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'*Candidatus* Moeniiplasma glomeromycotorum', an endobacterium of arbuscular mycorrhizal fungi

Mizue Naito[1], Alessandro Desirò[2a], Jonathan B. Gonzalez[3], Gang Tao[3b], Joseph B. Morton[4], Paola Bonfante[2], and Teresa E. Pawlowska[3]

[1]Graduate Field of Microbiology, Cornell University, Ithaca, NY 14853, USA; [2]Department of Life Sciences and Systems Biology, University of Torino, 10125 Torino, Italy; [3] School of Integrative Plant Science, Plant Pathology & Plant-Microbe Biology, Cornell University, Ithaca, NY 14853, USA; [4]Division of Plant & Soil Sciences, West Virginia University, Morgantown, WV 26506, USA.

[a]Present address: Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI 48824, USA.

[b]Present address: Guizhou Institute of Plant Protection, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China.

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Corresponding author:

Teresa E. Pawlowska
School of Integrative Plant Science
Plant Pathology & Plant-Microbe Biology
Cornell University
334 Plant Sciences Building
Ithaca, NY 14854-5904
Tel: (607) 254-8726
Fax: (607) 255-4471
E-mail: tep8@cornell.edu

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SUMMARY

Arbuscular mycorrhizal fungi (AMF, phylum *Glomeromycota*) are symbionts of most terrestrial plants. They commonly harbour endobacteria of a largely unknown biology, referred to as MRE (*Mollicutes*/mycoplasma-related endobacteria). Here, we propose to accommodate MRE in the novel genus '*Candidatus* Moeniiplasma.' Phylogeny reconstructions based on the 16S rRNA gene sequences cluster '*Ca.* Moeniiplasma' with representatives of the class *Mollicutes*, whereas phylogenies derived from amino acid sequences of 19 genes indicate that it is a discrete lineage sharing ancestry with the members of the family *Mycoplasmataceae*. Cells of '*Ca.* Moeniiplasma' reside directly in the host cytoplasm and have not yet been cultivated. They are coccoid, ~500 nm in diameter, with an electron-dense layer outside the plasma membrane. However, the draft genomes of '*Ca.* Moeniiplasma' suggest that this structure is not a Gram-positive cell wall. The evolution '*Ca.* Moeniiplasma' appears to be driven by an ultrarapid rate of mutation accumulation related to the loss of DNA repair mechanisms. Moreover, molecular evolution patterns suggest that, in addition to vertical transmission, '*Ca.* Moeniiplasma' is able to transmit horizontally among distinct *Glomeromycota* host lineages and exchange genes. On the basis of these unique lifestyle features, the new species '*Candidatus* Moeniiplasma glomeromycotorum' is proposed.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF, phylum *Glomeromycota*) are obligate biotrophs forming symbiotic associations with the roots of most terrestrial plants (Smith & Read, 2008; Gutjahr & Parniske, 2013). They improve plant mineral nutrient uptake in exchange for photosynthates and are important members of terrestrial ecosystems. Based on electron microscopy studies, it has been known for decades that AMF harbour endobacteria in the cytoplasm of their hyphae and spores, referred to as bacterium-like organelles, or BLOs (Mosse, 1970; MacDonald & Chandler, 1981; MacDonald *et al.*, 1982; Scannerini & Bonfante, 1991). These bacteria display diverse morphologies, including coccoid cells that remain unclassified and are referred to as *Mollicutes*/mycoplasma-related endobacteria or MRE, based on the 16S rRNA gene phylogenies that cluster them with members of the class *Mollicutes* (Naumann *et al.*, 2010). MRE have been found in AMF from nearly all major lineages of *Glomeromycota* surveyed to date (Naumann *et al.*, 2010; Desirò *et al.*, 2013; Desirò *et al.*, 2014; Toomer *et al.*, 2015). The MRE genomes are characterized by a highly reduced gene content that is indicative of metabolic dependence on the fungal host (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015). For example, MRE are incapable of amino acid and nucleic acid biosynthesis, and so these metabolites must be obtained from the AMF host cytoplasm. Similarly, the MRE genomes do not encode enzymes catalyzing the TCA cycle and oxidative phosphorylation. Remarkably, the MRE genomes harbour multiple genes horizontally acquired from AMF (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015). While the role of MRE in the biology of AMF is unknown, their broad distribution across the host taxa suggests that MRE may modulate the impact of AMF on terrestrial ecology. To recognize this unique

lineage of endosymbionts, we propose the new genus '*Candidatus* Moeniiplasma' and the new species '*Candidatus* Moeniiplasma glomeromycotorum.'

METHODS

16S rRNA gene and multilocus phylogenies. To elucidate the relationship between MRE and other lineages within the *Mollicutes* class, we conducted phylogenetic reconstructions based on the sequences of 16S rRNA gene and proteins encoded by 19 conserved genes (*dnaG, infC, nusA, rplA, rplB, rplC, rplE, rplF, rplM, rplN, rplP, rplT, rpmA, rpsB, rpsC, rpsE, rpsJ, rpsS, smpB*), selected based on the Genomic Encyclopaedia of Bacteria and Archaea, GEBA (Wu *et al.*, 2009). Sequences of these genes were extracted from the *de novo* sequenced metagenomes of MRE associated with *Dentiscutata heterogama* (Torres-Cortés *et al.*, 2015), *Racocetra verrucosa*, and *Rhizophagus clarus* (Naito *et al.*, 2015). Sequences from non-MRE species were obtained from IMG (Markowitz *et al.*, 2012). The 16S rRNA and amino acid sequences were aligned using MUSCLE (Edgar, 2004). Sequence alignments were adjusted manually. Amino acid sequence alignments were concatenated in Geneious 9.1.2 (Biomatters Ltd). Bayesian analyses were performed in MrBayes 3.2 (Ronquist *et al.*, 2012). 16S rRNA gene sequences were analyzed under the nucleotide substitution model GTR+I+ Γ (Tavaré, 1986) in a run of 1,000,000 generations with 25% burn-in. Amino acid sequences were examined under the model mixed+I+Γ in a run of 100,000 generations with 25% burn-in. The average standard deviation of split frequencies was used as a convergence diagnostic. Maximum Likelihood analyses were conducted using PhyML (Guindon *et al.*, 2010) run with 1,000 bootstrap. The GTR+I+Γ model was used for 16S rRNA gene sequences. The Rtrev+I+Γ (Dimmic *et al.*, 2002) model identified by MrBayes as the model that best fits these data was used for amino acid sequences.

Cultivation. In our cultivation attempts, we focused on MRE of *Rhizophagus clarus* NB112A, which originated in Namibia and its experimental population is maintained at the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi, INVAM (Morton *et al.*, 1993). Unlike many other AMF, *R. clarus* can be readily maintained *in vitro* in association with root-inducing T-DNA-transformed chicory roots grown on MSR medium (Cranenbrouck *et al.*, 2005) at 28ºC. In addition, a draft genome sequence is available for its MRE (Naito *et al.*, 2015) to inform media formulations. AMF filtrates containing MRE cells were subjected to different cultivation media, supplements, temperatures, and atmospheres. Media included Brain Heart Infusion, BHI (Bacto), PPLO Broth Base (BBL), 2x BHI, and 2x PPLO. They were supplemented with horse (Sigma), bovine (Sigma), and porcine serum (Sigma) at concentrations of 1 to 20% in 5% increments, yeast extract and TC yeastolate (Bacto) at concentrations of 0.1%, 0.25%, 0.5% and 1%, Tween®80 (Sigma) at concentrations of 0.05% and 0.5%, and AMF spore extracts. AMF spore extracts were made by harvesting spores and hyphae of *R. clarus* NB112A grown *in vitro* by manually removing all associated root structures, and dissolving the Phytagel-solidified medium in 10 mM sodium citrate buffer (pH 6; Fisher Scientific) at 30ºC for 20 min. Isolated spores and hyphae were then manually crushed, ground, and passed through a 0.22 μm filter. The filtrate was added directly to the MRE cultivation medium. Incubation conditions included ambient temperature, 28ºC, and 30ºC as well as ambient, microaerophilic, increased CO_2, and anaerobic atmosphere. All factors (cultivation medium, supplement, temperature and atmosphere) were tested combinatorially. Each medium and supplement condition was prepared as a liquid culture and inoculated at day 0 with AMF filtrate containing MRE cells, followed by incubation at every combination of temperature and atmospheric conditions. On day 0, 1, 3, 7,

14, 21, and 30, a portion of the liquid culture was subcultured onto a solid medium of the same type, solidified with agar Noble (Difco), and incubated for an additional 14 days, at the same temperature and atmospheric conditions as before. Any colonies that arose were genotyped by 16S rRNA gene sequencing, but none were identified as MRE.

Transmission Electron Microscopy. To explore MRE cell ultrastructure, spores of *R. clarus* NB112A were subjected to high-pressure/freeze-substitution in order to preserve fungal and bacterial cytology, processed as described in Desirò *et al.* (2016), and observed under transmission electron microscope.

Fluorescent *in situ* hybridization. Fluorescent *in situ* hybridization (FISH) was performed on fixed and crushed spores of *R. clarus* NB112A. The MRE-specific probe BLOgrBC (5'-GCCAATCCTACCCTTGTCA-3') (Naumann *et al.*, 2010) and the universal bacterial probe EUB338I (Amann *et al.*, 1990) were used as described by Naumann *et al.* (2010) with slight modifications. Specifically, AMF spores were immobilized in polyacrylamide pads for the procedure, and probes were hybridized at a stringency of 30% formamide. Cells were visualized using the DeltaVision RT system (Applied Precision).

16S rRNA gene sequence diversity. To explore the extent of MRE diversity across different *Glomeromycota* hosts, we reconstructed the genealogy of MRE using 16S rRNA gene sequences. In these reconstructions, we included MRE diversity from previously published reports (Naumann *et al.*, 2010; Desirò *et al.*, 2014; Naito *et al.*, 2015; Toomer *et al.*, 2015; Torres-Cortés *et al.*, 2015) as well as sequences newly generated from several populations of *R. clarus*

representing different geographic locations. We explored MRE diversity in *R. clarus* because this species is one of few AMF hosts that appear to harbour a homogenous MRE population (Naito *et al.*, 2015). Accessions of *R. clarus* AU402B, CL156, KR104, MG104A, ND269B, and WV219A were obtained from INVAM. AMF spores (isolates) were extracted from the cultivation medium by wet-sieving and sucrose centrifugation (Daniels & Skipper, 1982), followed by surface decontamination as described in Mondo *et al.* (2012), and whole genome (WG) amplified using Illustra™ GenomiPhi-V2 kit (GE Healthcare, Piscataway, NJ). WG amplification products were diluted 1:20 in water for subsequent PCR reactions. Bacterial 16S rRNA gene fragments were PCR-amplified using MRE-specific primers 109F1 (5'-ACGGGTGAGTAATRCTTATCT-3), 109F2 (5'-ACGAGTGAGTAATGCTTATCT-3), 1184R1 (5'-GACGACCAGACGTCATCCTY-3), 1184R2 (5'-GACGACCAAACTTGATCCTC-3), and 1184R3 (5'-GATGATCAGACGTCATCCTC-3) (Naumann *et al.*, 2010) and Phusion®High-Fidelity DNA polymerase (New England Biolabs). PCR reactions contained 1 μL diluted WG-amplified product, 0.02 U μL^{-1} Phusion polymerase, 1x Phusion HF Buffer with 1.5 mM $MgCl_2$, 180 μM each dNTP, and primers added as a 2:1 mixture of the two forward primers (0.75 μM and 0.375 μM) and a 2:1:1 mixture of the three reverse primers (0.75 μM, 0.375 μM, and 0.375 μM). Cycling conditions were 5 min initial denaturation at 98°C followed by 15 cycles of 10 sec at 98°C, 30 sec at 50°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C. The 1063 bp amplicons were purified using QIAquick PCR purification kit (Qiagen), and cloned using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen Life Technologies). Plasmid DNA from 16 recombinant bacterial colonies per sample was amplified using the Illustra TempliPhi 100/500 DNA Amplification Kit (GE Healthcare Life Sciences). Plasmid inserts were cycle-sequenced with the BigDye

Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) using T3 and T7 primers. Sequences were edited in Geneious 9.1.2 (Biomatters Ltd). To facilitate analyses and display of the MRE 16S rRNA gene data, we used MOTHUR (Schloss *et al.*, 2009) to cluster at a 94% similarity level gene fragments cloned and sequenced from each AMF spore (isolate) and to identify a sequence representative for each cluster. The 94% 16S rRNA gene sequence similarity level is recommended for delineation of species in the *Mollicutes* (Brown *et al.*, 2007). The representative MRE sequences were aligned in MUSCLE (Edgar, 2004). Phylogenies were reconstructed under the GTR+I+Γ (Tavaré, 1986) nucleotide substitution model implemented in MrBayes 3.2 (Ronquist *et al.*, 2012), with analyses conducted for 15,000,000 generations with 25% burn-in, and in PhyML (Guindon *et al.*, 2010) with 1,000 bootstrap replications.

RESULTS AND DISCUSSION

Phylogeny reconstructions based on 16S rRNA gene sequences cluster MRE with the representatives of the class *Mollicutes*, albeit without resolving their taxonomic position relative to individual mollicute lineages (Figure 1) (Naumann *et al.*, 2010). In contrast, phylogenies derived from amino acid sequences of 19 conserved genes indicate that MRE share ancestry with members of the *Mycoplasma pneumoniae* group in the family *Mycoplasmataceae* (Figure 2). MRE appear to be uncultivable. Therefore, they do not meet the minimal standards for description of a new species of the class *Mollicutes* (Brown *et al.*, 2007). Nevertheless, we recommend that MRE ubiquity and their potential ecological significance warrant a taxonomic proposal in accordance with the guidelines for a designation of a provisional *Candidatus* taxon (Murray & Stackebrandt, 1995).

Description of '*Candidatus* Moeniiplasma'

Moeniiplasma (Moe.ni.i.pla'sma. L. pl. neut. n. moenia, walls/fortifications; Gr. neut. n. plasma, that which is molded/shaped; N.L. neut. n. Moeniiplasma, shape surrounded by walls/fortifications). Representatives of '*Ca.* Moeniiplasma' inhabit hyphae and spores of *Glomeromycota* and are transmitted vertically from one host generation to the next (Naumann *et al.*, 2010; Naito, 2014). In addition, phylogenetic data suggest a history of horizontal transmission in '*Ca.* Moeniiplasma' (Toomer *et al.*, 2015). The occurrence of '*Ca.* Moeniiplasma' varies among host populations from different geographic locations. For example, in *Cetraspora pellucida*, *Gigaspora margarita*, *Gi. rosea*, and *Rhizophagus clarus,* '*Ca.* Moeniiplasma' was detected in some populations but not in others (Naumann *et al.*, 2010; Desirò *et al.*, 2014; Toomer *et al.*, 2015).

'*Ca.* Moeniiplasma' resides directly in the cytoplasm of *Glomeromycota* (Figure 3, Naumann *et al*., 2010, and Desirò *et al*., 2013). Cells are coccoid (diameter of 460 nm – 610 nm, measured in 8 cells) but may assume different shapes when, for example, compressed between the lipid bodies (not shown). A thin homogenous layer is present outside the cell membrane, an unusual feature for the wall-less *Mollicutes* class (Figure 3, Naumann *et al*., 2010, and Desirò *et al*., 2013). However, since the organization of such a layer changes depending on the sample preparation (from an electron-dense to a more transparent layer), and none of the draft genomes available for '*Ca.* Moeniiplasma' reveals genes involved in the peptidoglycan synthesis (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015), we suggest that this structure is not a Gram-positive cell wall.

The G+C content of '*Ca.* Moeniiplasma' DNA is 32-34% (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015), which is comparable to the 32% G+C content of the *M. genitalium* genome

(Fraser *et al.*, 1995). The draft genome assemblies span from 662,952 bp to 1,227,948 bp (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015), thus falling within the range of genome sizes exhibited by other members of the *M. pneumoniae* clade, from 580,070 bp in *M. genitalium* (Fraser *et al.*, 1995) to 1,358,633 bp in *M. penetrans* (Sasaki *et al.*, 2002). '*Ca.* Moeniiplasma' utilizes the UGA codon to encode tryptophan rather than as a stop codon (Naito *et al.*, 2015), codon usage shared with other SEM (*Spiroplasma*, *Entomoplasma*, and *Mycoplasma*) but not with AAA (*Asteroleplasma*, *Anaeroplasma*, *Acholeplasma,* and *Phytoplasma*) mycoplasmas (Razin *et al.*, 1998). Not unlike other *Mycoplasma* genomes (Marenda, 2014), the genomes of '*Ca.* Moeniiplasma' are extraordinarily plastic, a phenomenon related to the retention of recombination machinery and mobile genetic elements (Naito *et al.*, 2015; Naito & Pawlowska, 2016).

FISH experiments with probes specifically targeting '*Ca.* Moeniiplasma' (Naumann *et al.*, 2010) indicate that cells of these endobacteria are present in high numbers in the host cytoplasm (Figure 4). Quantitative PCR results support these observations, suggesting that '*Ca.* Moeniiplasma' can reach nearly 1000 cells per AMF spore (Desirò *et al.*, 2014), an estimate based on evidence of a single rRNA locus in the MRE genomes (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015).

Description of '*Candidatus* Moeniiplasma glomeromycotorum'

'*Candidatus* Moeniiplasma glomeromycotorum' (glo.me.ro.my.co.to'rum L. neut. n. *glomeromycotorum,* inhabitant of *Glomeromycota*). [(*Mollicutes*) NC; NA; C; NAS; oligonucleotide sequences of unique regions of the 16S rRNA gene 5'-GCCAATCCTACCCTTGTCA-3' (Naumann *et al.*, 2010) and 5'-

ATCCRTAGACCTTCMTCCTTC-3' (Desirò *et al.*, 2013); S (*Glomeromycota,* cytoplasm of mycelium and spores); M]. The phenotypic description is the same as that given above for the genus. Electron micrographs are shown in Figure 3.

Extensive intrahost diversity of '*Ca.* Moeniiplasma glomeromycotorum' 16S rRNA gene sequences is one of the most striking features exhibited by these organisms (Naumann *et al.*, 2010; Desirò *et al.*, 2014; Toomer *et al.*, 2015). Heritable endobacteria, such as '*Ca.* Moeniiplasma glomeromycotorum', are not expected to be diverse within host individuals because transmission bottlenecks limit the number of bacterial cells that are found in each new intrahost population (Moran *et al.*, 2008). In '*Ca.* Moeniiplasma glomeromycotorum', two factors appear to contribute to intrahost population diversity: (1) an ultrarapid rate of mutation accumulation (Naito & Pawlowska, 2016), which is likely related to the loss of DNA repair mechanisms (Naito *et al.*, 2015), and (2) recombination evident across DNA sequences sampled from '*Ca.* Moeniiplasma glomeromycotorum' populations associated with highly divergent AMF hosts (Toomer *et al.*, 2015; Naito & Pawlowska, 2016), consistent with retention of active recombination machinery in the '*Ca.* Moeniiplasma' genomes (Naito *et al.*, 2015).

The genealogy of '*Ca.* Moeniiplasma glomeromycotorum' reconstructed using 16S rRNA gene sequences (Figure 5) confirmed previous reports that, with few exceptions, '*Ca.* Moeniiplasma glomeromycotorum' sequences from a single host are dispersed across divergent clusters comprising '*Ca.* Moeniiplasma glomeromycotorum' associated with highly divergent *Glomeromycota* species (Naumann *et al.*, 2010; Desirò *et al.*, 2014; Naito *et al.*, 2015; Toomer *et al.*, 2015). Based on '*Ca.* Moeniiplasma glomeromycotorum' genome sequences (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015), this pattern appears to reflect the diversity of '*Ca.* Moeniiplasma glomeromycotorum' genotypes within a population, with a single rRNA locus per

genome, rather than diversity of multiple rRNA loci present in every genome of a genetically uniform population. In addition, no genetic differentiation is apparent among '*Ca.* Moeniiplasma glomeromycotorum' populations associated with isolates of a single AMF host from different geographic regions, *e.g. R. clarus* (Figure 5). This pattern is not unexpected given low genetic differentiation of AMF from different geographic locations (Rosendahl *et al.*, 2009; den Bakker *et al.*, 2010).

While only 10% of the *Glomeromycota* taxonomic diversity has been surveyed for the presence of '*Ca.* Moeniiplasma glomeromycotorum' thus far, the host taxa sampled represent the phylogenetic breadth of the phylum. Consequently, it is likely that a large portion of '*Ca.* Moeniiplasma glomeromycotorum' diversity has been discovered already, with the 16S rRNA gene sequences accumulated to date (Figure 5) displaying 79% similarity. While this degree of intraspecific sequence similarity is inconsistent with the recommendation that a 94% sequence similarity at the 16S rRNA gene should be used for separation of species in *Mollicutes* (Brown *et al.*, 2007), it reflects the unique biological properties of '*Ca.* Moeniiplasma glomeromycotorum'. In particular, all '*Ca.* Moeniiplasma glomeromycotorum' share: (i) the common habitat of the *Glomeromycota* cytoplasm, (ii) an ultrarapid mutation rate, and (iii) the ability to exchange genes across different genotypes. In addition, the present species delineation proposal for '*Ca.* Moeniiplasma glomeromycotorum' is consistent with species definitions in other heritable endobacteria, such as *Buchnera aphidicola* (Munson *et al.*, 1991) and *Wolbachia pipientis* (Lo *et al.*, 2007). These species share some of the molecular evolution patterns exhibited by '*Ca.* Moeniiplasma glomeromycotorum'.

While no type material designation is necessary for a provisional taxon (Labeda, 1997), we point out that AMF, which are hosts of '*Ca.* Moeniiplasma glomeromycotorum', are available at INVAM, http://invam.wvu.edu/.

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Figure Legends

Fig. 1. Phylogenetic placement of '*Ca.* Moeniiplasma glomeromycotorum' based on 16S rRNA gene sequences. Bayesian posterior probabilities greater than 0.90 are indicated above branches. Branches with Maximum Likelihood bootstrap support greater than 70% are thickened. MRc, '*Ca.* Moeniiplasma glomeromycotorum' of *Rhizophagus clarus;* MRv, '*Ca.* Moeniiplasma glomeromycotorum' of *Racocetra verrucosa;* MDh, '*Ca.* Moeniiplasma glomeromycotorum' of *Dentiscutata heterogama*.

Fig. 2. Phylogenetic placement of '*Ca.* Moeniiplasma glomeromycotorum' based on concatenated amino acid sequences of 19 conserved proteins. Bayesian posterior probabilities greater than 0.90 are indicated above branches. Branches with Maximum Likelihood bootstrap support greater than 70% are thickened. MRc, '*Ca.* Moeniiplasma glomeromycotorum' of *Rhizophagus clarus;* MRv, '*Ca.* Moeniiplasma glomeromycotorum' of *Racocetra verrucosa;* MDh, '*Ca.* Moeniiplasma glomeromycotorum' of *Dentiscutata heterogama*.

Fig. 3. Transmission electron micrographs of '*Ca.* Moeniiplasma glomeromycotorum' in the cytoplasm of *R. clarus* NB112A. **A.** Endobacteria (b) are directly embedded in the fungal cytoplasm (fc), near the fungal nucleus (n) and lipid bodies (lb). **B.** A homogenous electron-dense layer (arrowhead) is consistently present outside the membrane of the endobacteria, while many ribosomes populate their cytoplasm. Scale bars: A, 0.32 μm; B, 0.10 μm.

Fig. 4. FISH of '*Ca.* Moeniiplasma glomeromycotorum' within the cytoplasm of a crushed spore of *R. clarus* NB112A. **A**. MRE visualized with the MRE-specific probe, BLOgrBC (red). **B**. MRE visualized with the universal bacterial probe EUB338I (green). **C**. An overlay of A and B. Scale bars, 5 μm.

Fig 5. Genealogy of '*Candidatus* Moeniiplasma glomeromycotorum' based on 16S rRNA gene sequences. Bayesian posterior probabilities greater than 0.90 are indicated above branches. Branches with Maximum Likelihood bootstrap support greater than 70% are thickened. Each sequence represents '*Candidatus* Moeniiplasma glomeromycotorum' 16S rRNA genes sampled from a distinct *Glomeromycota* isolate and clustered at a 94% sequence similarity level.