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Revision of *Entrophospora* and description of *Kuklospora* and *Intraspora*, two new genera in the arbuscular mycorrhizal Glomeromycetes

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Summary

Five mycorrhizal fungal species of the Glomeromycetes which were organized in the genus *Entrophospora* are revised. They all form their spores within the hyphal stalk directly beneath or in some distance of a sporiferous saccule formed intercalary or terminally in the mycelium. Based on differences respective similarities in spore morphologies and root infection characteristics only *Entrophospora infrequens* and *Entrophospora baltica* remain in this genus. The genus is the type genus for the new family Entrophosporaceae. The other three species are organized in two new genera. *Kuklospora* gen. nov. with *Kuklospora colombiana* and *Kuklospora kentinensis* (formerly *Entrophospora colombiana* and *Entrophospora kentiniensis*) is placed into the family of the Acaulosporaceae. *Intraspora* gen. nov. so far contains only *Intraspora schenckii* (the former *Entrophospora schenckii*) and is included into the family of the Archaeosporaceae. The morphological differences between the genera and the distribution of these fungal species in ecosystems are discussed.

Introduction

Arbuscular mycorrhizal (AM) fungi are important soil microbiological genetic resources for the functioning of terrestrial ecosystems (see overview in: VAN DER HEIJDEN and SANDERS, 2002). The fungi are taxonomically organized in the recently described new phylum Glomeromycota (SCHÜSSLER et al., 2001). For ecosystem studies it is important that the fungi are correctly named and classified to understand the relationships between the ecosystem components. The Glomeromycota have one class, the Glomeromycetes (CAVALIER-SMITH, 1998). SCHÜSSLER et al. (2001) named four orden. Currently, seven families and nine genera are known in the Glomeromycota (http://tolweb.org/tree?group=Glomeromycota).

Of the five mycorrhizal species which were included in the genus, *Entrophospora* R.N. Ames & R.W. Schneid. (AMES and SCHNEIDER, 1979), *E. infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid. was originally described by HALL (1977) as *Glomus infrequens*. The reason was that its spores showed a short 'subtending hypha' giving the spores a glomoid appearance. AMES and SCHNEIDER (1979) discovered that spores of this species are subterminally formed 'in the stalk of a vesicle', i.e. directly beneath a terminally or intercalary swollen hypha. Such swollen structures were known from *Acaulospora* Gerd. & Trappe and were called 'vesicles' by GERDEMANN and TRAPPE (1974). They had originally been named 'mother spore' by MOSSE (1970) when she described 'honey coloured, sessile *Endogone* spores' . Those 'vesicles' were later designated 'hyphal inflated terminus' (JANOS and TRAPPE, 1982) or 'sporiferous saccule' (WALKER et al., 1984). The later term has been widely used until today (MORTON and BENNY, 1990; STÜRMER and MORTON, 1999; OEHL et al., 2006). At the site of the hyphal stalk (*sensus* support) of the sporiferous saccule where the spore of *Entrophospora* is formed, the hyphal stalk is inflated by the growing spore. The spore then appears to have two hyphal attachments – proximal and distal to the saccule – with the hyphal wall forming the outer layers of the spore wall. After degrading and detaching of these outer wall layers and of the sporiferous saccule, the spore of e.g. *E. infrequens* remains with a short stalk which was its connection with the sporiferous saccule.

The mycorrhizal status of the first species described of the genus *Entrophospora* was not clear as formation of mycorrhizae by *E. infrequens* in pot cultures failed (AMES and SCHNEIDER, 1979). It was a few years later that pure pot cultures were obtained at the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, confirming that *E. infrequens* forms mycorrhiza with *Pueraria phaseoloides* (Roxb.) Benth. The formation of sporiferous saccules and spores inside the roots and also of arbuscles, vesicles and hyphal structures in the roots was reported (SIEVERDING and TORO, 1985). Whereas pure pot cultures of three other *Entrophospora* spp., *E. colombiana* Spain & N.C. Schenck (SCHENCK et al., 1984), *E. schenckii* Sieverd. & S. Toro (SIEVERDING and TORO, 1987) and *E. kentinensis* C.G. Wu & Y.S. Liu (WU et al., 1995b) were repeatedly established (SIEVERDING and TORO, 1985; 1987; WU et al., 1995a; FRACCHIA et al., 2003), *E. baltica* Blasz. (BLASZKOWSKI et al., 1998) failed to grow in pure cultures. Several attempts have been started to again establish *E. infrequens* in pure cultures but failed over the last years. RODRIGUEZ et al. (2001) speculated that spores of *E. infrequens* may only be formed when the cultures are contaminated with other mycorrhizal fungi, or may form spores only in 2-3 years old pot cultures when growing in consortia with other mycorrhizal fungi. However, SIEVERDING and TORO (1985) had reported that their pure pot cultures of *E. infrequens* were established within nine months when they checked them for purity the first time.

Species described in the genus *Entrophospora* share one feature: They form a single spore within the hyphal stalk directly beneath or in some distance of a sporiferous saccule. Sporiferous saccules are known from the genera *Acaulospora* Gerd. & Trappe (GERDEMANN and TRAPPE, 1974) and *Archaeospora* J.B. Morton & D. Redecker (MORTON and REDECKER, 2001), but spores of both genera are formed laterally on the neck (i.e. on the hyphal stalk) of the sporiferous saccule. A closer look at the type of spore formation in addition to observations of the spore wall characteristics and investigations of the root infection structures make it obvious that three of the above mentioned *Entrophospora* spp. species are distinctly different from *E. infrequens*, and that they have important characteristics in common with other families of the Glomeromycetes. Of these three species, *Entrophospora colombiana* and *E. kentinensis* share three characteristics with Acaulosporaceae: their spores have three walls (STÜRMER and MORTON, 1999; OEHL et al., 2006) and a 'beaded' wall layer on the inner wall, and they form vesicular-arbuscular mycorrhizal structures in the roots which stain visibly blue with trypan blue. On the other hand, *Entrophospora schenckii* shares feature with *Archaeospora trappei* (R.N. Ames & Linderman) J.B. Morton & D. Redecker comb. nov. (MORTON and REDECKER, 2001): the relative thick inner wall, and the fungal structures that, except spores, do not stain or stain only weakly with trypan blue. The distinct differences justify the exclusion of *E. colombiana*, *E.*

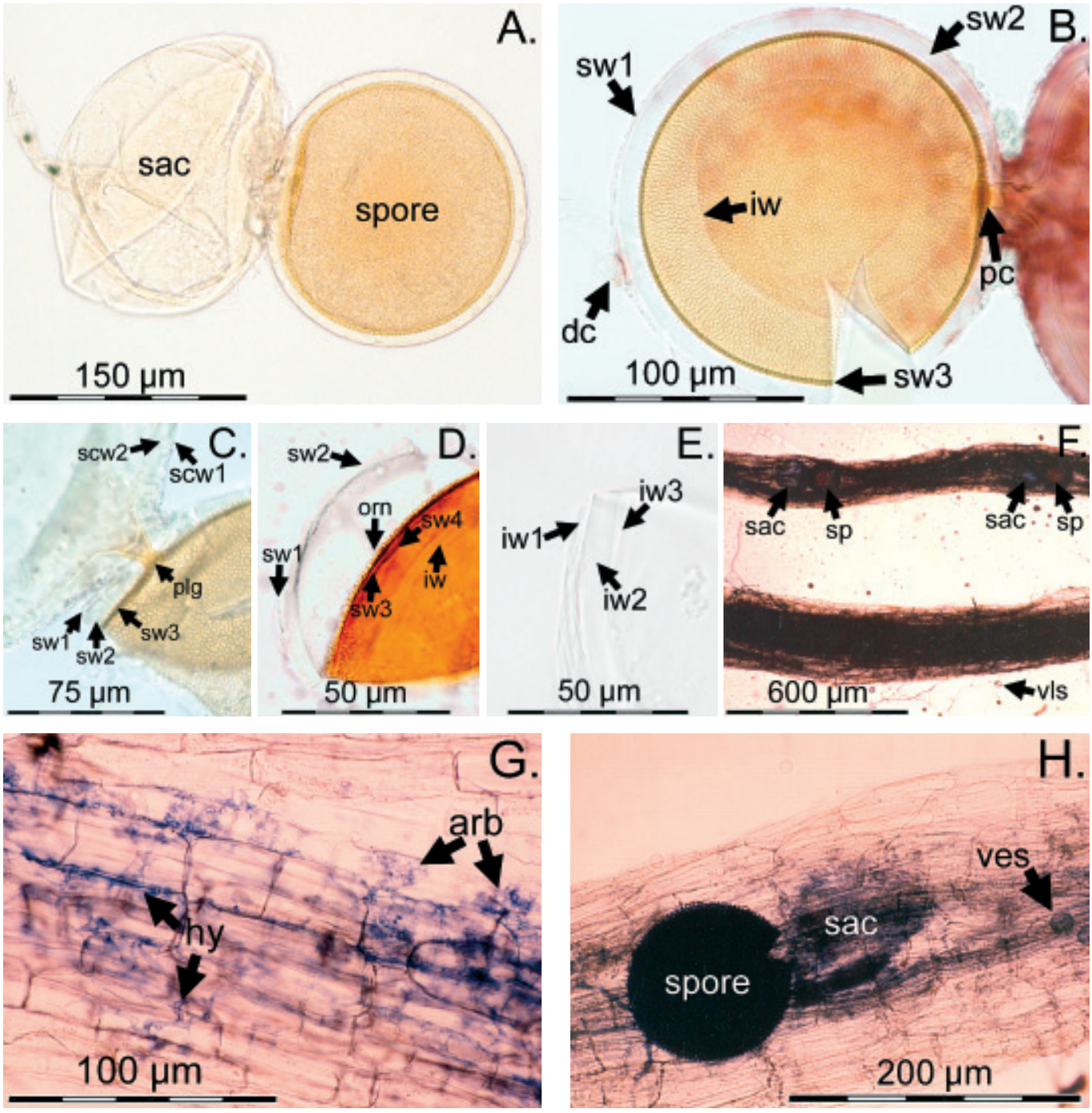

Fig. 1: *Entrophospora infrequens*: A. Young spore (spore) developed in the hyphal stalk of a intercalary formed sporiferous saccule (sac). B. Crushed spore with three layers of the outer spore wall (sw1, sw2, sw3) and inner wall (iw); saccule wall and parts of the outer spore wall layers staining in Melzer's reagent. Detached connection (dc) and intact connection proximal (pc) to the saccule. C. Broken spore with connection to the sporiferous saccule. The outer two spore wall layers (sw1-2) are continuous with the wall layers of the saccule (scw1-2). The layer sw3 is not ornamented at the connection to the saccule. Pore closed by a plug (plg) formed by sw3. D. Four-layered outer spore wall (sw1-4), inner wall (iw) and and rounded projections (orn) of sw3 in cross-view. E. Inner wall, isolated from spore, with three layers (iw1-3). F-H. Mycorrhizal fungal root structures staining blue to dark blue with trypan blue. F. Saccules (sac) and spores (sp) formed in root; extraradical vesicle-like structures (vls). G. Arbuscle trunks (hy) and arbuscles (arb), in process of degradation. H. Vesicles (ves) and crushed spore (spore) with ornamented spore surface and saccule (sac) attached.

kentinensis and *E. schenckii* from the *Entrophospora* genus and the description of two new genera within other known Glomeromycetes families. We include *E. colombiana* and *E. kentinensis* into a new genus in the Acaulosporaceae, and *E. schenckii* into a new genus in the Archaeosporaceae. The genus *Entrophospora* was formerly part of the Acaulosporaceae (Morton and Benny, 1990) because of similarities in spore ontology. However, the genus must be excluded from the Acaulosporaceae not only because of spore morphological differences but also because available and published molecular biological information show that *E. infrequens*, the type species of

the genus, is not related to *Acaulospora* spp. (RODRIGUEZ et al., 2001; MILLNER et al., 2001; WUBET et al., 2003). For *Entrophospora*, we thus erect a new family in the orden Diversisporales.

Material and methods

Specimen and isolates investigated

We re-investigated the spore wall structure, spore formation and also mycorrhizal root colonization structures from type and iso- and paratype specimen of *E. infrequens*, *E. colombiana* and *E. schenckii* deposited at OSC herbarium in Corvallis, Oregon, and specimen derived from pure cultures of these species maintained on host plants *Pueraria phaseoloides* (Roxb.) Benth. or *Brachiaria decumbens* Stapf at CIAT in Cali, Colombia, between 1982 and 1986. Additionally, specimen of E. *colombiana, E. kentinensis* (isolate TW111A originating from the type culture established by C.G. Wu, Taiwan) and *E. schenckii* were observed by Fritz Oehl at the International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi (INVAM), Morgantown, West Virginia, during his visit in 2002. Specimen of *E. baltica* were isolated from soils that derived from high alpine elevations in the Swiss Alps. These were compared with the description of its type material and its online presentation (www.agro.ar.szczecin.pl/~jblaszkowski/). Further specimen of *E. infrequens* isolated from soils from Bolivia, Benin and from Swiss mountainous and alpine regions, and specimen of *E. colombiana* and *E. kentinensis* from soils derived from Benin, Tropical West Africa, and those of *E. schenckii* from soils of alpine altitudes of Switzerland were also investigated.

Trap and single species cultures

The way of establishing the cultures at CIAT, Colombia, is explained in SIEVERDING (1991) and consisted in reproducing the native mycorrhizal spore population in trap pot cultures in an acidic organic Ultisol or a neutral fertile Mollisol (depending on the soil pH of the sampled soil). *Pueraria phaseoloides* was used as the trap plant. These initial cultures were sampled after about 6-9 months and of all species occurring several single species pot cultures were started by inoculating about 5-20 spores per pot. Pots were checked for purity of species starting at 3-9 months. In the case of *E. colombiana* and *E. schenckii*, species started producing spores quite early and pots yielded many spores when they were checked, 3-6 months after inoculation. The culture pots of *E. infrequens* were only checked for purity at 9 months after inoculation and not earlier. Two pure culture pots of the later species were maintained for another two months, and the soil was then stored in a cold room. Whereas cultures of *E. colombiana* and *E. schenckii* were frequently renewed, and new isolates from different locations were produced from 1984 until 1986, when the project at CIAT terminated, *Entrophospora infrequens* was not intended to be reproduced again due to lack of interest in this species at that time. More recent specimen of these species were isolated from arbuscular mycorrhizal consortia in trap cultures with *Plantago lanceolata* L., *Lolium perenne* L., *Trifolium pratense* L. and *Hieracium pilosella* L. as host plants (for methodology see e.g. OEHL et al., 2003; 2006) and maintained at the Botanical Institute of the University of Basel, Switzerland, or, in the case of *E. schenckii*, kindly provided by Samar Velho da Silveira (Porto Alegre, Brazil).

Spore wall terminology and analyses

The terminology of the spore wall structure is basically adapted from INVAM (see homepage: www.invam.caf.wvu.edu) and from STÜRMER and MORTON (1999) with some minor modifications (OEHL et al., 2006). We call INVAM's 'spore wall' 'outer spore wall' (sw), the central wall 'middle wall' (mw), and the innermost wall (from where usually the germination starts in the Glomeromycetes; SPAIN, 1992; SPAIN, 2003; OEHL and SIEVERDING, 2004) 'inner wall' (iw). The fungal structures were stained using trypan blue as described in SIEVERDING (1991). Spores were mounted in lactophenol, polyvinyl alcohol-lactic acid-lactophenol, polyvinyl alcohol-lactic acid-glycerol (PVLG; KOSKE and TESSIER, 1983), in a mixture of PVLG and Melzer's reagent (BRUNDRETT et al., 1994), a mixture of lactic acid to water at 1:1, Melzer's reagent, and in water. Analyses of the spore wall and mycorrhizal structures were performed with compound microscopes at 200-630 fold magnification. Most photographs in Figs. 1-5 were taken with a digital camera (Olympus model DP70-CU) on a Zeiss Axioplan compound microscope. The legends and scales on the photographs of the figures were inserted with Adobe Photoshop 6.0.

Revision of *Entrophospora* and erection of Entrophosporaceae

Emendations of species of *Entrophospora*

***Entrophospora infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd. (Fig. 1)**

Basionym: *Glomus infrequens* I.R. Hall, Trans. Br. Mycol. Soc. 68, 345-347. 1977.

Sporocarps unknown. Within the extraradical mycelium and sometimes in roots hyphae swell terminally, or intercalary at a septum, and form a sporiferous saccule. A spore grows then within the stalk of the saccule flowing the content of the saccule and of the hypha into the spore.

Sporiferous saccules usually with a slight tapering stalk (5-9 µm in diameter). Saccule hyaline, globose to subglobose, 100-180 x 120-200 µm in diameter (Fig. 1 A) with a bi-layered wall, 3-12 µm thick in total, that is continuous with the wall of the hyphal stalk. Debris sometimes attached to the saccule surface. The saccule usually collapses after spore formation and usually is not found in field soil samples. It may stain slightly pink to pink in Melzer's reagent.

Spores formed by inflating the hyphal stalk of the sporiferous saccule usually directly adjacent or in 5-10 µm distance to the saccule (Figs. 1 A-C). Spores are globose to subglobose, yellow-brown, orange-brown to brown, (90-)110-140(-175) µm in diameter.

Spore wall consists of two walls (sw and iw). The outer spore wall (sw; Fig. 1 B-D) has four layers that are together (8-)10-15(-20) µm thick. The first and second layer (sw1 and sw2) are continuous with the wall of the stalk of the sporiferous sacccule (Fig. 1 C). Layer sw1 is evanescent, hyaline and 1.5-4(-6) µm thick. The second layer is more resistant to degradation than sw1, but has often completely disappeared from old spores. It is hyaline, finely laminate and 2.5-6-(8) µm thick. Layers sw1 and sw2 may stain slightly pink in Melzer's reagent. The third layer (sw3) is laminate, golden yellow to orange-brown to brown, (1.5-)2.5-4(-5) µm thick and is ornamented with rounded projections, 1.5-4 µm high and 1-3 µm wide (Fig. 1 C, D) that grow into sw2. The projections often have a convex central depression, 0.6-1.2 µm deep, at the top. The spore wall layer sw3 forms a permanent pigmented part of the connection between the sporiferous saccule and the spore which remains also after degradation of the saccule (Fig. 1 C). The innermost layer sw4 is very thin (about 0.5 µm), concolorous and closely adherent to sw3 and thus, usually difficult to observe even in crushed spores (Fig. 1 D). The inner wall (iw) is hyaline, and 5-10 µm thick (Fig. 1 E) with one

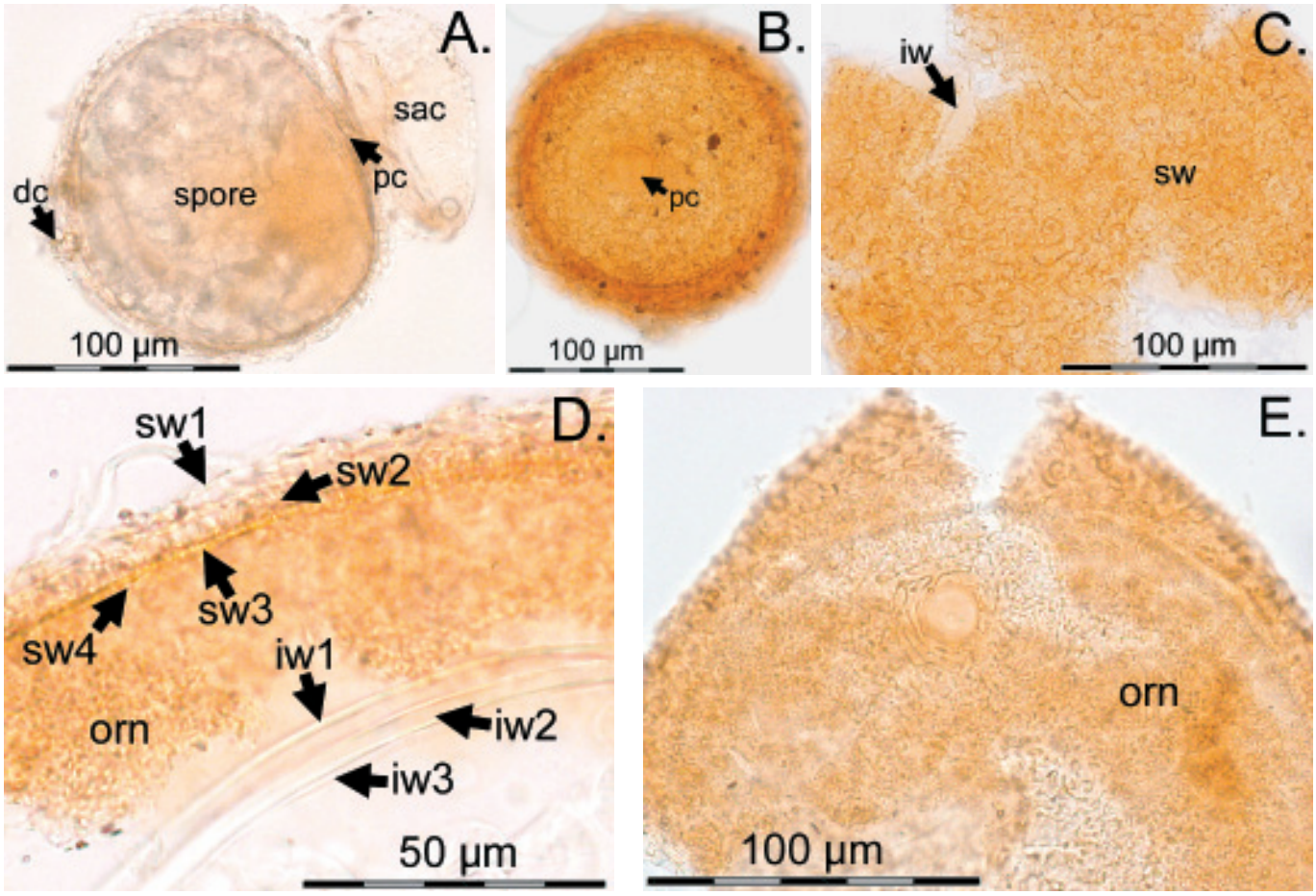

Fig. 2: *Entrophospora baltica*: A. Young spore developed in the hyphal stalk of a sporiferous saccules (sac); saccule collapsed. Spore with cicatrices formed by detached hypha (dc) and proximal (pc) connection to the saccule. B. Mature spore with cicatrix (pc) formed after detaching of saccule C. Crushed spore with the hyphal mantle of outer spore wall (sw) and inner wall (iw). D. Broken spore showing four layers of outer wall (sw1-sw4) and three layers of inner wall (iw1-iw3). The sw2 is the mantle formed by sinuous hyphae, sw3 is the ornamented wall with warts, sw4 is difficult to concern. E. Characteristic hyphal mantle (orn) with round cicatrix.

prominent central finely laminated wall layer (iw2). An outer and inner wall layer (iw1 and iw3) are tightly adherent to iw2. Both layers are hyaline and very thin (<0.5 µm), and thus are often hardly to detect.

Cicatrices: The pigmented wall layer sw3 is part of the connection between spore and saccule and continues for a short distance of 3-7(-10) µm into the saccules wall (Fig. 1 C). After detaching of the saccule the remaining rest resembles a wide cicatrix. The spore pore at this cicatrix is about 3-7 µm wide and closed by a plug arising from sw3 (Figs. 1 B-C), and by sw4. Distal to the saccule, a hypha is attached that is continuous with the two outer spore wall layers. When this hypha is broken off, a cicatrix is also visible as long as sw2 is not deteriorated (Fig. 1 B).

Germination: So far the germination structures in this species have not been detected.

Mycorrhizal formation: Forms vesicular-arbuscular mycorrhiza (Sieverding and Toro, 1985; Figs. 1 F-H). In some roots also hyphal coils were observed in root cells. Vesicle like structures, globose to subglobose, 15-40 µm in diameter with 0.5 µm thick walls are also formed intercalary or apically on root external hypha near roots (Fig. 1 F). These often collapsed in lactophenol. Sporiferous saccules and spores can be formed in roots (Figs. 1 F, H). Fungal structures stain light blue to visibly blue in trypan blue (Figs. 1 F-H).

Specimen observed: Paratype OSC #39,678 and OSC #42,409 deposited by Ames and Schneider (1979). Specimen from a pure pot culture, number C-132-1 at CIAT, Colombia, 1984-1985, nr. 2827-2831 and nr. 2835 of Sieverding collection, deposited at Z+ZT. Specimen nr. 71-7101 to 71-7110 of Oehl collection deriving from Bolivia, Benin and Switzerland, deposited at Z+ZT.

Distribution: This species was first isolated from forest soils in New Zealand (Hall, 1977), and thereafter from a horticultural field in California and from agricultural soybean and corn fields in Iowa, Illinois and Wisconsin (Ames and Schneider, 1979). There are many reports about the occurrence of this species, from acidic and neutral soils, and from many natural ecosystems and agricultural land use systems from all over the world, e.g. from Colombia (Sieverding and Toro, 1985), Brazil (Maia and Trufem, 1990), from Mexico (Oehl, unpublished), from the United States in Wyoming (Stahl and Christensen, 1982), Florida (Schenck and Smith, 1982) and Rhode Island (Koske et al., 1997), from Canada (Boyetchko and Tewari, 1993), Finland (Vestberg, 1995), Poland (Blaszkowski, 1993), Germany, France and Switzerland (Oehl et al., 2003; 2004;

2005), from India (SRIDHAR and BEENA, 2001), from Namibia (UHLMANN et al., 2004) and Australia (HALL and ABBOTT, 1984). We recently found it also in trap cultures inoculated with soils from tropical Bolivia (OEHL and PÉREZ-CAMACHO, unpublished data) and Benin (TCHABI and OEHL, unpublished), and also in montainous and high alpine areas of Switzerland and in the foreland of the Morteratsch glacier near Pontresina, Engiadina, Switzerland that was covered by the ice until about 30 years ago.

***Entrophospora baltica* Blasz., Madej & Tadych, Mycotaxon 68, 166-167. 1998 (Fig. 2)**

This species does not need an emendation. It is well described (BLASZKOWSKI et al., 1998) and was presented on-line (www.agro.ar.szczecin.pl/~jblaszkowski/). We recognize the spores formed within the stalk of a sporiferous saccule (Fig. 2 A), and the spores having an outer spore wall (sw) covered with a hyphal mantle with a round cicatrix visible when the saccule is broken off (Fig. 2 B), and an inner wall (iw; Fig. 2 C). The outer spore wall has four layers (sw1-4). The outer layers and the hyphal mantle are sw1 and sw2 (Fig. 2 D). The sinuous folding of the hyphal mantle and the pigmented spore wall layer sw3 with its fine warts on the outer surface are characteristic for this species (Figs. 2 C-E). Layer sw4 is very thin and closely adherent to sw3 and thus hardly to detect. For the inner wall (iw) it should be noted that a very thin innermost layer (iw3) was found (Fig. 2 D) that can also be observed in Fig. 4 of the original description of the species (BLASZKOWSKI et al, 1998). The pore at the spore base proximal to the sporiferous saccule is closed by a plug similar as in *E. infrequens*. The inner wall layer of the sporiferous saccule, the hyphal mantle and the hypha distal to the sporiferous saccule may stain slightly pink to pink with Melzer's reagent in some specimen what was not mentioned in the protologue.

Mycorrhizal formation: unknown

Specimen observed: Spores isolated from several locations in the Swiss Alps, e.g. in dolomitic soils at Piz Corvo (2700 m a.s.l., Cantone Ticino; Oehl collection, slides Nr. 72-7201 to 71-7204 deposited at Z+ZT).

Distribution: This species is known from Northwestern Poland (BLASZKOWSKI et al., 1998) and from Slowenski National Park (TADYCH and BLASZKOWSKI, 2000) in acidic to neutral soils (pH-3.6-6.7) and was recently found in Switzerland at several Alpine elevations (e.g. at 2600 m a.s.l. at Haldensteiner Calanda, Chur, Kanton Graubünden and at 2700 m a.s.l. in soils with pH 6.5-8.1 at Piz Corvo, Cantone Ticino, Oehl, unpublished). It also formed part of the mycorrhizal community of forest ecosystems near Valdivia in Southern Chile (CASTILLO and SIEVERDING, unpublished).

Emendation of genus description

***Entrophospora* R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd.**
Basionym: *Entrophospora* R.N. Ames & R.W. Schneid, Mycotaxon 2, 347-348. 1979.

Type species: *Entrophospora infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd.

Basionym: *Glomus infrequens* I.R. Hall, Trans. Br. Mycol. Soc. 68, 345-347. 1977.

Sporocarps unknown. Spores are formed by inflating the hyphal stalk directly beneath of a sporiferous saccule. Spores globose to subglobose and have two walls: an outer spore wall and an inner wall. Part of the outer layers of the outer spore wall are the wall layers of the hyphal stalk and the sporiferous saccule. The outer spore wall layers often degrade and are evanescent; the inner layers of the outer spore wall are more permanent and often extend for a short distance into the saccule. A plug formed by such a permanent wall layer closes the pore towards the saccule. The inner wall consists of a thick, finely laminated wall layer that might have a thin layer adherent to its outer and inner surface. None of the layers of the inner wall have a beaded appearance. Fungal structures in roots stain blue with trypan blue. Forming vesicular-arbuscular mycorrhizae.

Species remaining in the genus *Entrophospora*

Entrophospora infrequens (I.R. Hall) R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd.
Basionym: *Glomus infrequens* I.R. Hall, Trans. Br. Mycol. Soc. 68, 345-347. 1977.

Entrophospora baltica Blasz., Madej & Tadych, Mycotaxon 68, 166-167. 1998.

Species excluded from the genus *Entrophospora*:

Entrophospora colombiana Spain & N.C. Schenck, Mycologia 76, 693-694. 1984.

Entrophospora kentinensis C.G. Wu & Y.S. Liu, Mycotaxon 53, 283. 1995.

Entrophospora schenckii Sieverd. & S. Toro, Mycotaxon 28, 210. 1987.

Erection of new family

Entrophosporaceae fam. nov. Oehl & Sieverd.

Type genus: *Entrophospora* R.N. Ames & R.W. Schneid. (Mycotaxon 2, 348. 1979) emend. Oehl & Sieverd.

Latin diagnosis: *Spora singulatim efformata in hypham inflatam, anguste adiacetum ad sacculum sporiferum terminalem vel intercalarem. Sporae globosae vel subglobosae cum tunicis duabus: tunica exterior et tunica interior. Pauciores strata exteriores sporarum coniuncta tunica hyphae et sacculi. Strata interiores tunicae exterioris persistentes. Tunica interior sporae subtiliter laminata et perpetua, sine strato granulato. Formans structuras mycorrhizarum vesicular-arbuscularum. Structurae fungarum colorantes caeruleae cum* 'trypan blue'.

Spore formed by swelling directly beneath and within the hyphal stalk of a terminally or intercalary formed sporiferous saccule. Spores have two walls: an outer spore wall and an inner wall. Layers of the outer spore wall are continuous with the wall of the stalk and the saccule. The inner layers of the outer spore wall are persistent and may form part of the connection with the sporiferous saccule. A plug closes the pore proximal to the saccule. The inner wall consists of a thick, finely laminate layer that might have a thin layer adherent to its outer and inner surface. None of the layers of the inner wall have a beaded appearance. Forms vesicular-arbuscular mycorrhiza those fungal structures stain blue in trypan blue.

Discussion: The erection of the new Entrophosporaceae family is mainly based on the distinct morphological differences between spores of its type genus, *Entrophospora* N. Ames & R.W. Schneid.

emend. Oehl & Sieverd., and the two genera of the Acaulosporaceae, *Acaulospora* Gerd. & Trappe and *Kuklospora* gen. novum Oehl & Sieverd. (see below). MORTON and BENNY (1990) included the genus *Entrophospora* R.N. Ames & R.W. Schneid. into the Acaulosporaceae mainly due to parallelisms in spore ontogeny and organization of spore wall structures of those species which we place into *Kuklospora* (see below), with those of the genus *Acaulospora*. It is, however evident that there is no known parallelism between *E. infrequens* or *E. baltica* and any of the *Acaulospora* spp. Distinct morphological differences between *Entrophospora* and the genera of the Acaulosporaceae are: i) Spores in *Entrophospora* have two walls, and spores in the Acaulosporaceae have three walls. ii) In *Entrophospora* a plug is formed to close the connection between the sporiferous saccule and the spore after the content of the saccule is emptied to form the spore; such plug is not formed in Acaulospora. iii) *Entrophospora* has a relative thick inner wall absent in Acaulosporaceae. vi) Most Acaulosporaceae spores have a characteristic 'beaded' wall layer as part of the inner wall which is absent in *Entrophospora* spores. In addition to the morphological differences, all molecular biological data generated for the phylogenetic position of *Entrophospora infrequens* (MILLNER et al., 2001; RODRIGUEZ et al., 2001; WUBET et al., 2003) indicate that this species is not related to Acaulosporaceae. Hence, morphological and biochemical differences justify the exclusion of *Entrophospora* from Acaulosporaceae and the erection of the new family Entrophosporaceae.

Both the families, Entrophosporaceae and Acaulosporaceae have species which form vesicular-arbuscular mycorrhiza, and the fungal structures in roots stain visibly blue with trypan blue, an important differentiating characteristic which was used by Morton and Redecker (2001) when they described the Archaeosporaceae and Paraglomeraceae those hyphal structures stain not or only weakly in trypan blue.

New genus in Acaulosporaceae

***Kuklospora* gen. novum. Oehl & Sieverd.**

Type species: *Kuklospora colombiana* (Spain & N.C. Schenck) Oehl & Sieverd. comb. nov.
Basionym: *Entrophospora colombiana* Spain & N.C. Schenck, Mycologia 76, 693-694. 1984.

Sporocarpia ignota. Spora singulatim efformata subterminaliter vel intercalariter in hypham inflatam en distantiam ad sacculum sporiferum terminalem vel intercalarem. Sporae globosae vel subglobosae, tribus tunicis stratis pluribus. Stratum exterior tunicae exterioris coniunctum tunica hyphae et sacculi. Stratum interiorem laminatum tunicae exterioris duo pora sporae occludans. Stratum exterior tunica interioris granulatum, stratum medium tunicae interioris ex consuetudine colorans purpureum reagente Melzeri. Area germinationis in tunica interiore. Formans mycorrhizas vesicular-arbusculares. Structurae fungarum colorantes caeruleae cum 'trypan blue'.

Sporocarps unknown. Spores formed by inflating the hyphal stalk in some distance to a terminally or intercalary formed sporiferous saccule. Spores have three walls: an outer spore wall, a middle wall and an inner wall. One or a few layers of the outer spore wall are continuous with the wall of the stalk and the sporiferous saccule. These layers are often evanescent. Inner layers of the outer spore wall are permanent. When the connections of the hyphal stalk break off, the spore appears with two, often opposite, cicatrices which are closed by the permanent sublayers of the inner layers of the outer spore wall. The middle wall is thin and flexible. The inner wall consists of several thin layers of which the outer layer is ornamented having a characteristic 'beaded' appearance. The second layer of the inner wall usually stains deep purple in Melzer's reagent. The inner wall functions as germinal wall, a germination orb may be formed. Forming vesicular-arbuscular mycorrhizae. The fungal structures in the roots stain significantly blue with trypan blue.

Etymology: Greek: kuklo- (κυκλος, ring), and spora (σπορα, spore), referring to the two cicatrices which resemble circular depressions and the boarders of them wedding rings on the spore surface when the hyphal connection have detached from young spores.

Species included into the genus *Kuklospora*:

Kuklospora colombiana (Spain & N.C. Schenck) Oehl & Sieverd. comb. nov.
Basionym: *Entrophospora colombiana* Spain & Schenck Mycologia 76, 693-694. 1984.

Kuklospora kentinensis (C.G. Wu & Y.S. Liu) Oehl & Sieverd. comb. nov.
Basionym: *Entrophospora kentinensis* C.G. Wu & Y.S. Liu Mycotaxon 53, 283. 1995.

Notes to *Kuklospora colombiana* (Spain & N.C. Schenck) Oehl & Sieverd. comb. nov (Fig. 3)

The basionym is well described in SCHENCK et al. (1984). We want to give some additional remarks to the wall structure, applying our spore wall terminology. The spores are formed by swelling of the hyphal stalk of a terminal or intercalary formed sporiferous saccule (Figs. 3 A-C). The spores have three walls (Fig. 3 D-G): a three-layered outer spore wall (sw1-sw3), a thin, one to bi-layered middle wall (mw1 and mw2) and an inner wall that is three-layered (iw1-iw3). On the outer spore wall and on the inner wall usually only the two outer layers are easy to detect, while the very thin closely adherent inner layers of these two walls (sw3 and iw3, respectively) are difficult to observe – they are not mentioned in the protologue. The middle and the inner wall form *de novo* after the pore towards the sporiferous saccule is closed. Characteristic of *Kuklospora* are the 'beaded' appearance of iw1 (Fig. 3 G) and the dark purple reaction of iw2 in Melzer's reagent (Fig. 3 F). The same characteristics have only species of the genus *Acaulospora*.

The pore at the connection to the sporiferous saccule is closed by continuous sublayers of sw2 and by the adherent, thin layer sw3. After the hypha with the sporiferous saccule is detached from the mature spore, a circular depression is seen those boarder resembles a ring (Figs. 3 D-E). A second round cicatrix on the spore, opposite to the sporiferous saccule, remains when the distal hyphal connection breaks off. The outer hyaline layer of the spores frequently degrades and disappears on mature and older spores, and in such spores, in particular isolates from field samples, the cicatrices might be absent.

A germination orb between iw1 and iw2 was found by Spain (1992).

Mycorrhiza formation: Forming typical vesicular-arbuscular mycorrhizae (Figs. 3 H-I) with fungal root structures that stain significantly blue with trypan blue (Type specimen in OSC).

Specimen observed: Isotype specimen deposited at OSC, #42,497. Type and isotype specimen from CIAT (measured by SIEVERDING and TORO, 1985; Sieverding collection). Specimen from INVAM reference accession CL148 obtained by Fritz Oehl during his visit at INVAM in 2002. Spores isolated from trap cultures inoculated with soils from Benin and maintained in Basel by Atti Tchabi, in 2005.

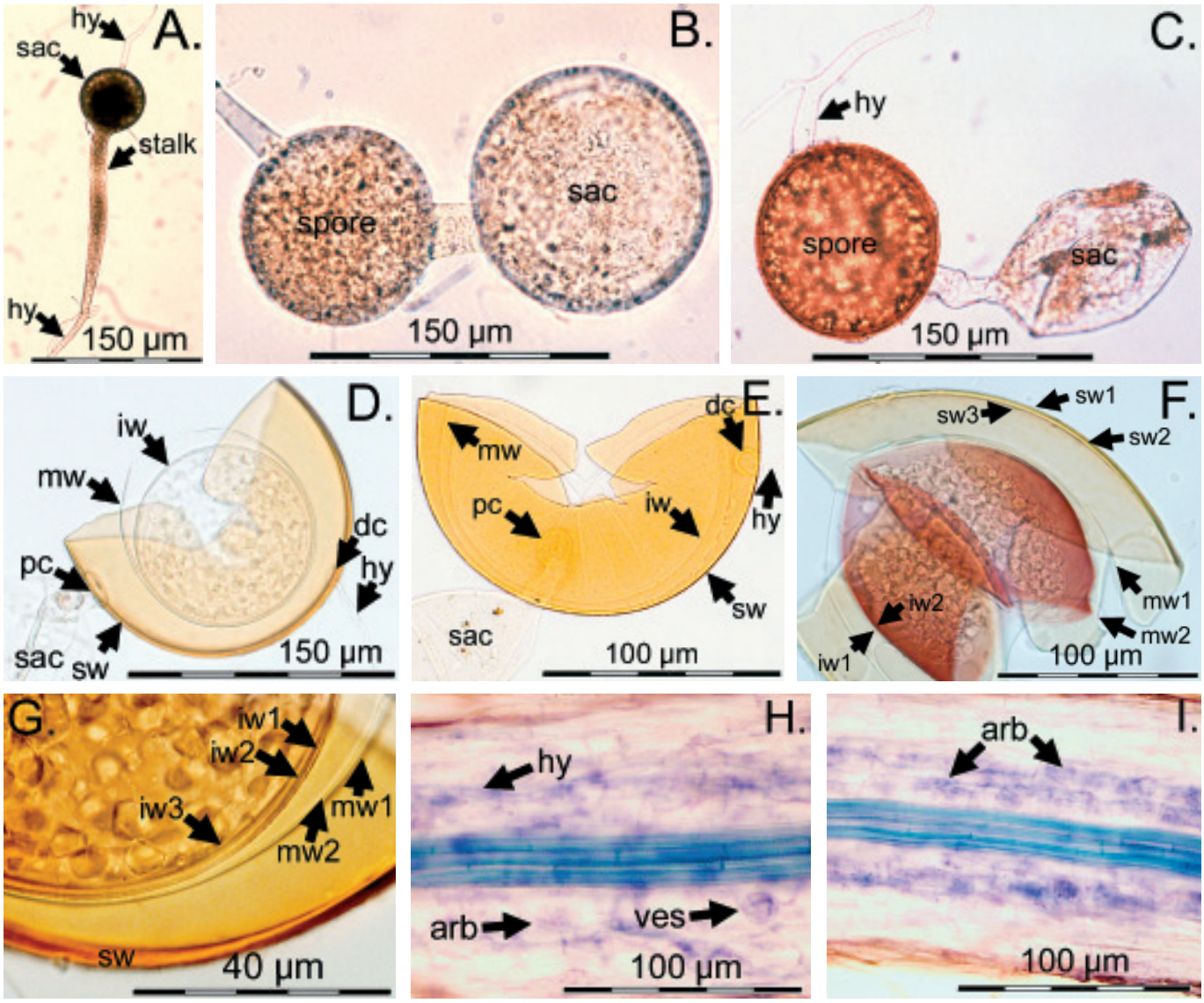

Fig. 3: *Kuklospora colombiana*: A. Intercalary in hypha (hy) formed sporiferous saccule (sac) with stalk (stalk) B.-C. Progressing spore (spore) formation by swelling in stalk in some distance from saccule (sac); hypha (hy) connected. D.-E. Broken spores with outer spore wall (sw), middle wall (mw), inner wall (iw) and two round connections proximal (pc) to the saccule (sac) and distal (dc) to the hypha (hy); the connections appear like rings from where the name *Kuklospora* derives. F. Broken spore with three-layered outer spore wall (sw1-3), bi-layered middle wall (mw1-2), and two layers of the inner wall (iw1-2); iw1 is beaded (granular) and iw2 stains purple in Melzer's reagent. G. Broken spore with two-layered middle wall (mw1-2) and three-layered inner wall (iw1-3). H.-I. Fungal structures in root staining blue with trypan blue; intraradical hyphae (hy), arbuscles (arb) and vesicle (ves).

Distribution: *Kuklospora colombiana* was first isolated from various locations in Colombia (Schenck et al., 1984; Sieverding and Toro, 1985). It was also reported from several States in Brazil (Balota et al., 1999; de Souza et al., 2002; Carrenho et al., 2002; Caproni et al., 2003; dos Anjos et al., 2005) and from India (Mehrotra, 1998; Selvam and Mahadevan, 2002) and the Philippines (Oba et al., 2004). We recently found it in trap cultures inoculated with tropical soils from Benin (Tchabi and Oehl, unpublished). We infrequently found this species in grasslands of lowlands and at mountainous elevations in Southern Germany and Switzerland. In Germany, *K. colombiana* was also isolated from an acidic sandy soil near Berlin (Baltruschat, pers. com.). So far, *K. colombiana* was only recorded from acidic soils.

Notes to *Kuklospora kentinensis* (C.G. Wu & Y.S. Liu) Oehl & Sieverd. comb. nov. (Fig. 4)

The sporogenesis of *K. kentinensis* was well described by Wu et al. (1995b) and has well been presented on-line (*http://www.tari.gov.tw/ACT/sporogenesis-EKTN.htm)* as well. Spore development is similar to *K. colombiana* (Fig. 4 A). Using our spore wall terminology the spores have three walls (Fig. 4 D) : a three-layered outer wall, a thin, bi-layered middle wall and a two- to three-layered inner wall that shows the typical 'beaded' appearance on the outer surface (Fig. 4 E-F). The beaded appearance was not mentioned in the protologue but in Wu et al. (1995a) and in the internet presentation. The layer iw2 of the inner wall stains purple in Melzer's reagent (Fig. 4 D-F),

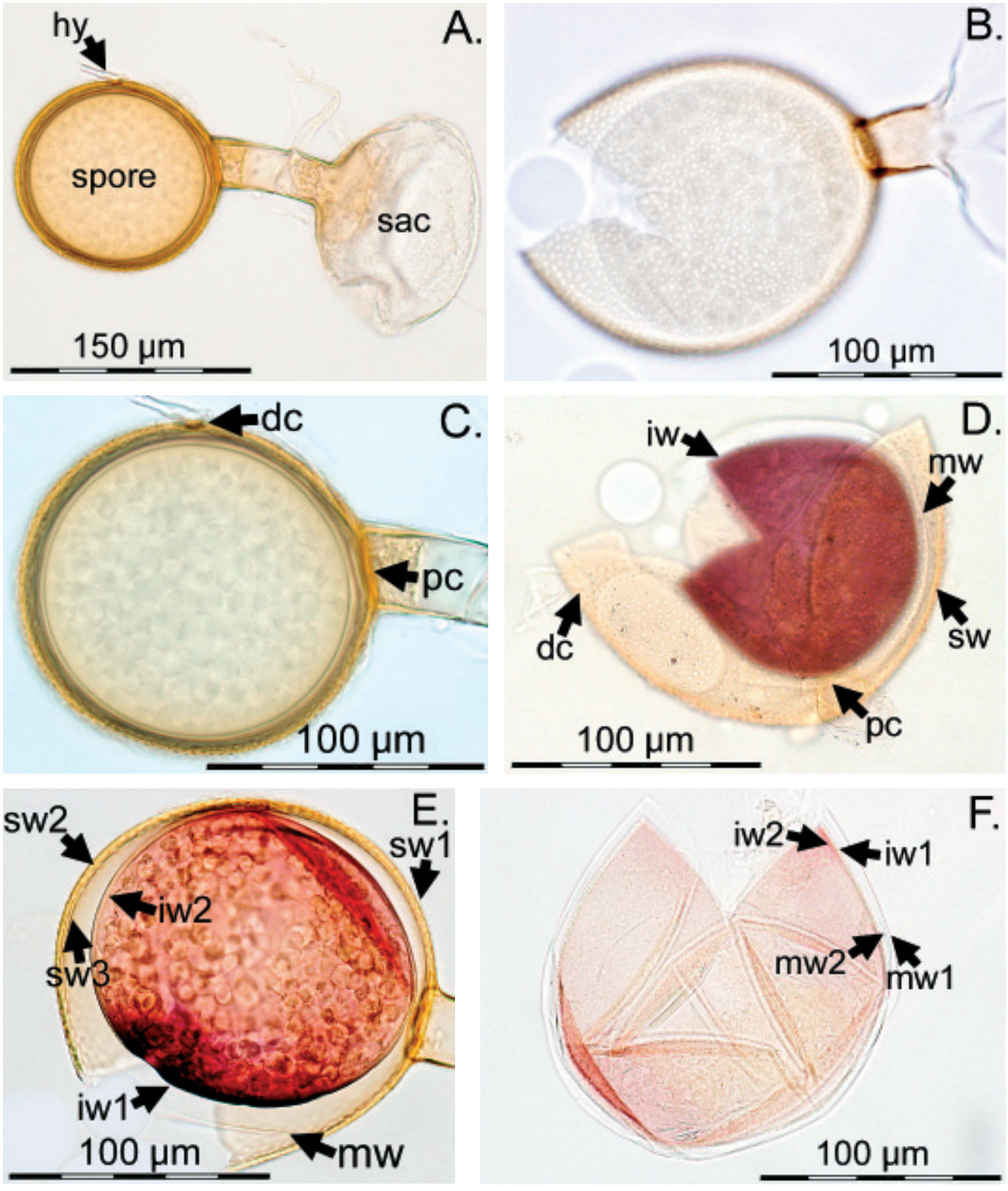

Fig. 4: *Kuklospora kentinensis*: A. Spore formed in the stalk in some distance of a terminally formed sporiferous saccule (sac). B. Young broken spore with characteristic pitted surface ornamentation. C. Connection of spore with hypha (dc) and sporiferous saccule (pc); three walls (sw, mw, iw); part of sw form the connection to the saccule, and part of sw closes the pore. D. Broken spore with three spore walls (sw, mw, iw) and broken connections to hypha (dc) and sporiferous saccule (pc). Part of iw stains dark purple with Melzer's reagent. E. Broken spore with three-layered outer wall (sw1-3), middle wall (mw) and two layers of the inner wall (iw1, iw2); iw2 stained by Melzer's. F. Middle and inner walls isolated from spore. Middle wall two-layered (mw1-2); inner wall with characteristic 'beaded' ornamentation (iw1) and iw2 staining purple in Melzer's reagent.

iw3 is very difficult to concern and is not shown in the figures. A prominent feature for *K. kentinensis* is the characteristic pitted ornamentation on the second outer spore wall layer sw2 (Fig. 4 B, C). In some spores the ornamented and pigmented sw2 extends over a distance of about 10-40(-100) µm into the connection to the sporiferous saccule (Fig. 4 C). The pore proximal to the saccule is about 15-40 µm wide and is closed by sublayers of sw2; the spore pore opposite to the saccule is smaller. When the sporiferous saccule and the distal hypha are detached, the two cicatrices on the spore surface can appear ring-like.

Mycorrhizal formation: Forming mycorrhizae with arbuscles and vesicles; the fungal structures stain blue to dark blue with trypan blue (Wu et al., 1995a; 1995b).

Specimen observed: Specimen #74-7401 (Oehl collection) obtained by Oehl during his visit at INVAM in 2002 originating from a pure culture that had been established with spores isolated from Taiwanese soils and having the INVAM reference accession number TW111A. Specimen #74-7411 to #74-7414 derived from trap cultures inoculated with agricultural soils from tropical Benin (Oehl collection, deposited at Z+ZT).

Distribution: *Kuklospora kentinensis* was first known from Taiwan (Wu et al., 1995a; 1995b). It was also reported from Brazil (Caproni et al., 2003; dos Anjos et al., 2005). We recently found *K. kentinensis* in soils from tropical Benin (Western Africa; Tchabi and Oehl, unpublished). So far, it is only known from tropical areas with a wide range of soil pH (4.0-8.0).

Discussion of *Kuklospora*

Kuklospora colombiana and *K. kentinensis* have morphological characteristics in common with *Acaulospora* spp. which were discussed for the basionym of *Kuklospora colombiana* (*E. colombiana*) by Morton and Benny (1990) so that a further discussion is not necessary. Morton and Benny (1990) refer to the potential parallelisms of spore formation in two different genera. Considering this, *K. colombiana* has its sister species in *A. dilatata* (Morton and Benny, 1990), and *K. kentinensis* has similarities with *A. scrobiculata* (Wu, on-line presentation: *http://www.tari.gov.tw/ACT/sporogenesis-EKTN.htm)*. The two genera can be easily distinguished by the formation of spores on the neck of a sporiferous saccule in *Acaulospora* and by the formation of spores within the stalk of the sporiferous saccule in *Kuklospora*. As shown above, species of the new genus *Kuklospora* differ phylogenetically from *Entrophospora* (e.g. Millner et al., 2001) and are related to *Acaulospora* (Redecker, 2000; Redecker et al., 2000; Nielsen et al., 2004). *Kuklospora* spp. are characterized by formation of vesicular-arbuscular mycorrhizal fungal structures in roots which stain blue to dark blue with trypan blue. This differentiates *Kuklospora* from the new genus *Intraspora* (see below).

New genus in Archaeosporaceae

Intraspora gen. nov. Oehl & Sieverd.

Type species: *Intraspora schenckii* (Sieverd. & S. Toro) Oehl & Sieverd. comb. nov.
Basionym: *Entrophospora schenckii* Sieverd. & S. Toro, Mycotaxon 28, 210. 1987.

Sporocarpia ignota. Sporae in solo vel in radice efformatae, singulatim in hyham inflatam, en distantiam ad sacculum sporiferum terminalem vel intercalarem. Sporae globosae vel subglobosae vel oblongatae, duabus tunicis. Stratum exterior sporae coniuncta tunica hyphae et sacculi; stratum interiorem tunicae exterioris duo pora sporae occludens. Tunica interior sporae semiflexibilisque stratis paucioribus. Formans mycorrhizam arbuscularem; vesiculae absentes vel rarae. Arbusculae hyphaeque non tinguntur vel ubi tinguntur pallidae reagente 'trypan blue'. *Tunica interior sporarum tinguntur caerulea vel obscura caerulea.*

Sporocarps unknown. Spores formed singly in soils and in roots, by swelling in the hyphal stalk in a short distance to a sporiferous saccule which is terminally or intercalary formed in the intra- or extraradical mycelium. Spores are globose to subglobose to oblong, with two walls: an outer spore wall and an inner wall. The outer layer of the outer spore wall is continuous with the wall of the hyphal stalk and the saccule. This outer layer is often evanescent on mature spores. The inner layer of the outer wall is permanent and closes the two spore pores. The inner wall of the spore is finely laminated and semi-flexible. The fungal structures in the roots do not or only very weakly stain with trypan blue. Forming arbuscular mycorrhizae; formation of vesicles is rare.

Etymology: Latin: *intra* (inside, within), *spora* (spore), referring to the spore formation within the hyphal stalk of the sporiferous saccule.

Species included into the genus *Intraspora*:

Intraspora schenckii (Sieverd. & S. Toro) Oehl & Sieverd. comb. nov. (Fig. 5).
Basionym: *Entrophospora schenckii* Sieverd. & S. Toro, Mycotaxon 28, 210. 1987.

The original description of the spore morphology of *I. schenckii* by Sieverding and Toro (1987) was confirmed by Fracchia et al. (2003). Using our spore terminology, *I. schenckii* forms small spores (45-75 µm) by swelling in the hyphal stalk of a terminally or intercalary formed sporiferous saccule (Figs. 5 A-B). Spore formation can be in soils and also in roots. Within roots the sporiferous saccule may be formed within one root cell and the spore in another cell. However, formation of sporiferous saccule and spore within one root cell appear also be possible. The spores have two walls: an outer bi-layered spore wall and an inner wall (Figs. 5 C-D). The outer layer sw1 of the outer spore wall is continuous with the hyphal and saccule wall. This wall layer can readily separate in broken spores from sw2 (Fig. 5C); sw1 is often absent on mature spores. Spore wall layer sw2 is permanent and closes the two pores of the hyphal attachments. There, two circular depressions (cicatrices) are visible on young spores when the sw1 is still present and the connections to the stalk have detached. The inner wall is finely laminated and semi-flexible. On the outer and inner side of wall layer iw2 very thin layers (iw1 and iw3) are sometime seen (Fig. 5 D); they are hard to detect in specimen mounted in PVLG but also appear on photos published by Fracchia et al. (2003) although they were not described. The inner wall of the spore stains blue to dark blue in trypan blue (Fig. 5 E).

Mycorrhizal formation: Mycorrhizae formation is concluded from spores formed in the cortical cells of roots. The inner wall of *I. schenckii* spores formed in roots or in soil stained dark blue with trypan blue (Figs. E-F) but none of the other structures stained in the studies of Sieverding and Toro (1985; 1987). Spores or fungal structures did not stain with acid fuchsin or methylen blue. *Intraspora schenckii* was reported by Fracchia et al. (2003) to form arbuscules and seldom small vesicles in roots. In their investigation, the fungal structures stained weak with trypan blue in roots of Vigna

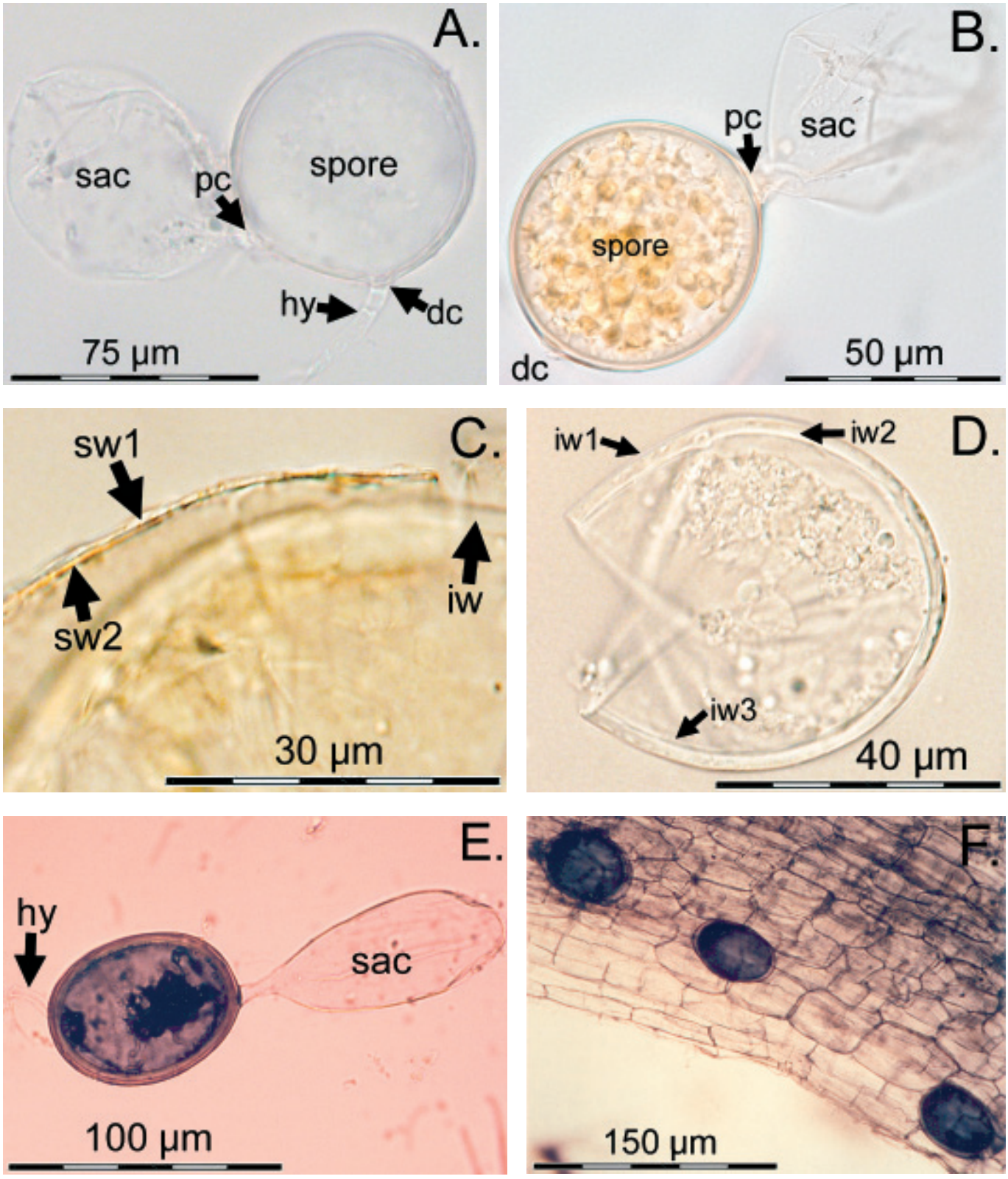

Fig. 5: *Intraspora schenckii*: A. Spore formed in the stalk of a sporiferous saccule (sac); hyphal connection (hy) opposite to sporiferous saccule; outer spore wall with two layers (sw1, sw2) and inner wall (iw). B. Spore with broken connection to hypha (dc) and with connection to sporiferous saccule (pc); the outer layer of the outer spore wall is continuous with the the saccule wall. C. Broken spore with two-layered outer wall (sw1-2) and inner wall (iw). Spore had been conserved in formalin for more than 20 years, sw2 stained slightly yellow in Melzer's reagent. D. Inner broken spore wall isolated from spore; three layers (iw1-3). E. Part of inner wall stains with trypan blue; hyphal wall (hy) and saccule wall (sac) do not stain. F. Spores formed in roots of tropical kudzu; only part of inner spore wall stains with trypan blue.

sp. but did not stain in tomato or clover. SIEVERDING and TORO (1987) described the species after culturing with *P. phaseoloides* (tropical kudzu).

Specimen observed: Type specimen, OSC #46,030. Spores obtained from the type culture pot # C-133-8 with *P. phaseoloides* (Sieverding collection #2858, 2863, 2871 and 2881 deposited at Z+ZT). Specimen from Estado do Rio Grande do Sul in Brazil (S. Velho da Silveira collection). Specimen from a single species culture maintained at INVAM (Oehl collection nr. 75-7511). Specimen obtained from a trap culture inoculated with a Jurassic limestone soil originating from high Alpine area in Switzerland (Oehl collection).

Distribution: So far, this species was found in a neutral phosphate rich soil in Cundinamarca, Colombia (SIEVERDING et al., 1985; 1987), in Southern Brazil (VELHO DA SILVEIRA and OEHL, unpublished), in Argentina (FRACCHIA et al., 2003), Poland (BLASZKOWSKI, personal communication), India (MOHANKUMAR et al., 1988; RAGUPATHY and MAHADEVAN, 1993), Thailand (BHADALUNG et al., 2005) and in the Swiss Alps (OEHL, unpublished).

Discussion of *Intraspora*

Intraspora schenckii is so far the only species in the new genus. It differs from the other two genera which form spores in the hyphal stalk of a sporiferous saccule firstly by the spore wall structure; *I. schenckii* has two walls like *Entrophospora* spp. and not three like *Kuklospora*. However, whereas *Entrophospora* has a much more complex outer spore wall and the spore formation directly adherent to the saccule, *Intraspora* has a rather simple outer spore wall and its spore formation is in some distance to the sporiferous saccule. The more important difference to *Entrophospora* and *Kuklospora* however is the absence or only weak staining of the root infection structures with trypan blue.

Such weak or faintly staining was the main characteristic described by MORTON and REDECKER (2001) for the Archaeosporaceae J.B. Morton & D. Redecker and Paraglomeraceae J.B. Morton & D. Redecker as *'arbusculae hyphaeque mycorrhizarum ubi tinguntur invisibiles vel pallidae'*. Indeed vesicles are not or only rarely formed by *I. schenckii* (FRACCHIA et al., 2003) who were able to faintly stain arbuscles in roots of *Vigna*. The absence of a reaction of the hyphae and arbuscles to trypan blue in our studies and the high similarity of the wall structure of *I. schenckii* with *Archaeospora trappei* (R.N. Ames & Linderman) J.B. Morton & D. Redecker comb. nov. (MORTON and REDECKER, 2001) led us to the conclusion that *I. schenckii* must be related to the Archaeosporaceae. *Archaeospora trappei* has a very similar spore wall structure (HAFEEL, 2004). Spores of these two species separated from field soils are almost not distinguishable when the outer wall layer is detached. Field collected spores of *I. schenckii* are also not distinguishable from field collected *A. myriocarpa* Spain, Sieverd. & N.C. Schenck (SCHENCK et al., 1986) those mycorrhizal structures also only faintly stain without vesicle formation (SCHENCK et al., 1986) and thus, is likely to be another small-spored *Archaeospora* species. The morphological similarities of *I. schenckii* with the small-spored *Archaeospora* spp. are strong and the difference between both genera is in the sporogenesis: the spore formation in the hyphal stalk instead laterally on the hyphal neck of a sporiferous saccule. The way of such different spore formation was also used in the past to differentiate related genera (MORTON and BENNY, 1990). We interprete *I. schenckii* as sister species of *Ar. trappei* as both species have almost identical spore wall structures, and mycorrhizal root infection and fungal staining characteristics in common. Hence, we consider *Intraspora* a sister genus to *Archaeospora* and, thus, include *Intraspora* in the Archaeosporaceae.

Since the formation of spores in the hyphal stalk of a sporiferous saccule was not described for Archaeosporaceae (MORTON and REDECKER, 2001), we here have to emend the description of this family.

Emendation of Archaeosporaceae

Archaeosporaceae J.B. Morton & D. Redecker, emend. Oehl & Sieverd.

Type genus: *Archaeospora trappei* (R.N. Ames & Linderman) J.B. Morton & D. Redecker. emend. Spain.
Basionym: *Acaulospora trappei* R.N. Ames & Linderman. Mycotaxon 3, 566. 1976.

Spores monomorph or bimorph; glomoid spores formed singly or in aggregations in soils or sometimes in roots, terminally on subtending hyphae, acaulosporoid and entrophosporoid spores formed singly, in clusters or in sporocarps in soils or in roots. Acaulosporoid spores formed laterally on the neck of a sporiferous saccule; entrophosporoid spores formed by swelling in the hyphal stalk of a sporiferous saccule. Glomoid spores with one spore wall of one to multiple layers, acaulosporoid and entrophosporoid spores with two or more walls with each mono- to multiple-layers. The fungal structures in the roots do not or only weakly stain with common dyes like trypan blue; forming arbuscular mycorrhizae; no or only very rare formation of vesicles and vesicle-like structures.

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