Morphological and Molecular Confirmation of Four Undescribed Oleaginous Species of *Mortierella* from Libya

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Research Article

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Abstract

A large number of undiscovered fungal species still exist on earth, which can be useful for the bioprospecting particularly the single cell oil (SCO) production. The present research study confirms four oleaginous fungal isolates from Libyan soil. These isolates (Barcoded as MSU-101, MSU-201, MSU-401 and MSU-501) were discovered and reported first time from diverse soil samples of district Aljabal Al-Akhdar in North-East Libya and fall in the class: *Zygomycetes*; order: *Mortierellales*. From the morphological and phylogenetic analysis, these isolates were identified and found as closest match with *Mortierella alpina* species. The present research study provides insight to the unseen fungal diversity and contributes to more comprehensive *Mortierella alpina* reference collections worldwide.

Introduction

Edible oils produced by oleaginous microorganisms are named as single cell oils (SCO). Majority of these oil accumulating microorganisms are species of yeast and fungi. *Mortierella* is one of the significant genera in this field and contains about hundred species [1–5]. Moreover, *M. alpina* is the main single cell oil producer / arachidonic acid producer at commercial scale under this genus. Wang et al. [6] described the *M. alpina* genome scale reconstructed metabolic model for higher production of arachidonic acid at industrial scale. Scientists are in continuous search for the new species / novel strains and trying hard to crack the reconstruction genome code to exploit these species, so that the arachidonic acid production can be simplified and commercialized with improved protocol [7–10].

Oleaginous fungi especially *Mortierella* species are ubiquitous, saprophytic and belong to zygomycetes class. The polyunsaturated fatty acids (PUFA) production potential makes these fungi unique and significant to oil producing industries. Modern ITS (rDNA) based taxonomical classification [11–14] of *Mortierella* genus categorized into seven groups: selenospora and parvispora", "verticillata-humillis", "lignicola", "mutabillis, globulifera and angusta", "strangulate and wolfii", "alpina and polycephala", and "gamsii".

During our studies on Libyan Mortierellaceous fungi, we isolated many diverse species, but surprisingly four species of *Mortierella* so far not reported in Libya were encountered. Thus, we claim that this is the first report on these oleaginous fungal species from this country. On the basis of morphological and molecular characteristics, these species were identified as *M. alpina* and barcoded as MSU-101, MSU-201, MSU-401 and MSU-501 subsequently.

Materials And Methods

Collection of soil samples and isolation of fungi

Four different locations viz; Marawah, Albayda, Faydiyah and Suluntah located in district Aljabal Al-Akhdar, North-East Libya were chosen and rhizosphere soil samples were collected by placing in sterilized

polybags from these locations in the January month of the year 2018. These soil samples transported to the microbiology laboratory of Management and Science University, Shah Alam, Malaysia and stored at 4°C for further processing. The fungal isolation was carried out by conventional serial dilution technique on potato dextrose agar medium (PDA) supplemented with 100 µg chloramphenicol /mL antibiotic. Morphological features of the fungus were observed on PDA medium after one-point inoculation in 9-cm petri dishes and incubation at 25^{o}C for 5–7 days [15].

Morphological identification

All four distinct isolated fungal species were inoculated with the help of sterilized inoculation needle by center point inoculation on the plastic Petri dishes (9 cm-diameter) containing potato dextrose agar media. Standard protocol was performed at the laminar air flow by maintaining all aseptic conditions to avoid any kind of contamination. These Petri dishes were sealed by parafilm and incubated for 5–7 days at 25^{o}C in the dark for the growth of novel fungal species. These plates were observed on daily basis and their morphological characteristics viz; colony appearance, pigmentation, growth pattern, colony colour (front and reverse), colony diameters were documented. Four isolates were identified by mycokeys and distinguished monographs [16].

The microscopic identification was performed by using distilled water and lactophenol solution under a light microscope (Olympus, Japan). The sporangiophore, sporangium and sporangiospores, shape and size, developmental pattern, mature and immature sporangiospores, intercalary chlamydospore were measured and documented [17]. Pure culturing and preservation were maintained in PDA slant tubes and stored in 20% glycerol at − 80^{0}C in cold chamber of university microbiology laboratory. Later, all four cultures were barcoded as MSU-101, MSU-201, MSU-401 and MSU-501 and deposited at MSU Culture Collection Center, Management and Science University, Shah Alam, Selangor, Malaysia as shown in Table 1.

Table 1
Comparison of morphological and cultural characteristics of fungal isolates obtained in this research study with reference, *Mortierella alpina*[a].

Characteristics	MSU-101 (No. MZ298831)	MSU-201 (No. MZ298832)	MSU-401 (No. MZ298834)	MSU-501 (No. MZ298835)	*Mortierella alpina*[a] M136 (ATCC 32222; CBS 528.72)
Colony	Rapidly growing at 25^0C on PDA, whitish colour; reverse colour of colony light yellowish white and little zonate pattern	Rapidly growing at 25^0C on PDA, Slightly cottony at the center with white margin; reverse colour of colony yellowish white with moderately zonate pattern	Rapidly growing at 25^0C on PDA, Cottony at the center with white margin; reverse colour of colony slightly yellowish white with irregularly zonate pattern	Rapidly growing at 25^0C on PDA, Cottony growth at the center with whitish margin; reverse colour of colony dark yellowish white overlapping in-distinguished zonate pattern	Cobweb to cotton-like White, arachnoid to cottony
Sporangiophores	Moderately branched, 2-3.5 µm wide at tip with variable length, Upto 245 (-370) µm long	Mostly branched, 3-3.5 (-2) µm wide at tip with variable length, Upto 250 (-400) µm long	Mostly branched, 3.3–3.8 (-2) µm wide at tip with variable length, Upto 250 (-390) µm long	Mostly branched, 3.3–3.8 (-2) µm wide at tip with variable length, Upto 250 (-400) µm long	1.5–3.5 µm wide at tip with variable length, 5–8 (-12), Upto 250 (-400) µm long
Sporangia	Globose, multi-spores, 16.5–33.5 X 18–32 µm	Globose, multi-spores, 16–32 X 19–32 µm	Globose, multi-spores, 14-33.5 X 18–32 µm	Globose, multi-spores, 16.5–33.5 X 18–32 µm	Globose, (15-) 20–30 µm
Sporangiospores	Oval, smooth, hyaline 8-15.5 X 5-8.5 µm	Ovoid, smooth, hyaline 7-14.5 X 4.8–8.3 µm	Ovoid, smooth, hyaline 7-14.5 X 5-8.5 µm	Ovoid, smooth, hyaline 7.5–15.5 X 5-8.5 µm	Ovoid, smooth, hyaline 5–11 X 5-9.5 µm

[a]Source of reference: Gams W. [15]

Characteristics	MSU-101 (No. MZ298831)	MSU-201 (No. MZ298832)	MSU-401 (No. MZ298834)	MSU-501 (No. MZ298835)	*Mortierella alpina*[a] M136 (ATCC 32222; CBS 528.72)
Chlamydospores	Present	Present	Present	Present	Present
Zygospores	Not observed	Not observed	Not observed	Not observed	Globose to sub-globose, (42-)55(-80) X (40-)52(-70) µm

[a]Source of reference: Gams W. [15]

Genomic DNA extraction and sequence alignment

Total Genomic DNA (gDNA) was extracted according to the standardized protocol [18]. ITS and rDNA conserved regions were amplified according to the standardized protocol [19]. The amplified products were examined by electrophoresis on 1% agarose gel and detected by using ethidium bromide stain. Afterwards, these products were purified by Gel band purification kit (First Base NGS KIT, Malaysia) and sequenced by ABI3100 sequencer at 1st BASE NGS (Malaysia) according to the manufacturer's instructions. The obtained sequences were compared against the earlier submitted NCBI database using the BLAST algorithm [19] to verify the percentage of identity corresponding to the analysed species as shown in Table 2. The fungal sequences were aligned by using Clustal_X v.2.1 [19–21] and neighbour joining based phylogenetic tree was constructed by using Mega (Molecular evolutionary genetic analysis) X software version 6.0, to observe the grouping of obtained novel fungal species sequences [19–21].

Table 2
GenBank accession numbers used for the phylogenetic analyses in the present study.

Location	Type of Sample	Barcode of Isolate	Accession Number	Percentage (%) of similarity by Clustal_X
Austria	Environmental sample	Uncultured *Mortierella* Clone IIS1-5	EU517021	100
Marawah, North-East Libya	**Soil sample**	Mortierella alpina **Strain MSU-101**	**MZ298831**	**100**
China	Soil sample	*Mortierella alpina* Strain QLF48	FJ025186	100
Aragon, Spain	Calcareous soil and Tuber Melanosporum ectomycorrhizal in the Mediterranean Zone	*Mortierella alpina* isolate MM3	KX343169	99.83
Suluntah, North-East Libya	**Soil sample**	Mortierella alpina **strain MSU-401**	**MZ298834**	**100**
Tongshan: New District, Xuzhou, Jiangsu, China	(Endophytic fungi) Seed sample	*Mortierella alpina* strain xsd08339	EU918703	99.83
Tianshui Lanzhou, Gansu, China	Endophytic fungi from the rhizosphere soils and roots of *Lycium barbarum* L.	*Mortierella alpina* strain GFRS11	MT447479	99.67
Tianshui Lanzhou, Gansu,China	Endophytic fungi from the rhizosphere soils and roots of *Lycium barbarum* L.	*Mortierella alpina* strain QLF60	FJ025143	99.67
Tianshui Lanzhou, Gansu, China	Endophytic fungi from the rhizosphere soils and roots of *Lycium barbarum* L.	*Mortierella alpina* strain QLF70	FJ025182	99.83
Mainz, Germany	Soil Sample	*Mortierella alpina* isolate A03ID2	KJ469805	98.85

The newly submitted *Mortierella* species sequences in GenBank are indicated in bold.

Location	Type of Sample	Barcode of Isolate	Accession Number	Percentage (%) of similarity by Clustal_X
Wageningen, Netherlands	Lyophilized spore material from the CBS-KNAW Fungal Biodiversity Centre in Utrecht, the Netherlands.	*Mortierella alpina* isolate d27	GQ922556	98.85
Lanzhou, Gansu, China	Endophytic fungi from the rhizosphere soils and roots of *Lycium barbarum* L.	*Mortierella alpina* strain QLF27	FJ025187	99.5
Qingdao, China	Soil Sample from Antarctica	*Mortierella* sp. strain HSX2#-13	MT367225	98.84
Asahikawa, Hokkaido, Japan	Samples from Walker glacier, Canadian High Arctic	*Mortierella alpina* GR8-3-20-1	LC515164	99.5
Larisa, Greece	Microbial community from rhizosphere soil sample	Uncultured zygomycete clone 1B6	FN689671	100
Lanzhou, Gansu, China	Endophytic fungi from the rhizosphere soils and roots of *Lycium barbarum* L.	*Mortierellales* sp. strain GFRS01	MT447469	99.84
Turin, ITALY	Environmental sample	Uncultured fungus clone 62	FN391358	99.84
Albayda, North-East Libya	**Soil Sample**	Mortierella alpina **strain MSU-501**	**MZ298835**	**100**
Faydiyah, North-East Libya	**Soil Sample**	Mortierella alpina **strain MSU-201**	**MZ298832**	**100**
Lanzhou, Gansu, China	Soil Sample from alpine grassland in eastern Qilian mountains	Mortierellales sp. QLF86	FJ025158	100
Larisa, Greece	Microbial community from rhizosphere soil sample	Uncultured Mortierellales clone 2B12	FN689675	100
Haidian district, Beijing, China	Soil Sample	*Mortierella alpina* strain XY01520	MT521795	100

The newly submitted *Mortierella* species sequences in GenBank are indicated in bold.

Location	Type of Sample	Barcode of Isolate	Accession Number	Percentage (%) of similarity by Clustal_X
Berlin, Germany	Fine airborne particles/spores, environmental Sample	*Mortierella alpina* isolate DSM100289_DF19_RLCS11	MT453274	100
Gronostajowa 7, Krakow, Malopolska, Poland	Rhizosphere Soil Sample Symbiotic microbes of Saxifraga stellaris sp. alpigena from the copper creek of the Schwarzwand (Austrian Alps)	*Mortierella* sp. isolate MMS	MF565377	100
Sevilla, Spain	Environmental sample	Uncultured *Mortierella* clone IB2_K7	FN812729	100
Viale Mattioli, Italy	Environmental sample	Uncultured fungus clone iO03_P_2_B12	FN397316	100
Anning District, Lanzhou, Gansu, China	Soil Sample from alpine grassland in eastern Qilian mountains	*Mortierellales* sp. QLF84	FJ025170	100
Viale Mattioli, Italy	Environmental sample	Uncultured fungus clone iE12_P_2_D7	FN397313	99.84
Innsbruck, Tyrol, Austria	Environmental sample	Uncultured *Mortierellaceae* clone IIS4-1	EU517031	100
Viale Mattioli, Italy	Environmental sample	Uncultured fungus clone 58	FN391354	99.84
Anning District, Lanzhou, Gansu, China	Soil Sample from alpine grassland in eastern Qilian mountains	*Mortierellales* sp. QLF15	FJ025162	100
Viale Mattioli, Italy	Environmental sample	Uncultured fungus clone 50	FN397151	100
Av. Monta nana, Zaragoza, Spain	Diversity of fungi isolated of calcareous Soil Sample and *Tuber melanosporum*	*Mortierella alpina* isolate 20PDA-D30	KX343151	99.84
Halle/Saale, Germany	Environmental sample	Uncultured Mortierella clone 09S50C12 (MOTU44)	HG936566	100
The newly submitted *Mortierella* species sequences in GenBank are indicated in bold.				

Results And Discussion

Morphological confirmation and diversity

On the basis of morphological and cultural characteristics, the fungal isolates were confirmed and belong to *Mortierella* genus. Colonies of oleaginous fungal isolates after seven days of incubation at 25°C on PDA, were sporulating, fast growing, producing concentric pattern, flower-shaped radial growth, yellowish to whitish in color as shown in Fig. 1. The detailed descriptions of morphological characteristics such as sporangiophores, sporangium, sporangiospores are shown in Table 1 with reference *M. alpina* (ATCC 32222; CBS 528.72) isolate. Distinguishing prominent features between four fungal isolates (Barcoded as MSU-101, MSU-201, MSU-401 and MSU-501) were growth pattern, margin and colour of the colony on PDA medium in front and back side as shown in Fig. 1, which draw attention for further investigation, thus these four isolates were further examined for molecular diversity and genetic characterization.

Molecular confirmation and diversity

In the ITS sequences analysis based on BLASTn (Basic Local Alignment Search Tool for nucleotides), MSU-101, MSU-201, MSU-401 and MSU-501 isolates were fall within the order *Mortierellales* as depicted in Figs. 3 and 4, which matches with morphological identification of isolates as described above. These four fungal isolates (Barcoded as MSU-101, MSU-201, MSU-401 and MSU-501) were compared and aligned with earlier submitted closely related species sequences by multiple sequence alignment (FASTA format) with software Clustal_X v.2.1. After that, the phylogenetic tree constructed by neighbour joining mode with 1000 bootstrap values, which had shown that four oleaginous fungal isolates were 100% similar with earlier *M. alpina* genomes sequences submitted in GenBank NCBI (Closest matching GenBank accession numbers were: EU918703; KX343169; FJ025186; FN689671; FN391358; FJ025158) as shown in Table 2; Figs. 3 and 4. Thus, these isolates were identified as *M. alpina* species. The ITS sequences of these fungal isolates were deposited in GenBank with accession number of MZ298831:MZ298835 (https://www.ncbi.nlm.nih.gov/nuccore/?term=MZ298831:MZ298835[accn]). These novel *Mortierella* isolates add on to a large contribution of fungal diversity collections all over the world.

Many species of *Mortierella* are potentialistic producers of C18 and C20 PUFAs (Poly unsaturated fatty acids) such as □-linolenic acid and arachidonic acid. *M. alpina* species is quite famous for the production of single cell oils as describes and reported by multiple scientist's time by time [21–29]. Shimizu and Sakuradani [30] reported *M. alpina* 1S-4 strain by extensive screening, for the large-scale production of variety of PUFAs. This isolate not only had the potential for SCO production but also had several advantages to work as a model for lipogenesis studies. Thus, we can anticipate from earlier published data that the isolates reported from present study can be useful for bioprospecting in terms of single cell oil production. However, the oil production potential of these oleaginous fungal isolates is under investigation and our research group is presently working in this direction to assess the SCO potential of these diverse isolates obtained from Libyan soil.

Conclusion

In the present study, four oleaginous fungal isolates barcoded as MSU-101, MSU-201, MSU-401 and MSU-501 were identified and confirmed by morphological and molecular analysis. These fungal isolates had shown highest similarity with *Mortierella alpina* species and can be potentialistic single cell oil producers, further research work is in progress for assessment and exploitation of these isolates in terms of oil production.

Declarations

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Author's contribution

FEME student from Libya, carried out the experiments at Microbiology Laboratory, MSU, Malaysia for the fulfilment of Ph.D. degree. KT and MAL drafted and written the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Yes

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Figures

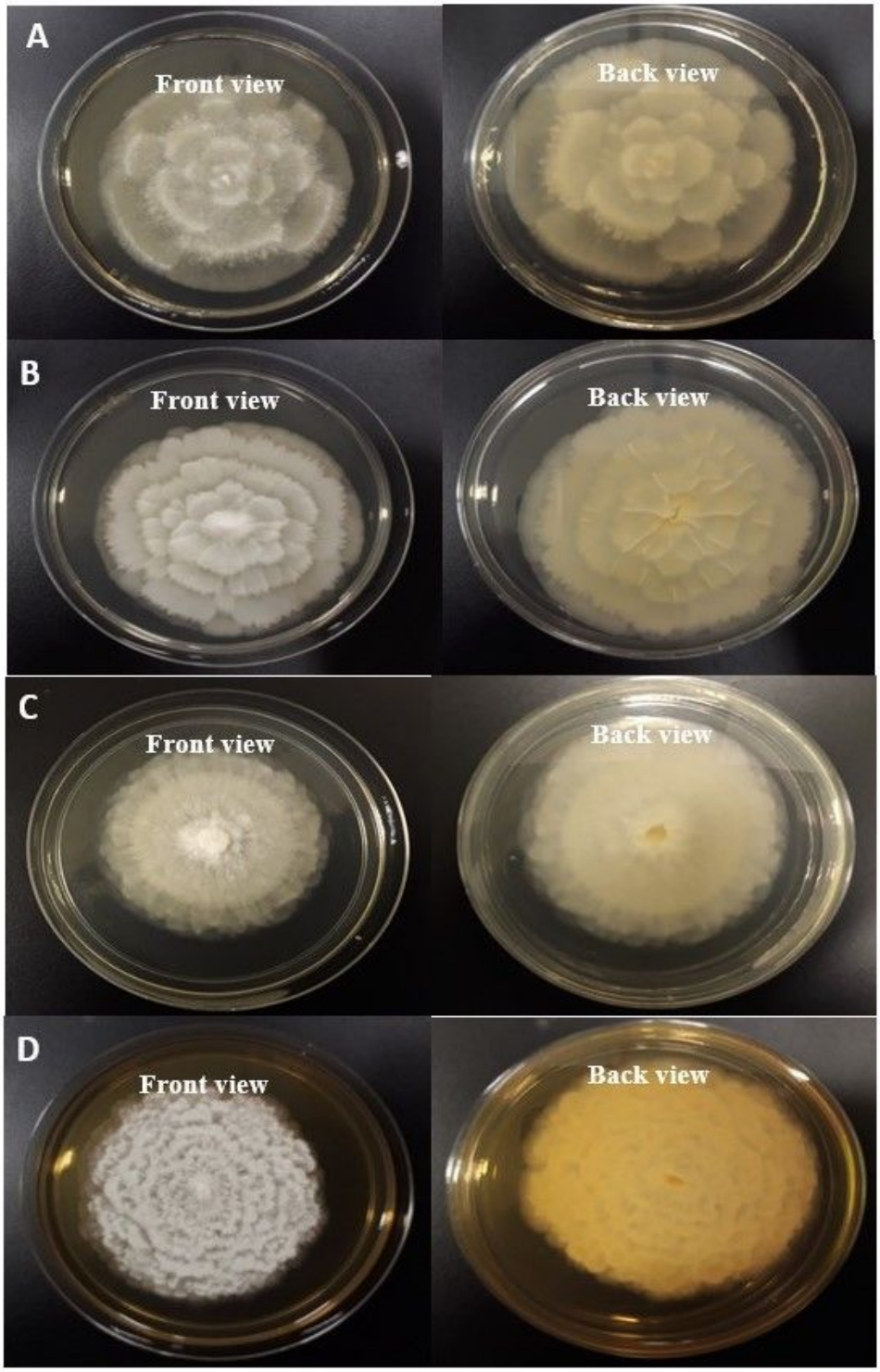

Figure 1

Colonies of fungal isolates on PDA medium (front view and back view) after 7 days of incubation at 25oC. 1A: MSU-101 colonies on PDA front and back view 1B: MSU-201 colonies on PDA front and back view 1C: MSU-401 colonies on PDA front and back view. 1D: MSU-501 colonies on PDA front and back view.

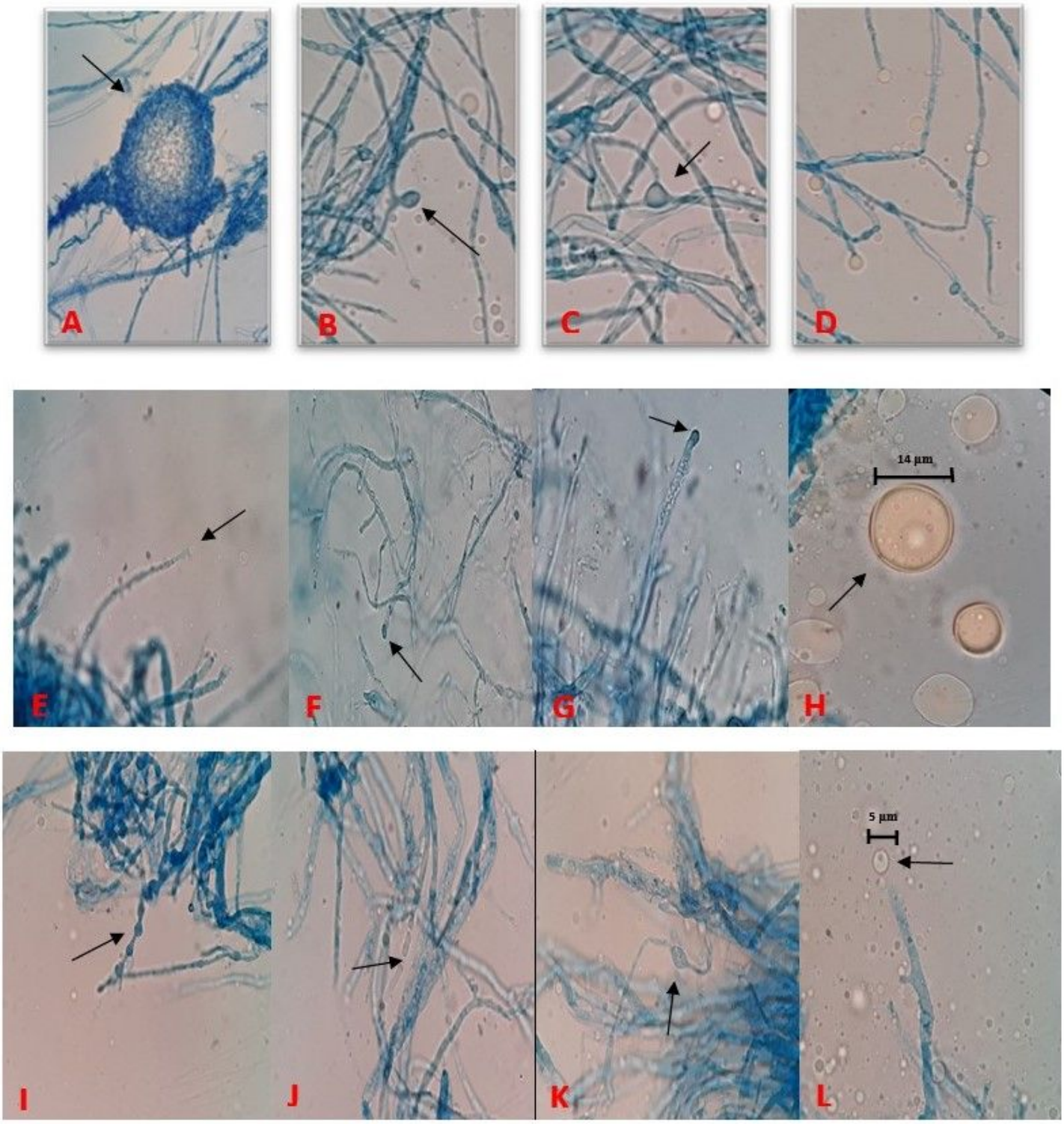

Figure 2

A: Meiospore of MSU-101 isolate. B: Immature sporangia from branched sporangiophore of MSU-101. C: Intercalary chlamydospore of MSU-101 isolate. D: Hyaline and ovoid sporangia, MSU-201 isolate. E: Developing sporangia on single sporangiophore, MSU-201 isolate. F: Immature young sporangia on highly branched sporangiophore, MSU-201. G: Immature young sporangia on highly branched sporangiophore, MSU-401. H: Mature globose sporangium containing sporangiospores, MSU-401. I:

Terminal chlamydospores with papillate ornamentation and hyphal segment remaining at the distal end, MSU-501. J: Net of hypha with branching and septation, MSU-501. K: Net of hypha with branching and chlamydospore, MSU-501. L: Developing sporangium at tip on sporangiophore, MSU-501.

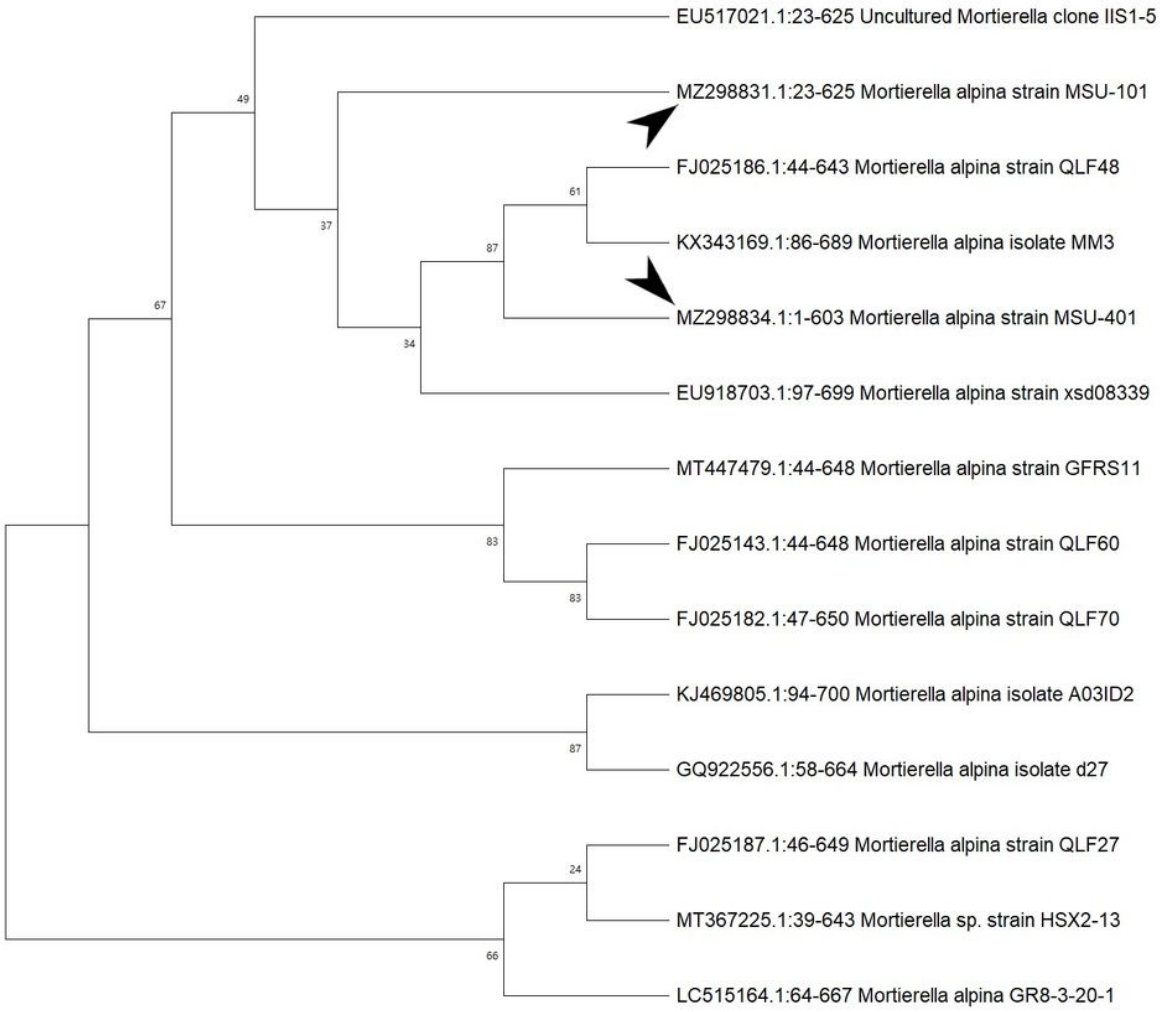

Figure 3

Neighbour joining method based internal transcribed rDNA sequences phylogenetic tree of isolate MSU-101 and MSU-401. Bootstrap support values are indicated at the nodes.

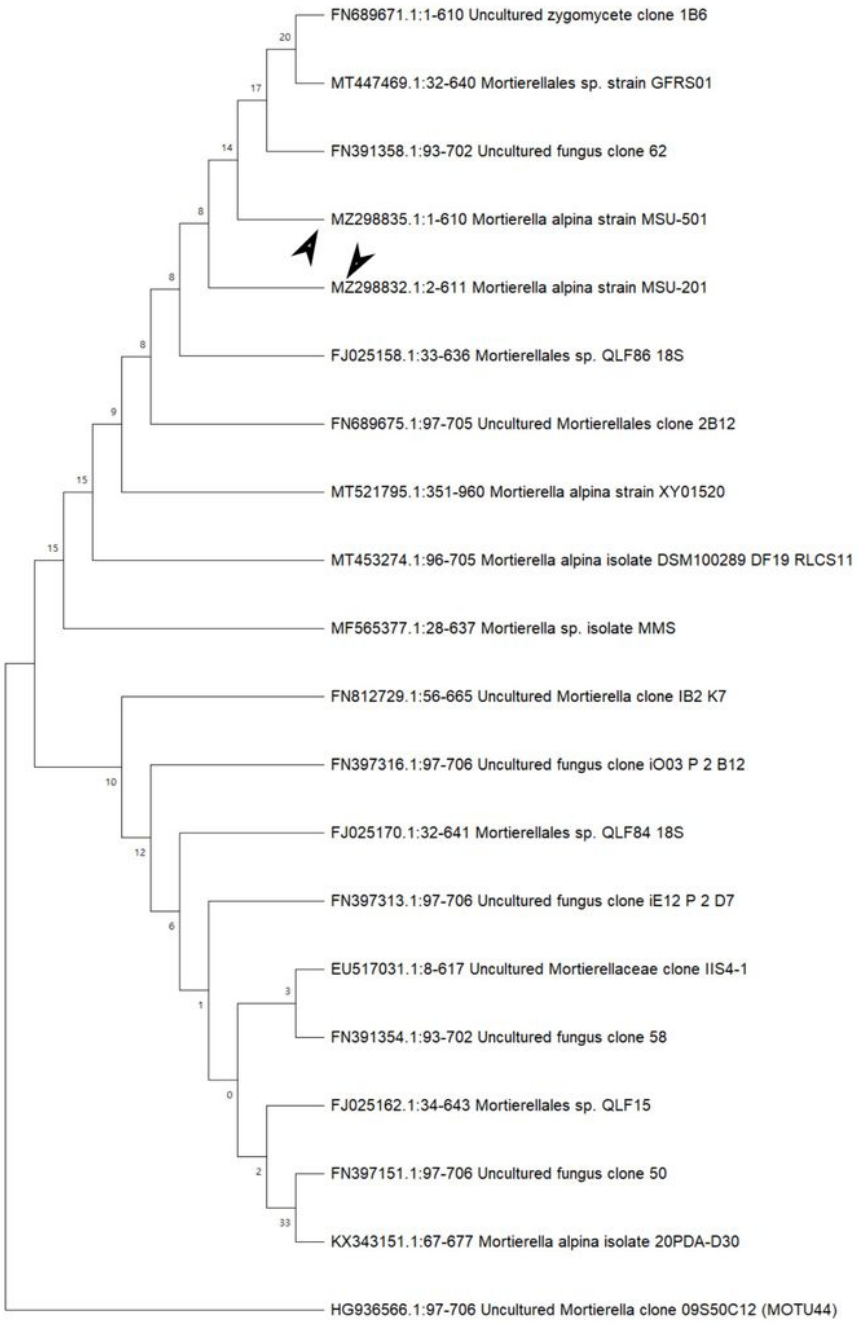

Figure 4

Neighbour joining method based internal transcribed rDNA sequences phylogenetic tree of isolate MSU-201 and MSU-501. Bootstrap support values are indicated at the nodes.