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Resolving the Mortierellaceae phylogeny through synthesis of multi-gene phylogenetics and phylogenomics

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Abstract

Early efforts to classify Mortierellaceae were based on macro- and micromorphology, but sequencing and phylogenetic studies with ribosomal DNA (rDNA) markers have demonstrated conflicting taxonomic groupings and polyphyletic genera. Although some taxonomic confusion in the family has been clarified, rDNA data alone is unable to resolve higher level phylogenetic relationships within Mortierellaceae. In this study, we applied two parallel approaches to resolve the Mortierellaceae phylogeny: low coverage genome (LCG) sequencing and high-throughput, multiplexed targeted amplicon sequencing to generate sequence data for multi-gene phylogenetics. We then combined our datasets to provide a well-supported genome-based phylogeny having broad sampling depth from the amplicon dataset. Resolving the Mortierellaceae phylogeny into monophyletic genera resulted in 13 genera, 7 of which are newly proposed. Low-coverage genome sequencing proved to be a relatively cost-effective means of generating a high-confidence phylogeny. The multi-gene phylogenetics approach enabled much greater sampling depth and breadth than the LCG approach, but has limitations too. We present this work to resolve some of the taxonomic confusion and provide a genus-level framework to empower future studies on Mortierellaceae diversity and evolution.

Keywords Mortierellaceae · Phylogenomics · Molecular systematics · Taxonomy · Multi-locus sequence typing

Introduction

Early diverging fungi belonging to Mortierellomycotina are diverse in ecology and species richness, and are classified within a single order (Mortierellales) and as belonging to a single family (Mortierellaceae). Phylogenetically, this lineage is closely related to arbuscular mycorrhizal fungi (Glomeromycotina) and Mucoromycotina, and are among the earliest diverging lineages of fungi to have independently evolved differentiated macroscopic fruiting body structures, in the form of ~ 1 cm sporocarps (Smith et al. 2013; Spatafora et al. 2016; Chang et al. 2019). Several species of *Mortierella* are prolific producers of polyunsaturated fatty acids and have relevance to nutraceutical industries and bioenergy research (Goyzueta et al. 2019). Mortierellaceae are commonly detected and isolated from soils, plant debris, insect guts, mosses and living plant roots (Dixon-Stewart 1932; Gams 1977; Domsch et al. 1980), and have been found on every continent, including Antarctica (Gams 1977; GBIF.org 2019). Yet, the inability to resolve phylogenetic relationships within Mortierellaceae has limited inferences pertaining to

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species distributions, diversity and functional ecology of Mortierellaceae (Gams 1977; Petkovits et al. 2011; Wagner et al. 2013).

Early efforts to classify Mortierellaceae were based on macro- and micromorphology, including colony growth patterns, hyphal branching, and spore production (Gams 1977). Most species within Mortierellaceae have distinct macromorphological growth patterns on agar media, with colonies forming rounded to slightly pointed rosette "petals", although some species grow in simple rings and others are completely devoid of visible growth rings (Fig. 1). Mortierellaceae fungi can produce three types of spores: asexual sporangiospores born in sporangia, asexual chlamydospores that can be terminal or intercalary, and sexual zygospores (Fig. 2). One or more spore types may be absent in some species, such as *M. chlamydospora* which lacks sporangiospores and *M. parvispora* which lacks chlamydospores (Gams 1977). In some species, chlamydospores may be decorated with spines or other ornamentations and are referred to as stylospores (Chien et al. 1974). Both heterothallism and homothallism have been observed in Mortierellaceae and sexuality varies by species, although mating is not commonly observed. Macromorphology, micromorphology, and the production of all three spore types may vary considerably between growth media and conditions, which can complicate morphological species identifications (Fig. 3).

Species and their groupings were repeatedly redefined in the mid 1900s, which was concluded by Gams in 1977, who divided the lineage into two subgenera: *Micromucor* and *Mortierella*. Within *Mortierella* subgenus *Mortierella*, Gams recognized 9 sections: *Alpina*, *Actinomortierella*, *Haplosporangium*, *Hygrophila*, *Schmuckeri*, *Simplex*, *Spinosa*, *Stylospora*, and *Mortierella*, the last of which contained the type genus and species for the Mortierellaceae, *Mortierella polycephala* (Gams 1977). *Micromucor* was later reclassified to belong within Mucoromycota in the genus *Umbelopsis* (Meyer and Gams 2003). Additional genera, *Gamsiella*, *Dissophora*, *Modicella*, and *Lobosporangium* were subsequently described and accepted as Mortierellaceae, but remained polyphyletic with respect to *Mortierella* (Thaxter 1914; Benjamin 1978; Benny and Blackwell 2004; Petkovits et al. 2011; Smith et al. 2013). The genus *Haplosporangium* was retired by Gams (1977) and all included species, including the type species *H. bisporale*, have been transferred to

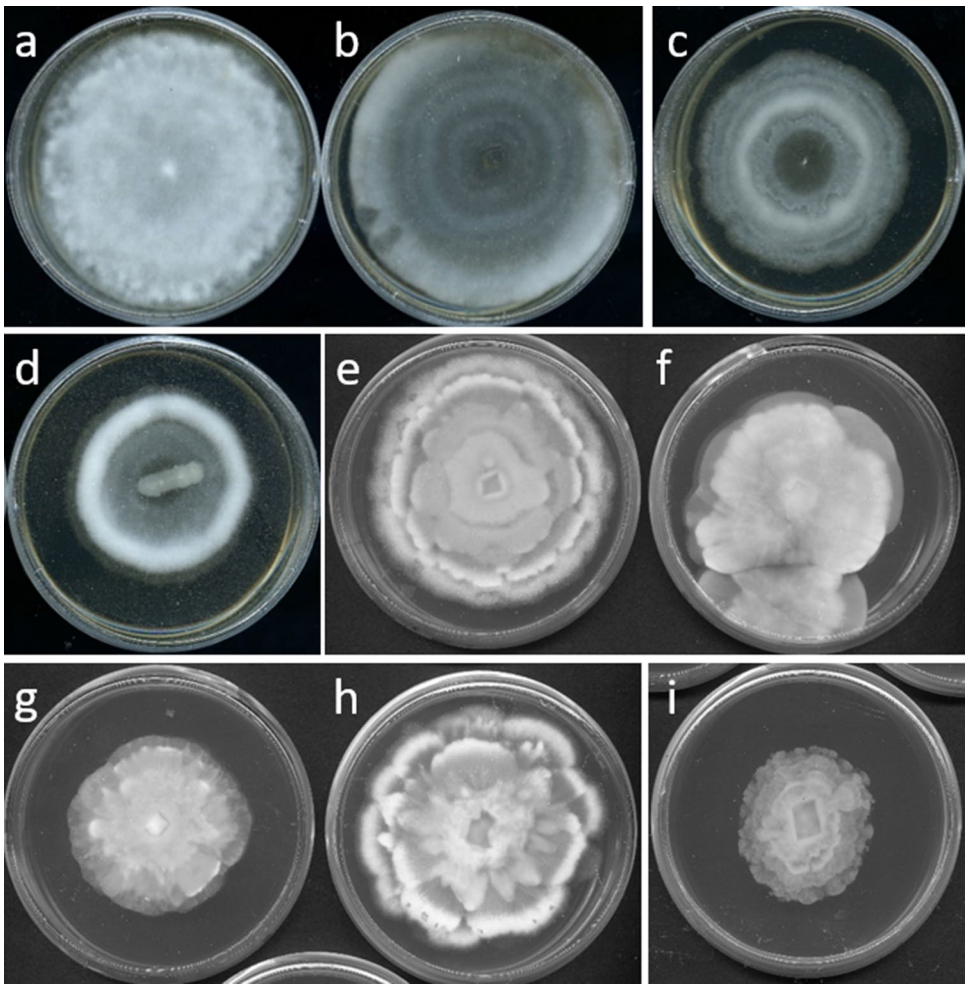

Fig. 1 Macromorphology species panel. **a** *Mortierella* sp. JL58 on MEA + YE, 11 days; **b–d** *Mortierella* sp. JL29, AP5, and JL1 on MEA + YE, 11 days; **e** *Mortierella elongata* NVP64-on PDA/2 + YE, 6 days; **f–h** *M. alpina* NVP153, JL109, and KOD1002 on PDA/2 + YE, 6 days; **i** *M. humilis* PMI1414-on PDA/2 + YE, 6 days

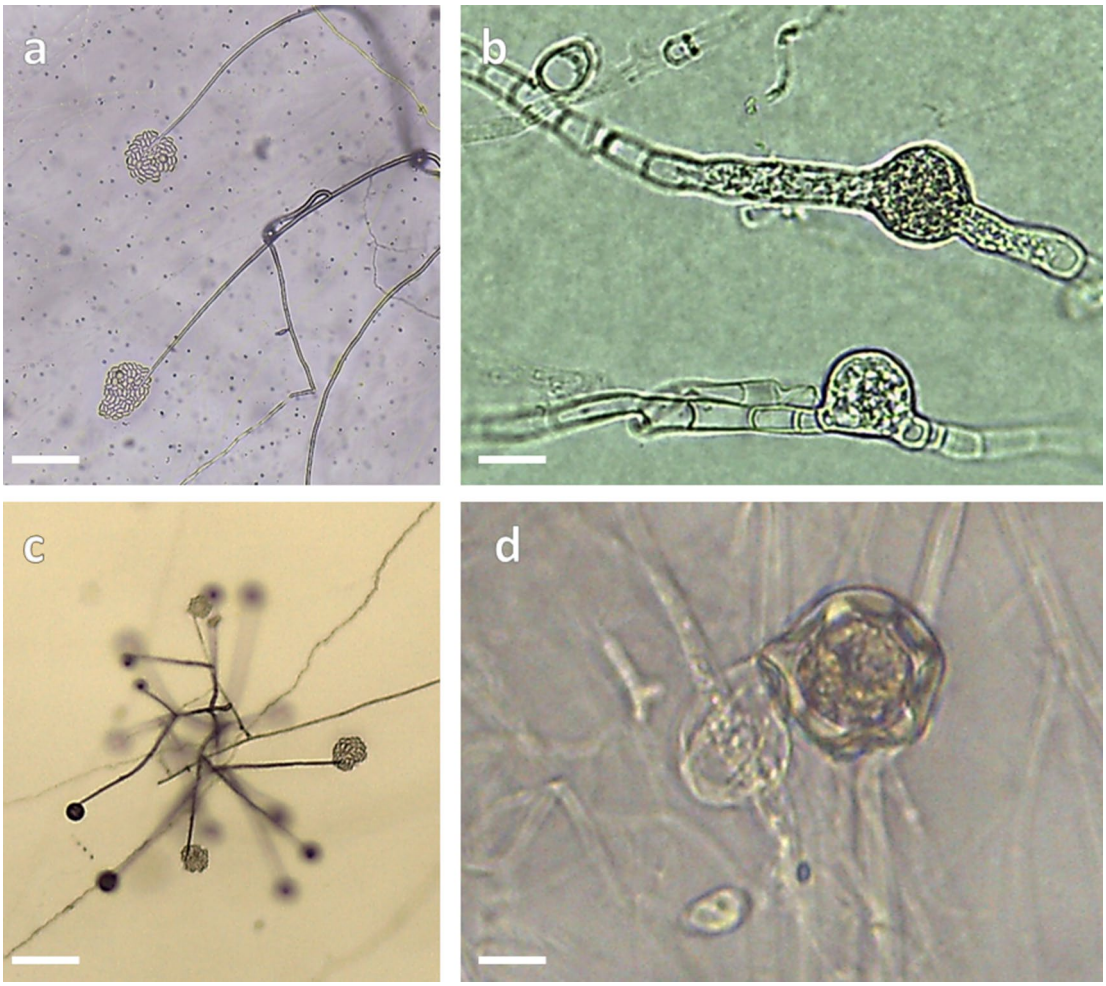

Fig. 2 Micromorphology panel. *Mortierella elongata* NVP64 **a** sporangiospores and bent sporangiophores on agar surface; **b** intercalary chlamydospore and septate, evacuated hyphae; **c** branched sporangiophore bearing sporangiospores; and **d** sexual zygospore from heterothallic mating with *M. elongata* NVP5. Scale bars: **a** 100 µm; **b** 10 µm; **c** 200 µm; **d** 20 µm

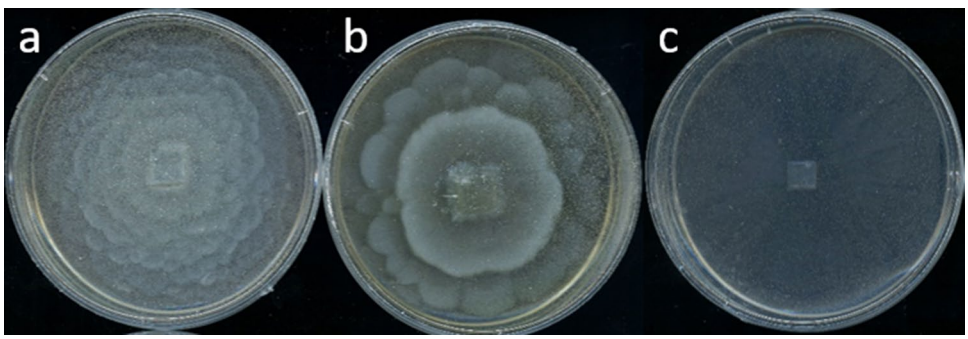

Fig. 3 Media-dependent morphology panel. *Mortierella* aff. *ambigua* JL86, 10 days, on three media **a** PDA/2 + YE, **b** MEA + YE, **c** CZA

Mortierella, in various clades. The first modern sequencing-based revision of the Mortierellaceae established that the morphological classification system was largely unsupported and defined 12 new clades (Petkovits et al. 2011). Only sect. *Schmuckeri*, sect. *Actinomortierella*, and sect. *Mortierella* were retained in the new clades. A second rDNA sequencing effort expanded the diversity of sequenced species and reorganized and combined several of the Petkovits et al. (2011) clades into a total of seven clades: selenospora, verticillata-humilis, lignicola, dissophora, capitata, alpina, and gamsii (Wagner et al. 2013).

Currently, there are close to 125 accepted Mortierellaceae species, and new species continue to be formally described each year (see Supplementary Table 1). Estimates based on environmental sequencing predict there to be more than 170 Mortierellaceae species worldwide, indicating at least one quarter of the species in this family remain to be described (Gams 1977; Benny 2009; Nagy et al. 2011; Smith et al. 2013; Wagner et al. 2013; Degawa et al. 2014; Takashima et al. 2018a, b). The current Mortierellaceae classification divides species into six genera: *Aquamortierella, Dissophora*, *Gamsiella*, *Lobosporangium*, *Mortierella*, and *Modicella* (Benny 2009). However, most of these genera are monotypic or ditypic and nearly all of the species in the family are classified as belonging to the polyphyletic genus *Mortierella* (Smith et al. 2013; Wagner et al. 2013).

Broad sampling of taxa, combined with robust phylogenetic analysis, and detailed morphological examinations, will underlie a phylogenetically-informed revision of Mortierellaceae classification and taxonomy. However, ribosomal data are unable to resolve phylogenetic relationships within Mortierellaceae (Wagner et al. 2013). That is because the Internal Transcribed Spacer (ITS) rDNA is too divergent to align across the family for phylogenetic reconstruction, though it can be useful as a DNA barcode for classifying isolates to ITS-based clades and is sometimes sufficient for species determinations. Conversely, large subunit (LSU) and small subunit (SSU) rDNA regions are too highly conserved to sufficiently resolve higher order phylogenetic relationships (Wagner et al. 2013). Thus, additional non-ribosomal markers are needed in order to identify monophyletic genera and increase genus-level taxonomic resolution.

In this study we applied two parallel approaches to resolve the Mortierellaceae phylogeny. In the first approach, we generated low-coverage genomes (LCG) from a phylogenetically diverse set of ingroup taxa to generate a robust and fully resolved phylogeny, which is a relatively new approach for phylogenomics (Olofsson et al. 2019; Zhang et al. 2019). We tested whether the LCG approach was suitable for fungi as a high-throughput method of genome sequencing to efficiently recover sequence data for phylogenomics. In the second approach, we developed and tested an automated pipeline to identify multiple lineage-specific markers for phylogenetic applications from a small number of representative genomes, and used multiplexed targeted amplicon sequencing to generate multi-gene sequence data across several hundred taxa. This allowed for an improved taxon sampling in terms of breadth and depth of as compared to the LCG approach. We then combined the concordant LCG and multi-gene phylogenetic (MGP) datasets to generate a resolved phylogeny and taxonomy for Mortierellaceae.

Materials and methods

Sampling, isolation, and culture conditions

Diverse isolates were obtained from established culture collections including the Agricultural Research Service Culture Collection (NRRL) and Westerdijk Fungal Biodiversity Institute (CBS-Centraalbureau voor Schimmelcultures) and from collaborators to broaden geographic and biodiversity sampling. Fresh isolates for this study were also obtained from roots, soils and plant substrates collected across Australia, Fiji, New Zealand, Uganda, and the United States (Supplementary Table 2). Isolates were obtained using three methods: soil baiting, shrimp baiting, and soil dilution plating or swabbing (Benny 2008; Finkelstein 2013; Nampally et al. 2015). Soil baiting involved placing substrates in squares of water agar (10 g/L BactoAgar (Difco)) supplemented with antibiotics (*i.e.* streptomycin, chloramphenicol) on the lid of an inverted water agar dish. Aerial hyphae able to colonize the upper plate were then transferred to new 1% malt extract agar (MEA: 10 g/L Malt extract, 1 g/L Yeast extract, and 10 g/L BactoAgar (Difco)) plates. Shrimp baiting refers to incubating soils with shrimp exoskeletons, which have been washed and sterilized (Shirouzu et al. 2012; Nampally et al. 2015). This substrate is enriched in chitin and selects for chitinolytic fungi, which includes many *Mortierella* species (Shirouzu et al. 2012; Nampally et al. 2015). After 1-week, colonized exoskeletons were surface sterilized with 3% hydrogen peroxide for 1 min, quenched with sterile water, and plated on squares of MEA or 1.2% potato dextrose agar (PDA: 12 g/L Potato dextrose broth and 10 g/L BactoAgar (Difco) on the lid of an inverted petri dish. Isolates whose macromorphology were consistent with *Mortierella* spp. were collected from the edge of growing colonies and transferred to new PDA or MEA plates until the cultures appeared to be pure. Soil dilution or swabbing involved either serial dilutions (1:100 and 1:1000) of soil in DI water plated on Saborauds Dextrose Agar (SDA (Thermo Scientific), or swabs streaked onto SDA (Warcup 1955). Individual isolates were picked at 1 day and 7 days and transferred to SDA.

In total, 318 isolates were studied with the aim of resolving the Mortierellaceae phylogeny (Supplementary Table 2). We included 59 strains from the ARS Culture Collection (NRRL) and 4 from the CBS strain repository selected to increase geographic diversity in our dataset. Sixteen of these were type strains. These included 21 isolates from across the United States, 12 from Europe, 3 from India, 2 from Mexico, 2 from Antarctica, and 1 each from Australia, Canada, Colombia, New Zealand, and Russia. We were unable to obtain metadata for 15 NRRL

strains. In total, the NRRL and CBS isolates comprised 36 previously identified species and 5 strains unidentified to species.

Preliminary isolate identification

To generate preliminary isolate identifications, DNA was extracted from mycelium using an alkaline extraction buffer (see Supplementary Methods for details). We PCR-amplified the ITS region and the 5′ portion of the LSU using the universal fungal primers ITS1-F and LR3 (Vilgalys and Hester 1990; Gardes and Bruns 1993). PCR products were separated by gel electrophoresis on a 1% agarose TAE gel containing ethidium bromide and visualized under ultraviolet illumination. The sizes of DNA fragments were estimated using a 100-bp ladder (ThermoFisher). Products with bands of the expected size were purified and template DNA was used in 10 µL sequencing reactions with BigDye® Terminator v3.1 (Applied Biosystems), using the primers ITS1-F and LR3 (Vilgalys and Hester 19900; Gardes and Bruns 1993). Sequences were generated on an Applied Biosystems 3730XL high throughput capillary sequencer at the Michigan State University Research Technology Support Facility Genomics Core. Sequences were de novo assembled with Geneious 8.1.3 and analyzed using the NCBI BLASTn tool (Johnson et al. 2008). Preliminary identifications were assigned based on sequence similarities and E-values. In the case of multiple equally high-quality BLAST hits to multiple Mortierellaceae species, the isolate was designated at the genus level (e.g. '*Mortierella* sp.'), with an indication of the clade to which it likely belonged as defined by Wagner et al. (2013).

Genomic DNA extraction

To prepare high-quality genomic DNA, isolates were grown in liquid 1% malt extract broth culture for 1–2 weeks. Mycelium was harvested by vacuum filtration and genomic DNA was extracted following a CTAB-based chloroform extraction protocol (Doyle 1991). DNA quality and concentration were estimated by gel electrophoresis and NanoDrop.

Low coverage genome (LCG) library preparation and sequencing

Plate-based DNA library preparation for Illumina sequencing was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Kapa Biosystems library preparation kit. 200 ng of sample DNA was sheared to 600 bp using a Covaris LE220 focused-ultrasonicator. The sheared DNA fragments were size-selected by double-SPRI and the selected fragments were end-repaired, A-tailed, and ligated with Illumina compatible sequencing adaptors from IDT containing a unique molecular index barcode for each sample library. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4, and Illumina's cBot instrument to generate a clustered flow cell for sequencing. Sequencing of the flow cell was performed on the Illumina HiSeq 2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2 × 100 indexed run recipe.

Low-coverage genome (LCG) sequence analysis

Automated genome assembly was performed with the tool Automatic Assembly For The Fungi (AAFTF) which performs read trimming and filtering against PhiX and other contaminants using BBMap v38.16 followed by genome assembly with SPAdes v3.13.1 (Bankevich et al. 2012; Bushnell 2014; Stajich 2018, 2019). Assemblies were further cleaned of vector sequences, screened for contaminant bacteria with sourmash using database Genbank Microbes 2018.03.29 (Brown and Irber 2016; Pierce et al. 2019). Duplicated small contigs were removed using minimap2 v2.17 alignment of contigs smaller than the assembly N50 (Li 2018). Contigs were further polished for the total Illumina read set with Pilon v1.10 and sorted by length and renamed (Walker et al. 2014). Each set of paired-end sequence reads for an isolate was automatically processed with AAFTF to produce a polished, vector screened, deduplicated, polished, and sorted genome assembly.

Genomes were annotated with funannotate v1.7.0, which used a combination of evidence from ab initio gene prediction and protein alignments to produce a predicted gene set (Palmer and Stajich 2017; Love et al. 2019). For each genome, the funannotate prediction step was run and allowed to train the augustus v3.3.2 gene predictor with BUSCO aligned core genes from the fungi_odb9 data set (Stanke et al. 2006; Palmer and Stajich 2017). Genemark.hmm-ES was run in self-training mode to produce additional predictions (Ter-Hovhannisyan et al. 2008). These ab initio predictions were combined with exon locations inferred by aligning proteins from the SwissProt database to the genome first with BLASTX v2.2.31+, followed by exonerate v2.4.0 to produce splice site-aware alignments. These data combined into consensus gene models for each genome using EVidenceModeler (NCBI and Camacho 2008; Slater and Birney 2005; Haas et al. 2008). All these analysis steps are run as part of the funannotate 'predict' procedure. The predicted gene models in each genome were further annotated with putative functional information using eggNOG

v1.03, CAZY, MEROPs, and Pfam databases searched with HMMER and DIAMOND (Eddy 1998; Buchfink et al. 2015). These annotated draft genomes were deposited in NCBI GenBank along with the primary Illumina sequences in NCBI SRA (Supplementary Table 3).

PHYling methods for genome analysis

To examine the phylogenetic relationships of the strains using these sequenced, assembled, and annotated genomes, the PHYling pipeline was applied to a set of conserved, typically single-copy markers that were previously developed (Spatafora et al. 2016; Beaudet et al. 2017; Stajich 2020). The predicted protein set from each strain was searched for each of the 434 single-copy markers in the "JGI_1086" set, which was developed from Joint Genome Institute's orthologous clusters from genomes in 2015. The best hits from each strain for each marker were aligned to the original HMM using hmmalign and the resulting alignments were trimmed with trimAL (Eddy 1998; Capella-Gutiérrez et al. 2009). Codon alignments were generated by back translating the protein alignments using the input coding sequences for each gene with the script bp_mrtrans.pl from BioPerl (Stajich et al. 2002) incorporated into PHYling. These individual alignments were concatenated together by PHYling using the script combine_multiseq_aln.py, recording the start/end of the alignment into a partition file. The concatenated protein and codon alignments were each used for phylogenetic analyses, initially in FastTree and later in RAxML (Price et al. 2010; JTT substitution model, Stamatakis 2014). In addition, individual gene alignments were subjected to phylogenetic analyses to estimate gene trees. All of the gene trees were combined and processed with ASTRAL v5.14.3 to infer a coalescent species tree from the individual gene trees (Mirarab et al. 2014).

Multi-gene phylogenetic (MGP) primer design and validation

We used a series of custom Python scripts to extract all exon sequences from the annotated de novo genome of *Mortierella elongata* AG77 (Uehling et al. 2017; https://github.com/natalie-vandepol/mortierellaceae_mlst). We then conducted a BLAST search for these exon sequences in the de novo *M. alpina* B6842 and *M. verticillata* NRRL6337 genomes (Wang et al. 2011; Spatafora et al. 2016). We filtered the results for high-identity, single-copy hits and used MUSCLE to align the sequences (Edgar 2004). From these, loci were selected based upon sequence identity and primers were manually designed using MEGA6 and OligoCalc (Kibbe 2007; Tamura et al. 2013). Primer sequences were tested in silico with iPCRess against several *Mortierella*, *Umbelopsis,* and *Mucor* genomes to estimate the likelihood of off-target amplification (Slater 2007; Supplementary Table 4).

Primer sets were selected for testing based on melting temperature compatibility. Primer sets were tested using genomic DNA from a panel of *Mortierella* isolates and DreamTaq Master Mix (MM) (ThermoFisher Scientific). PCR products were visualized through gel electrophoresis, as previously described, using a 1.2% agarose gel. Product sizes were estimated using a 100-bp ladder (ThermoFisher). Amplicons were Sanger sequenced and aligned in Mesquite 3.6. Thirteen loci showed primer specificity, robust amplification, and good alignment of sequences across the panel of Mortierellaceae diversity (Supplementary Table 4). These loci, together with the fungal ribosomal primers ITS1-F and LR5, were tested for multiplex compatibility (Hopple and Vilgalys 1994). Four sets of primer pairs were identified for multiplexed amplification of 3–4 loci in a single reaction. These sets were composed as follows: (1) RPB1, EF1a, 615, and 1870; (2) 370, 4955, and 10,927; (3) 5401, 4121, and ITS1-F/LR5; and (4) 5512, 2175, 5491, and 2451.

MGP multiplex amplification, library preparation, and sequencing

Multiplex PCR was performed using Platinum Multiplex PCR Master Mix (Applied Biosystems). 2.5 µl aliquots of the products were mixed with loading dye and subjected to electrophoresis on a 1.2% agarose gel and DNA was visualized by ethidium bromide staining. PCR products and their sizes were estimated based on a 100 bp ladder (ThermoFisher). Primer sets that failed to amplify in the MultiPlex reactions were amplified individually with DreamTaq MM and screened using gel electrophoresis. Amplicons were pooled and sent to NCAUR (USDA-ARS, Peoria, IL) for library preparation using the Nextera DNA Library Preparation and Nextera Index Kits (Illumina) and sequenced on a MiSeq platform instrument.

MGP sequence analysis

Demultiplexed paired-end sequences were filtered for PhiX using the filter_phix tool in the USEARCH pipeline and assembled using SPAdes v3.7.1 (Edgar 2010; Bankevich et al. 2012). The resulting contigs were identified to locus through a BLAST search against the genome sequences used for primer design and representative fungal and bacterial ribosomal sequences. A custom python script parsed the BLAST results to group contigs by locus. To minimize missing data, we defined "full-length" sequences as being at least 80% of the expected length for the locus. The count of full and partial length sequences for each sample and locus are summarized in Supplementary Table 5. Loci were selected for further analysis based on the frequency of duplicate

full-length sequences by comparing the total number of sequences for that locus to the number of samples (hereafter isolates represented by DNA sequence data) with at least one sequence. Loci with a sequence:sample ratio higher than 1.2:1 were excluded from the dataset, since this degree of over-representation would require extensive manual analysis to resolve, if possible, and could represent genuine paralogs or gene duplication events rather than barcode migration or cross-contamination between samples (Supplementary Table 5). Resolution of duplicate sequences in the case of a gene duplication or paralogs would likely result in different copies being retained for a given sample and the locus still being unusable.

The filtered contigs were aligned using MUSCLE and trimmed to conserved regions in Mesquite 3.6 (Edgar 2004; 2016). Additional loci were excluded if they had distinct sequence variants that could not be aligned. For the remaining 6 loci that were not excluded for the reasons described above, informative regions were identified using Gblocks 0.19b and analyzed with PartitionFinder 2 to determine the appropriate nucleotide substitution model (Castresana 2000; Lanfear et al. 2016). Phylogenetic trees for each marker were generated with RAxML using the CIPRES gateway [RAxML-HPC2 on XSEDE (8.2.12)] (Ronquist and Huelsenbeck 2003; Miller et al. 2010; Stamatakis 2014).

Taxonomic identification of isolates was verified through BLAST searches of ITS sequences and considered valid if the best hits were to the expected or closely related species. When the best hits were consistently to species in a different ITS-based clade, the isolate was indicated as "*Mortierella* sp." Isolates were removed from the dataset if sequence data was only present for one locus or if they consisted of multiple copies for the majority of the loci. The remaining isolates were screened for multiple copies of any locus. In cases of multiple copies, if one sequence was clearly consistent in phylogenetic placement compared to other loci, that sequence copy was assumed to be the orthologue and was kept, and any paralogues were deleted. Otherwise, all sequences for that locus were removed for that sample.

To improve the data matrix by increasing the number and diversity of isolates having four or more of the six loci, missing loci of several isolates were individually amplified with DreamTaq, screened via agarose gel electrophoresis, and Sanger sequenced. In addition, when genome sequences were available for a sample with missing loci, sequences from close relatives were used in a BLAST search against the raw genome sequencing reads to search for the target loci. This process was also used to construct MGP loci for LCG samples that had not been included in the MGP dataset. Matching reads were assembled and aligned to the query sequence in Geneious.

Three outgroup taxa were selected from published genomes available on JGI Mycocosm Portal: *Mucor circinelloides* CBS277, *Umbelopsis ramanniana AG#*, and *Lichtheimia corymbifera* FSU9682. The MGP loci were identified in these genomes using BLAST analyses in the MycoCosm portal and the sequences downloaded for inclusion in the alignments.

After finalizing the dataset and alignments, Gblocks 0.91b and PartitionFinder 2 were used to exclude characters (type = DNA, allowed gaps = with half) and identify the optimal model of evolution for phylogenetics for each locus (GTR + G+I) (Castresana 2000; Lanfear et al. 2016). SequenceMatrix 1.8 was used to generate a concatenated nucleotide matrix of the six loci (Vaidya et al. 2011). Four different phylogenetic tree building approaches were carried out. These included both constrained and unconstrained RAxML analyses of the concatenated matrix, a partially constrained MrBayes analysis of the concatenated matrix, and RAxML analyses of constrained and unconstrained single-gene alignments. A custom R script was used to prune the LCG tree to remove isolates not included in our concatenated and single gene datasets. The pruned genome tree provided a guide tree to constrain RAxML phylogenetic analyses. Key nodes were used to define partial constraints for MrBayes phylogenetic analyses. We did explore using ASTRAL to generate a consensus phylogeny as a counterpoint to our concatenated matrix analyses (Mirarab et al. 2014). However, we elected to not use this approach for our six gene dataset since this program was intended for datasets of several hundred genes.

Results

Geographic and biodiversity sampling

We improved geographic sampling of Mortierellaceae with our own isolates obtained from soils collected in Italy (1), Australia (32), Fiji (1), New Zealand (8), Uganda (13), and across the United States (53). These new isolates account for 110 cultures representing 14 previously described species and 44 strains that could not be resolved to species, 26 of which represent 4 putatively novel species. In most cases, isolates that could not be identified to species by ITS sequence could still be assigned to one of the ITS-based clades established by Wagner et al. (2013).

An additional 145 isolates were contributed by collaborators. Of those, 141 were from the United States, 1 from Argentina, and 3 where metadata was missing. The 145 contributed isolates represented 25 previously described species and 46 isolates that could not be identified to species.

The LCG dataset included 73 strains representing 28 described species and 21 isolates unidentified to species by ITS sequence analysis. Representatives were included from all seven ITS-based clades defined by Wagner et al. (2013)

and 11 of the 12 clades defined by Petkovits et al. (2011), the exception being the strangulata clade.

For the MGP approach, we PCR-amplified and sequenced the initial 13 loci across 332 isolates. Thirty-two isolates were excluded on the basis of quality filtering as described in detail later. These 32 excluded isolates are not reported in the sampling and metadata to avoid confusion about included versus excluded diversity, but are listed in Supplementary Table 6. We added 14 LCG isolates by mining the genomes for the MGP loci. Therefore, the final MGP dataset contained 314 isolates that represent 48 distinct Mortierellaceae taxa and three outgroup species (Supplementary Table 2). In this dataset, the gamsii, verticillata-humilis, and alpina clades were over-represented. Indeed, these are often the most commonly isolated Mortierellaceae. In addition, we specifically oversampled *M. elongata* and *M. alpina* isolates from disparate geographic regions in an attempt to better resolve these species complexes. In total, we included 117 isolates representing 11 of the 22 described species and one putative novel species from the gamsii clade, 69 isolates representing 7 of the 8 described species from the verticillata-humilis clade, and 70 isolates representing 7 of the 10 described species from the alpina clade. By comparison, the lignicola, dissophora, capitata, and selenospora clades were relatively undersampled. In the lignicola clade, we included 13 isolates representing 4 of the 8 described species, and 7 that were not previously identified to species. From the dissophora clade, we included 22 isolates representing 5 of the 7 described species and three putative novel species. For the capitata clade, 9 isolates represented 4 of the 9 described species and one unidentified species. For the selenospora clade, our dataset included 16 isolates, which represent 6 of the 12 described species. This study is the first modern phylogenetic analysis to include *Modicella*, represented by *M. reniformis* MES-2146. For the 81 described Mortierellaceae species that were not included in this study, we have summarized the current classification and, when possible, estimated their likely placement in our proposed classification based on high ITS sequence similarity to the species included in this study (Supplementary Table 1).

The low coverage genome approach

Molecular results

For two samples, the sequencing coverage was too low to assemble and be included in the study. One sample was found to be contaminated. On average, Illumina sequencing returned 588 (316–1217) GB of sequence data per isolate, which were assembled into ~ 3700 (1100–12,500) contigs (Supplementary Table 3). The average depth of genome coverage was 14.8X (8.5X–38.5X). Genome annotation identified an average of ~ 12,000 predicted proteins (7338–16,572) in each genome. Our search for the 434 phylogenetic markers in the annotated protein set for each isolate identified between 354 and 419 markers in each genome (Supplementary Table 3). Altogether there were a total of 109,439 characters in the concatenated LCG nucleotide matrix, which has been uploaded to TreeBase (submission 25,806).

LCG dataset and phylogeny

There are nine main branches in the LCG tree (Fig. 4). The wolfii-capitata clade is sister to the rest of the lineages and includes the taxa *Mortierella ambigua*, *M.* aff. *ambigua*, and *M. wolfii*. The next diverging lineage is *M. selenospora*. The third branch contains all representatives of the verticillata-humilis clade (17 isolates, 7 species). The single *M. cystojenkinii* isolate appears as a monotypic lineage. The fifth branch contains all included representatives of the gamsii clade (19 isolates, 7 described species) and a putative novel species that does not cluster with any known taxon in either ITS or phylogenomic analyses, represented by *Mortierella* sp. GBAus35, NVP41, and AD031. The sixth branch includes all representatives of the alpina clade (11 isolates, 2 species), and is divided into two subgroups containing predominantly *M. polycephala* and *M. alpina*, respectively, the former of which is the type species for *Mortierella*. The seventh branch is represented by the lignicola clade and the eight branch is the monotypic *Lobosporangium transversale*. The ninth and final branch contains *Gamsiella multidivaricata*, *Modicella reniformis*, *Dissophora ornata*, *Mortierella globulifera*, and several putative novel species that are sister to *Modicella*.

The multi-gene phylogenetics approach

Primer design

From the three reference genomes, 1269 exons met initial quality criteria related to length, copy number, and sequence similarity. Of those, 130 were classified as suitable candidates for primer design due to their internal moderate variability flanked at each end by highly conserved areas. Further analysis yielded 74 primer sets meeting target amplicon length, GC content, primer length, ambiguity, and self-compatibility criteria (Supplementary Table 4). Of those, 55 passed in silico PCR. As a positive control for this locus selection process, we checked our primer sets for exons from RNA polymerase subunit B (*RPB1*) and elongation factor 1-alpha (*EF1α*), which have been used successfully as fungal phylogenetic markers. We found both *RPB1* and *EF1α* among the 55 primer sets that had passed in silico PCR. We selected 22 loci, including *RPB1* and *EF1α*, for in vitro testing based on similar annealing temperatures (Supplementary Table 4). Thirteen were selected for continued use based on

Fig. 4 Low coverage genome Mortierellaceae phylogeny. Maximum likelihood analysis of a concatenated matrix of 109,439 nucleotide characters of protein-coding sequences from 434 genes. Clade colors indicate monophyletic groupings, lines and clade names denote previously defined clades for the purpose of discussion. Node numbers indicate bootstrap support

consistent amplification across a panel of Mortierellaceae isolates and alignment of the amplicon sequences.

Molecular results

Illumina sequencing generated a total of 711.5 GB of sequence data (2.8 million reads) that were demultiplexed into our initial 333 samples. Sequences were assembled into 7905 contigs across 329 isolates for a total of 7.6 GB unfiltered, assembled contigs having an average coverage of about 100×. Three isolates and the PCR negative control had little or no sequence data, could not be assembled, and were excluded from further analyses.

Filtering loci, sequences and isolates

Further quality control steps were taken to assess the consistency and validity of each locus as a phylogenetic marker. Loci 4121 (hypothetical protein, predicted amino acid transporter), 2175 (CTP synthase), and 615 (chitin synthase) were found to exceed the 120% sequence over-representation cutoff described in the methods and were therefore excluded (Supplementary Tables 4 and 5). Loci 10,927 (hypothetical protein, class V myosin motor head), 5401 (adenosylmethionine-8-amino-7-oxononanoate transaminase, biotin/cofactor biosynthesis), and 5491 (delta-12 fatty acid desaturase) each had two very distinct sequence variants that could not be aligned, so these loci were excluded from further analysis. Locus 370 (acyl-CoA oxidase) was found to be a member of a gene family, with no one homologue consistently amplified across sampled taxa. Therefore, this locus was excluded from further analysis.

Some isolates had more than one full-length sequence for a given locus, referred to here as duplicates (Supplementary Tables 5 and 7). In most cases, we attribute this to cross-contamination of isolates during post-PCR sample handling. Duplicate sequences were resolved by identifying where the sample was placed phylogenetically by other loci, and then determining which of the duplicates sequences were congruent. We resolved 2 of 5 duplicates in locus 1870 (xanthine dehydrogenase), 1 of 1 in locus 2451 (calcium-translocating P-type ATPase), 4 of 7 in locus 4955 (hypothetical protein, DNA replication licensing factor), 2 of 5 in locus *EF1*α (elongation factor 1-alpha), and 11 of 13 in locus *RPB1* (RNA polymerase II subunit RPB1). Locus 5512 (glycosyltransferase Family 21 protein) had no duplicates.

Thirty-two isolates were completely excluded from further analysis for various reasons detailed here and summarised in Supplementary Table 6. Four isolates were completely excluded from the dataset because all but one locus had duplicate sequences, therefore no trends could be used to resolve duplicated sequences. Eleven isolates were removed for having zero full length sequences or only ITS sequences. Five isolates were removed because only one locus amplified and there was no corroboration from other loci to verify the placement of the sample. Two isolates were excluded because they were contaminated by *Umbelopsis* and the loci were strongly incongruent. Five additional isolates were removed because placement according to the non-ribosomal loci radically disagreed with the species identification by ITS sequence. Four isolates were found to be duplicated in our dataset, due to having been assigned new isolate ID numbers after having been shared between labs. One isolate was excluded because the only loci for which it had sequences were deleted loci. We mined reference genomes and the unassembled LCG dataset for MGP loci and were able to fill in several "holes" in the dataset from loci that had failed to amplify/sequence. We also added 15 new isolates that were not included in the initial MGP dataset by successfully recovering at least two full-length MGP loci from genome sequences.

Primer performance

Many isolates did not have all 6 loci represented in the final dataset. This was particularly true for isolates that were not closely related to the species from which the primers were designed. We attribute this result to sequence mismatch preventing the primer from binding, hereafter referred to as "primer mismatch", rather than absence of the target genes. Sequence reads for all of the loci were detected in genome sequences of isolates across all clades of the Mortierellaceae, although incomplete sequencing often meant that the assembled reads did not meet the minimum sequence length and therefore could not be included in our analyses (Supplementary Table 8). The effect of primer mismatch varied by loci (e.g., 1870, 5512 had lower recovery; *EF1*α, *RPB1* had higher recovery), as seen by inconsistent percentages of isolates amplified across the clades. In contrast, locus 2451 amplified poorly across all clades.

Unconstrained MGP phylogeny

The MGP dataset included 314 isolates representing 48 unique Mortierellaceae taxa and three outgroup species. These included 69 of the 73 Mortierellaceae LCG isolates, the exceptions being *Mortierella wolfii* NRRL 66265, *Mortierella* sp. GBAus43, *M. chlamydospora* NRRL 2769, and *Mortierella* sp. NRRL A-10996. The final 6 gene concatenated matrix contained a total of 8181 characters, which has been uploaded to TreeBase (submission 25806). No strong incongruence was observed between the unconstrained MGP phylogeny and the LCG tree, but some weak incongruences were evident (Supplementary Fig. 1). For instance, in the MGP phylogeny, the wolfii-capitata clade was split into several basal branches. The verticillata-humilis, gamsii, and

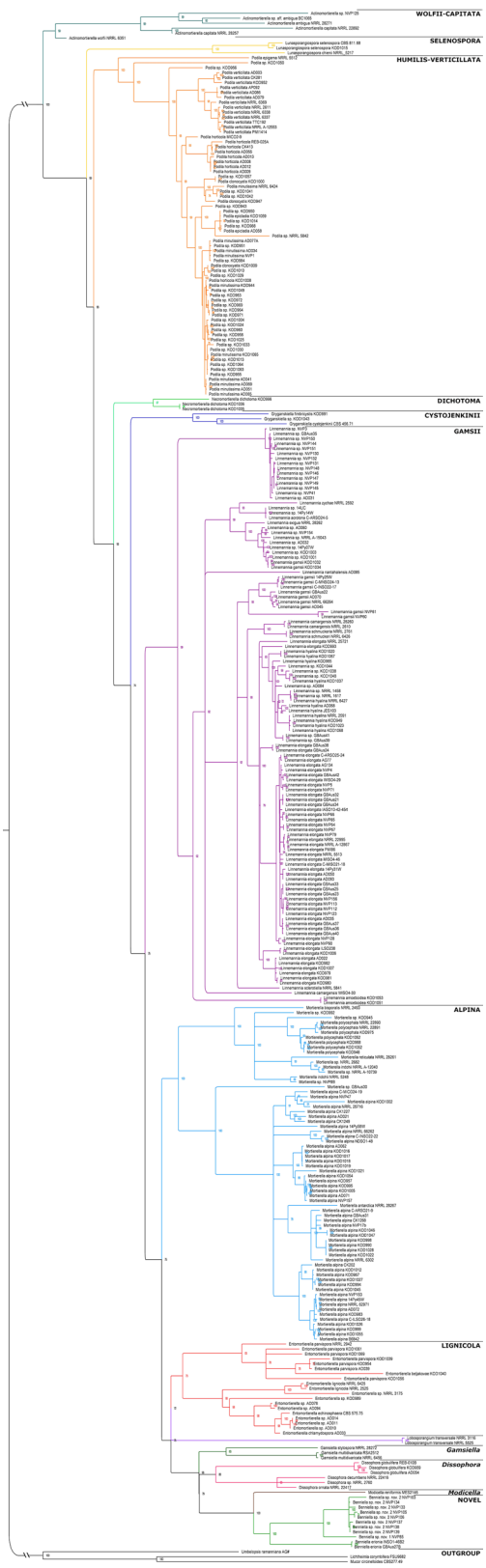

Fig. 5 Constrained MGP Mortierellaceae phylogeny. Maximum Likelihood analysis of the concatenated 6-gene MGP dataset composed of 8181 nucleotide characters and constrained by the LCG phylogeny. Taxa are named according to the initial ITS-based species identification and proposed genus-level taxonomy. Clade colors indicate monophyletic groupings according to the proposed taxonomy, lines and names denote previously defined clades for the purpose of discussion. *Actinomortierella* (wolfii-capitata)—blue-green, *Lunasporangiospora* (selenospora)—yellow, *Podila* (humilis-verticillata)—orange, *Necromortierella* (dichotoma)—lime green, *Gryganskiella* (cystojenkinii)—dark blue, *Linnemannia* (gamsii)—magenta, *Mortierella* (alpina)—light blue, *Entomortierella* (lignicola)—red, *Lobosporangium*—lavender, *Gamsiella*—dark green, *Dissophora*—pink, *Modicella*—brown, *Benniella* (novel)—leaf green

alpina clades were still internally cohesive and consistently placed along the backbone. However, the alpina clade was split between two separate branches. *Mortierella dichotoma* was not represented in the LCG dataset. Our analyses place it apart from the other "clade 1" species, as defined by Wagner et al. (2013), where it comprised a monotypic branch between the verticillata-humilis and cystojenkinii clades. The MGP phylogeny also placed the selenospora clade within the second alpina branch and *M. parvispora* and *M. beljakovae* were grouped with *M. dichotoma*, rather than the rest of the lignicola clade. The lignicola clade, *Gamsiella*, *Dissophora*, *Modicella*, *Lobosporangium*, and the novel group detected in this study were still clustered together. All together, the internal group structure was generally consistent with the LCG analysis, although *Lobosporangium* was placed within the lignicola clade, possibly due to long branch attraction with *Mortierella* sp. NRRL3175. There were several weakly supported nodes (40–71%) along the backbone of the unconstrained MGP phylogeny, indicating that (similar to rDNA) the MGP loci were generally able to resolve relationships within, but not between, the major groups.

LCG-constrained MGP phylogeny

Since the unconstrained MGP phylogeny was unable to resolve the backbone placement of the major clade of Mortierellaceae, we synthesized our datasets to leverage the high confidence of the LCG phylogeny with the sampling depth of the MGP dataset. To obtain a constraint tree, we "pruned" the LCG tree to remove the 4 isolates not represented in the MGP dataset. Since MrBayes does not accept a constraint tree as a direct input, we defined major nodes of the LCG tree as partial constraints. However, the resulting tree topology was clearly inconsistent with the genome tree, though somewhat consistent with the unconstrained RAxML tree (Supplementary Fig. 2). We then generated an LCG-constrained RAxML phylogeny from the concatenated MGP matrix. This phylogeny had strong support (74–100% at major nodes), was congruent with the LCG phylogeny, and

will be used henceforth as our best estimate of the Mortierellaceae phylogeny (Fig. 5).

Resolving the Mortierellaceae phylogeny into monophyletic lineages resulted in a total of 14 clades (Fig. 5). To help stabilize Mortierellaceae taxonomy we have resurrected *Actinomortierella* and erected seven novel monophyletic genera to accommodate supported clades: *Benniella, Entomortierella*, *Gryganskiella*, *Linnemannia*, *Lunasporangiospora*, *Necromortierella*, and *Podila*.

We erected the genus *Linnemannia* to include the monophyletic gamsii clade, which contains the *L. elongata* complex, *L. gamsii*, *L. amoeboidea* and related species. *L. elongata* isolates do not appear to cluster by geographic origin, indicating that *L. elongata* may be cosmopolitan globally. Different genotypes of isolates within the *L. elongata* complex could not be distinguished using the ITS sequence data, and even with a global sampling provide poor resolution of the species within this complex. *L. gamsii* isolates were split between two branches: one monophyletic and the other shared with *L. zychae*, *L. exigua*, and *L. acrotona*. Lastly, *L. amoeboidea* originally clustered with *M. alpina* in ITS-based studies but consistently was resolved in *Linnemannia* in our analyses (Wagner et al. 2013; Petkovits et al. 2011).

Since *M. polycephala* is the type species for *Mortierella*, the genus *Mortierella* is conserved for the alpina clade, which was split into two main groups. The first included *M. polycephala*, *M. bisporalis*, *M. reticulata*, and *M. indohii*. The other branch was composed of the *M. alpina* complex and *M. antarctica*.

Podila includes species within the verticillata-humilis clade, which contains *P. verticillata*, *P. minutissima*, and related species. *Podila* species have historically been difficult to resolve or identify by ITS analysis, as the ITS sequences of species within this group usually share 98–99% identity. Although *P. horticola* and *P. minutissima* ITS sequences share 99% identity, they were distinguished in our analyses.

The deeply diverged clade in the Mortierellaceae phylogeny that includes *Lobosporangium*, *Dissophora*, *Gamsiella*, *Modicella*, and the newly erected genera *Entomortierella* and *Benniella* also has the highest diversity of ecologies and morphologies in the family. *Entomortierella* includes the lignicola clade and *E. parvispora*. *Lobosporangium* was placed between *Entomortierella* and the deepest branch with *Gamsiella, Dissophora, Modicella*, and *Benniella*.

The MGP loci as phylogenetic markers

In this study, we found *EF1α* to be the least informative of the tested loci, as even the constrained gene tree had extremely low support (1–50% at most major nodes) and many misplaced taxa (e.g., *Mortierella alpina* KOD1046 grouped with *Podila* and *M. alpina* NRRL 6302 grouped with *Entomortierella*, both separate from the dominant *M. alpina* cluster). However, *EF1α* and *RPB1* had the highest recovery rate across all isolates, including for outgroup taxa. *RPB1* was the best performing individual locus of the MGP dataset, both in terms of its consistent amplification and ability to distinguish species. For example, the ITS1f-LR5 sequences of *Linnemannia elongata* NVP64 and *L. gamsii* NVP61 shared 98% similarity and 97% similarity with *L. hyalina*. However, *RPB1* sequences of these species showed 85–96% interspecific sequence similarity, with intraspecific sequence similarity usually 98–99% (Supplementary Tables 9 and 10). The other loci provided additional resolution of species within other genera. For example, locus 2451 provided the best resolution of species in *Podila*, with generally 96–99% sequence similarity within species and 89–94% similarity between species. *P. epicladia* and *P. minutissima* shared the highest sequence similarity (96–98% similarity within, 95–96% similarity between). For more reliable separation of these two species, locus 5512 could be used, where *P. epicladia* and *P. minutissima* were each 98–99% similar within species and 96% similar between species.

Taxonomy

The proposed classification for Mortierellaceae follows general principles promoted in the Hibbett et al. (2007) phylogenetic classification of Kingdom Fungi, and followed by Spatafora et al. (2016) in their reclassification of zygomycetous fungi. Below are the accepted and proposed genera, each in alphabetical order, a brief discussion of each genus, and the species included in this study. Following each genus description are any comb. nov. descriptions for each species transferred to that genus. Species characteristics, synonyms, basionyms, and MycoBank numbers are also summarized in Supplementary Table 11.

Accepted Genera

Actinomortierella Chalab. 1968

≡ *Mortierella* Coem., Bulletin de l'Académie Royale des Sciences de Belgique Classe des Sciences 15: 536 (1863)

≡ *Carnoya* Dewèvre, Grevillea 22 (101): 4 (1893) [MB#20101]

≡ *Naumoviella* Novot., Chung-kuo Ti Chen-chun [Fungi of China]: 155 (1950) [MB#20362]

MycoBank No: MB#20012

Description: Phylogenetically basal within the Mortierellaceae. Sporangiophores have an apical inflation in the uppermost portion from which short branches arise. The main sporangiophore and branches may or may not form terminal sporangia. Sporangiospores are globose to ellipsoid; chlamydospores are absent.

Habitat: Isolates of this genus have been reported from Fiji, India, New Zealand, across North America, and the

United Kingdom. *A. ambigua* and *A. capitata* are most commonly isolated from soil and dung. *Actinomortierella.* sp. aff *ambigua* BC1065 was isolated from decaying wood and fungivorous millipedes (Macias et al. 2019). In contrast, *A. wolfii* is generally isolated from compost, decaying hay and the lungs of diseased animals, usually cattle, where it causes mycotic pneumonia and abortion. *A. wolfii* is the only Mortierellaceae species known to be pathogenic to mammals and thus grows well at unusually high temperatures (Seviour, Cooper, and Skilbeck 1987).

Type species: *Actinomortierella capitata* (Marchal) Vandepol & Bonito

Species in this study:

Actinomortierella ambigua (B.S. Mehrotra) Vandepol & Bonito **comb. nov.**

MycoBank No.: MB#835795

Basionym: *Mortierella ambigua* B.S. Mehrotra, Mycologia 55 (3): 289 (1963)

Type specimen: INDIA, Allahabad, isolated from garden soil. Botany Department, University of Allahabad, M-80.

Actinomortierella capitata (Marchal) Vandepol & Bonito **comb. nov.**

≡ *Mortierella vesiculosa* B.S. Mehrotra, Baijal & B.R. Mehrotra, Mycologia 55 (3): 295 (1963)

≡ *Carnoya capitata* (Marchal) Dewèvre, Grevillea 22 (101): 5 (1893)

MycoBank No.: MB#835796

Basionym: *Mortierella capitata* Marchal, Bull. Soc. R. Bot. Belg.: 134 (1891)

Type specimen: No type known

Actinomortierella wolfii (B.S. Mehrotra & Baijal) Vandepol & Bonito **comb. nov.**

MycoBank No.: MB#835797

Basionym: *Mortierella wolfii* B.S. Mehrotra & Baijal, Mycopathologia et Mycologia Applicata 20 (1–2): 51 (1963)

Type specimen: INDIA, Jobner, Rajasthan, isolated from sandy soil. Botany Department, University of Allahabad, M-82.

Notes: *Mortierella capitata* Marchal (1891) was reassigned to *Carnoya* by Dewèvre (1893) and then transferred to *Actinomortierella* by Chalab (1968) along with several other Mortierellaceae species. The genus *Actinomortierella* was subsequently reduced to a subsection of *Mortierella* by Gams (1977). Since all but one of the species formerly in *Actinomortierella* were clustered together in our analyses (the exception being *Lunasporangiospora chienii* as *Actinomortierella umbellata*), we are resurrecting *Actinomortierella*. However, while *Actinomortierella* was validly described by Chalabuda in Griby Roda Mortierella (1968), the novel combinations for *A. ambigua*, *A. capitata*, and *A. wolfii* were in violation of Article 41.5 of the Shenzhen Code. Therefore, we provide valid combinations for each of these species.

Aquamortierella Embree & Indoh 1967

MycoBank No: MB#20047

Description: Hyphae highly branched. Sporangiospores uniquely reniform (kidney-shaped) to allantoid (sausage-shaped). Zygospores unknown and chlamydospores not mentioned. Suggested to be the only known member of the Mucorales, wherein the Mortierellaceae were then presumed to belong, to normally form sporangia and discharge spores under water.

Habitat: Initially isolated from midge larvae in a freshwater stream in New Zealand. It was also found in Japan.

Type species: *Aquamortierella elegans* Embree & Indoh

Notes: No living material of this taxon currently exists.

Dissophora Thaxter 1914

MycoBank No: MB#20187

Description: Fertile, septate aerial stolons are abruptly differentiated from fine vegetative hyphae. Sporangiophores arise as buds from these stolons in intervals behind the advancing apex. *D. globulifera* comb. nov., was not originally described in this genus, but it does produce sporangiophores from aerial stolons, in accordance with the original diagnostic characteristic. Unlike the other *Dissophora* species, *D. globulifera* sporangiophores appear as outgrowths from the bases of older sporangiophores, forming "tufts", rather than singly along the length of the fertile aerial stolon.

Habitat: All *Dissophora* species have been isolated from forest litter and soil. *D. globulifera* has also been isolated from agricultural soil. *D. decumbens* isolates are from North America, *D. ornata* isolates are from South America, and *D. globulifera* has been isolated from Europe and Japan.

Type species: *Dissophora decumbens* Thaxter

Species in this study:

Dissophora decumbens Thaxt., Botanical Gazette Crawfordsville 58: 361 (1914) [MB#160412]

Dissophora globulifera (Rostr.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella ericetorum* Linnem., ZentBl. Bakt. Parasit-Kde, Abt. 2: 228 (1953)

MycoBank No: MB#833727

Basionym: *Mortierella globulifera* O. Rostr., Dansk botanisk Arkiv 2 (5): 2 (1916)

Type specimen: Isolated from decaying root of *Dactylis glomerata*, MBT#8101 (**Neotype**)

Dissophora ornata (Gams) Gams, Studies in Mycology 31: 91 (1989) [MB#135572]

≡ *Mortierella ornata* W. Gams, Caldasia 13: 715 (1983)

Gamsiella Benny & M. Blackwell 2004

≡ *Mortierella* subgen. Gamsiella R.K. Benj., Aliso 9: 157 (1978) [MB#530804]

MycoBank No: MB#28820

Description: This genus was originally monotypic and defined based on the sporangiophore morphology of *G. multidivaricta*: "branched aerial hyphae form intercalary, lateral enlargements which become several times successively di- or tridivaricately branched, the ultimate branches forming two-spored sporangia on slender, elongate, attenuated pedicels" (Benjamin 1978). *G. stylospora*, does not form sporangiospores, instead making stylospores. As Dixon-Stewart described (1932) "Stylospores very well developed on Czapek's medium, borne on fine aerial upright hyphae.. No sporangia have been seen".

Habitat: Isolates have been reported from soil, decaying wood, and dung in Australia and Russia.

Type species: *Gamsiella multidivaricata* (R.K. Benj.) Benny & Blackwell

Species in this study:

Gamsiella multidivaricata (R.K. Benj.) Benny & Blackw., Mycologia 96 (1): 147 (2004) [MB#488121]

≡ *Mortierella multidivaricata* R.K. Benj., Aliso 9: 158 (1978)

Gamsiella stylospora (Dixon-Stew) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833728

Basionym: *Mortierella stylospora* Dixon-Stew., Trans Br Mycol Soc 17 (3): 218 (1932)

Type specimen: AUSTRALIA, Victoria, isolated from sandy loam soil, CBS 211.32 [MBT#8202]

Lobosporangium M. Blackwell & Benny 2004

MycoBank No: MB#28819

Description: Sporangiophores are branched, sporangia transversely elongate with 1–5 spines at the apex. Sporangiospores are irregularly shaped. Zygospores are absent.

Habitat: The type strain of this monotypic genus was isolated from Nevada soil in 1964. It has only been reported since from soils in Texas and the Sonoran Desert.

Type species: *Lobosporangium transversale* (Malloch) Blackwell & Benny

Species in this study:

Lobosporangium transversale (Malloch) M. Blackw. & Benny, Mycologia 96 (1): 144 (2004) [MB#488122]

≡ *Echinosporangium transversale* [as 'transversalis'] Malloch, Mycologia 59: 327 (1967)

Modicella Kanouse 1936

MycoBank No: MB#20336

Description: Species in this genus are the only Mortierellaceae known to produce macroscopic fruiting bodies, in the form of small, whitish, round sporocarps. Spores can be germinated on artificial media and grown axenically. They are morphologically Mortierellaceae-like in their acolumellate sporangium and garlic-like odor that is similar to that of other Mortierellaceae species.

Habitat: *Modicella* specimens are found growing saprotrophically on soils and decaying plant matter. *M. malleola* has been recorded in Europe, New Zealand, North America, and Taiwan, whereas *M. reniformis* has only been found in South America.

Type species: *Modicella malleola* (Harkn.) Gerd. & Trappe

Species in this study:

Modicella reniformis (Bres.) Gerd. & Trappe, Mycologia Memoirs 5: 68 (1974) [MB#317772]

≡ *Endogone reniformis* Bres., Hedwigia 35: 297 (1896)

Mortierella Coemans 1863

MycoBank No: MB#20345

Description: Sporangiospores are absent in some species, when present they can range from smooth and ellipsoid to reticulated and/or irregular, depending on the species. Chlamydospores, where present, are scarce and either smooth or spiny. When known, zygospore production is by heterothallic mating, though some homothallic *M. polycephala* isolates have been reported (de Hoog et al. 2000).

Habitat: Most *Mortierella* species prefer to grow at cooler temperatures (Carreiro and Koske 1992). Several species of *Mortierella* are known to be mycoparasitic to varying degrees. The type species of this genus, *M. polycephala*, was originally isolated from a mushroom. One strain in the present study was isolated from the surface of a truffle. *M. bisporalis* is a facultative biotrophic mycophile that first competes with its host for substrate, then causes lysis of the host mycelium and penetrates the host to live biotrophically (Rudakov 1978). *Mortierella alpina* has also been shown to parasitize oospores of members of Oomycota (Phylum Heterokontophyta) (Willoughby 1988).

Type species: *Mortierella polycephala* Coemans

Species in this study:

Mortierella alpina Peyronel, I germi astmosferici dei fungi con micelio: 17 (1913) [MB#170280]

≡ *Mortierella oblatispora* W. Gams & G.J. Bollen (?)

≡ *Mortierella acuminata* Linnem., Pflanzenforschung 23: 21 (1941)

≡ *Mortierella renispora* Dixon-Stew., Trans Br Mycol Soc 17 (3): 214 (1932)

≡ *Mortierella monospora* Linnem., Flora (Regensburg) 130: 210 (1936)

≡ *Mortierella thaxteri* Björl., Botaniska Notiser 1936: 116 (1936)

Mortierella antarctica Linnem., Mucorales, eine Beschreibung aller Gattungen und Arten dieser Pilzgruppe: 198 (1969) [MB#317880]

Mortierella bisporalis (Thaxt.) Björl., Botaniska Notiser 1936: 126 (1936) [MB#258541]

≡ *Haplosporangium bisporale* Thaxt., Botanical Gazette Crawfordsville 58: 364 (1914)

Mortierella indohii C.Y. Chien, Mycologia 66: 115 (1974) [MB#317900]

Mortierella polycephala Coem., Bulletin de l'Académie Royale des Sciences de Belgique Classe des Sciences 15: 536 (1863) [MB#145769]

≡ *Mortierella vantieghemi* Bachm. (1900)

≡ *Mortierella vantieghemii* Bachm., Jahrbücher für Wissenschaftliche Botanik 34: 279 (1900)

≡ *Mortierella raphani* Dauphin, Ann. Sci. Nat., Bot.: 30 (1908)

≡ *Mortierella vantieghemi var. raphani* (Dauphin) Linnem., Pflanzenforschung 23: 31 (1941)

Mortierella reticulata Tiegh. & G. Le Monn., Annales des Sciences Naturelles Botanique 17: 350 (1873) [MB#236117]

Novel Genera

Benniella Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833778

Etymology: In honor of Gerald Benny, an American mycologist who has dedicated his career to the study of zygomyceteous fungi. Dr. Benny made significant contributions to Mortierellaceae taxonomy, among others. This included establishing the subphylum Mortierellomycotina, elevating *Gamsiella* to generic level, and renaming *Echinosporangium* as *Lobosporangium*.

Description: Colonies on MEA or PDA are pure white to off white in color and do not produce rosette or colony patterns when young. With age, some slight growth rings may develop in the mycelium along the agar surface. Aerial mycelium is very abundant, over 1 cm thick. Hyphae are sterile, without observed sporangiophores or zygospores.

Habitat: These fungi have been isolated from dried soils collected in the United States (Indiana and Ohio), Australia, and Uganda. Isolates were baited from soils using sterilized shrimp exoskeletons.

Type species: *Benniella erionia* Liber & Bonito

Benniella erionia Liber & Bonito, **sp. nov.**

MycoBank No: MB#833779

Etymology: "erionia" - from the Greek "erion" meaning "wool". This describes the appearance of the mycelium as unpatterned, light colored, and wooly.

Description: Colonies on MEA are pure white, and do not produce rosettes or patterning. Hyphae are sterile, without observed sporangiophores or zygospores, and are 3.63 ± 0.09 µm (mean ± SEM) in diameter. Terminal structures borne on axillary hyphae are swollen and irregularly branched, and in older cultures, these become darkened and resemble chains of spherical chlamydospores, each spore 10.7 ± 1.89 µm. Growth rates on PDA ½ + YE are 6.6–9.5 µm/min (min and max of 3 replicates) at room temperature (RT), and 5.0–8.5 µm/min at 30 °C. On MEA + YE, growth rates are 4.9–8.4 µm/min at RT, and 4.9–5.2 µm/min at 30 °C.

Habitat: The type specimen (isolate GBAus27B) was cultured from soils collected in woodland of *Eucalyptus marginata* and *Corymbia calophylla* in Australia, on sandy gravels on low divides in the subhumid zones. Isolates were baited from soils using sterilized arthropod exoskeletons. The other *B. erionia* isolate in this study, INSO1-46B2, was isolated from soybean field soil in Indiana, USA.

Type specimen: AUSTRALIA, Western Australia, Camballan, sub-humid upland forest woodlands dominated by *Eucalyptus marginata* and *Corymbia calophylla*. 24 Sept. 2014, G.M. Bonito, FLAS-F-66497 (**holotype**) [MBT#392648].

Entomortierella Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833613

Etymology: entomon- (insect) refers to the insect association common to the species in this genus.

Description: Most of the species produce sporangiospores, usually globose and smooth, but spiny in the case of *E. lignicola*. *Entomortierella beljackovae*, *E. chlamydospora*, and *E. echinosphaera* produce chlamydospores, the latter two of which are usually spiny. Almost all of the species are known to produce zygospores, but are divided between hetero- and homothallic sexual lifestyles.

Habitat: Species in this genus appear to be arthropod and/or worm associates, as they are commonly isolated from ant pellets, termite nests, and vermicompost. They are also frequently isolated from soil, roots, and rotting plant matter.

Type species: *Entomortierella lignicola* (Martin; Gams & Moreau) Vandepol & Bonito

Species in this study:

Entomortierella beljakovae (Milko) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833729

Basionym: *Mortierella beljakovae* Milko, Novosti Sistematiki Nizshikh Rastenii 10: 85 (1973)

Type specimen: UKRAINE, Rovensk region, Sarna, from coniferous forest soil, 1971, CBS 123.72 [MBT#8042].

Entomortierella chlamydospora (Chesters) Vandepol & Bonito **comb. nov.**

≡ *Mortierella chlamydospora* (Chesters) Plaats-Niterink, Persoonia 9 (1): 91 (1976)

MycoBank No: MB#835762

Basionym: *Azygozygum chlamydosporum* Chesters, Trans Br Mycol Soc 18 (3): 213 (1933)

Type specimen: Isolated from *Antirrhinum majus* infected by *Rhizoctonia solani*, MBT#8049 (**Syntype**).

Entomortierella echinosphaera (Plaats-Niterink) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835750

Basionym: *Mortierella echinosphaera* Plaats-Niterink, Persoonia 9 (1): 91 (1976)

Type specimen: THE NETHERLANDS, Aalsmeer, isolated from Begonia sp., CBS 575.75 (**Holotype**) [MBT#8059].

Entomortierella lignicola (G.W. Martin) Vandepol & Bonito **comb. nov.**

≡ *Mortierella lignicola* (G.W. Martin) W. Gams & R. Moreau, Annales Scientifiques Université Besançon 3: 103 (1960)

≡ *Mortierella sepedonioides* Linnem., Pflanzenforschung 23: 23 (1941)

MycoBank No: MB#835763

Basionym: *Haplosporangium lignicola* G.W. Martin, Mycologia 29 (5): 618 (1937)

Type specimen: COLOMBIA, Sierra Nevada de Santa Marta, isolated from rotten wood, CBS 207.37 [MBT#15136].

Entomortierella parvispora (Linnem.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella gracilis* Linnem., Pflanzenforschung 23: 38 (1941)

MycoBank No: MB#835751

Basionym: *Mortierella parvispora* Linnem., Pflanzenforschung 23: 53 (1941)

Type specimen: GERMANY, isolated from soil, MBT#8163 (**Syntype**).

Gryganskiella Vandepol, Stajich & Bonito, **gen. nov.**

MycoBank No: MB#833857

Etymology: In honor of Andrii Gryanskyi, a Ukrainian-American mycologist, for his contributions in research, training and genomics of fungi in Mucoromycota.

Description: Sporangiospores are smooth and elliptical to cylindrical. Chlamydospores are lightly pigmented, light brown or ochre/orange. While this characteristic is not unique to this genus, it is conserved within this group.

Habitat: These species have been reported from agricultural soil & moss in the Netherlands & South America.

Type species: *Gryganskiella cystojenkinii* (W. Gams & Veenb.-Rijks) Vandepol & Bonito

Species in this study:

Gryganskiella cystojenkinii (Gams & Veenb.-Rijks) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833858

Basionym: *Mortierella cystojenkinii* W. Gams & Veenb.-Rijks, Persoonia 9 (1): 137 (1976)

Type specimen: THE NETHERLANDS, Wageningen, isolated from agricultural soil, CBS 456.71 [MBT#8054].

Gryganskiella fimbricystis (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833859

Basionym: *Mortierella fimbricystis* W. Gams, Persoonia 9 (1): 138 (1976)

Type specimen: ARGENTINA, South Patagonia, Puerto Edwards near Beagle Canal, isolated from the center of moss cushion in a very wet bog, CBS 943.70 [MBT#8084].

Linnemannia Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833612

Etymology: In honor of Germaine Linnemann, a German mycologist who contributed many Mortierellaceae species descriptions and early hypotheses on their evolutionary relationships.

Description: Nearly all known species of *Linnemannia* produce sporangiospores, with the exception of *L. acrotona*. When produced, sporangiospores are usually ellipsoid, but can also be spherical to cylindrical. Production of chlamydospores is irregular between species. When produced, most chlamydospores are various shades of brown. *L. amoeboidea* makes irregular amoeba-like chlamydospores. The species for which the sexual reproductive mode is known are heterothallic.

Habitat: This genus contains some of the most widely distributed Mortierellaceae species. *L. elongata*, *L. hyalina* and *L. gamsii*, are especially common in neutral or calcareous soils. Most of the species in this genus are isolated from soils and are usually associated with plant rhizospheres or decaying plant matter.

Type species: *Linnemannia hyalina* (Harz; Gams) Vandepol & Bonito

Species in this study:

Linnemannia acrotona (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833769

Basionym: *Mortierella acrotona* W. Gams, Persoonia 9 (1): 133 (1976)

Type specimen: INDIA, Rajasthan, Jaipur, Rambagh Palace Hotel, isolated from soil, CBS 386.71 [MBT#8005].

Linnemannia amoeboidea (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833770

Basionym: *Mortierella amoeboidea* W. Gams, Persoonia 9 (1): 116 (1976)

Type specimen: GERMANY, Teutoburger Wald, Beller Holz, isolated from Chromelosporium sp. in September 1972, CBS 889.72 [MBT#8022].

Linnemannia camargensis (Gams & Moreau) Vandepol & Bonito **comb. nov.**

≡ *Haplosporangium gracile* Nicot, Bulletin de la Société Mycologique de France 73: 90 (1957)

MycoBank No: MB#835745

Basionym: *Mortierella camargensis* W. Gams & R. Moreau, Annales Scientifiques Université Besançon 3: 103 (1960)

Type specimen: FRANCE, Camargue, Bois des Rièges, isolated from sandy soil, CBS 221.58 [MBT#18848].

Linnemannia elongata (Linnem.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella debilis* E. Wolf, Zentralblatt für Bakteriologie und Parasitenkunde, Abteilung 2 107: 528 (1954)

MycoBank No: MB#833768

Basionym: *Mortierella elongata* Linnem., Pflanzenforschung 23: 43 (1941)

Type specimen: GERMANY, MBT#140592.

Linnemannia exigua (Linnem.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella indica* B.S. Mehrotra, Indian Phytopathology 13: 68 (1960)

≡ *Mortierella sterilis* B.S. Mehrotra & B.R. Mehrotra, ZentBl. Bakt. ParasitKde, Abt. 2: 178 (1964)

MycoBank No: MB#835752

Basionym: *Mortierella exigua* Linnem., Pflanzenforschung 23: 44 (1941)

Type specimen: GERMANY, MBT#140594.

Linnemannia gamsii (Milko) Vandepol & Bonito **comb. nov.**

≡ *Mortierella spinosa* Linnem., Flora (Regensburg) 130: 214 (1936)

MycoBank No: MB#835747

Basionym: *Mortierella gamsii* Milko, Opredelitel mukoralnykh gribov [Key to the identification of Mucorales]: 76 (1974)

Type specimen: THE NETHERLANDS, Baarn, Maarschalksbos, isolated from soil, CBS 749.68 [MBT#8087].

Linnemannia hyalina (Gams) Vandepol & Bonito **comb. nov.**

≡ *Hydrophora hyalina* Harz, Bulletin de la Société Impériale des Naturalistes de Moscou 44: 144 (1871)

≡ *Mortierella candelabrum var. minor* Grove, Journal of Botany, British and Foreign 23: 131 (1885)

≡ *Mortierella hygrophila* Linnem., Flora (Regensburg) 130: 212 (1936)

≡ *Mortierella hygrophila var. minuta* Linnem., Pflanzenforschung 23: 50 (1941)

MycoBank No: MB#833682

Basionym: *Mortierella hyalina* (Harz) W. Gams, Nova Hedwigia 18: 13 (1970)

Type specimen: UNITED KINGDOM, North of London, Rothamsted, isolated from *Triticum aestivum* roots in July 2003, MBT#56360 (**Isotype**).

Linnemannia nantahalensis (C.Y. Chien) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835746

Basionym: *Mortierella nantahalensis* C.Y. Chien, Mycologia 63 (4): 826 (1971)

Type specimen: UNITED STATES OF AMERICA, North Carolina, Joyce Kilmer Memorial Forest in the Nantahala National Forest, isolated from soil, CBS 610.70 [MBT#8154].

Linnemannia schmuckeri (Linnem.) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835748

Basionym: *Mortierella schmuckeri* Linnem., Archiv für Mikrobiologie 30: 263 (1958)

Type specimen: MEXICO, Queretaro, isolated from soil, MBT#8193 (**Syntype**).

Linnemannia sclerotiella (Milko) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835749

Basionym: *Mortierella sclerotiella* Milko, Novosti Sistematiki Nizshikh Rastenii 4: 160 (1967)

Type specimen: UKRAINE, near Kiev, isolated from mouse dung in 1964, CBS 529.68 [MBT#8195].

Linnemannia zychae (Linnem.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella brachyrhiza* E. Wolf, Zentralblatt für Bakteriologie und Parasitenkunde, Abteilung 2 107: 532 (1954)

≡ *Mortierella zychae var. japonica* J.Y. Lee, Bull. Sugadaira biol. Lab. 5: 47(1972)

≡ *Mortierella zychae var. simplex* Linnem., Pflanzenforschung 23: 46 (1941)

MycoBank No: MB#835753

Basionym: *Mortierella zychae* Linnem., Pflanzenforschung 23: 46 (1941)

Type specimen: GERMANY, Allgäu, isolated from decaying wood of *Populus tremula*, CBS 316.52 [MBT#8235].

Lunasporangiospora Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833611

Etymology: luna- (crescent) refers to the lunate sporangiospores unique to the two species in this genus.

Description: Sporangiospores are smooth and characteristically lunate. Chlamydospores are terminal and scarce in *L. selenospora* and absent in *L. chienii*. Mating and zygospores are unknown in both species.

Habitat: Isolates of this genus have been reported from mushroom compost and forest soil from North America, Europe, and Asia.

Type species: *Lunasporangiospora chienii* (P.M. Kirk) Vandepol & Bonito

Species in this study:

Lunasporangiospora chienii (P.M. Kirk) Vandepol & Bonito, **comb. nov.**

≡ *Mortierella umbellata* C.Y. Chien, Mycologia 64 (1): 99 (1972)

≡ *Actinomortierella umbellata* (C.Y. Chien) Chalab., Griby Roda Mortierella Coemans: 199 (1973)

MycoBank No: MB#833681

Basionym: *Mortierella chienii* P.M. Kirk, Index Fungorum 2: 1 (2012)

Type specimen: GEORGIA, Athens, isolated from cultivated soil, CBS 124.71 [MBT#8211].

Lunasporangiospora selenospora (W. Gams) Vandepol & Bonito, **comb. nov.**

MycoBank No: MB#833724

Basionym: *Mortierella selenospora* W. Gams, Persoonia 9 (1): 128 (1976)

Type specimen: THE NETHERLANDS, Horst, isolated from mushroom compost together with *Entomophthora coronata* and *Aphanocladium album*, CBS 811.68 [MBT#8196].

Necromortierella Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833725

Etymology: necro- (death) refers to the necrotrophic mycophilic lifestyle of the type species, in that it kills and consumes the cells of other fungi.

Description: Sporangiophore narrow, tapering quickly to a narrower apex with irregular dichotomous branching. Sporangiospores are ellipsoidal to cylindrical. Chlamydospores are elongated or irregular.

Habitat: *N. dichotoma* is the only known necrotrophic mycophile (kills fungal cells and feeds saprotrophically on the dead tissue) in Mortierellaceae. This species has only been reported from mouse dung in Germany.

Type species: *Necromortierella dichotoma* (Linnem. ex W. Gams) Vandepol & Bonito

Notes: There may be additional species in this genus that were not included in this study. Additional work must be done to determine whether related species are also necrotrophic mycophiles.

Species in this study:

Necromortierella dichotoma (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#833726

Basionym: *Mortierella dichotoma* W. Gams, Persoonia 9 (1): 128 (1976)

Type specimen: GERMANY, isolated from mouse dung, MBT#8056 (**Syntype**).

Podila Stajich, Vandepol & Bonito, **gen. nov.**

MycoBank No: MB#833766

Etymology: In honor of Gopi Podila (1957–2010), an Indian American biologist who advanced the fields of plant–microbe interactions, plant genetics and biotechnology in bioenergy crops. In particular, Podila researched the genetic basis of the poplar microbiome and metabolome.

Description: All species produce sporangiospores, though with variable morphologies: globose to fusoid, smooth to spinulose or verrucose. Chlamydospores are absent in some species and scarce or unknown in the others. When known, zygospore morphology ranges from naked to smooth and globose and mating is usually heterothallic, though at least one species is homothallic.

Habitat: Species of *Podila* are frequently isolated from forest and agricultural soil, compost, dung, and municipal waste. *P. minutissima* has been isolated from *Populus* roots (Bonito et al. 2016). They have been reported from Europe, New Zealand, and North America. *P. minutissima* is a semi-saprotrophic mycophile (saprotrophically consumes dead fungal tissue) it is possible that additional species in this genus are also mycophilic/mycoparasitic to some degree (Rudakov, 1978).

Type species: *Podila minutissima* (Tiegh.) Vandepol & Bonito

Species in this study:

Podila clonocysits (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835720

Basionym: *Mortierella clonocysits* W. Gams, Persoonia 9 (1): 132 (1976)

Type specimen: SPAIN, Gran Canaria, isolated from soil under *Apollonias canariensis* in April 1976, CBS 357.76 [MBT#8053].

Podila epicladia (Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835721

Basionym: *Mortierella epicladia* W. Gams & Emden, Persoonia 9 (1): 133 (1976)

Type specimen: SPAIN, Gran Canaria, isolated from soil under *Apollonias canariensis* in April 1976, CBS 355.76 [MBT#8074]

Podila epigama (Gams & Domsch) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835722

Basionym: *Mortierella epigama* W. Gams & Domsch, Trans Br Mycol Soc 58 (1): 11 (1972)

Type specimen: GERMANY, isolated from municipal waste, CBS 489.70 [MBT#8076].

Podila horticola (Linnem.) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835723

Basionym: *Mortierella horticola* Linnem., Pflanzenforschung 23: 21 (1941)

Type specimen: GERMANY, MBT#8105 (**Syntype**).

Podila humilis (Linnem. ex W. Gams) Vandepol & Bonito **comb. nov.**

MycoBank No: MB#835724

Basionym: *Mortierella humilis* Linnem. ex W. Gams, Beitrag zu einer Flora der Mucorineae Marburgs, Diss. (1963)

Type specimen: MEXICO, isolated from *Pinus* forest soil, MBT#8109 (**Syntype**).

Podila minutissima (Tiegh.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella minutissima var. dubia* Linnem., Mucor.-Gatt. Mortierella Coem.: 39 (1941)

MycoBank No: MB#833767

Basionym: *Mortierella minutissima* Tiegh., Annales des Sciences Naturelles Botanique 4: 385 (1878)

Type specimen: no type known

Podila verticillata (Linnem.) Vandepol & Bonito **comb. nov.**

≡ *Mortierella marburgensis* Linnem., Flora (Regensburg) 130: 211 (1936)

≡ *Haplosporangium fasciculatum* Nicot, Bulletin de la Société Mycologique de France 73: 90 (1957)

≡ *H. attenuatissimum* F.J. Chen, Mycosystema 5: 19 (1992)

MycoBank No: MB#835725

Basionym: *Mortierella verticillata* Linnem., Pflanzenforschung 23: 22 (1941)

Type specimen: GERMANY, MBT#140598.

Discussion

To our knowledge, this study provides the most extensive and in-depth sampling of Mortierellaceae diversity to date, that extends to new isolates from Africa, Australia, and the United States, where several novel species and lineages were discovered. We also included *Modicella*, sampled from a sporocarp, in the first application of low coverage genome sequencing to large-scale fungal phylogenetic systematics (Petkovits et al. 2011; Smith et al. 2013; Wagner et al. 2013). We further developed and tested a pipeline for identifying non-ribosomal phylogenetic markers. By combining these approaches, we were successful in resolving the phylogeny of Mortierellaceae to provide a phylogenetic-based framework for their taxonomy.

There is considerable uncertainty concerning Mortierellaceae species diversity that remains to be sampled (Hibbett and Glotzer 2011; Nagy et al. 2011). Nagy et al. (2011) estimated the rate of novel species discovery in Mortierellaceae by comparing the diversity represented in sequence repositories to diversity within putatively novel sequences and those of described species. They concluded that most Mortierellaceae diversity was already discovered and they estimated a total of approximately 126 species in the family. Given that 102 of the 125 currently accepted species in Mortierellaceae were described prior to 1980, and only 9 more between 1990 and 2000, this might seem to be a reasonable conclusion (Supplementary Tables 1 and 11). However, taking into account that vast regions of the world are still unsampled, and the limited resolution of ITS and/or 28S rDNA regions in metabarcoding diversity studies, this estimate may be low. There are several examples of distinct species of *Mortierella* that have very similar ITS sequence similarity, e.g. *Podila horticola* and *P. minutissima*. The rate of species discovery in Mortierellaceae has increased in the last decade, with at least seven new species being described in the family between 2011 and 2019 from Poland, Japan, Taiwan, and Korea (Hibbett and Glotzer 2011; Ariyawansa et al. 2015; Li et al. 2016; Supplementary Table 1). Moreover, our sampling efforts in Africa and Australia for this study yielded multiple novel species an entirely novel lineage at the generic level. From our deep sampling efforts in Illinois caves, we recovered 119 isolates. These represented 8 genera and 9 under-represented species, which include isolates of *L. amoeboidea* and *N. dichotoma*. These species are rarely isolated and the collection of new strains is inherently valuable to understanding the ecology, genetics, and distribution of these fungi (Gams 1977). For the purposes of exploring the diversity and distribution of Mortierellaceae, we expect more species to be discovered in undersampled locations such as South America, Africa, and Asia.

We found low coverage genome sequencing is a relatively cost-effective means of generating a high-confidence fungal phylogeny. Further, it requires fewer assumptions, and less upstream handling and preparation time than traditional genome sequencing or multi-locus sequence typing, as only a single high quality, high concentration DNA preparation is needed. The approximately 15X sequencing coverage we achieved was sufficient to recover several hundred orthologous genes for our phylogenomic analyses. Occasional misassembly of target loci necessitated mining MGP loci from the raw genome reads, rather than the assembled contigs. Nonetheless, we were still able to detect and recover full length MGP loci from 15 isolates entirely from genome sequences. The LCG approach has been applied successfully in other systems as well, including insects and olive trees, with both low and high quality specimens and genome coverage between 0.5 and 30× (Olofsson et al. 2019; Zhang et al. 2019). All LCG phylogenomic approaches have relied on first identifying existing phylogenetic markers in assembled whole genome sequence data. Olofsson et al. (2019) built phylogenies with two classical markers and compared these to phylogenies based on SNPs obtained from additional orthologous gene sets. They also demonstrated the capability of an LCG approach to extract phylogenetic information from degraded herbarium specimens with extremely low coverage (< 0.5X), which encourages LCG sequencing of fungal herbarium specimens as well (Olofsson et al. 2019). The approach used by Zhang et al. (2019) was more similar to the LCG approach described here, including the breadth of phylogenetic diversity represented in their Hexapoda dataset, but with higher genome coverage than our dataset (20x-30x vs. 15X) and with fewer genomes (21 vs. 73).

Non-ribosomal (nuclear) phylogenetic markers should be single-copy genes that are not under selective pressure and contain sufficient sequence variation to make phylogenetic inferences. Identification of nuclear markers has historically been done manually, starting from protein sequence and characteristics, as in the case of *RPB1* (Jokerst et al. 1989;

Sidow and Thomas 1994). Even with the advent of genome sequencing, discovery and evaluation of novel nuclear markers has been a largely manual process (Blair et al. 2008). There has been at least one other effort to automate the discovery and evaluation of nuclear markers, a program called DIscoMark, which uses a similar approach to the unbiased MGP locus identification method developed here but starting with orthologous genes instead of raw genomes (Deteringet al. 2016). Both approaches are dependent on the availability of high-quality input genomes. In this study, our pipeline did successfully identify single-copy loci, some of which were phylogenetically informative that we used to improve DNA-based species identification. *Elongation Factor 1 alpha* (EF1α) and *RNA polymerase II large subunit* (RPB1) have previously been used as phylogenetic markers in Fungi (James et al. 2006; Stockinger et al. 2014). The six MGP markers were sufficient to sort Mortierellaceae species into clades and provide structure at the species level, however, the 6 loci were insufficient to resolve the higher-level organization of clades along the Mortierellaceae backbone. The limited number of high-quality genomes available for the locus selection pipeline made it difficult to screen loci and primers in silico for off-target amplification or gene paralogs prior to in vivo use. Additional reference genomes would also inform the primer design process, reducing primer mismatch, locus bias and off-target amplification.

The main trade-off between the LCG and MGP approaches is sampling depth versus breadth. The high capacity of Illumina sequencing platforms meant that there was a minimum sample number needed for the MGP approach to be cost-effective. Therefore, we were able to include "lower priority/higher risk" isolates than in the genome sequencing project, including a high proportion of isolates that could not be identified by ITS sequence data. However, even with multiplex PCRs, there was significantly more sample handling and bench time required for the MGP protocol compared to the LCG. In light of these issues, we suggest that the LCG approach is a superior method for resolving the broad phylogeny of such a diverse lineage. By combining LCG and MGP approaches, we were able to resolve higher-level phylogenetic relationships using the LCG-derived data, while improving sampling depth and breadth with the MGP approach to place taxa and improve diversity sampling within the phylogeny.

This study upheld the majority of the modern rDNA clades defined by Petkovits et al. (2011) and Wagner et al. (2013) (Supplementary Table 12). To resolve the polyphyly of *Mortierella*, we have erected seven novel genera in Mortierellaceae. These include *Podila* (verticillata-humilis clade), *Mortierella* (alpina-polycephala clade), and *Linnemannia* (gamsii clade) (Petkovits et al. 2011; Wagner et al. 2013). However, ITS-clades 1 (selenospora-parvispora), and 5 (strangulata and wolfii) as described by Wagner et al. (2013) are not supported. *Lunasporangiospora*, *Actinomortierella*, *Gryganskiella*, and *Necromortierella* more closely correspond to the selenospora, wolfii, and parvispora clades as described by Petkovits et al. (2011). The MGP dataset places *E. parvispora*, originally part of selenospora-parvispora, in the newly erected genus *Entomortierella* with the retained lignicola clade. The LCG dataset places *Actinomortierella* species (Petkovits/wolfii) at the base of the tree, apart from *Lobosporangium transversale* (strangulata clade), which is still near the middle of the phylogeny. The composition of clade 4 (globulifera, angusta, and mutabilis) was retained, although the species were resolved as separate genera, *Dissophora* and *Gamsiella*. The monophyletic genus *Mortierella* has two main subgroups, the previously defined alpina and polycephala clades (Petkovits et al. 2011). These two clades were widely separated in the Bayesian analyses of Petkovits et al. (2011) and distinct groups within clade 6 as defined by Wagner et al. (2013).

Some of the new genera described here have loosely conserved ecological niches (Supplementary Table 11). For example, taxa now classified as *Entomortierella* have almost all been isolated from insect nests or bodies (Gams 1977; Watanabe et al. 1998). Several members of the re-defined genus *Mortierella* are known to be mycophilic and/or have been isolated from mushrooms and truffles (Domsch et al. 1980). These associations are not unique to these genera, as demonstrated by *Actinomortierella capitata*, *Actinomortierella* aff. *ambigua*, and *Necromortierella dichotoma*, but genus-level conservation may represent specialization and evolution of an ancestral trait (Gams 1977; Macias et al. 2019). While much remains unknown about the ecological function of most Mortierellaceae, these trends inspire some additional confidence in the groupings defined by our phylogenetic analyses.

Existing ITS-based species identifications, or lack thereof due to highly similar ITS sequences, are not fully resolved by this study, as this will require the inclusion of type specimens to confidently identify correct ITS classifications. This is most notable in *Podila*, *Mortierella*, and *Linnemannia* due to extensive sampling, high species number, and ITS sequence similarity. Rather, we provide a genus-level framework that will empower future studies to thoroughly resolve individual genera.

Conclusions

Previous research has estimated that the majority of Mortierellaceae diversity has already been discovered and may reside in culture collections (Nagy et al. 2011). However, our research reveals novel species and genera in both thoroughly sampled and historically undersampled regions, including Michigan, USA and Uganda, respectively. Based

on these results, we believe that there is a need for continued geographic sampling efforts to identify new species and to establish the ranges and ecological niches of recognized species of Mortierellaceae, including *L. elongata* (Ozimek et al. 2018; Liao et al. 2019).

While greatly improved by our study, ecological data to accompany sequence data are still scarce for Mortierellaceae. One of the valuable contributions of this work is the curation of reference sequences with updated taxonomy, supported by multiple independent loci, that will be integrated into NCBI and UNITE reference sequence database. These vouchered sequence data could also be used to seed non-ITS reference databases. Together, these data will improve the ability to accurately identify taxa and novel species and thereby improve understanding of the diversity and ecology of these fungi. Further consolidation of global geographic and environmental records of Mortierellaceae isolates would help resolve the range and ecology of these species. We recommend future efforts prioritize sequencing of non-ribosomal markers from type isolates, additional culture collections, and isolates from under-sampled regions.

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Author contributions NV-primer design & validation, MLST PCR, MLST sequence analysis, & writing. JL-microscopy, photography, species & genus description, isolate troubleshooting. AD-DNA extraction & strain isolations. HN-LCG sequencing. MK-LCG sequencing. KB-LCG sequencing coordination. IVG-LCG sequencing coordination. ANM-metadata for shared strains & proofreading. KO-idea, strains, research support, MLST sequencing, & proofreading. JES-idea, software development, LCG sequence assembly, annotation, analysis, & writing, data deposition. GB–research ideas, research support, & writing.

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Data availability All amplicon sequences and genome sequences have been deposited to Genbank. All trees have been deposited to Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S25806?x-access-code=16dea5a74941bc1aa812c9ad125aed0&format=html).Code availability All custom scripts and pipelines are available on GitHub and/or Zenodo.

Compliance with ethical standards

Conflict of interest None to report.

Consent to participate All authors have agreed to participate in this research

Consent for publication All authors have read and approved the submitted manuscript.

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