

***Cetraspora auronigra*, a new glomeromycete species from Ouro Preto (Minas Gerais, Brazil)**

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Cetraspora auronigra, a new fungus of the Racocetraceae (Gigasporales, Glomeromycota), was found in a tropical mountainous forest and an adjacent ironstone outcrop located in the neighborhood of a mining area of Ouro Preto (Minas Gerais, southeastern Brazil). It forms bright yellow to golden-yellow, three-walled spores terminally on sporogenous cells and single, multiply lobed, hyaline to subhyaline germination shields on the outer surface of the inner (germinal) wall. The germinal wall stains dark purple to purple black in Melzer's reagent. Spores of *C. auronigra* are most similar to the 'apricot yellow' to yellow brown spores of *C. armeniaca*, whose outer spore wall stains garnet red when exposed to Melzer's reagent, while in *C. auronigra* this wall does not show any staining reaction. The name of the new fungus is dedicated to its spore colors, especially when exposed to Melzer's reagent, and to the historical city Ouro Preto where the fungus was found in the rhizosphere of several annual and perennial plant species.

Keywords: arbuscular mycorrhizal fungi, biodiversity, Gigasporaceae, Glomeromycetes, *Scutellospora*.

The so-called Iron Quadrangle in the state Minas Gerais, situated in southeastern Brazil, embraces one of Brazil's largest urban centres represented by the city of Belo Horizonte, where continued mining for precious and industrially valuable metals has a long-term tradition and gave the name and wealth to the state (Jacobi *et al.* 2007). The city Ouro Preto, near Belo Horizonte, belongs to the world cultural heritage of the UNESCO because of its history and beauty. Mining for gold has been performed above all in the 16th to 18th century. The gold found in river terraces and later in quartz veins is rich in palladium which, when oxidized, develops a black

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crust, which gave the name to the city. Within the last 150 years, the region has become known for several kinds of metal mining, especially for iron and bauxite (IBRAM 2010).

Along with the financial benefits, the intense mining activity in the region has led to profound changes in the landscape and damage to the environment. Indeed, landscape and vegetation restoration has become a major task and challenge, especially since in most of the time, soil superficial organic layer has not been preserved or stored, making the establishment of plants in post-mined areas difficult (Machado *et al.* 2013). Arbuscular mycorrhizal (AM) fungi might play an important role in the re-colonization and survival of native plants at mined sites (Allen & Allen 1980, Totolá & Borges 2000, Frost *et al.* 2001, Silva *et al.* 2012 a, Souza *et al.* 2012), and therefore it is of major importance to know the native AM fungi in disturbed and undisturbed sites of the region, which might be responsible for the success of restoration measurements.

During studies of the AM fungal diversity in a tropical mountainous forest environment and an adjacent ironstone outcrop (port. campo rupestre ferruginoso), which both were located in the neighborhood of mined sites in the surrounding of Ouro Preto (Minas Gerais), a new AM fungus with characters fitting taxa of the Racocetraceae (Oehl *et al.* 2008) was detected. The objective of this work was to thoroughly study this fungus. According to the results obtained, it is described hereafter under the epithet *Cetraspora auronigra*.

Materials and methods

Study sites, soil sampling and chemical soil analyses

The study sites were an iron-rich outcrop ('campo rupestre ferruginoso') and a semi-deciduous seasonal mountainous forest (Valim *et al.* 2013), established on Plinthosols according to FAO (2006) soil classification. The climate according to Köppen is Cwb, i.e., mesothermic, with a rainy south hemispheric summer (November–March) and a dry winter. The annual mean precipitation is around 1,250 mm and the annual mean temperature is 20 °C. The fragments of the semi-deciduous seasonal forest and the iron-rich rock outcrop form a mosaic of vegetation structural types growing over iron duricrusts that in Brazil occur mainly in the so-called Iron Quadrangle and Serra de Carajas regions of the states of Minas Gerais and Pará, respectively (Castro 2008). These ecosystems are known for their high floral diversity, containing a high number of endemic plant species (Jacobi *et al.* 2007).

Field soil samples (0–20 cm depth) were taken in the iron-rich outcrop and in the semi-deciduous seasonal mountainous forest in September 2011 and March 2012 as described in Mello (2012), with six field plot replicates per site and sampling date (plot size 10 × 10 m²). The samples from September 2011 were analyzed for selected chemical soil characteristics as described in Silva *et al.* (2008). The pH (H₂O) at the two sites were 5.6 and 5.8, organic

carbon contents were 7.4 and 10.9 g kg⁻¹, available P were 3.5 and 5.5 mg kg⁻¹ and available K were 45 and 63 mg kg⁻¹, respectively.

AMF bait cultures

To cultivate the new fungus within the native AM fungal communities, per field soil replicate each one sterilized 2L pot was filled with a mixture of each sampled field soil and sterilized Quartz sand (1:1, v/v). Thereafter the soil-sand mixture was moistened, and each three plantlets (pre-germinated seeds) each of *Zea mays* and *Panicum miliaceum* were planted. The plants grew for a four months cycle under 25/20 °C (day/night) temperature in the greenhouse of the Federal University in Ouro Preto, with three daily manual irrigations (2 minutes long each). Every week, the pots received 20 mL of 1× Hoagland solution (Hewitt 1952). After four months, the pot substrates were dried out for five days, and the substrates were used for a second growth cycle under identical growth conditions on newly seeded *Zea mays* and *Panicum miliaceum*. After each cycle, 50 g of substrate were separated to extract the spores newly formed in the pots (see below). The fungus, however, did not reproduce any spores in the 24 AMF bait cultures initially established.

Morphological analyses

About 200 spores were extracted from the field soils by wet sieving and sucrose centrifugation following the methodology of Sieverding (1991). The spores were mounted in polyvinyl-alcohol-lactic acid-glycerin (PVLG), in PVLG + Melzer's reagent, and in water (Spain 1990, Sieverding 1991), and microscopically examined. Photographs were taken with a digital camera (Leika DFC 295) on a compound microscope (Leika DM750) using Leica Application Suite Version V 4.1 software. The terminology of Oehl *et al.* (2008, 2011 a) and Silva *et al.* (2012 b) for spore morphology and germination of gigasporalean species is followed. Specimens mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at the mycological herbaria of ETH Zurich, (Z+ZT, Switzerland) and of the Federal University of Pernambuco (URM, Recife, Brazil).

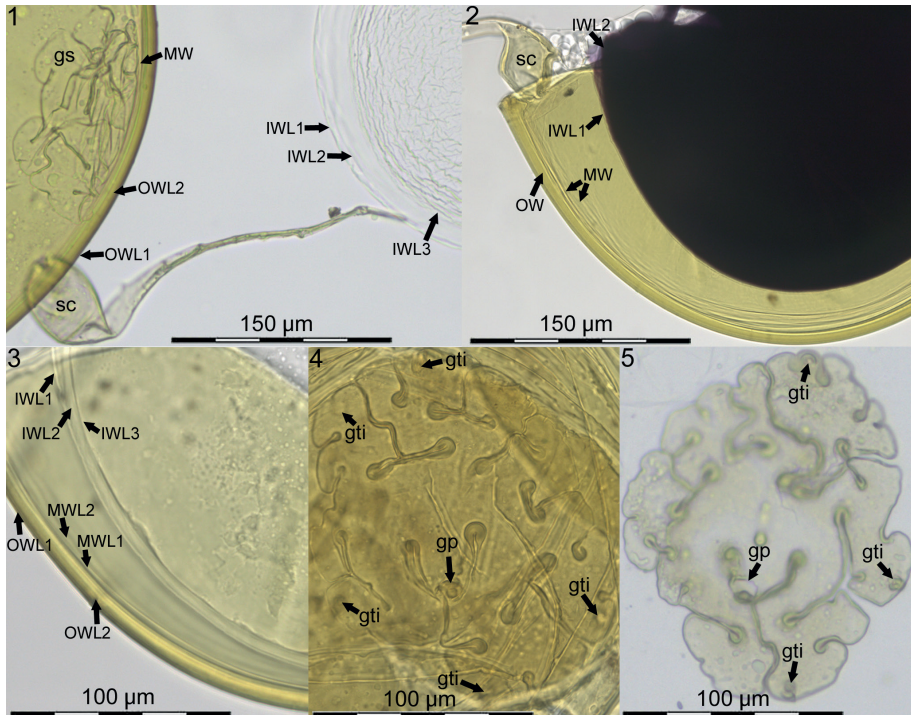
Results

Taxonomy

***Cetraspora auronigra* Oehl, L. L. Lima, Kozovits, Magna & G. A. Silva, sp. nov.** – Figs. 1–5

Mycobank no.: MB 808817

Diagnosis. – Sporae claro-flavae ad aureae, 235–348 × 237–365 µm; tunicis tribus; tunica interior obscuro-purpurea vel purpureo-nigra reagente Melzeri; scutellum germinale hyalinum ad subhyalinum ad rare albo-flavum, subglobosum vel ovale vel ellipsoidum, 135–156 × 140–185 in diametro, lobatum, paucioribus (6–)8–12 lobis. Differt a *Cetraspora armeniaca* in colore tunicae exterioris in aqua, PVLG et reagente Melzeri.



Figs. 1–5. *Cetraspora auronigra*. 1–3. Crushed spores: triple walled (OW, MW & IW) with multiple layers (OWL1–2, MWL1–2 & IWL1–3), formed on sporogenic cells (sc), and differentiating a germination shield (gs). IWL2 becomes dark purple to purple black when exposed to Melzer's reagent (Fig. 3). 4–5. Multiply lobed, hyaline to subhyaline germination shields in planar view with central germ pore (gp) and germ tube initiations (gti) that are visible in the periphery of several lobes.

Type. – BRAZIL, Minas Gerais, Ouro Preto (20°22'21"S; 43°31'04"W), isolated from a ironstone outcrop vegetation ('campo rupestre'), 95–9501 (holotype, ZT Myc 52468); 95–9502–9510 (isotypes, ZT Myc 52469); 95–9511–9513 (isotypes, URM 87581). Other specimens (paratypes ZT Myc 52470 and URM 87582) from an adjacent natural tropical mountain forest (20° 22' 16" S; 43° 31' 02" W).

Etymology. – Latin *auronigra* dedicated to Ouro Preto ('black gold'), the historical city and world cultural heritage of the UNESCO, and also referring to the golden spores of the new species that become purple-black on the inner wall when exposed to Melzer's reagent.

Characters. – Glomerospores formed singly in soil, terminally on subterminal or sometimes intercalary bulbous suspensor cells (= 'sporogenous' cells; Figs. 1–2). Spores are bright yellow to golden yellow, rarely whitish yellow. They are globose to subglobose to elliptical (235–348 × 237–

365 μm) and have three walls (outer, middle, and inner wall) of which only the inner wall becomes dark purple to purple black when exposed to Melzer's reagent (Fig. 2).

Outer wall is 9.1–14.6 μm thick in total and consists of three layers: outermost wall layer (OWL1) is subhyaline to light yellow, semi-persistent and 1.1–1.6 μm thick. OWL2 usually is brilliant golden yellow, rarely whitish yellow, and 7.5–12.0 μm thick. Third layer (OWL3) is concolorous to OWL2, semi-flexible, 0.5–1.0 μm thick, and usually difficult to observe since the middle wall often does not readily separate from OW. None of the three OW layers stains in Melzer's reagent. The straight pore channel at the spore base (about 2.4–4.0 μm broad) is often closed by a plug formed by spore wall material of OWL2 and by OWL3, but it also can appear open.

Middle wall (MW) is 1.8–2.7 μm thick in total and consists of two hyaline layers, a semi-flexible outer layer MWL1 and a semi-flexible layer MWL2, which often appear as one layer (Figs. 2–3). MWL1 is 1.1–1.5 μm thick and generally does not separate from underlying MWL2 which is 0.7–1.2 μm thick. Both layers often show several folds in crushed spores (Figs. 3–4).

Inner wall (IW) is triple-layered (Figs. 2–3), 3.2–5.9(17) μm thick, bearing a germination shield on the outer surface. The outer IW layer (IWL1) is hyaline, semi-flexible and 0.6–0.8 μm thick. The second layer (IWL2) is semi-flexible, unit to finely laminate, amorphous when expanding in lactic acid based mountants, and is 2.0–2.7(4.5) μm thick, expanding up to 12–15 μm under pressure in the lactic acid based mountants (Fig. 1). The innermost layer (IWL3) is relatively thin (0.6–1.2 μm thick), flexible, mostly tightly adherent to IWL2, and therefore generally difficult to observe. IWL2 stains dark purple to purple black in Melzer's reagent.

Sporogenous cells (sc) are globose to elongate, 54–71 μm long and 35–51 μm broad (Figs. 1–2) and generally dark yellow to yellow golden. Two wall layers are visible on young sporogenous cells, which are continuous with OWL1 and with laminated OWL2 of the spore wall. On the sporogenous cells, OWL1 is 0.4–1.0 μm thick and semi-persistent, and OWL2 is 1.5–2.8 μm thick and persistent as long as sc remains attached on the spore. Single 'hyphal pegs' are sometimes formed on the sporogenous cells. They are 4–10 μm thick at the sporogenous cell base tapering to 2.7–4.0 μm within 25 μm length. The sporogenous hyphae attached to the cells are also bi-layered, 10–21 μm thick and tapering to 5–7 μm within 60–320 μm distances from the sporogenous cell. Within these distances, the sporogenous hyphal wall tapers from 1.5–2.5 μm to 1.1–1.6 μm , and 2–8 septa originating from OWL2 may be visible in the sporogenous hyphae.

Germination shield is hyaline to subhyaline (Figs. 1, 4–5), infrequently light yellow in aged spores, subglobose to oval to rarely ellipsoid, 135–156 \times 140–185 μm in diameter. It generally has (6)8–12 lobes. Large folds (~7–30 μm long) arising from the shield wall separate the lobes. The one-layered shield wall and the folds are hyaline to subhyaline and generally only 0.9–1.8 μm thick. Each lobe potentially bears one rounded germ tube initiation, 2.6–

5.1 μm in diameter. However, several germ tube initiations (gti's) may remain undetected in young spores or in crushed mature spores due to the pressure applied on the cover slide, especially when the shields are completely separated from the spores by applying harsh pressure (Fig. 5). Single germination tubes may simultaneously emerge from 1 to 2 gti's during early germination. They penetrate the OW and branch in the spore periphery within short distances.

Distribution. – The new fungus has so far been found only in rhizospheric soils in the two study sites mentioned above. In the ironstone outcrop vegetation, *Tibouchina heteromalla* (Melastomataceae), *Baccharis serrulata* and *Eremanthus erythropappus* (Asteraceae), *Dyckia rariflora* (Bromeliaceae), *Paliavana sericiflora* (Gesneriaceae), *Psylocarpus laricoides* (Rubiaceae), *Senna reniformis* and *Periandra mediterranea* (Fabaceae), *Sporobolus metallicolus*, *Dichantherium sciurotoides*, and *Axonopus siccus* (Poaceae) are the most frequent or dominant species. In the adjacent natural tropical mountain forest, species as *Eremanthus erythropappus* (Asteraceae), *Leandra australis*, and *Miconia coralina* (Melastomataceae), *Byrsonima variabilis* (Malpighiaceae), *Senna reniformis* (Fabaceae), and *Myrcia splendens* (Myrtaceae) are commonly found. At these sites, *C. auronigra* co-occurred with other AM fungal species such as *Acaulospora mellea*, *Glomus macrocarpum*, *Claroideoglomus etunicatum*, *Gigaspora margarita*, and *Scutellospora spinosissima*, among others.

Discussion

Racocetraceae species are characterized by the spore formation on sporogenous cells and the formation of multiply-lobed, hyaline to sub-hyaline germination shields positioned on the outer surface of the inner, germinal wall (Oehl *et al.* 2008, Silva *et al.* 2012 b). *Cetraspora auronigra* can easily be distinguished from all other species in the family Racocetraceae by the brilliant yellow to yellow golden, triple-walled spores that become dark purple to purple black on the inner wall due to the staining reaction in Melzer's reagent.

All 12 *Racocetra* species known so far differentiate bi-walled spores on sporogenous cells and multiply-lobed, hyaline to subhyaline germination shields, and they do not stain on the inner wall when exposed to Melzer's reagent, while the colors of the outer wall generally changes, depending on the species, e.g. from white to yellow or from yellow to red or dark reddish-brown (Oehl *et al.* 2008, Tchabi *et al.* 2009, Goto *et al.* 2011, Lin & Yen 2011, Silva *et al.* 2012 b). So far, five species have unambiguously been attributed to the genus *Cetraspora* that has triple-walled spores and also multiply-lobed, hyaline to subhyaline germination shields. Two of these species form spores with roughened surfaces or surface projections (*C. helvetica* Oehl *et al.* and *C. nodosa* (Błaszk.) Oehl *et al.*; Błaszkowski 1991, Oehl *et al.* 2010, 2011 c, Silva *et al.* 2012 b), while the three other species have smooth spore

surfaces (*C. armeniaca* (Błaszk.) Oehl *et al.*, *C. gilmorei* (Trappe & Gerd.) Oehl *et al.* and *C. pellucida* (T.H. Nicolson & N.C. Schenck) Oehl *et al.*; Gerdemann & Trappe 1974, Nicolson & Schenck 1979, Ferrer & Herrera 1980, Błaszkowski 1992, Oehl *et al.* 2008). Spores of *C. pellucida* are brilliant white, those of *C. gilmorei* are hyaline to creamy, and those of *C. armeniaca* are apricot yellow to yellow brown (Gerdemann & Trappe 1974, Nicolson & Schenck 1979, Błaszkowski 1992, Oehl *et al.* 2008), while those of *C. auronigra* are bright yellow to golden yellow. The diagnostic feature for *C. auronigra*, however, is that it is the only *Cetraspora* species whose outer wall does not react at all in Melzer's reagent, while the outer wall of *C. gilmorei* turns reddish brown, the OW of *C. armeniaca* turns garnet red, and the OW of *C. helvetica*, *C. nodosa* and *C. pellucida* stains pinkish purple to dark purple (Błaszkowski 1992, Oehl *et al.* 2008, 2010). Hyaline to subhyaline germination shields of other gigasporalean species such as *Scutellospora calospora*, *S. spinosissima*, and *S. alterata* are only bi-lobed (Pontes *et al.* 2013), or mono-lobed, as in *Orbispora pernambucana* and *O. projecturata* (Kramadibrata *et al.* 2000, Silva *et al.* 2008, Oehl *et al.* 2011 b, Silva *et al.* 2012 b). Sometimes *Scutellospora* species form additional 'false lobes', especially as artifacts when pressure is applied on the shields, but these 'false lobes' never form germ tube initiations (gti's), and thus, such shields (Pontes *et al.* 2013) can hardly be confused with those of *Cetraspora* and *Racocetra* spp., that differentiate multiple (4–12) 'true lobes' with multiple (>2) gti's per shield (Silva *et al.* 2012 b, Figs. 4–5). According to the findings of Pontes *et al.* (2013), the shield morphology and phylogeny of *Scutellospora striata*, provisionally attributed to the genus *Cetraspora*, will need more careful attention than given initially by Cuenca & Herrera-Peraza (2008) and Oehl *et al.* (2008).

Molecular spore analyses had been performed on *C. auronigra* using the primers designed for the amplification of the partial LSU rDNA sequences of multiple known AMF species (Silva *et al.* 2006). More recently, these primers had also been used successfully on several new glomeromycotan species belonging to a broad range of AMF genera, families and orders (e.g. Goto *et al.* 2012; Mello *et al.* 2012, 2013; Furrázola *et al.* 2013). However, so far the trials to amplify DNA from the field collected spores of *C. auronigra* failed. Using the system for Glomeromycota of Oehl *et al.* (2011 a), AMF taxa can generally be identified readily on the genus level by morphological spore identification. This kind of identification might not be so easy for glomoid species but it is reliable and can be recommended, especially when concomitant molecular analyses on spores of a specific species fail or cannot be performed for whatever reason. The morphological identification is especially reliable for genera within Scutellosporaceae and Racocetraceae, such as *Scutellospora*, *Racocetra*, and *Cetraspora* species, and has been confirmed by the newest advances of Silva *et al.* (2012 b), and was further developed by Pontes *et al.* (2013; see also above). Furthermore, the spore morphology of *C. auronigra* is well delimited. For many other species, however, e.g. for glomoid species, it might be highly advantageous to indispensable to confirm the mor-

phological conclusions directly by molecular analyses on spores obtained from monosporic pot cultures (e.g. Błaszczkowski *et al.* 2008, Oehl *et al.* 2014 a, b).

Hitherto, *C. auronigra* is only known from the two study sites in Ouro Preto (Minas Gerais). Future studies have not only to reveal the molecular phylogeny but also its biogeographical distribution, first for the Iron Quadrangle of Minas Gerais and for SE Brazil, but also on a more continental to global level.

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