Ranges National Park, along Wildflower Track near Butchers Road (37.88278°S 144.19966°E) on 9 October 2022, under a Parks Victoria permit issued to the University of Melbourne.

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Approximately 1 g of material was suspended in 50 ml sterile water, then aliquots spread onto 10 potato dextrose agar (PDA) plates containing rifampicin (10 µg/ml) and chloramphenicol (34 µg/ml) to inhibit bacterial growth. Species in the Mucorales growing on the plates were identified by the production of aerial sporangiophores, and strains further purified on PDA medium supplemented with the same antibiotics to form strains derived from single asexual spores. Growth rates were measured on PDA cultured from temperatures between 8 °C and 37 °C. The colours of the colonies were defined using an established palette (Kornerup & Wanscher 1978).

Mycelium was cultured in potato dextrose broth, lyophilized, and then used for genomic DNA extraction (Pitkin *et al.* 1996). To assess carbon assimilation properties, spores were suspended in yeast nitrogen base medium (*i.e.*, containing trace elements, a nitrogen source, but not carbon) and added to the wells of API 50 CH strips (bioMérieux, France) as used previously (Schwarz *et al.* 2007). Growth was assessed for up to four weeks.

Three microscopes were used to examine the strain: a Leica DM6000B compound microscope, Leica M205 FA dissecting microscope and a Hitachi TM4000Plus scanning electron microscope. For the latter, sporangia were first placed on carbon stubs and then sputter-coated with gold using a Quorum Q150T ES Plus machine.

To generate molecular markers from the genomic DNA, a region of the large ribosomal subunit (*LSU*) was amplified with primers LR3 (5′-GGTCCGTGTTTCAAGAC-3′) and NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the internal transcribed spaces + 5.8S ribosomal subunit (*ITS*) with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) using ExTaq DNA polymerase (Takara, Japan). Because Sanger sequencing initially from the PCR products directly after amplification yielded mixed chromatograms, indicative of multiple copies being present in the strain, the *ITS* amplicon was cloned into a plasmid (using the TOPO pCR2.1 kit; Invitrogen, USA), subsequent *Escherichia coli* colonies cultured, plasmids isolated, and multiple individual inserts in the plasmid sequenced using the universal M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers at the Australian Genome Research Facility (AGRF). To generate whole genome sequencing information, genomic DNA was sequenced by the Victorian Clinical Genetics Services as 150 nucleotide paired-end reads on an Illumina NovaSeq 6000 instrument. DNA sequences were analyzed in Geneious, Sequencher or the Galaxy platform. A genome assembly was conducted using Velvet (Zerbino & Birney, 2008) with a *k*-mer length of 80 in Galaxy (Afgan *et al.*, 2018). The genes encoding actin (*ACT1*), translation elongation factor 1α (*TEF1*) and the small ribosomal subunit (*SSU*) were assembled from the Illumina sequences, and the Illumina reads used to confirm the presence of multiple copies of the rDNA region as inferred from Sanger sequencing.

To resolve gaps in the assembly of the Illumina reads near the mating type (*MAT* or *sex*) locus, the DNA regions corresponding to the missing sequences were amplified with primer pairs MAI0899 (5′-TAGCTTACTCTCTAGAGC-3′) and MAI0900 (5′-CCATTTAGTATCACATTCC-3′), MAI0901 (5′-GAGCATGACATGATATAG-3′) and MAI0902 (5′-TGTCAATGTACCATCACTGC-3′) or MAI0903 (5′- GGATCATAAGGAATGTGC-3′) and MAI0904 (5′-GTCGCCTTCCTTGCTTGC-3′). The amplicons were purified from agarose gels and Sanger sequenced at AGRF with the primers used for amplification.

Phylogenetic trees were generated for five DNA regions separately as well as from a concatenated alignment. To do this, comparable sequences were downloaded from GenBank based on those examined by Hoffmann *et al.* (2013). A species to represent each genus was selected, primarily based on availability of sequences for as many regions as possible and having been used in previous phylogenetic studies. *Absidia repens* and *Halteromyces radiatus* were selected as outgroups. The DNA sequences were aligned using clustalW, then a maximum likelihood phylogeny generated using MEGA 11 using the optimal model selection of the general time reversible model +G +I (Tamura *et al.* 2021).

Results

A fungal strain was obtained into pure culture with the appearance of asexual reproduction by many members of the order Mucorales, *i.e.*, rapid growth and the production of aerial sporangiophores.

Genomic DNA was extracted from the strain, and either by PCR or whole genome sequencing used to generate DNA sequence barcodes that are commonly used to test the inter-relationships within this order. Individual DNA regions are deposited to GenBank as accessions for *ACT1* OR965928, *TEF1* OR965929, *ITS* OR990597-OR990598, *SSU* OR978257-OR978258 and *LSU* OR978255-OR978256. The Illumina sequencing reads are deposited in the GenBank Short Read Archive in BioProject PRJNA1052770.

The placement of the new strain was poorly defined in the phylogenetic trees created for each of the five individual

regions, *i.e.*, showing limited bootstrap support. The five regions were therefore concatenated and the analysis repeated (Fig. 1). Both single DNA and combined DNA approaches revealed that the species could not be placed within a current genus.

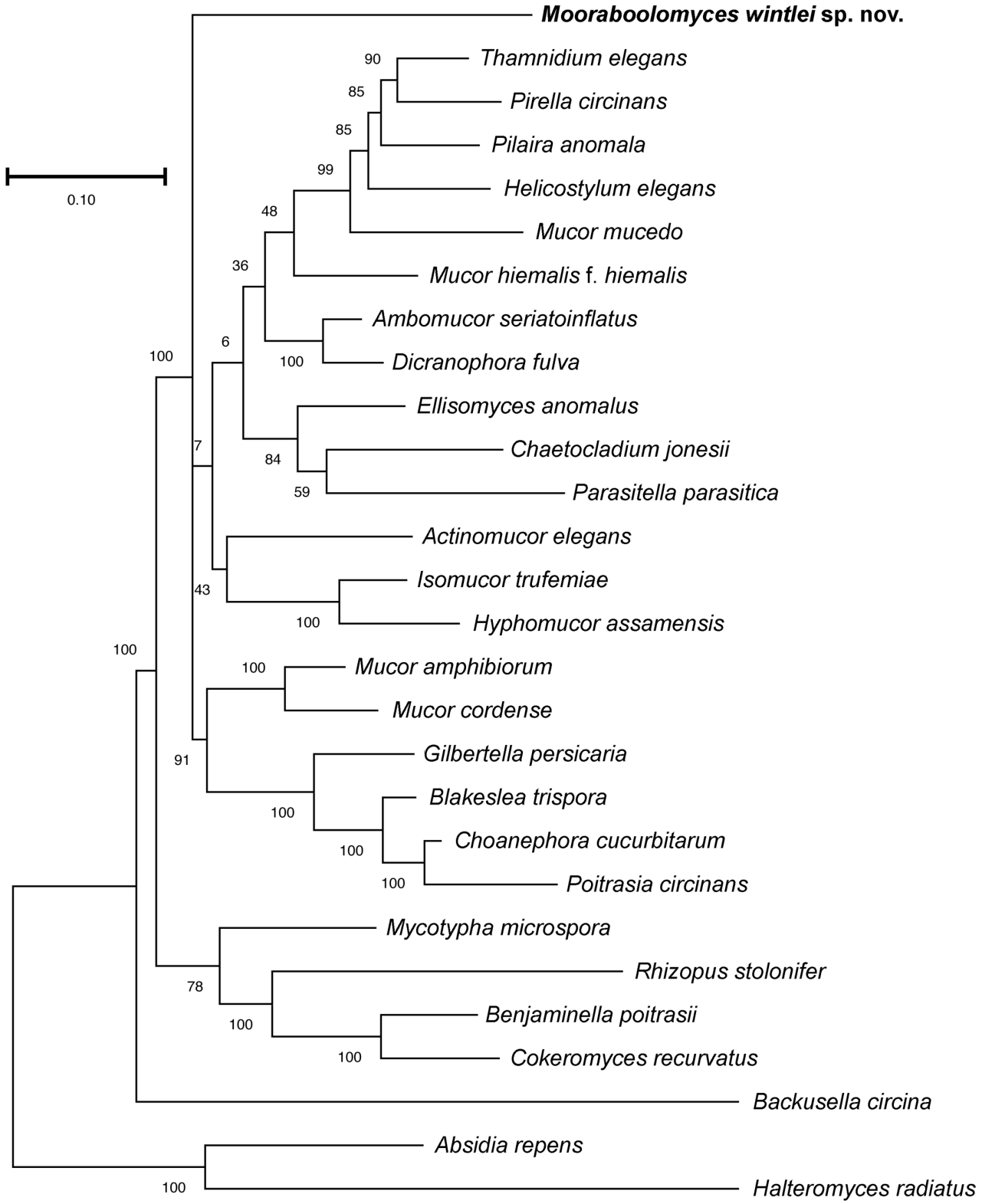

FIGURE 1. Relationship of *Mooraboolomyces wintlei* relative to closest relatives in the Mucorales, as inferred from a phylogeny using five DNA regions (*ACT1*, *ITS*, *LSU*, *SSU*, *TEF1*) using maximum likelihood. Bootstrap support from 100 trials as percentages are provided.

The putative mating type (*MAT*/*sex*) locus was assembled from the Illumina reads along with additional targeted PCRs and Sanger sequencing, and used as another comparison to this region in other Mucorales species (Fig. 2). The sequence suggests a heterothallic mode of sexual reproduction because only one of the two genes (*sexP*) conferring

the mating types was present in the strain. The sequence for this region is deposited to GenBank as accession OR965930.

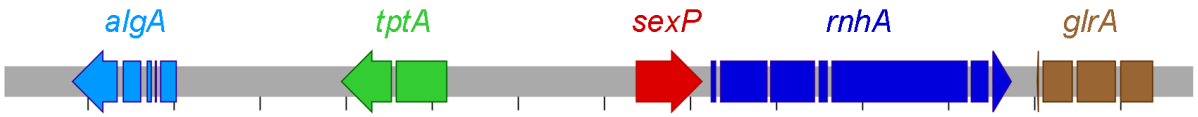

FIGURE 2. Diagram of the putative *sex* locus in *Mooraboolomyces wintlei*. The strain contains only one copy of the *sex* gene, in this case *sexP*. The surrounding genes are commonly found adjacent to the locus in other Mucorales species. The lines below the regions indicate 1.2 kb intervals.

Taxonomy

Mooraboolomyces C.P. Hull, A.S. Urquhart & A. Idnurm, *gen. nov.* (Fig. 3)
MycoBank: MB 851394

Etymology:—*Moorabool* (Wadawurrung language) for one of the local councils managing the Brisbane Ranges National Park + *myces* (Greek) for fungus.

Type species:—*Mooraboolomyces wintlei* C.P. Hull, A.S. Urquhart & A. Idnurm.

Description:—Distinguished by DNA sequence variation with other genera (see information below) because growth *in vitro* is similar to other genera in the Mucorales, producing abundant mycelia and aerial asexual sporangia.

Mooraboolomyces wintlei C.P. Hull, A.S. Urquhart & A. Idnurm, *sp. nov*. (Fig. 3)
MycoBank: MB 851395

Etymology:—*wintlei* in recognition of the contribution of Brendan A. Wintle in the exploration and preservation of Australian biodiversity.

Holotype:—AUSTRALIA. Victoria: The University of Melbourne Herbarium (MELU) F155157a as a sample of a culture of mycelia, sporangia and sporangiospores preserved on filter paper derived from the Brisbane Ranges National Park, from leaf litter under a canopy dominated by *Eucalyptus obliqua*, *E. macrorhynchus* and *Xanthorrhoea australis*, as isolated into culture on potato dextrose agar.

Ex-type strains:—UoMD22-5 = JMRC:SF:015237. These strain names refer to the University of Melbourne Diversity collection of 2022 isolate 5 = in the Jena Microbial Resource Collection (Hans Kröll Institute and the Friedrich-Schiller-University, Germany) with their reference system and number.

Description:—Radial growth rates *in vitro* on potato dextrose agar per day are 0.7 mm 8 °C, 1.7 mm 14 °C, 1.6 mm 20 °C, 2.0 mm 22 °C, 1.2 mm 28 °C, and no growth above 30 °C. Colonies with irregular edges, due to sporangiophore movement and subsequent germination of sporangiophores to form satellite colonies. Colonies initially white, becoming pale grey from the middle outwards with age as asexual sporulation occurs, while from the reverse side colonies have greyish yellow pigmentation that fades over time. Sporangiophore height up to 6 mm from the surface; sporangiophores unbranched. Sporangiophores spherical, acquiring a dark-brown pigmentation, with pigmented forms continuing through a developmental range of diameters (29.7–45.6 µm), as an average 36.7 µm. Sporangiophore hyphal width average 5.0 µm (range 4.1–6.2 µm). Columellae with a distinct pigmented collar at their base. Columellae average width 14.7 µm (range 13.8–16.3 µm) and from collar to tip length an average of 18.3 µm (range 16.3–20.6 µm). Asexual spores are ovoid, hyaline and smooth, length an average of 4.04 µm (3.49–4.47 µm) and width average of 2.34 µm (2.07–2.47 µm). No zygospores observed. Able to grow on D-xylose, D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, L-arabitol, xylitol and L-arabinose, and weak growth on D-galactose, as sole carbon sources, with absence of growth on the other carbon sources present in commercial API 50 CH strips.

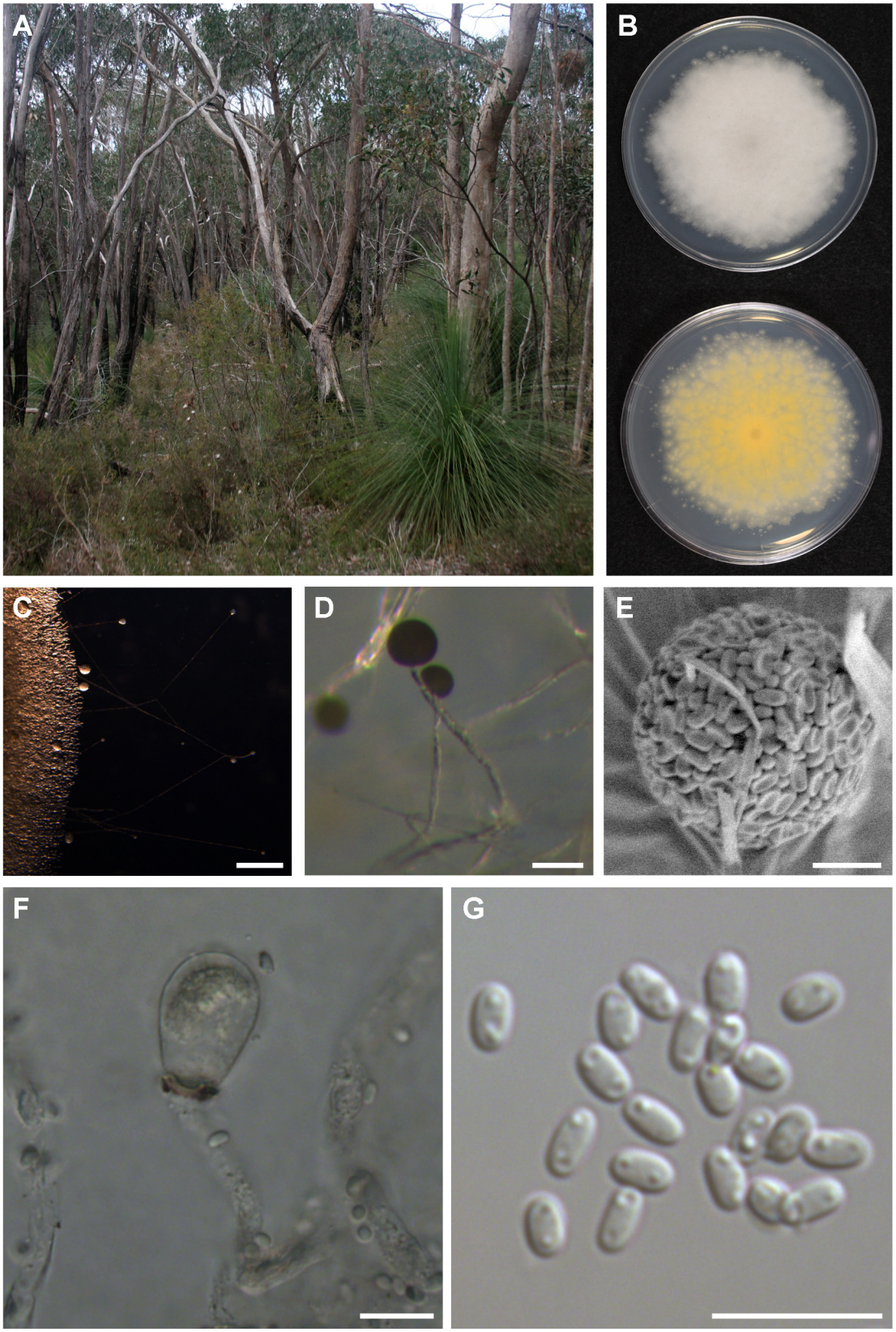

FIGURE 3. Physical properties of *Mooraboolomyces wintlei*. A. Photograph of the location from where the species was obtained. B. Petri dish plates (9 cm diameter) from above (top) or below (bottom) after 13 days at 23 °C on PDA. C. Edge of growing culture with the fungus illuminated to show examples of sporangia and spores extending beyond the starting culture to initiate new growth to account for the images in B. D. Two sporangia. E. Scanning electron microscopy of the spores in a sporangium. F. Columella, featuring the dark structure at its base. G. Asexual spores. Scale bars: C = 1 cm, D = 40 µm, E = 6 µm, F, G = 10 µm.

Discussion

In this study we identify a new genus and species in one of the early diverging lineages of fungi. The species was initially identified by growth that was distinct from others *in vitro*. The Mucorales currently comprise a relatively small group of fungal species, that nonetheless count amongst their members some well-known species that are commonly found as food contaminants, agents of disease or used in food production. *Mooraboolomyces wintlei* is presumably a saprophytic species, based on its isolation from leaf litter.

The designation of this being the first species in a new genus is based on several factors, primarily focused on distances in DNA sequences from other members of the Mucorales. The first indication of such divergence came from BLASTn searches with the five different regions indicating a difference from those previously characterised. That is, the following sets of similarities have best matches of: *ITS* 90.91% to a *Mucor* sp., *LSU* 90.34% to *M. hiemalis* f. *silvaticus*, *SSU* 96.56% to a different *Mucor* sp., *ACT1* 88.59% to *M. hiemalis* f. *corticola*, and *TEF1* 90.87% to *M. moelleri* (note for *ITS* the % depends on the length of the alignment, while against *ACT1* the mRNA sequences were excluded to reduce bias by removing the less well conserved introns). The five separate phylogenies could not place this new organism with any strong affinity to a genus, and the concatenated phylogeny (Fig. 1) also places the genus within the Mucoraceae of the Mucorales and unique to the other genera.

Only a single strain was isolated from the environmental sample, and which did not produce zygospores in culture. The genome sequence of the strain contains a single candidate *sex* locus with one of the two transcription factors that confer one of the two *sex* types in the Mucorales (Fig. 2). These two observations therefore suggest that the species is most likely heterothallic. Additional isolates would need to be obtained to confirm this prediction about the *M. wintlei* lifecycle, and also to examine the conditions to trigger the production and subsequent analysis of the morphology of zygospores as another trait for comparison within these species.

Previous studies exploring Australian diversity in the Mucorales have found species which belong to established genera found in other parts of the world [*i.e*., *Absidia*, *Backusella*, *Pilaira* and *Synchephalastum*, as discussed (Urquhart *et al.* 2021)]. To date one other genus in the Mucorales, *Halteromyces*, was first found in Australia (Shipton & Schipper 1975). It will be valuable to explore further Australian biodiversity. Fungal biogeography is also poorly understood, and hence determining distribution patterns of *M. wintlei* or other Mucorales species remains to be established.

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