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Congruence of ribosomal DNA sequencing, fatty acid methyl ester profiles and morphology for characterization of the genus *Rhizophagus* (arbuscular mycorrhiza fungus)

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Abstract Difficulties in obtaining sterile axenic cultures and heterogeneity in nuclear-encoded ribosomal DNA (n-rDNA) sequences within a single arbuscular mycorrhizal spore make genetic analysis of arbuscular mycorrhizal fungi (AMF) a complicated task, and currently available methods of genotyping are inadequate for identification to the species level. Therefore, we applied a multipronged approach on different isolates grown in root organ culture (ROC) belonging to the genus *Rhizophagus* which were not characterized at species level. Each strain was characterized using the fatty acid methyl ester profile (FAME), partial sequencing of a small subunit-internal transcribed spacer (SSU-ITS) and a large subunit (LSU) region of n-rDNA, and morphological examination of spores. Neighbor-joining trees obtained from the SSU-ITS rDNA sequences were broadly similar to those obtained from the LSU rDNA sequences. FAME profiles of the same isolates used for molecular characterization were obtained using fatty acid datasets, and results were compared to a neighbor-joining tree of n-rDNA sequence. Based on the results of these studies, a combination of morphology, biomarkers (FAME), and molecular sequencing (of highly variable D1-D2 of LSU and ITS) is recommended for phylogenetic analysis and characterization of species/strain of Glomeromycota.

Keywords Spore morphology · *Rhizophagus irregularis* · *Rhizophagus proliferus* · Electron microscopy · Root organ culture

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Introduction

Arbuscular mycorrhizal fungi (AMF) belong to the fungal phylum Glomeromycota (Schüßler et al. 2001a) and form obligate symbiotic relationships with more than 80 % of land plants (Smith and Read 1997). Currently, with 230 morpho-species having been described using morphological characters, AMF are the most important and widespread mycorrhizal fungi worldwide. Although a large number of AMF species have been described using morphological characters, it is difficult to compare species of AMF that are morphologically similar (Morton et al. 1993). Difficulties in identification and culture, along with problems in obtaining sufficiently pure DNA from spores maintained in pot culture, have greatly hampered studies on the ecology and genetic diversity of this group (Corradi et al. 2004). However, root organ cultures (ROC) of *Glomus* and *Gigaspora* have served to overcome the constraint to some extent (Tiwari and Adholeya 2002), and provide abundant high quality material for molecular and biochemical characterization. Molecular characterization among different species of AM fungi is mostly based on SSU and ITS region of ribosomal DNA. Very little genetic diversity was generated by using n-LSU rDNA sequences retrieved from GenBank (da Silva et al. 2006), and even some phylogenetic analysis has been reported using protein gene sequences (Helgason et al. 2003; Corradi et al. 2004). It is known that AMF

species with very similar morphology are generally separated using sequencing analysis of ITS rDNA (Lanfranco et al. 2001). However, it was shown by Krüger et al. (2009), and Stockinger et al. (2009, 2010) that the ITS region alone is not sufficient to separate closely related species, and especially species that fell within the GlGrAa and GlGrAb clade (sensu Schwarzott et al. 2001). The present investigation focuses on sequencing of SSU-ITS and LSU rDNA of ribosomal DNA along with fatty acids analysis for separation of closely related isolates of the genus *Rhizophagus*. It is also known that the fatty acid methyl ester (FAME) profile can be a powerful tool for systematic studies in addition to DNA sequencing in organisms, particularly with limited morphological datasets (Bentivenga and Morton 1994). However, very little is known about the fatty acid profiles of the genus *Rhizophagus* (earlier known as the *Glomus* group Ab; Schwarzott et al. 2001) grown under *in vitro* conditions. To gain more insight into fatty acid profiles and for correlation with n-rDNA, we characterized this feature for several *Rhizophagus* isolates.

Classification and nomenclature of AM fungi using congruent morphological and molecular methods were earlier confirmed by Redecker and Raab (2006) and Walker et al. (2007), as well as some from fatty acid methyl ester profiles (Bentivenga and Morton 1996) and others from rRNA sequences (Walker and Schüßler 2004). More recently, Morton (2009) suggested that the phylogeny of fungi in the Glomeromycota must be analyzed within the framework of a balanced multidisciplinary approach. The approach taken in this study is based on biochemical, molecular, and morphological datasets obtained from pure cultures and is one to greatly influence, and bring about considerable changes in, conventional concepts. We hypothesized that the separation of AM fungi to lower taxon levels using molecular analysis will be congruent to morphological and biochemical datasets. Even minor differences in any of the data obtained for the different parameters could highlight the variation among isolates. For testing the hypothesis, closely related isolates of AM fungi belonging to the genus *Rhizophagus* were selected from the Centre for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, India. We restricted our investigation to contamination-free AM spores collected from root organ cultures so that we could clearly establish the difference in fatty acid profiles between closely related *Rhizophagus* isolates. We also studied the morphology and ultrastructure of these selected *Rhizophagus* isolates in order to compare results with that of n-rDNA. Keeping this in view, we sought to characterize spores of the genus *Rhizophagus* grown under *in vitro* condition by (1) construction of FAME profiles, (2) morphology and ultrastructure studies, and (3) sequencing analysis of ribosomal DNA

Materials and methods

Root organ cultures of AMF isolates

All AMF isolates (Table 1) were grown in root ROC on Ri-T-DNA-transformed carrot roots (Bécard and Fortin 1988). Each isolate was subcultured every 3–4 weeks on fresh bi-compartment Petri plates. The set-up allowed spores to proliferate only in the compartment with sucrose-deficient minimal (M) medium (Chabot et al. 1992). Spores were recovered after 3 months of incubation from the bi-compartment Petri plate by using 10 mM citrate buffer (Doner and Becard 1991).

Morphological analysis

Diagnostic slides, each with 15–20 broken and unbroken spores, were prepared using Melzer's reagent and polyvinyl alcohol (PVLG) plus Melzer's reagent (1:1) as described by Walker and Trappe (1993). The slides were observed under a Zeiss compound microscope equipped with a digital imaging system, and photographed using a Zeiss Axiacam RTC (Germany). The dimensions of spores wall thickness was measured using Axio Vision (v.4.7) attached to the compound microscope (Zeiss Axicam). For transmission electron microscopy (TEM), the spores were fixed at 4 °C for 18 h in 2.5 % glutaraldehyde and 2 % p-formaldehyde (PF) in 0.2 M phosphate buffer (pH 7.4), washed, centrifuged first in 0.1 M phosphate buffer and then in double-distilled water, post-fixed in 2 % aqueous osmic acid before dehydration, stained in uranyl acetate, and embedded in LR white. Ultrathin sections were mounted on grids and observed under a Morgagni 268D (Fei Electron Optics) electron microscope. Features of the spore morphology were

Table 1 Summary table of identification profiles of *Rhizophagus* isolates based on molecular, biochemical, and morphological characters

CMCC isolate	Origin	Identification[a]
ROC1	Tropical soil, India	*Rhizophagus irregularis*
ROC2	Tropical soil, India	*Rhizophagus irregularis*
ROC3	Tropical soil, India	*Rhizophagus irregularis*
ROC4	Tropical soil, India	*Rhizophagus irregularis*
ROC5	Temperate field, Belgium	*Rhizophagus proliferus*
ROC6	Temperate field, Belgium	*Rhizophagus irregularis*
ROC7	Tropical soil, India	*Rhizophagus irregularis*
ROC8	Temperate field, Iran	*Rhizophagus proliferus*
ROC9	Tropical soil, India	*Rhizophagus irregularis*
ROC10	Tropical soil, India	*Rhizophagus irregularis*

[a] Identification based on: closet reference species (SSU-ITS rDNA and LSU rDNA), light and electron microscopy, and FAME analysis

compared with the recent classification described by Oehl et al. (2011b).

Molecular analysis

Single spores from ROC isolates were collected and crushed in 10 μl of 10× PCR buffer (Invitrogen, Grand Island, NY, USA), 10 μl of 20 % chelex 100 resin (Bio-Rad, USA) was added immediately, and the contents were briefly vortexed. The extract was incubated at 95 °C for 15 min and then centrifuged at 8,000g for 3 min. The supernatant (5 μl) was used as a crude DNA template for PCR amplification. LSU rDNA amplification of eight AM isolates out of the ten was done by single-step PCR using primer pair 28 G1 and 28 G2, according to the condition described by da Silva et al. (2006), with 5 μl of crude DNA extract. Single spore DNA extracts of two ROC isolates were amplified by two-step nested PCR using the eukaryote-specific primers ITS3 + NDL22 in the first step (White et al. 1990) and the eukaryotic specific primers LR1 (Van Tuinen et al. 1998) and fungus-specific primer FLR2 (Trouvelot et al. 1999) in the second. The product of the first PCR was diluted and used for the second PCR. The PCR was carried out under the conditions described by Van Tuinen et al. (1998b) with 58 and 60 °C as annealing temperatures in the first and the second PCR, respectively.

The end region of an SSU with complete internal transcribed spacer (ITS) was amplified using parameters for the PCR described by Redecker (2000) with 56 °C as the annealing temperature. The amplicon of first PCR was diluted 10-fold and used as a template for the second step PCR using the primer pair GLOM1310/ITS4i (specific for Glomeraceae) according to the conditions described by Redecker (2000). PCR or nested PCR products were purified using Qiagen PCR purification system (Qiagen, USA). The purified PCR product of the SSU-ITS and LSU rDNA fragment was cloned using the pCR4-TOPO vector supplied with TOPO TA cloning kit for sequencing (Invitrogen). Two or three transformed colonies were picked and plasmid was extracted using Wizard *Plus* SV Mini Kit (Promega, USA). SSU-ITS and LSU rDNA insert was used for cycle sequencing reaction with PCR primers. The sequencing was performed on an automated multicapillary DNA sequencer, namely ABI Prism 3130×l Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator ver. 3.1 Ready Reaction Cycle Sequencing Kit (Applied Bio systems) at the sequencing laboratories of TERI, New Delhi. The sequences were assembled and edited using Bioedit and deposited in the NCBI database with their respective accession number. Sequence similarities were determined using the Blastn similarity search algorithm (Atschul et al. 1990) available at the NCBI home page. The phylogenetic tree was constructed using MEGA v.4.0 (Tamura et al. 2007).

Biochemical analysis

FAME was extracted from each isolate using, with some modifications, the method described by Madan et al. (2002) and run on a GC-MSD (Agilent) under the following conditions: injector temperature, 240 °C; pressure, 20 kPa; column, DB23 30 m × 320 μm × 0.25 μm; and oven ramp temperatures, 70 °C (4 °C/min), 160 °C (3 °C/min), and 220 °C (2 °C/min). The analysis was performed using Chemstation v.1.0 and the retention time of each FAME was compared with FAME37 mix (Sigma Aldrich). FAME was also confirmed by comparing the mass spectrum of the fatty acid with the library of DB 23FAME and Wiley 8 (Agilent).

Results

Light and electron microscopy revealed that AMF isolates cultivated in vitro in this study belonged to the genus *Rhizophagus*. Eight of ten AM isolates showed close affinity with *R. irregularis*, isolates CMCC ROC5 and CMCC ROC8 being the exceptions (Table 1). Light microscopy revealed (Fig. 1) that the spore wall of all AM isolates consisted of two morphologically distinct layers: an outer mucilaginous layer (L1) and an inner semi-flexible hyaline layer (L2). All the ten isolates except CMCC ROC5 and CMCC ROC8 when examined under a TEM, showed a three-layered wall with the innermost layer comprising 3–4 membranous sublayers. Spore walls of CMCC ROC5 and CMCC ROC8 showed four layers with the fourth layer displaying the laminated sublayers, a feature associated with *R. proliferus* (Fig. 2). The fourth wall layers of the isolates CMCC ROC5 and CMCC ROC8 were observed to be membranous under the compound microscope and several times laminated when observed under TEM (Fig. 2).

Single spore DNA extracts of the ten isolates were amplified using the primer pair GLOM1310/ITS4i, which led to an amplification product about 1,000 bp long. Blastn result showed that all isolates had close similarity (>95 %) to SSU-ITS rDNA sequences retrieved from known AMF. Phylogeny based on 31 SSU-ITS n-rDNA sequences including known sequences of *Rhizophagus intraradices*, *Rhizophagus irregularis*, *Rhizophagus proliferus* and *Funneliformis geosporum* from GenBank showed three major clusters of Glomeraceae (Fig. 3). SSU-ITS n-rDNA sequences obtained from all the ten isolates except CMCC ROC5 and CMCC ROC8 clustered with known *R. irregularis*. However sequence from isolate CMCC ROC5 and CMCC ROC8 clustered with known *R. proliferus* retrieved from GenBank (Fig. 3).

All sequences obtained by sequencing the variable domain of LSU rDNA showed >97 % homology with known *R. irregularis* retrieved from GenBank, except isolates CMCC ROC5 and CMCC ROC8, which showed >98 %

homology with known *R. proliferus* instead (Table 1). The tree topology obtained by using LSU rDNA sequences was broadly similar with the topology obtained by using the SSU-ITS region of rDNA. LSU rDNA sequences obtained from all the isolates except from CMCC ROC5 and CMCC ROC8 clustered with known *R. irregularis* sequences retrieved from GenBank with 75 % bootstrap support. CMCC ROC5 and CMCC ROC8, however, grouped with known *R. proliferus* retrieved from GenBank, as was the case with phylogeny based on SSU-ITS rDNA (Fig. 4). LSU rDNA phylogeny indicated that rDNA sequences from two different clones fell within the same subcluster (Fig. 4).

In all the isolates studied, the predominant saturated fatty acids in the spores were tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), and octadecanoic acid (C18:0); the three together constituted 35–60 % of the total fatty acid profile (Table 2). Non-significant levels (<1 %) of two saturated fatty acids, namely pentadecanoic (C:15) and henicosonoic (C 21:0), were also detected in CMCC ROC2. Three unsaturated fatty acids, namely cis-9-hexadecenoic C16:1(α-c-9), cis-9-octadecenoic [C18:1(α-c-9)], and α-c-9,12-octadecenoic [C18:2(α-c-9,12)], accounted for 40–60 % of the total fatty acids in all the isolates studied (Table 2). In addition, α-c-9,12,15 octadecadienoic unsaturated fatty acid C18:3(α-c-9,12,15) was found in significant amount in CMCC ROC5 and CMCC ROC8 (Table 2).

Discussion

This study was conducted to assign the different selected isolates to their correct taxon, but it was clear from the start that any single method would not be adequate for the purpose. Accordingly, for each isolate, a combined dataset was obtained from the different approaches to highlight the similarities and differences between each AM isolate and to emphasize their unique position.

It is well known that ITS rDNA polymorphism has been reported within single AMF spores (Sanders et al. 1995). In the present investigation, unlike in the ITS region we observed low rDNA variation within LSU region of ribosomal DNA in single AM spores (Fig. 4). As there is usually lower LSU rDNA variation within a single AM spore, it seems to be a robust region of interest to study for AM phylogeny and molecular marker development (Clapp et al. 2001).

The spores of all the ten isolates of AMF contained significant amounts of 16:(1α-c-9), 18:1(α-c-9) C18:2(α-c-9,12) fatty acids, which are widely distributed among *Rhizophagus* spp. (Olsson et al. 1995); however, the higher carbon-unsaturated fatty acids C18:1ω9c and C20:1ω9c were detected in substantial amounts in *Gigaspora* sp. (Madan et al. 2002). We observed significant amounts of 16:1(α-c-9) FAME biomarker for identification of AM

Fig. 1 **a–l** Morphology of *Rhizophagus* sp. used in this study observed ▶ under a light microscope. **a** Mature spores of CMCC ROC1 with a three-layered wall (*Sw1*, *Sw2*, *Sw3*), staining only innermost wall layers with Melzer's reagent. **b**, **c** Mature spores of CMCC ROC2 broadly obovoid to ellipsoid with a double-layered wall, hypha base showing wall structure and constricted hypha (*arrow*), wall layer (*Sw2*, *Sw3*) stained by Melzer's reagent, Sw1 and hypha showing no reaction to Melzer's reagent. **d**, **e** CMCC ROC3 and CMCC ROC4 spores are irregular in shape with three-layered wall (*Sw1*, *Sw2*, *Sw3*), SW1 nonreactive to Melzer's reagents. **f** Hypha of isolate CMCC ROC5 showing pigmentation with Melzer's reagent (*arrow*). **g** Mature spores of CMCC ROC5 are globose to subglobose in shape with 4 walls layer (*Sw1*, *Sw2*, *Sw3* and *Sw4*), layer Sw4, 0.5–0.1 μM thick and several times laminated, 0.8–1.2 μM thick subtending hypha with open pore, laminated innermost layer nonreactive in Melzer's reagent. **h** Mature spore of CMCC ROC6 with a three-layered wall (*Sw1*, *Sw2*, *Sw3*) and cylindrical to slightly flared subtending hypha, outer wall layer (*Sw1*) not stained with Melzer's reagent, SW3 reactive in Melzer's reagent. **i** Intense reactivity of innermost spore wall (*Sw3*) in Melzer's reagent, Inner layer (*Sw3*) of a mature CMCC ROC7 spore slightly separated from spore wall (*Sw1*, *Sw2*). **j** Mature spores of CMCC ROC8 with membranous inner spore wall (*Sw4*) with straight subtending hypha, mature spores and hypha showing very little or no reaction to Melzer's reagent. **k**, **l** Mature spores of CMCC ROC9 and CMCC ROC10 with a three-layered wall (*Sw1*, *Sw2*, *Sw3*), smooth mucilaginous outer wall layer not reactive in Melzer's reagent. *Bars* (**a**, **c–e**, **g–l**) 20 μM; **b**, **f** 50 μM

fungi which was also observed by Peng et al. (1993). This fatty acid is possibly misidentified in most of the literature as 16:1(α-c-11) marker fatty acid. Larsen et al. (1998) observed that the presence 18:2 fatty acid was the dominant fatty acid of saprotrophic fungi while it was negligible in the mycelium of *Rhizophagus* species originating from environmental soil. In contrast, the present investigation showed that this fatty acid was present in significant amounts in *Rhizophagus* spores grown under monoxenic conditions, establishing that this fatty acid is present not only saprophytic fungus but also in Glomeromycota.

Using SSU-ITS rDNA phylogeny, we found that isolate CMCC ROC2 grouped with known *R. irregularis* with a very low bootstrap support, and therefore the rDNA sequence was genetically more diverse than other *R. irregularis* isolates used for the present investigation. LSU rDNA topology also revealed that isolate CMCC ROC2 showed a wider genetic divergence than other *R. irregularis* isolates (CMCC ROC1, CMCC ROC3, and CMCC ROC4) originating from the same tropical plots. Koch et al. (2004), using amplified fragment length polymorphism (AFLP), reported similar wide genetic and phenotypic diversity among *R. intraradices* isolates originating from a field in Tanikon, Switzerland. In addition, we detected significantly low amounts of C16:1 and C18:0 fatty acids (Table 2) and thus, in consequence, biochemically also showed different fatty acid profiles than other isolates collected from the same site. Moreover, electron microscopy studied also showed that the spore walls of CMCC ROC2 had only two layers whereas those of almost all the isolates had three layers.
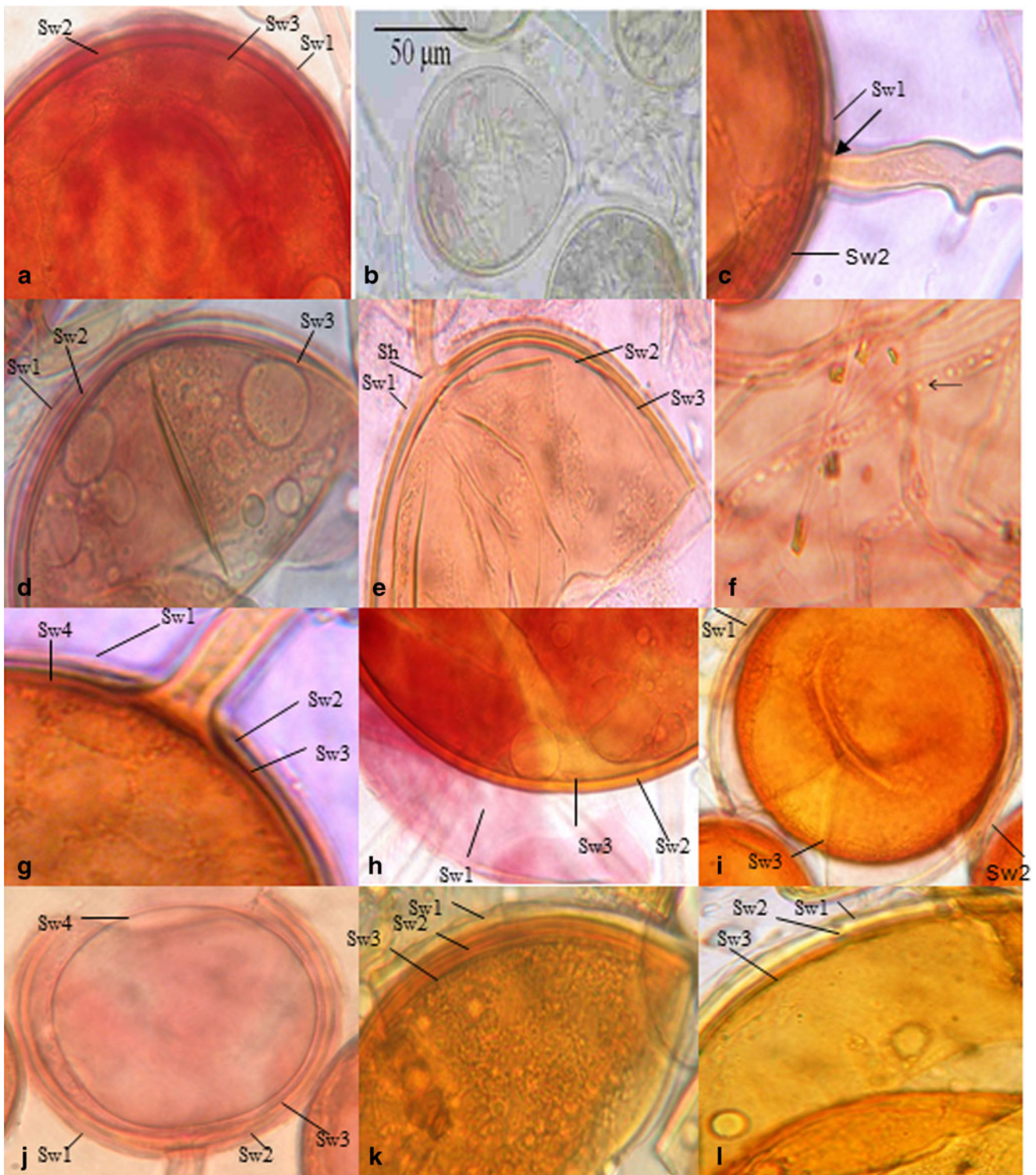
Maia and Kimbrough (1994) observed an intervening electron-dense layer in *R. intraradices* but this feature was absent between the two layers of the spore wall of CMCC ROC2. As a result, molecular phylogeny placed CMCC ROC2 within the *R. irregularis* clade of *Rhizophagus* with low bootstrap support, while FAME and morphological data also supported this identification. The combination of approaches that we used placed this isolate in the *R. irregularis* group and suggested that this isolate is more diverse than other *R. irregularis* isolates collected from same site.

Fig. 2 a–h Spore wall of
Rhizophagus sp. used in this
study observed under a
transmission electron
microscope (TEM). **a** Mature
spore of isolate ROC1 with outer
wall layer (*Sw1*) and inner wall
layer showing more distinct
lamination (*arrow*). **b**, **c** Mature
spores of isolate ROC2 showing
a double-layered wall (*Sw1*,
Sw2), inner wall layer (*Sw2*) of
CMCC ROC2 showing indis-
tinct lamination and an electron-
dense layer (*arrow*). **d** Mature
spore of ROC4 showing a three-
layered wall (*Sw1*, *Sw2*, *Sw3*),
laminated layers generally in-
separable. **e** Mature spore of
CMCC ROC5 with four distinct
wall layers (*Sw1*, *Sw2*, *Sw3* and
Sw4), wall laye, Sw4 clearly
separated from layer Sw3. **f**
Membranous layer (*Sw3*) of
CMCC ROC6 not separated
from layers *Sw1* and *Sw2*). **g**,
h Mature CMCC ROC8 spore
with a four-layered wall (*Sw1*,
Sw2, *Sw3* and *Sw4*), innermost
wall layer (*Sw4*) showing
lamination
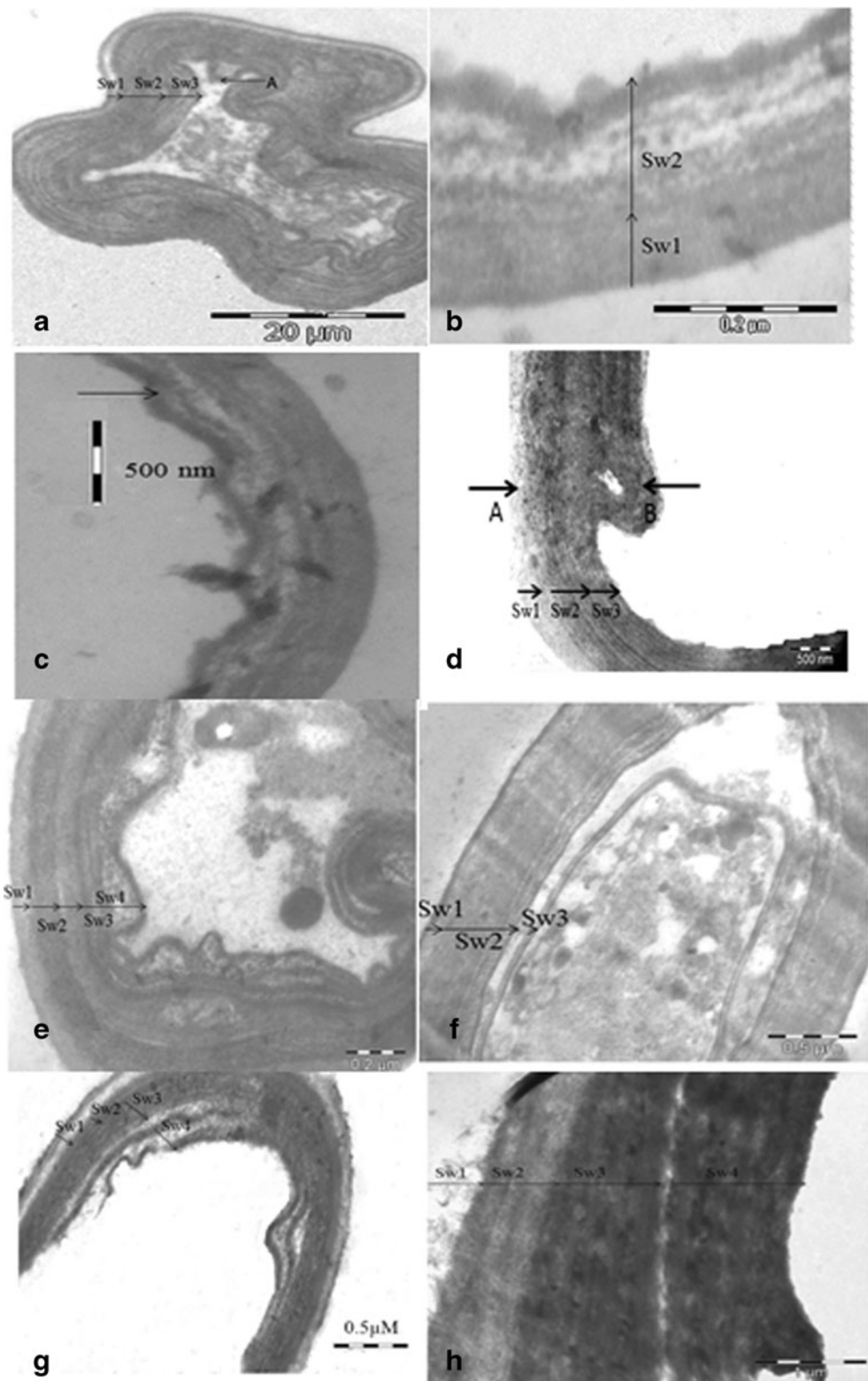
The LSU n-rDNA sequence of CMCC ROC8 placed the isolate within the cluster of known *R. proliferus* with 92 % bootstrap support; however, phylogeny based on SSU-ITS n-rDNA sequence revealed greater genetic distance from the reference *R. proliferus* sequences with a bootstrap value of 89 %. This variation between the genetic distances obtained by sequences from two different regions of n-rDNA stresses the importance of analyzing both the n-rDNA regions for a definitive identification. Furthermore, the FAME extracts of this isolate consist of 16:0 and 16:1(α-c-9) fatty acids

Fig. 3 Neighbor-joining tree obtained from alignment of 31 n-rDNA sequences, each about 1,000 bp long, from partial small subunit (SSU) and internal transcribed spacer (ITS) regions with *Funneliformis geosporum* sequences as the outgroup. The numbers in the branches are percentage bootstrap values (out of 500 trials). *Names followed by accession no.* represent sequences retrieved from GenBank. *Names preceded by a triangle* represent the sequence obtained in this work. Non-significant values (<50%) omitted. There were 600 positions in the final datasets
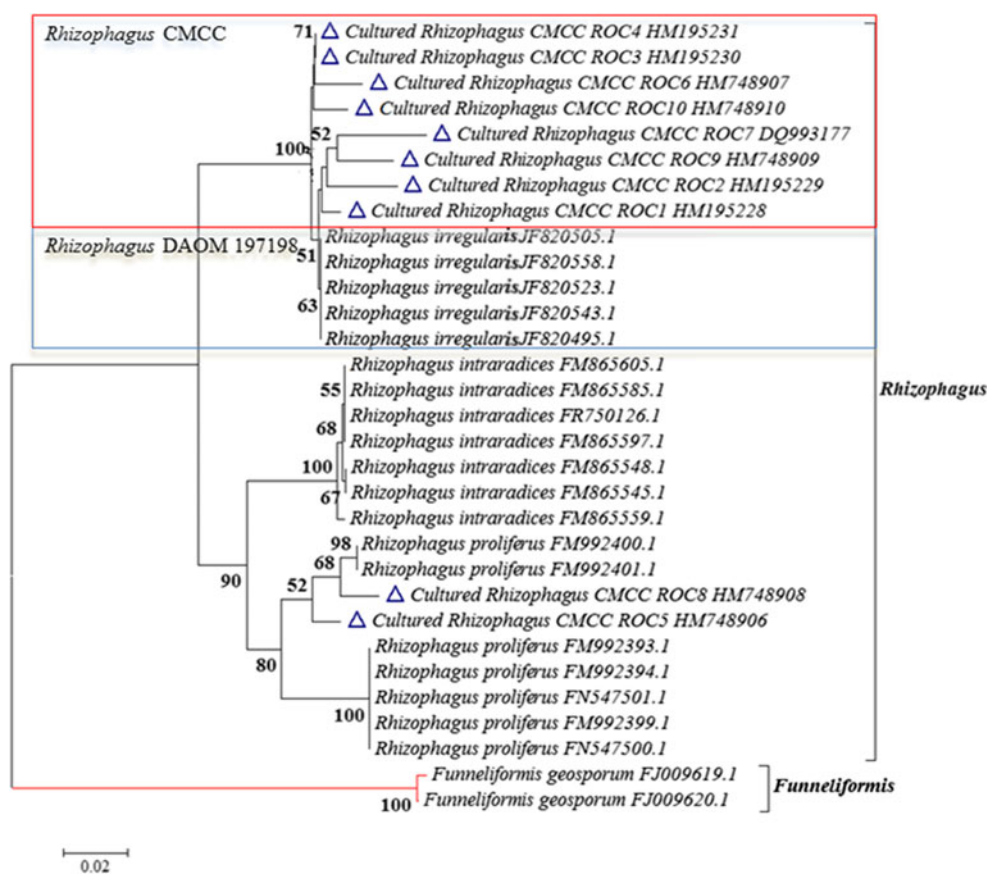
representing 31 and 36 % of the total fatty acids, respectively. Fatty acids with 18 carbon were also present in significantly low amounts in this isolate as similarly reported by Declerck et al. (2000): the actual quantities being as follows: C18:0 (5 %), C18:1 (6.79 %), C18:2 (4 %), and C18:3 (4 %). A significantly low amount of C17:1 (α-c-10) was found only in the isolate CMCC ROC8, but this fatty acid was absent in all the *R. irregularis* isolates used in the present study. Furthermore, the ultrastructure study revealed that the spore walls of CMCC ROC8 consisted of four layers. The innermost spore layer and the subtending hypha of this isolate showed very little or no reactivity to Melzer's reagent and, under the electron microscope, also showed a pattern of spore wall differentiation similar to that of *R. proliferus* described by Declerck et al. (2000). Considering the above points, isolate CMCC ROC8 was identified as *R. proliferus*, and the presence of C18:3 fatty acids in very low amounts (3–4 %) may serve as a biomarker of *R. proliferus* (Table 2).

Phylogenetic analysis using a 1,000-bp fragment of SSU-ITS rDNA and a 500-bp fragment of LSUrDNA placed the CMCC ROC5 isolate within the *R. proliferus* clade. Phenotypic and ultrastructure data also support these molecular results. Compound and electron microscopy studies revealed that this isolate showed a similar spore morphology and wall layer organization as defined for *R. proliferus*

(Fig. 2e). On comparing the FAME, this isolate could be further distinguished. A total of eight FAME were observed in this isolate; however, only 6–7 FAME were observed in other *R. irregularis* isolates used for the present investigation (Table 2). This isolate consisted of C16:0, C16:1c-9, C18:0, C18:1, and C18:2 representing 80 % of total fatty acids. This spore's fatty acid composition is in agreement with previously reported profiles of *R. proliferus* grown under in vitro conditions (Declerck et al. 2000). We also observed significantly greater amounts of C16:1c-9 fatty acids representing ≈40 % of the total profile in this isolate (Table 2), similar to the *R. proliferus* species reported by Declerck et al. (2000). According to the total data obtained from different approaches, isolate CMCC ROC5 can be placed in the *R. proliferus* group.

Molecular phylogenetic analysis using the SSU-ITS and LSU region of ribosomal DNA showed that CMCC ROC9 and CMCC ROC10 fell in the clade of *R. irregularis* (Figs. 3, 4), whereas the FAME profile revealed the presence of significantly greater percentages of AM marker fatty acid C16:1 (α-c-9) (25–30 %) in these isolates in comparison with the low amounts (10–20 %) in *R. irregularis* isolates used in the present investigation (Table 2). Therefore, these isolates were further characterized using spore morphology which was compared with the reference *R. irregularis* as described by Błaszkowski and Czerniawska (2008) (Fig. 1).
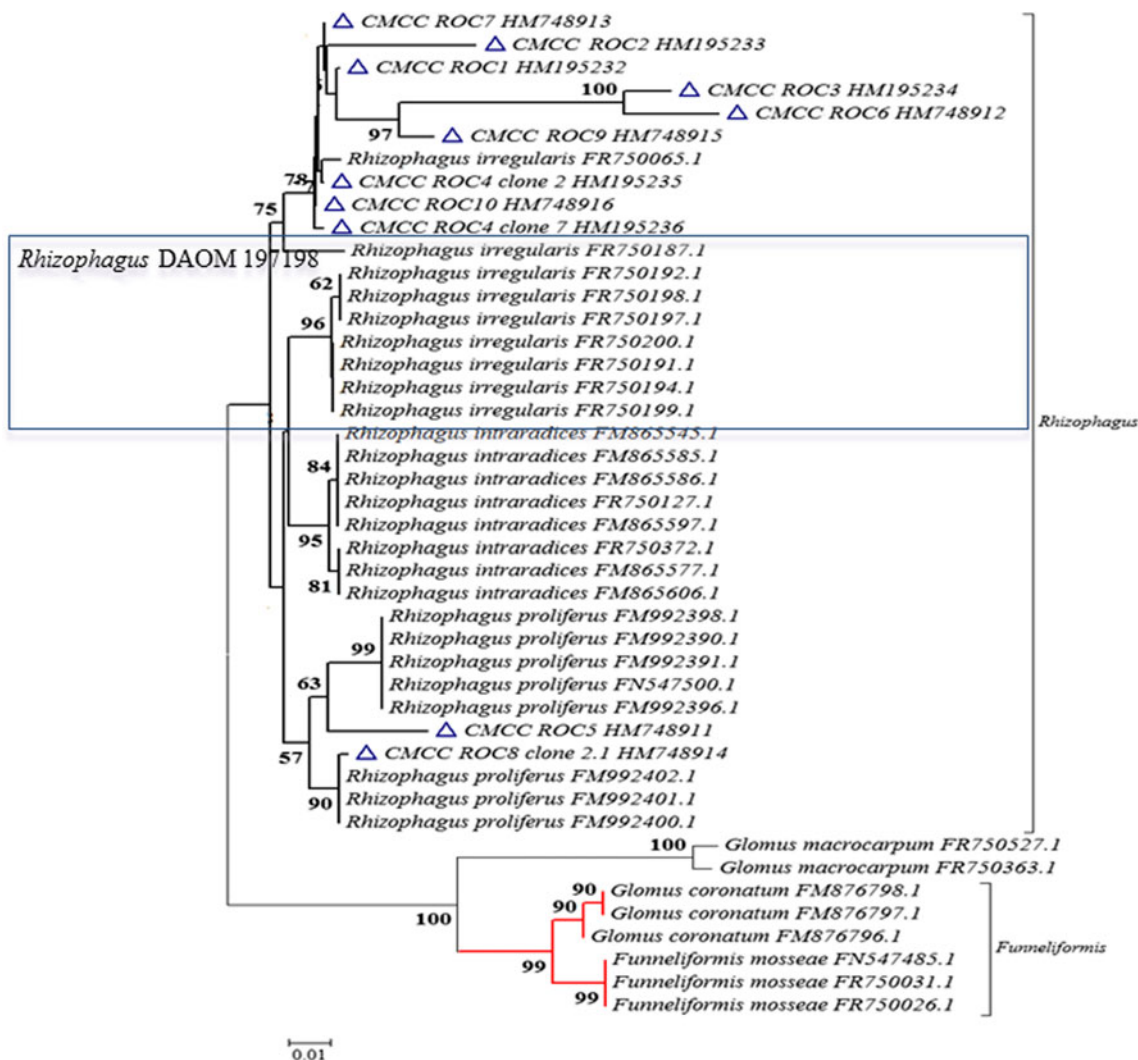
Fig. 4 Neighbor-joining tree obtained from alignment of the 44 n-rDNA sequences, each about 600–800 bp long, from partial large subunit (LSU) region with *Funnelliformis* as the out group. The numbers in the branches are percentage bootstrap value (out of 500 trials). *Names followed by accession no.* represent sequences retrieved from GenBank. *Names preceded by a triangle* represent the sequence obtained in this work. Non-significant values (<50%) omitted. There were a total of 488 positions used for phylogenetic analysis

Table 2 Multi-way ANOVA analyses using the mean percentage of fatty acid of three replicates per *Rhizophagus* isolate using JMP 5.1.1 software (SAS Institute, Cary, NC, USA, 1989–2002) and differences between the means were analysed using Tukey's HSD multiple comparisons with $P < 0.05$

Fatty acid	ROC1	ROC2	ROC3	ROC4	ROC5	ROC6	ROC7	ROC8	ROC9	ROC10
C14:0	20.68 abc	12.87 cd	30.18 a	24.08 ab	10.58 cd	29 a	15.55 bcd	11.453 cd	11.795 cd	14.621 d
C16:0	18.67 c	16.69 c	18 c	16.96 c	37.55 a	18.34 c	18.08 c	29.55 a	35.76 a	27.66 b
C 16:1 (α-c-9)	18.53 c	10.03 d	18.63 c	19.62 c	38.45 a	19.13 c	19.11 c	39.24 a	25.21 b	24.01 b
C 17:1 (α-c-10)	0	0	0	0	6.6 a	0	0	4.6 a	0	0
C18:0	8.22 bc	5.8 c	8.22 bc	6.89 bc	6.8 bc	8.29 bc	6.55 bc	6.794 bc	10.75 bc	17.125 a
C18:1 (α-c-9)	21.69 c	37.13 a	18.3 d	26.5 bc	0.8 f	15.65 d	37.27 a	0.8 f	14.06 d	8.926 e
C18:2 (α-c-9,12)	12.2 b	16.89 a	6.74 e	6.3 e	4.3 e	9.51 cd	3.09 fg	4 e	2 fg	7.521 g
C18:3 (α-c-9,12,15)	0	0.59 b	0	0	3.7 a	0	0.61 b	3.7 a	0	0

Non-significant differences between ROC isolates are indicated by identical letters and were determined by Tukey HST

We observed that isolates CMCC ROC9 and CMCC ROC10 showed more affinity with *R. irregularis* (Fig. 1). Based on this combined view of morphological and molecular analysis, we identified CMCC ROC9 and CMCC ROC10 as *R. irregularis* (Figs. 1k, l, 3, 4) whereas FAME profiles did not confirm these results. It is suggested that characterization of AM fungal isolates based on a single approach (FAME biomarker) sometimes failed to separate closely related AM taxa.

Phylogenetic analysis using 1,000 bp of SSU-ITS rDNA conclusively indicated that *R. irregularis* and *R. intraradices* were separated (Fig. 3). The data obtained did not differ from the previous analyses by Stockinger et al. (2009) and suggested that the 1,500-bp fragment consisting of SSU-ITS-LSU rDNA clearly separated AM species belonging to the genus *Rhizophagus*. The present study also indicated that sequence analysis ≈600 bp of n- LSU rDNA clearly separated *R. irregularis* and *R. intraradices* into different
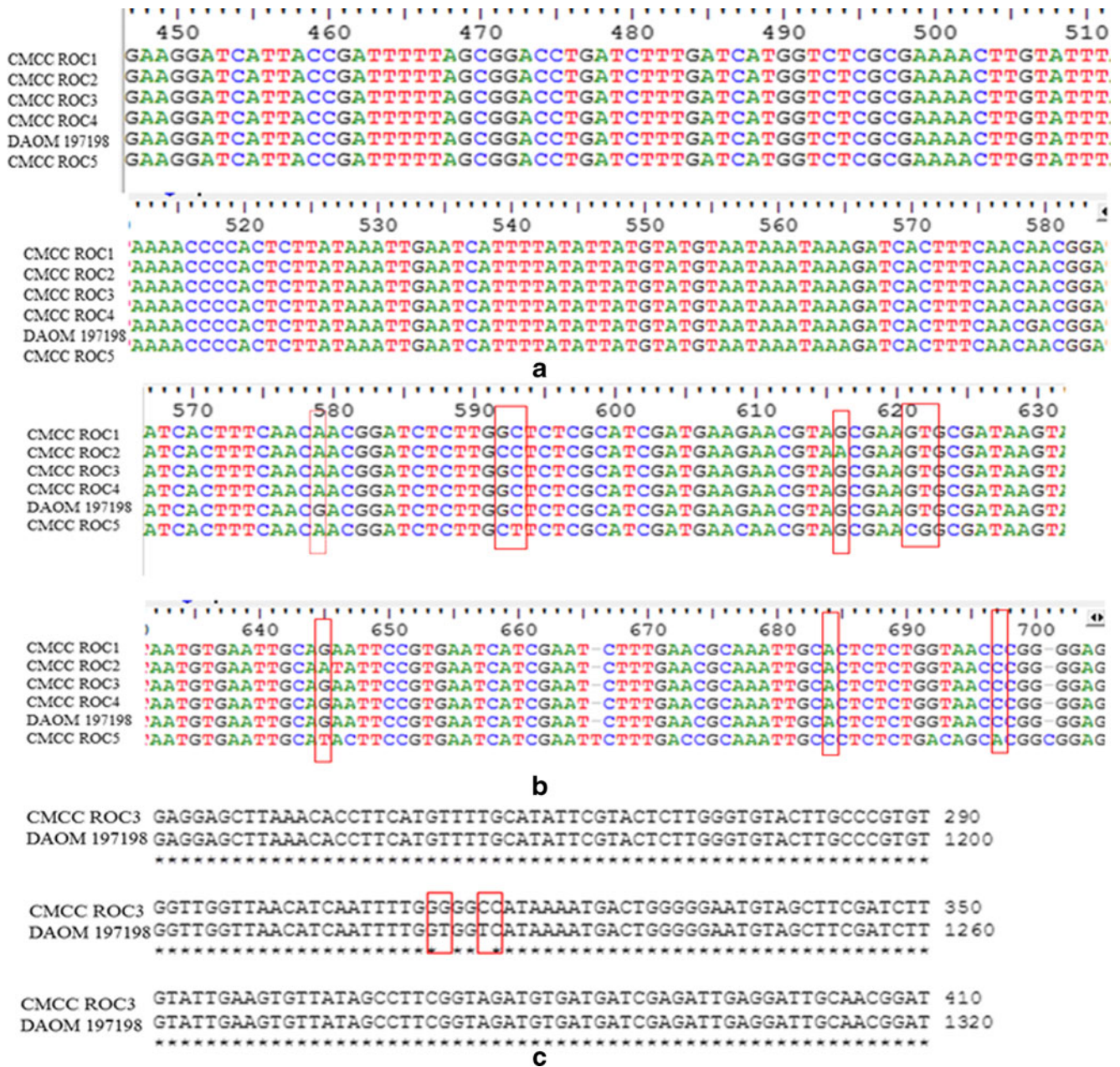

Fig. 5 a–c Representative region of the nucleotide alignment of the ITS1, 5.8S and LSU region of rDNA from different *Rhizophagus* strains. **a** Alignment of ITS1 region of ROC1-5 and reference isolate DAOM 197198. **b** Alignment of 5.8S rDNA regions of ROC1-5 and reference isolate DAOM 197198; variable 5.8S rDNA region indicated by *square* **c** Alignment of CMCC ROC3 with reference isolate DAOM 197198 using LSU region of ribosomal DNA; variable LSU rDNA region indicated by *square* (313 and 316 bp) of isolate CMCC ROC3

subclades (Fig. 4). However, Stockinger et al. (2009, 2010) observed little resolution between *R. intraradices* and *R. irregularis* when ≈600 bp of LSU n-rDNA was used for phylogenetic analysis.

It was also observed that the ITS1 rDNA fragment spanning nucleotide from 446 to 567 bp of isolate CMCC ROC1-4 showed 100 % homology with model AM fungi (*R. irregularis* DAOM 197198, FM865551) retrieved from the NCBI GenBank (Fig. 5a). In contrast, variants were observed within the 5.8S region (span nucleotide 568–722) of rDNA (Fig. 5b). We observed variants in 5.8S rDNA on the 580-bp position where there is the 'A' nucleotide in CMCC ROC1-4 and the 'G' nucleotide in DAOM 197198 (Fig. 5b). Using these datasets, we could differentiate model the isolate DAOM 197198 with CMCC ROC1-4, although using ITS1 region these isolates were not differentiated. Additionally, using 5.8S rDNA variants, two groups were differentiated, mainly ROC2, ROC5 (group1) and ROC1, ROC3, and ROC4 (group 2) shown in (Fig. 5b).

The present study using SSU-ITS rDNA phylogeny revealed that eight ROC isolates out of ten shown statistically 100 % bootstrap support with model *R. irregularis* DAOM isolates (Fig. 3); however, the same isolates using LSU rDNA sequences did not show tight clustering with reference DAOM isolates. For example, we could separate CMCC ROC3 with reference strain DAOM 197198 when the D1-D2 domain of LSU rDNA fragment (Span nucleotide 900–1,476 bp) was used (Fig. 5c). Although it is well known that the ITS1 region is suitable for resolving closely related species or isolates, the ITS1 region from four isolates failed to show variation, whereas the conserved domain of 5.8S rDNA and the variable domain of LSU rDNA could separate these isolates. These analyses suggested that different regions of ribosomal DNA showed different resolution powers for clear separation of AM fungal species/isolates. This analysis forecasts that a combination of ITS2, 5.8S rDNA and LSU region of ribosomal DNA reveals higher resolution and, if worked extensively, species/isolate level differentiation is possible and achievable, including through barcode research.

Previous studies have documented that molecular phylogeny of AM fungi shows congruence of morphological characters. Declerck et al. (2000) have characterized single AM species using more than one approach. Morton and Msiska (2010) and Kruger et al. (2011) revised the classification of family Gigasporaceae (Glomeromycota) and *Acaulospora brasiliensis* using a combination of morphological and molecular characters, respectively. Moreover, Oehl et al. (2011a) proposed the classification of AMF based on partial sequence analysis of beta tubulin, and SSU and LSU regions of rRNA gene with a combination of conserved phenotypic characters. The current study indicates that no single character can be adequate for resolving AM isolates; only with the use of combined data retrieved from FAME, molecular,

and morphological profiles can the distinctions between them become obvious.

Conclusion

As more and more AMF isolates are brought into ROC, the availability of pure AM species/isolates will increase, making it feasible to build a complete and comprehensive profile of each isolate, which will serve as its signature profile. Our study demonstrates the feasibility of this approach with ten isolates from ROC and emphasizes that relying on any one character for accurate identification is fraught with difficulties, as it takes a combination of molecular, biochemical, and morphological characters to describe differentiation among isolates. As we hypothesized, the present study shows that using sequences from the most variable regions (ITS2 and D1-D2 LSU rDNA), conserved domain (5.8S rDNA) along with spore morphology and ultrastudies observations provides a more dependable and holistic approach to assign isolates of AMF to the correct species. However, FAME profiles revealed only additional datasets for characterized AM isolate up to family/genera level.

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