Mycorrhiza (1996) 6:161–166

ORIGINAL PAPER

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Redescription of *Glomus caledonium* based on correspondence of spore morphological characters in type specimens and a living reference culture

Accepted: 12 February 1996

Abstract *Glomus caledonium* accession UK301 from Rothamsted, England was designated a living reference culture of the species based on close correspondence of spore morphological characters with those of preserved holotype (Farlow Herbarium) and paratype (Oregon State University) specimens. Morphological characters were defined and interpreted according to their origin and sequence in spore differentiation. Three discrete stages were discriminated by simultaneous appearance of new layers in the spore wall and in the wall of the subtending hypha. An outer mucilaginous layer nonreactive in Melzer's reagent and a more rigid hyaline layer were present initially in the most juvenile spore wall, followed by de novo formation of a granular layer and and a yellow-brown laminate layer. Spore wall differentiation terminated with the innermost sublayer of the laminate layer occluding the hyphal pore. A germ tube, when present, originated in the lumen of the subtending hypha near the occluding sublayer. A preserved voucher of an isolate from France and a living culture from Denmark possessed corresponding subcellular characters to those of isolate UK301, thus establishing definitive morphological criteria to group different geographic isolates of the species.

Key words Development · Taxonomy · Vesicular-arbuscular mycorrhizal fungi

Introduction

Taxonomic decisions based on morphology of spores of arbuscular mycorrhizal fungi (Glomales, Zygomycetes) are most problematical in the genus *Glomus* for a number of reasons. First, the number of species described is almost triple that in any other genus (Morton 1988), but the subcellular morphological characters defining species are less discrete and, therefore, more difficult to separate and interpret. Many diagnostic characters, such as arrangement of types of "walls" of the spore (sensu Morton 1988; Walker 1983) intergrade in a bewildering range of variation. Second, many diagnostic surface "walls" slough with age or degrade from hyperparasitism, abiotic factors, and preservatives (Morton 1993). Third, early descriptions of *Glomus* species were written with little available information on the range of variation in morphological characters, so that documentation often was not sufficiently detailed. Last, type specimens tend to change their appearance from the time they are collected, due to effects of preservation media such as lactophenol (Morton 1993). A combination of these factors has hindered unequivocal identification of many species in large, living culture collections, such as the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) (Morton et al. 1993).

An operational taxonomy to catalog and inventory accessions in INVAM was implemented utilizing "reference cultures", each one representing a putative species defined by one or more unique morphological characters in reproductive spores. All other accessions with correspondence in these characters were grouped as members of the same "species". A species was validated and assigned a Latin name only when defining (or diagnostic) morphological characters of the reference organism matched those of types and/or published descriptions. For unnamed species, the record number of the reference culture in the INVAM Culture Database (Microsoft Access, Version 7) was assigned as a temporary specific epithet. The crucial objective in these comparative analyses, therefore, was to reconcile fresh specimens from cultures with preserved materials and descriptions.

In this paper, the INVAM reference culture of *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd. is

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designated by correspondence with type specimens in sufficient condition to match diagnostic structural features. Spore characters of living and preserved specimens are defined and interpreted according to their patterns of differentiation during spore ontogenesis and the species is redescribed.

Materials and methods

Spores of the holotype specimen of *G. caledonium* from the Farlow Herbarium, which had been preserved in lactophenol, were collected and mounted permanently on a glass slide in PVLG, a mixture of polyvinyl alcohol, glycerin and lactic acid (Koske and Tessier 1983), as well as in PVLG mixed 1:1 (v/v) with Melzer's reagent. Similarly preserved spores from the Oregon State University Herbarium (OSC 29,432) were mounted permanently on a glass slide and broken. One voucher slide contributed by Peter Wyss (isolate LPA12, from V. Gianinazzi-Pearson, Dijon, France) consisted of broken spores permanently mounted in PVLG.

UK301 (contributed by R. D. P. Cargeeg, Agricultural Genetics, Rothamsted, England) was designated as the INVAM reference culture of the species *G. caledonium*. INVAM culture DN986 (contributed by Ivar Jakobsen, RISØ National Laboratory, Roskilde, Denmark, isolate RIS 42) produced spores of similar morphology and thus was included in the comparison. All cultures were started from whole inoculum (growth medium, chopped roots, spores, hyphal fragments) provided by the contributor mixed 1:10 (v/v) with a potting medium consisting of a sandy loam soil (Lily series) premixed 1:2 (v/v) with quartzite sand (average grain size = 0.9 mm) and limed to pH 6.2 after steaming. This mix was placed in 15-cm diameter plastic pots and seeded with sudangrass [*Sorghum sudanense* (Piper) Staph.] at a rate of 80–100 seeds/pot. Plants were grown in a greenhouse under fluorescent lights (photosynthetically active radiation of 245 μmol m^{-2} s^{-1} at pot level) with a 14-h photoperiod. Room temperatures ranged from 19 to 30 °C during the culture period. Mature spores were extracted from a pot sample 3 months after plants emerged (1 month prior to drying and harvest) by sucrose density-gradient centrifugation (Daniels and Skipper 1982) to assess purity (presence of only one spore morphotype) and abundance.

Patterns of ontogenesis of subcellular characters were defined by sequential sampling of a culture outside the region containing the starter inoculum (with its abundance of mature/degrading spores). Whole inoculum of *G. caledonium* UK301 obtained after one propagation cycle (to verify culture purity) was diluted 1:10 (v/v) with sand, placed in two 4 × 21-cm Cone-tainers (Stuewe and Sons, Corvallis, Ore.), and seeded with sudangrass. Cultures were grown for 10 weeks, after which the contents of each cone-tainer were transferred to a nylon 100-μm mesh sleeve (Tetko, Briarcliff Manor, N.Y.). Each sleeve was transplanted into the center of a 15-cm diameter plastic pot surrounded by the same growth medium described above and seeded with sudangrass. A soil core was removed from opposite sectors of each pot beginning 6 weeks after plant emergence and again at three additional weekly intervals to extract newly formed spores. A minimum of 50 spores from each sample was washed, examined, and mounted within 24 h of collection.

Color of whole spores and subcellular spore characters was measured by examining specimens and a printed chart (available from the author) illuminated simultaneously with a two-branch fiber optic illuminator at 3400°K. Color values are reported as percentage cyan, magenta, yellow, and black. Juvenile spores were distinguished by their bright white color and dense opaque contents. Older spores were cream-colored (0-0–20–0) to yellow-brown (0–10–80–0) with translucent contents.

Juvenile and older spores were collected separately using a Pasteur pipette and mounted in PVLG and PVLG mixed with Melzer's reagent (1:1 v/v) on glass slides. The following voucher specimens of *G. caledonium* isolates are stored in a permanent collection in INVAM at West Virginia University: R. Cargeeg, s. n., (INVAM UK301), slides 2814–15, 2866–67, 2870, vials (0.5% sodium azide) 1536, 1569, color photos 202–205; I. Jakobsen, RIS 42 (INVAM DN986), slides 1741, 2340, 2843, vial 940; P. Wyss, LPA12, slide 1717.

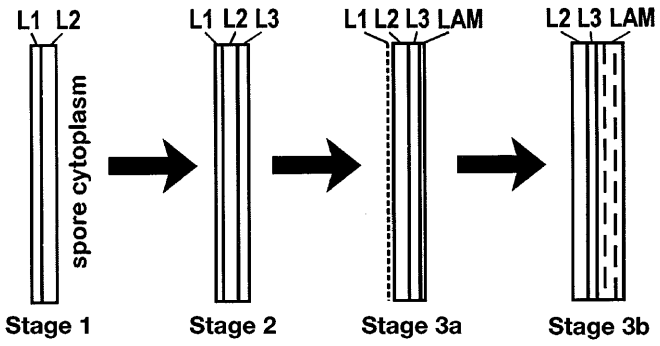


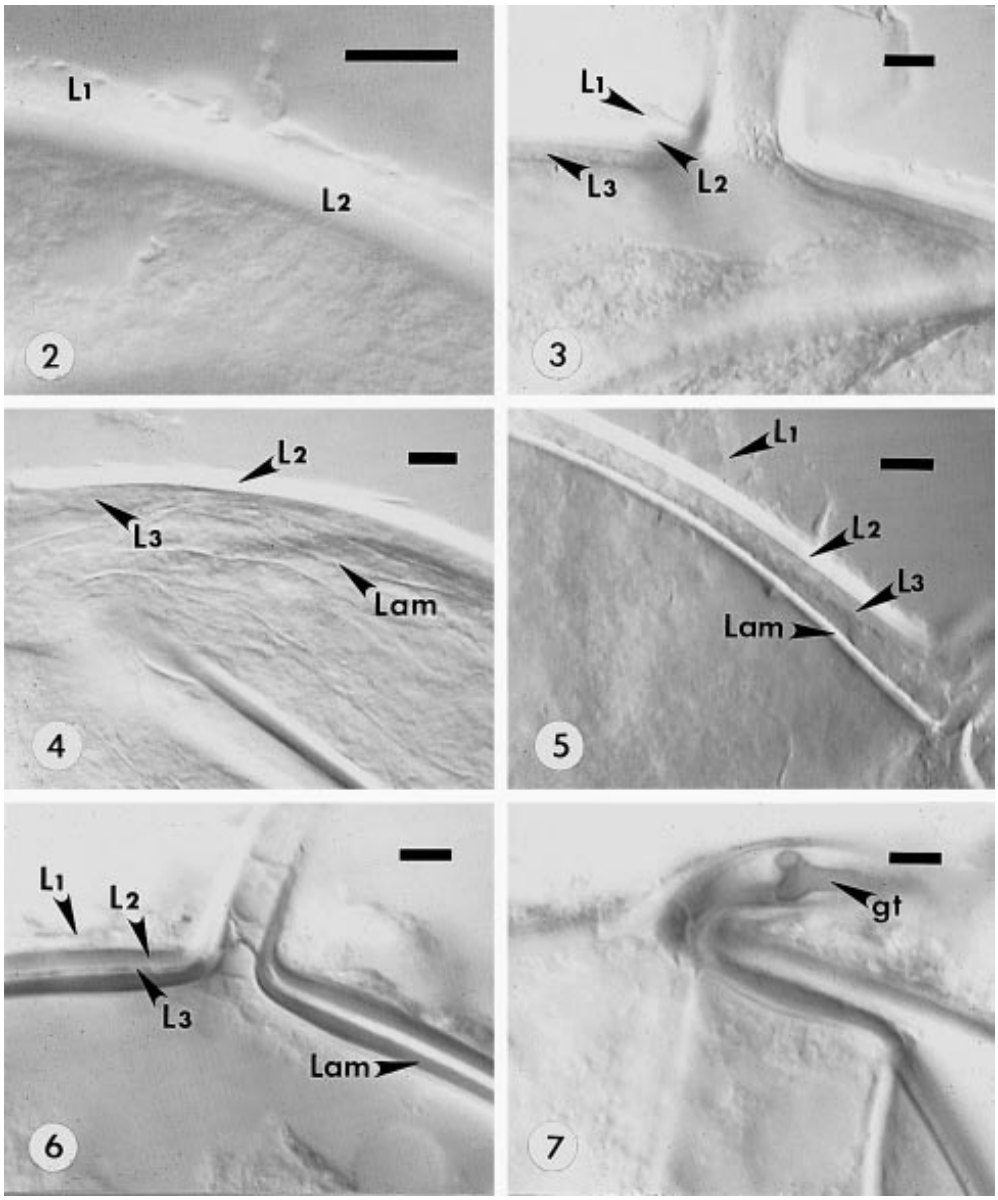
Fig. 1 Murographic illustration of discrete stages in ontogenesis of spores of *Glomus caledonium* reference culture UK301. Stage 3 is divided into early (3a) and late (3b) substages based on first and subsequent appearance of sublayers (*dashed lines*) in the laminate layer (*LAM*). *L1* Mucilaginous hyaline layer nonreactive in Melzer's reagent, *L2* rigid hyaline layer, *L3* granular layer birefringent in polarized light. The *outer dotted line* indicates sloughing

Results

Developmental patterns

Differentiation in spores of *G. caledonium* occurs in three discrete stages, where each stage is distinguished by the appearance of a new layer in the spore cell wall (Fig. 1). All of these characters originate in common with the wall of the fertile subtending hypha, and so they are considered to be distinctive layers of only one structure, the spore wall. Each discrete layer produces a unique phenotype at the time it has completed differentiation. The laminate layer of the spore wall is somewhat different from other layers in that it consists of a variable number of sublayers of identical phenotype. The terminology employed in this paper approximates traditional usage (Morton 1988; Walker 1983), except that "layer" is substituted for "wall" to reflect the common developmental origins of spore wall components (Berch 1986; Franke and Morton 1994).

In stage 1, the spore wall consists of two hyaline adherent layers (L1 and L2) which are a continuation of the juvenile wall of the subtending hypha. L1 is mucilaginous and 2–3.5 μm thick in the most juvenile spores (Fig. 2). Thickness is difficult to measure in older spores because the outer boundary becomes more plastic and indistinct in the mounting media. It either is nonreactive in Melzer's reagent or occasionally stains a pale pink color (0–20–20–0) on the wall of the subtending hypha. L2 is rigid, 1.5–3.5 μm in thickness, and also does not react in Melzer's reagent. Granular material



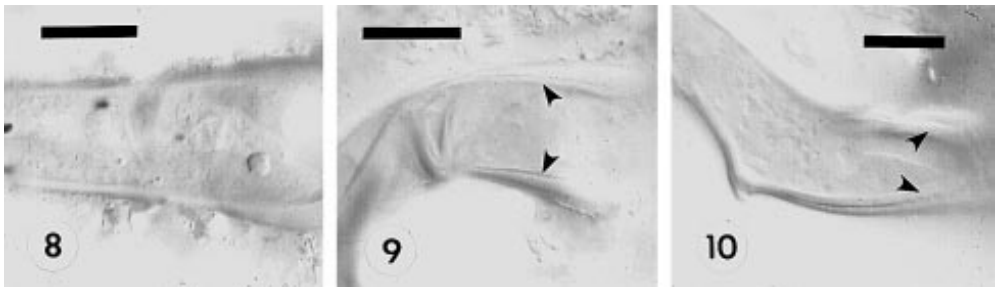
Figs. 2–7 The sequence of morphological character differentiation in spores of *G. caledonium* UK301 mounted in PVLG + Melzer's reagent (1:1 v/v) and photographed with differential interference contrast (DIC) optics; *bars* 10 μm. **Fig. 2** Stage 1, with two layers of the spore wall formed (*L1* and *L2*, see Fig. 1). **Fig. 3** Stage 2, with formation of a new granular layer (*L3*). **Fig. 4** Beginning of stage 3 (denoted as 3a in Fig. 1), with synthesis of the first sublayer of the laminate layer (*Lam*). **Fig. 5** Later stage 3 (denoted as 3b in Fig. 1), with more sublayers in the laminate layer. **Fig. 6** Mature spore with all four layers in the spore wall (*L1–L3*, *Lam*) fully differentiated. **Fig. 7** Germ tube (*gt*) formation near the region of the septum

accumulates on the inner surface of the rigid, hyaline layer in stage 2 to form an intact layer (L3) which is 2–3.5 μm thick (Fig. 3). L3 originates as part of the subtending hyphal wall, but only in the region proximal to the spore.

Pale yellow-brown (0–10–20–0) sublayers synthesized in stage 3 together constitute the laminate layer (LAM) of the spore wall. The first sublayer originates de novo as part of both the differentiating subtending hyphal wall and the spore wall (Figs. 4, 9). It is very thin (<0.5 μm) and produces many folds in a broken spore (Fig. 4). Rigidity is imparted to this layer as additional adherent sublayers are synthesized (Fig. 5). L3 now is sandwiched between two rigid components (L2 and LAM) and appears as a thinner (<1 μm) birefringent layer in polarized light (Fig. 6). During this stage, L1 undergoes degradation and either sloughs (Fig. 5) or accumulates some organic debris. Spore contents are occluded near completion of LAM differentiation, with the innermost sublayer bridging the hyphal pore (Fig. 6). At the end of stage 3, the subtending hyphal wall is a continuation of L2 and the LAM of the spore wall (Fig. 10). The outer mucilaginous layer (L1) usually is sloughed. Germ tube development originates near the occluding septum (Fig. 7), with no evidence of a pregermination structure. Germination occurs when a germ tube emerges from the lumen of the subtending hypha.

Comparative taxonomy

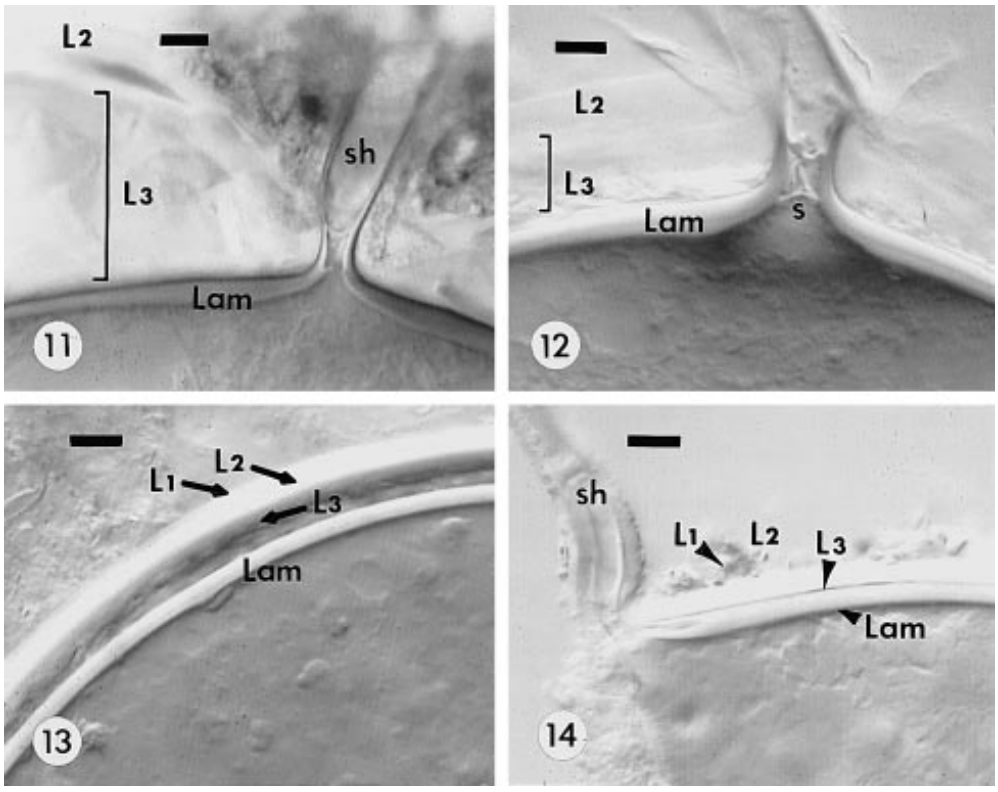
Spores of *G. caledonium* from holotype (Farlow) and paratype (OSU) material contain all subcellular characters of fresh spores found in reference culture UK301, with the exception of the outermost mucilaginous layer



Figs. 8–10 Sequence of differentation in the subtending hypha of spores of *G. caledonium*UK301 in PVLG + Melzer's reagent; photographed with DIC optics; *bars* 10 μm. **Fig. 8** Two layers continuous with L1 and L2 in stage 1 of spore wall differentiation (see Fig. 2). **Fig. 9** Synthesis of a yellow-brown layer (*arrows*) continuous with the laminate layer of the spore wall in stage 3 (see Figs. 4, 5). **Fig. 10** Thickened yellow-brown layer (*arrows*) continous with laminate layer of the spore wall

(Figs. 11–12). Differences in properties of these characters were noted, but they did not contrast sufficiently to be considered uniquely different. The hyaline layer (corresponding to L2) is less intact and separates frequently from the laminate layer when spores are mounted and broken. The layer corresponding to L3 does not have the granular appearance noted in fresh spores, appearing instead as an amorphous matrix of highly birefringent fragments under polarized light. Without a clearly defined edge, thickness of L3 varies with degree of separation from adjacent layers of the spore wall. Other preserved specimens, namely mounted spores of isolate LPA12, contained all component layers of the spore wall, including the outer mucilaginous layer (Fig. 13).

Spores in the original dried inoculum of accession UK301 are 200–320 μm in diameter and thinly coated with soil particles which impart a yellow-orange (0–20–40–0) to orange-brown (0–30–100–0) color. Fresh spores extracted from two subsequent propagation cycles were lighter, ranging in color from light yellow (0-0–30–0) to yellow brown (0–10–80–0). Size of mature spores varied between propagation cycles, from 180–360 μm (mean of 274 μm) in the first cycle to 160–280 μm (mean of 214 μm) in the second cycle. Freshly extracted spores of INVAM accession DN986 (RISØ isolate RIS 42, I. Jakobsen) were within the same size (220–320 μm) and color (yellow-brown) range as UK301. The spore wall consisted of identical layers (Fig. 14) to those found in spores of UK301. Minor variation was expressed in a slightly thinner outer mucilagenous layer (0.6–2 μm), when present, and more frequent separation of all outer layers from the laminate



Figs. 11–14 Comparison of spore subcellular structure in isolates of *G. caledonium* other than reference culture UK301. Spores were mounted in PVLG + Melzer's reagent and photographed with DIC optics. *S* Septum, *sh* subtending hypha, other abbreviations as in Figs. 2–7; *bars* 10 μm. **Fig. 11** Preserved holotype specimen from Farlow Herbarium. **Fig. 12** Preserved paratype specimen from the Oregon State University Herbarium (OSC 29,432). **Fig. 13** Voucher specimen from P. Wyss, isolate LPA12 (Dijon, France). **Fig. 14** Freshly extracted spore from INVAM culture DN986-1 (I. Jakobsen isolate RIS 42)

layer in broken spores such as that found in type specimens.

Biogeographic patterns

Despite the absence of some morphological details in the species description of *G. caledonium*, the species has been identified on all continents except Antarctica based on its origin in experiments (Abbott 1982; Daft and Hogarth 1983; Hetrick et al. 1984; Joner and Jakobsen 1994; Lioi and Giovannetti 1987; Walker and Vestberg 1994; Wyss and Bonfante 1993), in survey lists (Blaskowski 1994; El-Giahmi et al. 1993; Gerdemann and Trappe 1974; Hall 1977; Koske 1987; Saito and Vargas 1991), and in origin of INVAM accessions (this paper). This redescription and the presence of a living reference culture provides a species concept to validate these and other biogeographic data.

Discussion

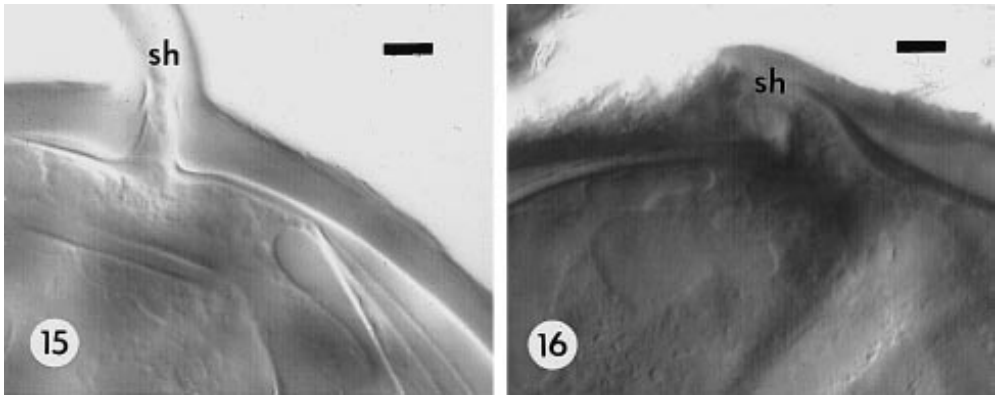
Differentiation of layers in the spore wall of *G. caledonium* spores from reference culture UK301 fits patterns of a developmental model proposed for Glomales by Morton et al. (1995). The sequence is linear, with each layer of the spore wall developing its own unique phenotypic properties before the next layer begins to form. However, some layers of the spore wall (e.g. L3, LAM) arise de novo and concurrently in the spore and the wall of the subtending hypha. This pattern differs uniquely from that found in spores of *Gigaspora* and *Scutellospora* species, where no new layers of the subtending hyphal wall are formed during spore wall differentiation (Bentivenga and Morton 1995; Franke and Morton 1994; Morton 1995).

Juvenile stages of spore wall differentiation (stages 1 and 2) of *G. caledonium* were recovered only from samples collected at 6 and 7 weeks post-plant emergence. Studies with four other *Glomus* species (S. Stürmer and J.B. Morton, unpublished) verify a short "window" between 4 and 8 weeks after culture initiation to collect early juvenile spores in this genus. In contrast, juvenile stages are obtained at all sampling dates from cultures of different *Scutellospora* species (Franke and Morton 1994; Morton 1995).

Spores from living cultures of INVAM accessions UK301 and DN986 had a narrower size range than geographic isolates described by Gerdemann and Trappe (1974) and Nicolson and Gerdemann (1968). Similar trends in size variation were found in species of *Scutellospora* (Morton 1995). Described spore wall structure (Gerdemann and Trappe 1974; Hall 1977; Nicolson and Gerdemann 1968) also did not exactly match that of fresh or preserved spores. The outer mucilaginous layer and the granular layer are not mentioned, but they are understandable omissions given that the former often is sloughed in mature spores (especially those from field collections) and the latter is difficult to detect in intact or slightly broken spores using only brightfield optics.

Correspondence between type and fresh specimens of *G. caledonium* was clearly evident once spore characters were defined from ontogenetic comparisons. Type specimens showed no reaction of any layers to Melzer's reagent, which was due either to inherent properties or inhibition by the preservation medium (Morton 1986). Examination of developing spores clearly established that the outer mucilaginous layer was nonreactive on spores of *G. caledonium* (Figs. 2–3), an important exception diagnostically since this layer produces a strong reddish-purple (0–20–80–0) reaction in a large proportion of known *Glomus* species (Figs. 15, 16; Morton and Benny 1990). The granular layer (L3) was more friable in preserved spores and was separated into many birefringent fragments. The extent to which this variation is a direct effect of the lactophenol preservative was not determined, but it seems to be the most likely cause. Spores stored dry with roots in pot culture medium for longer than 1 year do not exhibit this variation.

The two *Glomus* species most easily confused with *G. caledonium* are *G. clarum* Nicol. & Schenck and *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe because of close resemblance in size and color under a dissecting microscope. However, spores of *G. clarum* have a much wider color range, from bright white to dark yellow-brown. Spores of *G. mosseae* also often have a noticeably flared subtending hypha and a recurved occluding septum invaginating some distance



Figs. 15, 16 Comparison of subcellular structure in spores from INVAM reference cultures of species sometimes confused with *G. caledonium*. Spores were mounted in PVLG and Melzer's reagent and photographed with DIC optics; *bars* 10 μm. **Fig. 15** Freshly extracted spore of *G. clarum* FL239. **Fig. 16** Freshly extracted spore of *G. mosseae* UK115

into the hyphal lumen. Spore wall structure of these two *Glomus* species also differs from that of *G. caledonium*. In spores of *G. clarum*, the outer mucilaginous layer reacts strongly in Melzer's reagent, the hyaline layer is thick (5–12 μm) and laminate instead of unitary, a granular birefringent layer is not present, and another thinner (2–4 μm) pale yellow to yellow laminate layer also is present (Fig. 15). The spore wall of *G. mosseae* is similar to that of *G. caledonium* in having an outer mucilaginous layer, a birefringent layer, and a yellow-brown laminate layer. However, the mucilaginous layer reacts strongly in Melzer's reagent and the hyaline layer (L2) found in *G. caledonium* spores is absent (Fig. 16).

In summary, ontogenetic interpretations of spore wall subcellular characters provided a definitive basis to reconcile differences between fresh and type specimens. Correspondence among isolates from different geographic origins also establishes an unambiguous morphological species concept of *G. caledonium*.

Acknowledgements I thank Drs. Steve Bentivenga, Jim Graham, John Klironomos, Rick Koske, and Frank Pfleger, Mr. Sidney Stürmer, Ms. Kelly Heldreth, and an unnamed reviewer for valuable comments. The kind assistance of Drs. Aaron Liston and Richard Halse at Oregon State University and Donald Pfister at Farlow Herbarium in providing type specimens is appreciated. This work was carried out as taxonomic studies for NSF grant DIR-9015519 and the West Virginia Agricultural and Forestry Experiment Station.

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