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Glomus hyderabadensis, a new species: its taxonomy and phylogenetic comparison with related species

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Abstract—A novel *Glomus* species was isolated from rhizosphere soils supporting *Allamanda cathartica* from Hyderabad, India. The spores proliferate and bud out a daughter spore terminally which is connected to the mother spore by a small connective. Sequence analysis of D1D2 variable domain of 26S ribosomal RNA gene of this *Glomus* sp. shows that it is different from other known AM fungi. The morphological and molecular phylogenetic analyses support the creation of *Glomus hyderabadensis* sp. nov.

Key words—morphotaxonomy, molecular sequencing, phylogeny

Introduction

The genus *Glomus* Tulsane & Tulsane represents by far the largest genus within the Glomerales and is placed in the monogeneric family Glomeraceae (Schüßler et al. 2001). Based on rRNA SSU analysis and on the basis of natural relationship of AM fungi, a new fungal phylum Glomeromycota has been erected (Schüßler et al. 2001). Taxonomy and identification of glomalean fungi has traditionally relied on the morphology of their large multinucleate spores. Phylogenetic analysis based on ribosomal RNA gene sequences (Redecker et al. 2000, Schwarzott et al. 2001) indicate that the genus is non-monophyletic in nature.

During a survey of the rhizosphere soils of medicinal plants an interesting arbuscular mycorrhizal (AM) fungus belonging to genus *Glomus* was collected. The spores of this AM fungus are unique, as they proliferate / bud out a daughter spore terminally. The wall layers in the daughter spore are similar to the mother spore. This type of proliferation or bud out spore production is not reported in any of the existing AM fungi (Morton & Benney 1990; Schenck & Perez 1990; Manoharachary et al. 2002). Further the other spore characteristics do not match with any of the described species under genus *Glomus*. Sequence analysis of the D1/D2 variable domain of 26S ribosomal RNA gene shows that the sequence of this fungus is different from all the existing AM fungi reported in the literature, hence it is described here as a new taxon.

Materials and Methods

Collection of AM fungal spores

The AM fungal spores were isolated by wet sieving and decanting method (Gerdemann & Nicolson 1963) from the rhizosphere soil of *Allamanda cathartica* L. (Apocyanaceae).

DNA isolation

Twenty spores were washed thoroughly three times with sterile distilled water. DNA was isolated using GenElute™ Plant Genomic DNA kit, G2N10 (Sigma Chemical Company, St. Louis, USA). The fungal spores were mechanically disrupted in a 1.5 ml microfuge tube using disposable pistel (Sigma Chemical Company, St. Louis, USA). The lysis of spores and purification of DNA was performed as per manufacturer's instructions.

Amplification of ITS region and D1D2 region of 26S ribosomal RNA gene

The entire ITS and D1D2 variable domain of 26S rRNA was amplified with primers pITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') (White et al. 1990) and pNL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman & Robnett 1998). The primers were obtained from Integrated DNA Technologies, Inc., USA. Polymerase Chain Reactions were performed in a final reaction mixture (100μl) containing 50ng genomic DNA, 25pmols each of primers (pITS1 and pNL4R), 200 mM each of dATP, dTTP, dGTP and dCTP (Promega Corporation, USA); 2.5mM $MgCl_2$ and 2.0 units of Taq polymerase (Promega); and 10 μl of 10X reaction buffer (Promega). The amplification reactions were performed in a PTS 100 Mini Cycler (MJ Research, USA) with the following cycling parameters; initial denaturation for 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1.0 min at 72°C, with a final extension for 10 minutes at 72°C and cooled at 4°C. The amplified products were separated on 1.2%

agarose (Sisco Research Laboratories, India) gel by electrophoresis and visualized by staining with ethidium bromide (0.5μg/ml). Gel photographs were taken using VDS Image Master (Pharmacia Biotech, USA).

Sequencing of D1D2 region

Direct sequencing of PCR-amplified ITS and D1/D2 domains was performed by using ABI Big Dye Terminator kit. The D1D2 variable domain of 26S ribosomal RNA was performed with 2 primers, pNL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4. Sequencing reactions were purified by ethanol and sodium acetate precipitation, the pellet was washed twice with 70% ethanol, which considerably improved the removal of dye terminators from the reaction. Processing of the samples for loading on to ABI 310 model sequencer was performed as per manufacturer's instructions. The oligonucleotide primers used for sequencing were procured from Integrated DNA Technologies (IDT), USA.

Molecular phylogenetic analysis

The sequences were aligned using Clustal X program (Thompson et al. 1997) and manually corrected. Sequence similarity search was done using GenBank BLASTN search (Altschul et al. 1990), sequences of closely related taxa were retrieved and used for phylogenetic analysis. The GenBank accession numbers of the related species are given in the phylogenetic tree. For the Neighbour-Joining analysis (Saito & Nei 1987), the distances between the sequences were calculated using Kimura's two-parameter model (Kimura 1980). Sites where gaps existed in any of the sequences were excluded.

Nucleotide sequence accession number

The D1D2 domain sequence determined in this study has been deposited in GenBank database with the following number: AY211274

Results and Discussion

Glomus hyderabadensis Swarupa, Kunwar, Prasad et Manohar ***sp. nov.*** **Figs. 1-7**

Sporocarpia ignota. Sporae singulae, globosae, subglobosae vel ellipsoidae, 97-136 μm diametro, melleus vel aurantius-brunneus; sporae tunica e stratis tribus in uno terma (terma A). Hypha sustinens singulus, infundibuliformis. Maturus sporae prolificus terminalis, procreans gemmiformis sporae filiales; sporae filia sessilis, singulae, globosae, vel sub-globosae vel ellipsoideus. Sporae filialis consociatus cum nater sporae ab apertus connectivum.

[***Etymology***: Specific epithet derived from the place of collection (Hyderabad)].

HOLOTYPUS HIC DESIGNATUS ex ralix solum *Allamanda cathartica,* Hyderabad, India. 30. 5. 2001, leg. Swarupa, Herb. No. HCIO 43,918.

Sporocarps unknown. Spores formed singly, globose, subglobose to ellipsoidal, 97-136 μm in diam, honey coloured to orange-brown; spore wall as three walls in a single group (group A). Composite spore wall 3.3-5.9 μm thick, adherent, outer wall smooth or roughened, dull yellow, 1.1-2.4 μm, in older spores walls perforated, with an aperture of 1-1.5 μm diam, middle wall single, non layered, rigid, unit, orange-brown, 1.1-2.7 μm, inner wall rigid, non layered, dull yellow, 1.1-2.1 μm. Subtending hypha single, slightly flared toward the point of attachment, 15-32 μm thick, 136-233 μm long, rarely branched, pore in the subtending hypha occluded by a thick straight septum. Mature spores proliferating terminally, producing bud-like daughter spore; daughter spore sessile, single, globose, subglobose, ellipsoidal, 18-48.4 μm in diam, honey coloured to orange-brown; composite daughter spore wall 3-5.6 μm thick, number of wall groups one (group A), adherent, three wall layers, outer wall smooth or roughened, dull yellow, 1-2.2 μm thick, middle wall single, non layered, rigid, unit, orange-brown, 1-2 μm thick, inner wall rigid, non layered, dull yellow, 1-1.8 μm thick. Daughter spore linked to mother spore by an open connective, 5.6-9.5x8.2-11 μm.

Glomus hyderabadensis is characterized by the production of sessile, single, daughter spores from the mother spore and perforations in the spore wall. The spore measurements and wall characteristics also differ from the known species of *Glomus* (Schenck & Perez 1990). These observations merit its description and erection as a new taxon of species rank.

Glomus pustulatum Koske, Friese, C. Walker & Dalpe produces numerous hyaline, circular to irregular blister like thickenings on the outermost spore wall (Koske et al. 1986). In the present fungus only one daughter spore is produced per mother spore and the colour and wall structure of the daughter spore is like the mother spore. In *G. etunicatum* W.N. Becker & Gerd. the outer wall is smooth or roughened from decomposition of outer wall and adherent debris but has two groups (A and B) of spore walls and the composite wall is thicker (4-13 μm) (Becker & Gerdemann 1977), whereas in the new taxon only one group (A) of spore wall is present and it is thinner (3.3-5.9 μm).

Perforations in the wall are reported in *G. geosporum* (T.H. Nicolson & Gerd.) C.Walker also, but these perforations are sometimes absent from mature specimens and the middle wall is laminated. The inner wall is membranous and forms a septum separating the spore contents from the lumen (Walker 1982). However, in the present fungus perforations are present in the mature spores, outer wall is dull yellow and is always intact even at maturity unlike in *G. geosporum* where it is

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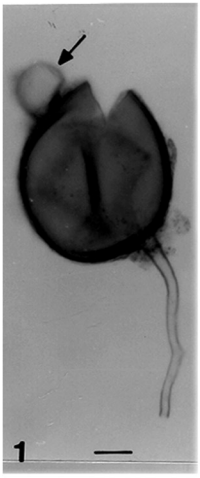
Figs. 1-7. *Glomus hyderabadensis* Scale bar = 30 μm. 1. Mother spore with young terminal daughter spore (arrow). 2. Daughter spore detached from mother spore, subtending hypha flared at the point of attachment with the spore (arrow). 3. Ellipsoidal spore. 4. Subglobose spore. 5. Daughter spore connected with the mother spore with a connective (arrow), septum (arrow) at the point of attachment of subtending hypha with the spore. 6. Similar three layered wall in mother and daughter spore (arrows). 7. Perforations (arrows) in the spore wall.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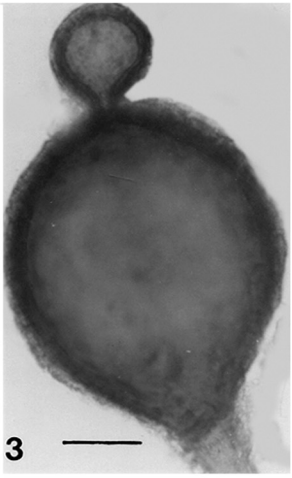
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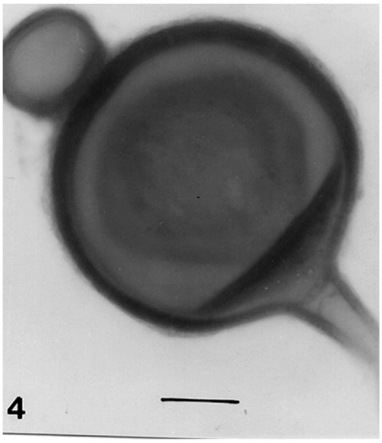
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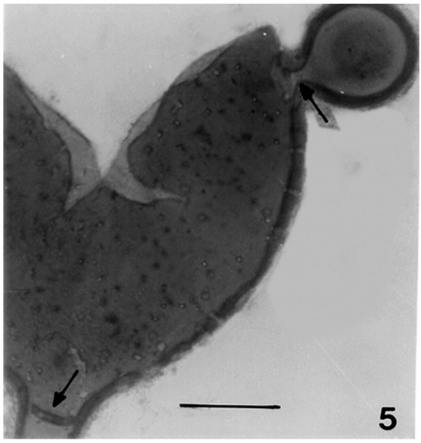
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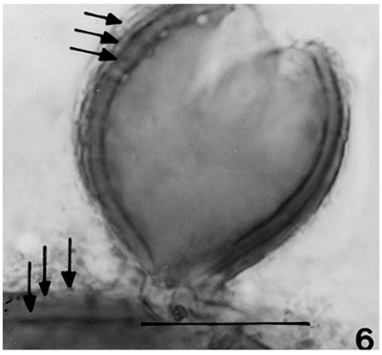
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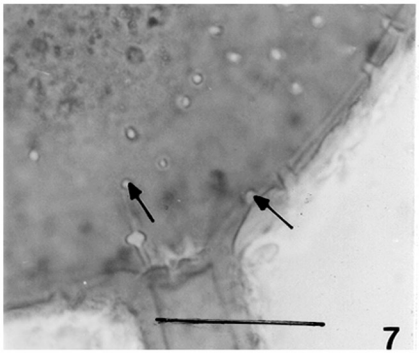
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hyaline and sometimes absent from mature specimens. In *G. multisubstensum* Mukerji, Bhattacharjee & J.P. Tewari and *Gigaspora rosea* T.H. Nicolson & N.C. Schenck (= *Gi. candida* Bhattacharjee, Mukerji, J.P. Tewari & Skoropod) perforations are reported but they seem to be caused due to hyperparasitism. In. *G. multisubstensum* the perforations were seen in surface view while in sectional view transverse fissures were seen (Mukerji et al. 1983). In *Gi. rosea* the perforations in the spore wall appeared to be caused by the parasitic action of soil microorganisms since its inner surface showed numerous hypha like structures passing through the perforations (Bhattacharjee et al. 1982). The perforations were either dispersed or in groups and varied in size indicating that more than one type of parasite may have been involved, while in our study of *G. hyderabadensis* perforations were uniformly distributed and were mostly uniform in size.

In *G. globiferum* Koske & C. Walker the spores are formed singly or in pairs or triplets adhering to each other by common peridial hyphae, bearing numerous terminal or intercalary, globose or ovoid vesiculate swellings (Koske & Walker 1986). Wheras in *G. hyderabadensis* peridium is absent with single daughter spore attached to the mother spore.

Phylogenetic analysis

The ITS and D1/D2 region was amplified with primers ITS1 and NL4, the amplicon was about 1.2 kb. Attempts to sequence the ITS region with primers ITS1, ITS2, ITS3 and ITS4 were unsuccessful for unknown reasons. As an alternative, we have sequenced the D1/D2 domain of 26S rRNA gene. The 26S rRNA domain D1/D2 have been determined for all the known yeast taxa (Kurtzman & Robnett 1998; Fell et al. 2000) and are extensively used for describing new yeast species along with morphological and physiological characters. With few exceptions, the yeast taxa that differ by more than 1% variation in the D1/D2 domain are considered as separate species (Kurtzman & Robnett 1998; Fell et al. 2000). The D1/D2 sequence of PCR amplified ITS-D1D2 fragment was determined using primers NL1 and NL4. Both the strands of the PCR product were sequenced. The determined sequence is 510 bases in length.

GenBank search using BLAST (Altschul et al. 1990) resulted in retrieval of several algal sequences. However, these sequences showed 23.6 to 35.1% variation with *G. hyderabadensis* sequence and appear to be not closely related to *G. hyderabadensis*. The first 10 hits were as follows, GYNRGNP *Gymnodinium catenatum* Graham, AF409122 *Mallomonas asmundae* (Wujek and van der Veer) Nichols, AF409121 *Chrysolepidomonas dendrolepidota* Peters and Anderson, AF318261 *Dinophyceae* gen. sp. *antifer* Cons4, AF409125 *Vacuolaria virescens* Cienkowski, AF210742 *Vacuolaria virescens,* AF417673 *Nitzschia laevis* Hust., AF417670 *Nitzschia alba* (Kütz) W. Smith, AF210743 *Olisthodiscus luteus* Carter and OD28SRRNA *Ochromonas danica* Prings. *Glomus* sp. HCIO – 43,918 differs from the algal taxa by 23.6 to 35.1% variation. The sequence divergence among the

algal taxa used in the analysis ranged from 15.5 to 34.3%, suggesting that they are highly divergent sequences. Our attempts to culture algae from the crushed spores of *Glomus* sp. HCIO-43,918 on different media did not yield any algal species.

As the morphotaxonomic features of the new fungus suggest that it may be a new *Glomus* species, we restricted our next BLAST search to Glomeromycota. The first hits in the BLAST search were *Archaeospora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) J.B. Morton & D. Redecker AJ510234, AJ271712 and AJ510233 followed by *Glomus occultum* C. Walker AJ271713. Phylogenetic position of *G. hyderabadensis* in relation to other AM fungi based on D1/D2 variable domain sequences is shown in Figure-8. Based on the sequence analysis of D1/D2 domain *Ar. gerdemannii* is the nearest relative of *Glomus* sp. HCIO – 43,918, but the

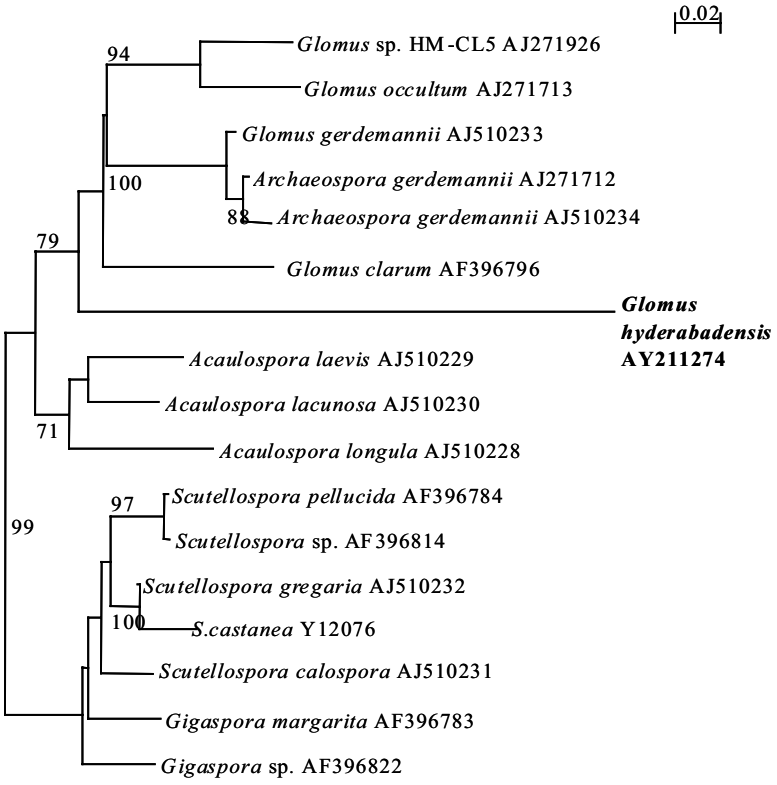

Fig. 8. Phylogenetic tree depicting the relationship between *Glomus hyderabadensis* and other reference taxa. The tree was constructed based on D1/D2 variable domain of 26S rDNA using program N J Plot. Bootstrapping was done in 1000 replications. Values for frequencies less than 70% are not given.

new fungus differs from its nearest relative by more than 29.2% sequence variation. The sequence variation between two isolates of *Ar. gerdemannii* was 0.8%. Variation among the three *Acaulospora* sp. viz. *A. lacunosa* J.B. Morton, *A. longula* Spain & N.C. Schenck and *A. laevis* Gerd. & Trappe ranged from 9.3 to 9.8% indicating that they are distinct species.

Among the *Glomus* species, *Glomus* sp. strain HM-CL5 has more sequence imilarity to *Glomus* sp. HCIO – 43,918, however both of them differ from each other by 30.5% variation. *G. occultum* is the nearest relative of *Glomus* sp. strain HM-CL5 (6.8% variation). Interestingly *Glomus* sp. strain HM-CL5 differs from other *Glomus* species used in the sequence analysis by 16.1 to 22.2% variation. These results indicate that D1/D2 sequences are highly divergent among *Glomus* species.

These results together with the novel morphotaxonomic features of *Glomus* sp. HCIO – 43,918 support the description of a new taxon *G. hyderabadensis*.

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