Rhizoglomus venetianum, a new arbuscular mycorrhizal fungal species from a heavy metal contaminated site, downtown Venice in Italy

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Abstract

Rhizoglomus venetianum, a new arbuscular mycorrhizal fungal species, has been isolated and propagated from a heavy metal contaminated site in Sacca San Biagio island, downtown Venice, Italy. Interestingly, under the high levels of heavy metals occurring in the site, the new fungus was able to grow only intraradically. In greenhouse trap and single species cultures under low heavy metal levels, the fungus produced innumerous spores, clusters and sporocarps extraradically, which were formed terminally on subtending hyphae either singly, in small spore clusters, or, preferably, in loose to compact non-organized sporocarps up to 2,500 × 2,000 × 2,000 µm. Spores are golden-yellow to bright yellow brown, globose to subglobose to rarely oblong, 75–145 × 72–140 µm in diameter and have four spore wall layers. Morphologically, the new fungus is similar to *R. intraradices*, and phylogenetically it forms a monophyletic clade next to *R. irregulare*, which generally forms irregular spores and lacks, like *R. intraradices*, the flexible innermost wall layer beneath the structural/persistent third wall layer. A key for the species identification is presented comprising all 18 *Rhizoglomus* species, so far described or newly combined.

Keywords

Arbuscular mycorrhizal fungi; Heavy metals; *Rhizoglomus*; Morphology; Molecular phylogeny; SSU–ITS–LSU nrDNA

Introduction

The number of species of arbuscular mycorrhizal (AM) fungi (AMF, Glomeromycota, Tedersoo et al. 2018) greatly increased during the last decades, passing from about 120 to ca. 300 species since nineties (Schenck and Pérez 1990; Giovannetti et al. 1990; Oehl et al. 2011a; Błaszkowski 2012; Krüger et al. 2012). The continuous progress in the morphological identification of AMF species (e.g. Sieverding et al. 2014; Błaszkowski et al. 2015, 2018) and in the setup of suitable molecular tools based on more informative regions of nuclear rDNA (Silva et al. 2006; Krüger et al. 2009), resolving even very closely related taxa (Stockinger et al. 2010; Krüger et al. 2012), allowed the discovery and separation of new species (ca. 10 per year in the last 20 years), covering diverse genera and families, some ubiquitous, but others rare or associated with particular plants or habitats (Turrini and Giovannetti 2012). However, the huge AMF diversity, evidenced on the basis of environmental DNA sequences, which do not correspond to formally described species (Öpik et al. 2010, 2014), is still far to be fully described. Actually, it was calculated that probably less than 5% of existing AMF in the world have formerly been described so far (Krüger et al. 2009), most of which have not yet been cultivated in pure cultures. Indeed, the difficulty in cultivating AMF out of their original environment still represents one of the major limits to the isolation and description of new species. Nevertheless, AMF research focused on invaluable "hot spots" for species diversity (Myers et al. 2000), distributed around the world, together with studies on AMF occurring in extreme habitats (high-altitude habitat, high-salinity habitat, thermal habitat, desert, wetland, polluted soils) would probably lead to the discovery of novel endemic genetic resources (Turrini and Giovannetti 2012; Sousa et al. 2018). In very recent years, new species were isolated from extreme habitats, i.e. *Rhizoglomus melanum* from wetland (Sudová et al. 2015), *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizoglomus arabicum* from hyper arid environments of the Arabian Peninsula (Symanczik et al. 2014, 2018), and *Acaulospora pustulata* and *Acaulospora tortuosa* from high altitude habitat in Sierra Nevada, Spain (Palenzuela et al. 2013).

Among extreme habitats, those contaminated by heavy metals (HM) represent invaluable sites for the recovery of peculiar AMF isolates and species. HM are extremely toxic to microbial communities, modifying the structure of some essential enzymes, causing oxidative damage and DNA injury and altering plasma membranes (Hassan et al. 2011).

During last years HM-polluted habitats have been largely studied in relation to AMF occurrence, due to the their beneficial effects in enhancing plant tolerance to such toxic habitats (Pawłowska et al. 1996; Hildebrandt et al. 2007). It has been shown that AMF play a fundamental role in HM phytostabilization and phytoextraction (Gohre and Paszkowski 2006) by trapping HM in their hyphae and spores, and, consequently, reducing metal availability for the plants (González-Chávez et al. 2002, 2009; Cornejo et al. 2013). Many studies

have described different AMF communities occurring in HM-polluted soils (Turnau et al. 2001; Vogel-Mikuš et al. 2006; Regvar et al. 2010; Hassan et al. 2011; Ban et al. 2015; Yang et al. 2015, Sánchez-Castro et al. 2017) and in the roots of plants spontaneously growing in HM-polluted areas (Whitfield et al. 2004; Vallino et al. 2006; Zarei et al. 2008, Bedini et al. 2010). Molecular analyses of AMF communities of HM-polluted sites detected several sequence types new to science (Vallino et al. 2006; Sánchez-Castro et al. 2017), including sequences from Sacca San Biagio, an ash disposal island located downtown Venice (Italy; Bedini et al. 2010).

Sacca San Biagio, which can be considered a model system for the study of remediation and requalification of polluted environments, has been spontaneously colonized by pioneer plants, animals and microorganisms during the past 30 years, since incinerator plant's closure in 1984. Most of the plants occurring on the island showed a high level of AM fungal colonization (67%), but no spores were retrieved in bottom ashes. The molecular analysis, carried out on the roots of three mycorrhizal plants growing on the island, evidenced the occurrence of sequences corresponding to the '*Rhizoglomus intraradices*/*Rhizoglomus fasciculatum*' complex, together with sequences detected so far only *in planta* and sequences new to science (Bedini et al. 2010).

The goal of the present work was to obtain in pure culture AMF spores, after transplanting the host plants from Sacca San Biagio island to a non-polluted environment, and to describe a new AM fungal species associated to the ruderal host plant *Senecio inaequidens*. Both morphological and molecular techniques were used to characterize the AM fungus, which was named *Rhizoglomus venetianum*.

Material and Methods

Study site

The new glomeromycotan species originated from Sacca San Biagio island, downtown Venice (latitude 45° 42′ N, longitude 12° 30′ E). The island is a tideland, that was elevated after the Second World War, filling the lagoon sand with inert construction waste, which has been subsequently covered by bottom ashes produced by an incinerator plant of municipal solid waste (MSW), operating for more than ten years, since 1973 up to 1984. Ashes form layers of about 1.5-3 m and occupy a volume of about 60,000 m^3 (Bedini et al. 2010). They are highly toxic, since the incineration process enhances the concentration of some pollutants, expecially heavy metals, which represent a risk for human health and the environment (Clijsters et al. 2000). Ashes contains high concentrations of different heavy metals: Al (25,941.6 mg kg^{-1}), As (24.8 mg kg^{-1}), Cd (3.4 mg kg^{-1}), Cr (101.6 mg kg^{-1}), Cu (1820.1 mg kg^{-1}), Ni (83.6 mg kg^{-1}), Pb (2000.8 mg kg^{-1}), Zn (3210 mg kg^{-1}) (Bedini et al. 2010). Moreover they contain 85.1% sand, 10.1% silt, 4,8% clay, 5.1% total organic C, a pH (KCl) of 8 and the

following total nutrient concentrations: 2.8 ‰ total N, 1215.3 mg kg^{-1} total P, and 24.6 mg kg^{-1} available P (Olsen) (Bedini et al. 2010). After the year 1984 the incinerator was demolished and no activities were carried out on the isle, which has been naturally colonized by both herbaceous and woody plants for more than 30 years. Plant communities detected on the isle were mostly ruderal and nitrophilous, (class *Artemisietea vulgaris*, including perennial ruderal xerophilous phytocenoses, typical of temperate or Mediterranean regions) (Mucina et al. 1997). Among plant species occurring in Sacca San Biagio island, *S. inaequidens* plants, belonging to the Asteraceae family, were selected and collected with their intact root system. They are ruderal plants originating from South Africa, very adaptable to different environments, invasive, spreading in many countries also in Europe. *Senecio inaequidens*, widely distributed on the isle at the sampling time, was chosen for spore isolation, since it was shown to be highly mycorrhizal and able to host in its roots different AMF species (Bedini et al. 2010).

Sampling, establishment and growth of trap and pure cultures.

Senecio inaequidens plants and rhizosphere toxic ashes were collected in Sacca San Biagio island in May 2011 (Figs 1-2). Plants and ashes were transplanted in 8 L pots filled with a steam-sterilized 1:1 volumetric mixture of agricultural field soil collected at the Interdepartmental Centre for Agri-environmental Research Enrico Avanzi (CIRAA), University of Pisa, S. Piero a Grado, Pisa, Italy (latitude 43° 40′ N, longitude 10° 19′ E) and TerraGreen (calcinated clay; OILDRI, Chicago, IL, USA), then transferred in greenhouse to induce AMF sporulation of fungi occurring in *S. inaequidens* roots, which were used as "trap cultures" for the isolation of AM fungal spores from the heavy metal toxic island (Fig. 3). At different time points, after 6, 9, 12 months of culture, rhizosphere soil (50 g) from different trap cultures was sieved through a set of nested sieves down to a mesh size of 50 μm (Gerdemann and Nicolson 1963) for spore collection and analysis and the spores were extracted by a water-sucrose gradient and centrifugation (Sieverding 1991). Spores investigated in this study were obtained from spore clusters (max 5 spores connected with a common hypha). Pure cultures were achieved making use of the "sandwich system", useful to allow the interaction between AMF and plant roots (Giovannetti et al. 1993). Briefly, small spore clusters were let to germinate for 10 days in sterile microwells containing sterile water, in a growth chamber at 24°C in the dark. Germinated spores were then placed in contact with the roots of *Trifolium alexandrinum* plantlets placed on 47-mm diameter cellulose ester MilliporeTM membranes (0.45 μm diameter pores). Another 47-mm membrane was placed on spores and root systems to close the sandwich. The "sandwich system" was buried in sterile 10 cm pots containing steam-sterilized quartz grit, then maintained in suntransparent bags (Sigma, Milan, Italy) under controlled conditions (18-24°C, 16-8-h photoperiod of

irradiance 100 μEm^{-2} s^{-1}, 60% RH). Plants were supplied weekly with 10 ml half strength Hoagland's solution. After 6 weeks the "sandwich systems" were opened, plants were gently removed and the occurrence of extraradical mycelium was assessed under a stereomicroscope (Wild, Leica, Milan, Italy) (Figs. 4-5). When colonization check was positive, plants were transferred to 700 cm^3 plastic pots containing a 1:1 mixture of soil and Terragreen, and moved to the greenhouse. Pots were regularly checked for spore formation, as described above. Sporulation was obtained after 4 months of culture (Fig. 6). The new species was maintained and renewed under greenhouse conditions together with *T. alexandrinum* and *Lactuca sativa* as host plants in the International Microbial Archive (IMA) collection of the Department of Agriculture Food and Environment, University of Pisa, Pisa, Italy.

Morphological analyses

Spores were isolated from the pot-culture soil by wet sieving and decanting through a set of nested sieves down to a mesh size of 32 μm (Sieverding 1991), then transferred into Petri dishes and examined under a stereomicroscope (Wild, Leica, Milan, Italy). Spores were isolated by using capillary pipettes, mounted on microscope slides in polyvinyl alcohol lacto-glycerol (PVLG; Koske and Tessier 1983), in PVLG + Melzer's reagent (1:1, v:v; Brundrett et al. 1994) and in water (Spain 1990). Qualitative spore traits (sporocarp and spore shape, color and size, spore wall structure, including color and size of each wall layer of the spores and their subtending hyphae) were examined on > 25 sporocarps and > 100 spores.

The terminology of the spore structure basically is that presented for species with glomoid spore formation in Oehl et al. (2011b), Sieverding et al. (2014) and Błaszkowski et al. (2018). Photographs were taken with a digital camera (Leika DFC 295) on a stereomicroscope (Leica S8APO) and a compound microscope (Leica DM750) using Leica Application Suite Version V4.1 software. Specimens mounted in PVLG and a (1:1) mixture of PVLG and Melzer's reagent were deposited at Z+ZT (ETH Zurich, Switzerland), and at the Botanical Garden of the University of Pisa, Italy.

Molecular analysis and phylogeny

Intact, healthy spores were manually collected with a capillary pipette under a dissecting microscope (Wild, Leica) and cleaned by sonication (120 s) in a B-1210 cleaner. After three rinses in sterile distilled water (SDW), spores were surface sterilised with 2% chloramine T supplemented with streptomycin (400 μg/ml) for 20 min and rinsed five times in SDW. Intact sterilized single spores were selected under a laminar flow hood, individually transferred into Eppendorf PCR tubes, crushed with a glass pestle and their DNA directly amplified

using the nested protocol of Krüger et al. (2009), focused on a fragment of about 1500 bp covering partial SSU, the whole ITS and the D1 and D2 variable regions of the LSU sequences of rDNA. In the first PCR reaction crushed spores were amplified in 25 µl of PCR reaction mix using 0.125 U GoTaq Flexi DNA Polymerase (Promega, Milan, Italy), 0.4µM of each primer (SSUmAf1 and LSUmAr3, Krüger et al. 2009), 0.2 mM (each) dNTPs, 1.5 mM MgCl2 and 1× manufacturer‘s reaction buffer. The thermal cycler was programmed as follows: a manual hot start at 95°C for 3 min, 35 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 10 min. The nested PCR reactions were performed by diluting (1:100) the first PCR amplicons and using 2 µl of dilutions as template for the second reaction in a final volume of 50 µl. The primer pair, SSUmCf1-LSUmBr3 (Krüger et al. 2009; 0.4µM), was added to the PCR mix. Taq DNA polymerase, dNTPs, buffer and MgCl2 concentrations were the same as those described above. Amplification conditions were as follows: a manual hot start at 95°C for 3 min, 35 cycles at 95°C for 30 s, 63°C for 45 s, 72°C for 1,5 min and a final extension step at 72°C for 10 min. PCR products (10 µl) were separated on 1% agarose gels containing ethidium bromide (0.5 µg/ml).

Amplified DNA fragments of SSU-ITS-LSU regions were purified by Wizard SV Gel and PCR Clean-Up System according to the manufacturer‘s instructions (Promega), with a final elution volume of 20 µl and purified products (2 µl) were quantified by a BioPhotometer (Eppendorf). Purified products were cloned into pGem®-T Easy vector according to the manufacturer‘s instructions (Promega). Putative positive clones were screened by standard SP6/T7 amplifications, followed by a nested PCR using the SSUmCf1-LSUmBr3 primer pair. Concentration of PCR mix components and PCR conditions were the same as those described above for PCR reactions. Six clones from single spores (two clones/spore) were purified by Wizard® Plus SV Minipreps (Promega). Recombinant plasmids were sequenced using SP6/T7 vector primers at GATC Biotech (Köln, Germany). Sequences reported in this study were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession numbers LS974594-LS974599. The alignment for the tree obtained in this study was deposited at TreeBase under the ID: 23045.

Sequences were edited in MEGA6 (Tamura et al. 2013) and their similarities determined using the Basic Local Alignment Search Tool (BLASTn) provided by NCBI. Then they were aligned with those corresponding to the closest matches from GenBank as well as with some sequences from the Glomeraceae family covering the entire SSU-ITS-LSU region or portion thereof, using MUSCLE as implemented in MEGA6. Phylogenetic tree was inferred by Bayesian and Maximum-likelihood analyses. The Bayesian analysis was carried out in MrBayes version 3.2 (Ronquist et al. 2012), using the General Time Reversible sequence evolutionary model and branch support values corresponded to the posterior probabilities of two Markov chain

Monte Carlo samplings over 500,000 generations and a tree sampling every 100 generations after discarding the initial 10%. For the maximum-likelihood analysis the evolutionary rate differences among sites were computed in MEGA6 using a discrete Gamma distribution + G method. The confidence of branching was assessed using 1000 bootstrap resamplings. The generated phylogenetic tree was drawn in MEGA6 and edited in Adobe Acrobat XI.

Results

Taxonomic analyses

Rhizoglomus venetianum Oehl, Turrini, & Giovann., **sp. nov.**, Figs 7–18

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DIAGNOSIS - Differs from *Rhizoglomus irregulare* and *R. intraradices* by having an additional, fourth wall layer, below the structural wall layer.

ETYMOLOGY: *venetianum* referring to the city of Venice to which the island Sacca San Biagio belongs.

TYPE: Holotype, deposited at Z+ZT (accession ZT Myc 58970), derived from a pure culture established on the host plant *Trifolium alexandrinum* in the greenhouse of the Microbiology Laboratory in Pisa, at the Department of Agriculture, Food and Environment, University of Pisa, Italy. Mycorrhizal mycelia and intraradical mycorrhizal structures, occurring in the roots of *Senecio inaequidens* plants, were collected in Sacca San Biagio island in the central lagoon of Venice Italy (45°25’36’’N; 12°18’34’’E). The island hosted a Municipal Solid Wastes incinerator operating since 1973 up to 1984, producing bottom ashes that were disposed all over the island area. Since 1984 no significant activities were carried out on the island and in 2003 the furnace was demolished. In the last 30 years the highly polluted soil of the island has been naturally colonized by spontaneous vegetation. Collector of the host plant *S. inaequidens* was A. Turrini and collection date 31.05.2011. Isotypes deposited at Z+ZT (ZT Myc 58971) and at the Botanical Garden of the University of Pisa (PI-MH-Z11 to Z18). The living culture is currently maintained in the International Microbial Archives in Pisa under the accession number IMA10.

DESCRIPTION: Spores formed terminally on subtending hyphae (SH) either singly, in small spore clusters, or, preferably, in loose to compact non-organized sporocarps with (10–)40–150 up to hundreds to a few thousands of spores per sporocarp, with sporocarps up to 2,500 × 2,000 µm. Large sporocarps may consist of several small sporocarps. Spores are golden yellow-brown to yellow brown, globose to subglobose to rarely oblong or rarely irregular, (75–)85–130(–145) × (72–)84–125(–140) µm. Mycelial hyphae staining pinkish to purple in Melzer’s reagent.

Spore wall has four layers. Outer layer (SWL1) is hyaline, evanescent, 0.6–1.3 µm thick. Second layer (SWL2) is hyaline to subhyaline, evanescent, 0.8–1.4 µm thick. Third layer (SWL3) is structural, persistent, laminate, golden-yellow to bright yellow brown, 2.0–3.5 µm thick, expanding up to 7.5 µm under pressure in lactic acid based mountants. Innermost layer (SWL4) flexible, light-yellow to bright yellow, 0.6–1.4 µm thick, usually tightly adherent to SWL3, sometimes separating or showing several folds in crushed spores. In Melzer's reagent, SWL1 and SWL2 stain pinkish to pinkish purple, while SWL3 stain purple.

Subtending hyphae (SH) of spores cylindrical to slightly funnel-shaped, sometimes recurved, 9.1–13.2 µm broad and 15–100 µm long, and without introverted wall thickening toward the spore base. The base generally is not closed by a septum formed by SWL3 or SWL4 but open. Such septa can more often be found in some distance from the spore bases within the intrasporocarpic hyphae (ISH), which are 8–15 µm thick, golden yellow to bright yellow brown. SWL2-4 continue in the ISH. The extrasporocarpic hyphae (ESH, or mycelia hyphae) are hyaline, 5–12 µm thick and have 1–2 hyphal wall layers. In the transition zone between ISH and ESH hyphae are slightly pigmented and generally show several septa within a rather short distance of 100 to 200 µm.

MYCORRHIZA FORMATION: forming AM associations with *Trifolium alexandrinum* L. and *Lactuga sativa* as plant host in pot cultures. The mycorrhizal structures consist of arbuscules, vesicles, and intra- and extraradical hyphae and stain dark blue in 0.05% trypan blue.

Molecular analyses

Phylogenetic analyses of the partial SSU, ITS and the partial LSU of the rDNA placed *R. venetianum* sequences in the genus *Rhizoglomus* Sieverd. et al., typified by *R. intraradices* (N.C. Schenck & G.S. Sm.) Sieverd. et al. (Sieverding et al. 2014). Sequences of the new species formed a separate, strongly supported clade (BI = 0.99; ML =95%), sister to that comprising *Rhizoglomus irregulare* (Fig. 19). Actually, blast analyses showed 95-98% homology with *R. irregulare* sequences (isolates DAOM197198, MUCL46241, accession numbers HE817882, FR750130), representing the closest species in our molecular analyses.

Erection of a new combination in *Rhizoglomus*

In this paragraph, a recently described species, *Rhizophagus neocaledonicus* (Crossay et al. 2018), which morphological and phylogenetically belongs to the genus *Rhizoglomus*, is transferred to *Rhizoglomus* in a new combination:

Rhizoglomus neocaledonicum (D. Redecker, Crossay & Cilia) Oehl, Turrini & Giovann. **comb. nov.**

MycoBank 827095

Basionym: *Rhizophagus neocaledonicus* D. Redecker, Crossay & Cilia, Mycological Progress 17: 739. 2018. https://doi.org/10.1007/s11557-018-1386-5.

Key to the species in *Rhizoglomus*

Here, we are presenting an updated morphological identification key for all 18 species belonging to the genus *Rhizoglomus* according to Sieverding et al. (2014), including the recently described or newly combined species *Rh. dunense*, *Rh. vesiculiferum* and *Rh. neocaledonicum* (e.g. Sieverding et al. 2014; Al-Yahya'ei et al. 2017; Błaszkowski et al. 2018):

1 Species with two spore wall layers: 2

1 Species with > two spore wall layers: 3

2 Spores whitish yellow to yellow; species with 1–2 laminae on structural wall layer: 4

2 Spores light brown to red brown; 50–90 µm in diam, globose to subglobose, formed singly, in small clusters or large, dense sporocarps (up to 15 × 10 × 10 mm); SWL1 hyaline, evanescent, 1–1.5 µm; SWL2 red to dark brown, 3-6 µm, with several laminae: *R. invermaium* (I.R. Hall) Sieverd., G.A. Silva & Oehl

3 Species with three spore wall layers: 5

3 Species with > three spore wall layers: 6

4 Spores generally < 50 µm; whitish yellow to yellow, 15–50 µm in diam, globose to subglobose, formed singly or in clusters; SWL1 hyaline to light yellow, evanescent, 0.5–1.2 µm; SWL2 whitish yellow to yellow, 0.5–2.0 µm: *R. microaggregatum* (Koske, Gemma & P.D. Olexia) Sieverd., G.A. Silva & Oehl

4 Spores generally > 50 µm; hyaline to yellow, 60–110 µm in diam, globose to subglobose, formed in small clusters to dense sporcarps, up to 1.8 x 1.4 x 1.4 mm; SWL1 hyaline, evanescent, 0.5–1.2 µm.; SWL2 yellow to yellow brown, 1.2–2.4 µm, consisting of two laminae, that might separate under pressure applied: *R. aggregatum* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl

5 SWL3 is structural, laminate layer: 7

5 SWL3 is flexible layer, adherent to structural, laminate layer SWL2: 8

6 Spores generally < 75 µm; spores hyaline to yellowish white or subhyaline, 40–75 µm, globose to subglobose, formed in clusters; spore wall with four layers, 3.3–5.8 µm in total, SWL1 & SWL2 2.5–3.0 µm in total, SWL3 & SWL4 laminate, but each is only 0.5–1.0 µm thick: *R. proliferum* (Dalpé & Declerck) Sieverd., G.A. Silva & Oehl

6 Spores generally > 75 µm: 9

7 Spores yellowish white to yellow brown: 10

7 Spores chestnut brown to dark brown; 137–285 µm in diameter, globose to subglobose, formed singly or in small clusters; SWL1 is evanescent, hyaline, 0.8–1.2 µm thick; SWL2 is semi-persistent to evanescent, 2.8–5.1 µm thick and shows many fissures in degraded stages. Under pressure on the cover slide single, irregular pieces of SWL2 (about 5–15 × 5–15 µm) may split from the spore surface. SWL3 is dark chestnut brown to black brown, laminate, 7.2–12 µm thick: *R. melanum* Sudová, Sýkorová & Oehl

8 Spores generally < 90 µm: 11

8 Spores generally > 90 µm: 12

9 Spores without flexible inner layer beneath the pigmented, structural layer: 13

9 Spores with flexible inner layer beneath the pigmented, structural layer: 14

10 Spores ovoid, oblong to often irregular; hyaline to pale yellow, 60–130 × 80–240 µm; they may have deep wall depressions and apical cap-like swellings; SWL1 (0.5–1.5 µm thick) & SWL2 (0.6–5.0 µm thick) hyaline and semi-permanent; SWL3 hyaline to pale yellow, with inseparable laminae, 1.5–4.4 µm thick, staining pale orange to deep red in Melzer's reagent: *R. irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl

10 Spores usually globose to subglobose, rarely irregular, without deep wall depressions and apical cap-like swellings: 15

11 Spores hyaline to creamy, 70–90 µm, globose to subglobose, formed singly, in clusters or large sporocarps, SWL1 hyaline, 0.5–1.2 µm, evanescent; SWL2 laminate, 2.0–7.5 µm, pesistent; SWL3 (semi-)flexible, 0.5–1.4 µm thick; all layers staining dark purple in Melzer's: *R. fasciculatum* (Thaxt.) Sieverd., G.A. Silva & Oehl

11 Spores dark chestnut to coffee brown, mostly globose to subglobose, 61−83 µm in diam; SWL1 hyaline, mucilaginous, SWL2 laminate, dark brown, 5.4−6.5 µm thick, SWL3 fine, tightly attached to SWL2, sub-hyaline to bright brown: *Rh. neocaledonicum* (D. Redecker, Crossay & Cila) Oehl, Turrini & Giovann.

12 Spores generally > 100 µm, hyaline or white to creamy or light yellow to brownish yellow: 16

12 Spores 50–70 µm, yellow to yellow brown, globose to subglobose, formed singly, in clusters or sporocarps, 1.0 × 0.6 × 0.5 mm; three layered wall up to 12 µm thick; SWL1 hyaline, 0.8–2.0 µm, evanescent; SWL2 light brown, laminate, 4.0–8.0 µm; SWL3 hyaline, 1.5–2.0 µm thick: *R. antarcticum* (Cabello) Sieverd., G.A. Silva & Oehl

13 Hyaline outer spore wall layers difficult to differentiate; spores pale yellow to brownish yellow, 110–172 µm, globose to subglobose, formed singly or in small clusters (2–5 spores); SWL1 hyaline, mucilaginous, 0.8–2.5 µm

311 thick, reddish in Melzer's, SWL2 hyaline, rigid, 1.6–2.8 µm thick, SWL3 hyaline to pale yellow, semi-flexible,
312 easily separated from SWL2, 1.5–2.0 µm thick, non reactive to Melzer's reagent; SWL4 yellow to brownish
313 yellow, laminated, 2.6–3.8 µm thick, staining dark red in Melzer's: *R. custos* (C. Cano & Dalpé) Sieverd., G.A.
314 Silva & Oehl
315 13 Pigmented inner wall layer separating into several layers/laminae under pressure; spores, pale yellow to
316 grayish yellow, 30–85 × 50–125 µm, globose to subglobose, formed in clusters up to 0.8 × 1.0 mm; SW difficult
317 to interpret; SWL1 hyaline, evanescent, 0.6–2.5 µm, SWL2 readily separating in 2–4 laminae, which might be
318 counted as SWL2-5, in total up to 5.5 µm thick: *R. arabicum* (Błaszk., Symanczik & Al-Yahya'ei) Sieverd.,
319 G.A. Silva & Oehl
320 14 Spores pastel yellow to light yellow, 75–131 µm, globose to subglobose, formed single or in loose clusters up
321 to 1.2 mm in diam; SWL1 hyaline, semi-permanent, 1.0–5.3 µm; SWL2 hyaline, permanent and unit, 0.8–1.5
322 µm; SWL3 laminate, pastel yellow to light yellow, 6.3–14.0 µm, consisting of laminae up to 0.8–1.0 µm thick,
323 frequently easily separating from each other in crushed spores; SWL4 hyaline and flexible, 0.8–2.0 µm. SWL1
324 and SWL3 stain reddish white to greyish red and brownish violet to violet brown in Melzer's reagent,
325 respectively: *R. natalense* (Błaszk., Chwat & B.T. Goto) Sieverd., G.A. Silva & Oehl
326 14 Spores golden-yellow to bright yellow brown, globose to subglobose, 75–145 × 72–140 µm in diam, formed
327 singly, in small spore clusters, or, preferably, in loose to compact sporocarps up to 2.5 × 2.0 × 2.0 mm; SWL1,
328 hyaline, evanescent, 0.6–1.3 µm thick; SWL2, hyaline, evanescent, 0.8–1.4 µm thick; SWL3 structural,
329 persistent, laminate, golden-yellow to bright yellow brown, 2.0–3.5 µm thick, expanding up to 7.5 µm under
330 pressure in lactic acid based mountants; SWL4 flexible, light-yellow to bright yellow, 0.6–1.4 µm thick, usually
331 tightly adherent to SWL3, sometimes separating or showing several folds in crushed spores. In Melzer's reagent,
332 SWL1 and SWL2 stain pinkish, while SWL3 stain purple: *R. venetianum* Oehl, Turrini, & Giovann.
333 15 Spores colourless, hyaline, 39−125 µm, globose to subglobose, formed singly in soils; SWL1 semi-
334 permanent, smooth or slightly roughened, 1.0–5.0 µm; SWL2 finely laminate, 4.0–8.8 µm; SWL3 uniform, to
335 laminate, when up to 2.0 µm thick, (semi-)flexible; SWL1 stains pinkish white to dark red in Melzer's, while
336 SWL3 turns pale yellow: *R. dunense* Błaszk. & Kozłowska
337 15 Spores pigmented, pastel yellow to light yellow or yellow brown to grey brown: 17
338 16 Spores hyaline (to white), becoming, whitish yellow with age, 120–290 µm, globose to subglobose, formed
339 singly or in loose clusters; SWL1 1.2–2.3 µm, evanescent to semi-persistent, staining purple in Melzer's, SWL2
340 5–20 µm laminate, persistent; SWL3 2.0–9.0 µm, often becoming yellow with age: *R. clarum* (T.H. Nicolson &
341 N.C. Schenck) Sieverd., G.A. Silva & Oehl

16 Spores light yellow to brownish yellow, 145–450 µm, globose to subglobose, formed singly or in loose clusters; SWL1 hyaline, evanescent, 2.0–5.0 µm, staining light purple in Melzer's; SWL2 hyaline to light yellow, persistent, 10–16 µm; SWL3 yellow to yellow brown, flexible, consisting of 1–2 laminae, 0.5–2.0 µm: *R. manihotis* (R.H. Howeler, Sieverd. & N.C. Schenck) Sieverd., G.A. Silva & Oehl

17 Spores pastel yellow to light yellow, globose, (69–)90(–108) µm diam., rarely egg-shaped, 75–83 × 80–91 µm, formed in loose clusters of 4–18 spores (Błaszkowski et al. 2018) or in sporocarps up to 13 × 10 mm in diam (Thaxter 1922; Gerdemann and Trappe 1974), rarely singly in soil; SWL1 finely-laminate, semi-permanent, hyaline, (1.8–)3.1(–4.5) µm thick, usually slightly swelling in PVLG. SWL2 uniform (not divided into visible sublayers), permanent, hyaline, (1.0–)1.3(–2.3) µm thick, tightly adherent to layer 3, usually difficult to see; SWL3 laminate, permanent, pastel yellow to light yellow, (10.5–)12.0(–13.0) µm thick; the laminae usually easily separate from each other even in spores slightly crushed in PVLG; SWL1 staining yellowish white to pale yellow, SWL2 yellowish red to reddish white & SWL3 staining dark ruby in Melzer's reagent: *Rh. veisculiferum* (Thaxt.) Błaszk., Kozłowska, Niezgoda, B.T.Goto & Dalpé

17 Spores yellow brown to grey brown, often with a greenish tint, 90–135 µm, globose to subglobose, without depressions or swelling at the spore apex, formed singly, in clusters or sporocarps; SWL1 (0.5–1.3 µm thick) & SWL2 (0.8–2.0 µm thick) hyaline and evanescent; SWL3 yellow brown to grey brown, laminate, 3.2–12 µm comprises frequently separating sublayers, which are each 0.5–1 µm thick; SWL1 staining purple in Melzer's reagent: *R. intraradices* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl

Discussion

The new AM fungus *Rhizoglomus venetianum* can be easily distinguished from all other *Rhizoglomus* spp. by the combination of spore size, color and spore wall structure. It forms a four-layered spore wall, such as *R. natalense*, *R. custos* and *R. proliferum*, which form either smaller spores (*R. proliferum*; < 75 µm), spores of a different spore wall structure (*R. custos*) or less-pigmented spores (*R. natalense*; light yellow to bright yellow spores; see identification key above). Morphologically it resembles *R. intraradices*, which lacks, as also known for *R. irregulare*, the innermost, fourth spore wall layer, which is always present in *R. venetianum*.

R. venetianum is a fungus isolated from a very toxic environment, containing high levels of heavy metals, expecially Zn, Pb and Cu, the latter occurring at very high concentrations, compared with other metal polluted environments in which studies on AMF diversity were performed (Turnau et al., 2001; Vallino et al., 2006; Abdel-Azeem et al., 2007; Khade et al. 2009; Long et al. 2010; Alguacil et al. 2011; Hassan et al. 2011; Krishnamoorthy et al. 2015). Interestingly, also another new species in the genus *Rhizoglomus* (*R. custos*) was

isolated from a naturally heavy-metal polluted environment, i.e. the bank side of the Rio Tinto River in southern Spain (Cano et al. 2009), showing high levels of Fe (2g/L), Mg (1.3g/L), Cu (390 mg/L), Zn (280 mg/L) and Mn (100 mg/L). *R. custos*, as *R. venetianum*, forms a multi-stratified wall structure, a trait that might represent a possible barrier to toxic elements for the spores and might help the survival of the species in very toxic environments. Other species in the genus *Rhizoglomus*, such as *R. clarum* or *R. intraradices*, have been shown to possess different attributes linked to the ability of developing strategies, such as avoidance, compartmentalization or resistance to stress, allowing them to live and grow in environments with high levels of heavy metals (Ferrol et al. 2009). Such traits, accompanied with the attitude of Glomeraceae to colonize roots by hyphal fragments or pieces of mycorrhizal roots rather than by germinated spores, might have helped also *R. venetianum* to be more competitive and widespread in the roots of *S. inaequidens* plants collected on the island. Interestingly, on Sacca San Biagio, *R. venetianum* appeared to grow entirely intraradically, since no spores of any AMF species were previously detected within ashes (Bedini et al. 2010). We can speculate that AM fungal species, living in very disturbed soil, may complete their life cycle in a privileged ecological niche (within plant roots), avoiding the direct contact with toxic compounds. In our previous study on AMF diversity occurring on Sacca San Biagio (Bedini et al. 2010), the most abundant sequence types detected in the roots of three plant species collected on the island corresponded to R. *irregulare*/R. *fasciculatum* and to *R. intraradices*. Since SSU sequences encompassing the V3-V4 region were not analyzed in this work, we are not able to confirm the occurrence of *R. venetianum* within the VeGlo8 cluster of our previous work. The genus *Rhizoglomus*, to which the identification key to species is proposed in this work, is the prevalent genus in studies on AMF diversity occurring in HM-contaminated sites, since it was dominant in colonized roots of *Fragaria vesca* L. collected in zinc wastes in southern Poland (Turnau et al. 2001), in a HM-contaminated soil along South Tyne River (Whitfield et al. 2004), in a chemical industry contaminated site in Northern Italy (Vallino et al. 2006), in soils of mines in Asia (Zarei et al. 2008; Yang et al. 2015; Sun et al. 2016; Wu et al. 2010; Wei et al. 2015; Park et al. 2016) and Europe (Alguacil et al. 2011; del Mar Montiel-Rozas et al. 2017; Sanchez-Castro et al. 2017).

In conclusion, Sacca San Biagio represents an interesting site, contributing to our knowledge of AMF diversity in extreme habitats. Future works will disclose the concentrations of heavy metals able to inhibit the growth of the isolate *R. venetianum* IMA10, compared with other AMF species and isolates, and the occurrence of the new fungus in other environments.

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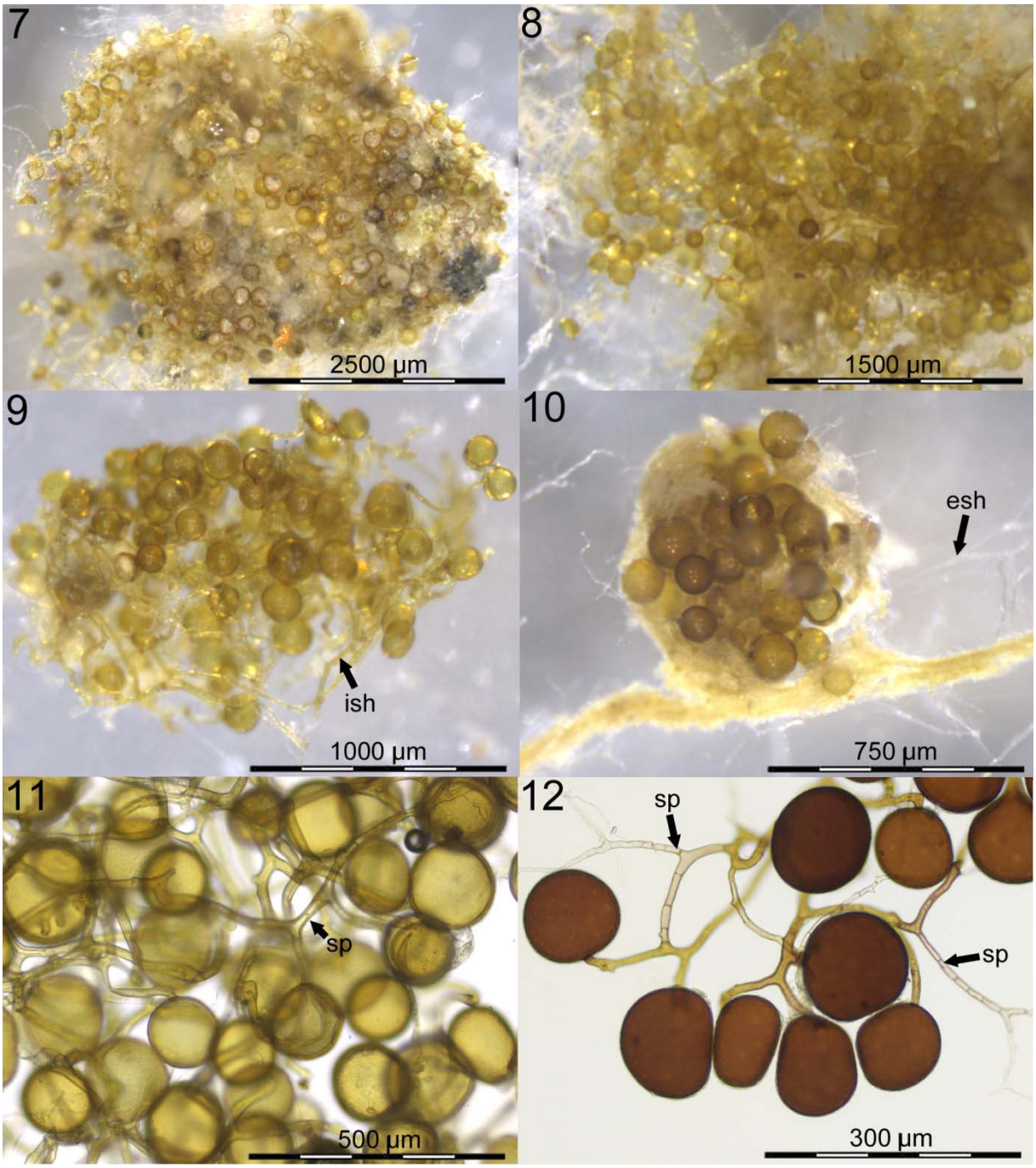
578

Figure legends

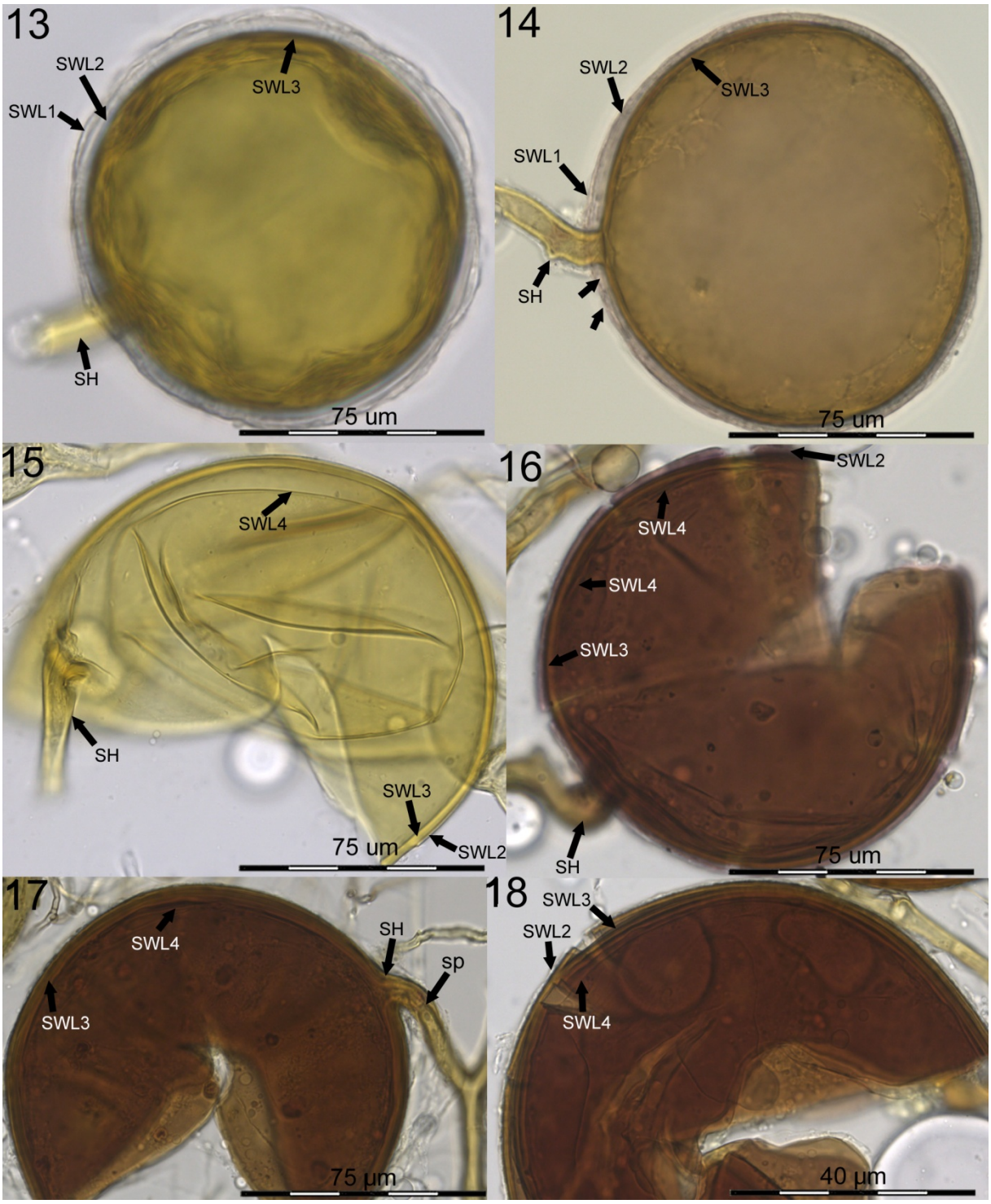
Figs 1-6

Isolation of *Rhizoglomus venetianum* from Sacca San Biagio island, Venice, Italy. 1. Sacca San Biagio island (on the left) with the perimetral embankments and the vegetation coverage and Venice's living quarters in the background. 2. Ashes collected from the island. 3. *Senecio inaequidens* trap cultures using plants collected in the island. 4-5. Production of extraradical mycelium from *Trifolium alexandrinum* roots used as bite plants in the sandwich system (root diameter ca. 500 µm). 6. Spores of *R. venetianum* produced in the type culture (single spore diameter ca. 80–140 µm).



Figs 7-12

Rhizoglomus venetianum. 7–10. Sporocarps in water isolated from the rhizosphere of *Trifolium alexandrinum* from the single species culture of the type. Intrasporocarpic hyphae (ish) are generally pigmented, while extrasporocarpic hyphae (esh) are hyaline to subhyaline. 11. Sporocarp fragment mounted in PVLG. 12. Sporocarp fragment mounted in PVLG + Melzer's reagent. Several septa (sp) can be observed on intrasporocarpic hyphae and especially at the transition between extra- and intrasporocarpic hyphae.



595

596 **Figs 13-18**

597 *Rhizoglomus venetianum*. 13–15. Uncrushed and crushed spores in PVLG. Spores with four layers (SWL1-4),
598 cylindrical to slightly funnel-shaped subtending hyphae (SH) and open pores at the spore base. Sometimes a
599 septum (sp) can be detected at some distance to the spore base. 16–18. Spores mounted in PVLG + Melzer's
600 reagent. SWL1 and SWL2 stain pinkish to pinkish purple, while SWL3 stain purple.

601

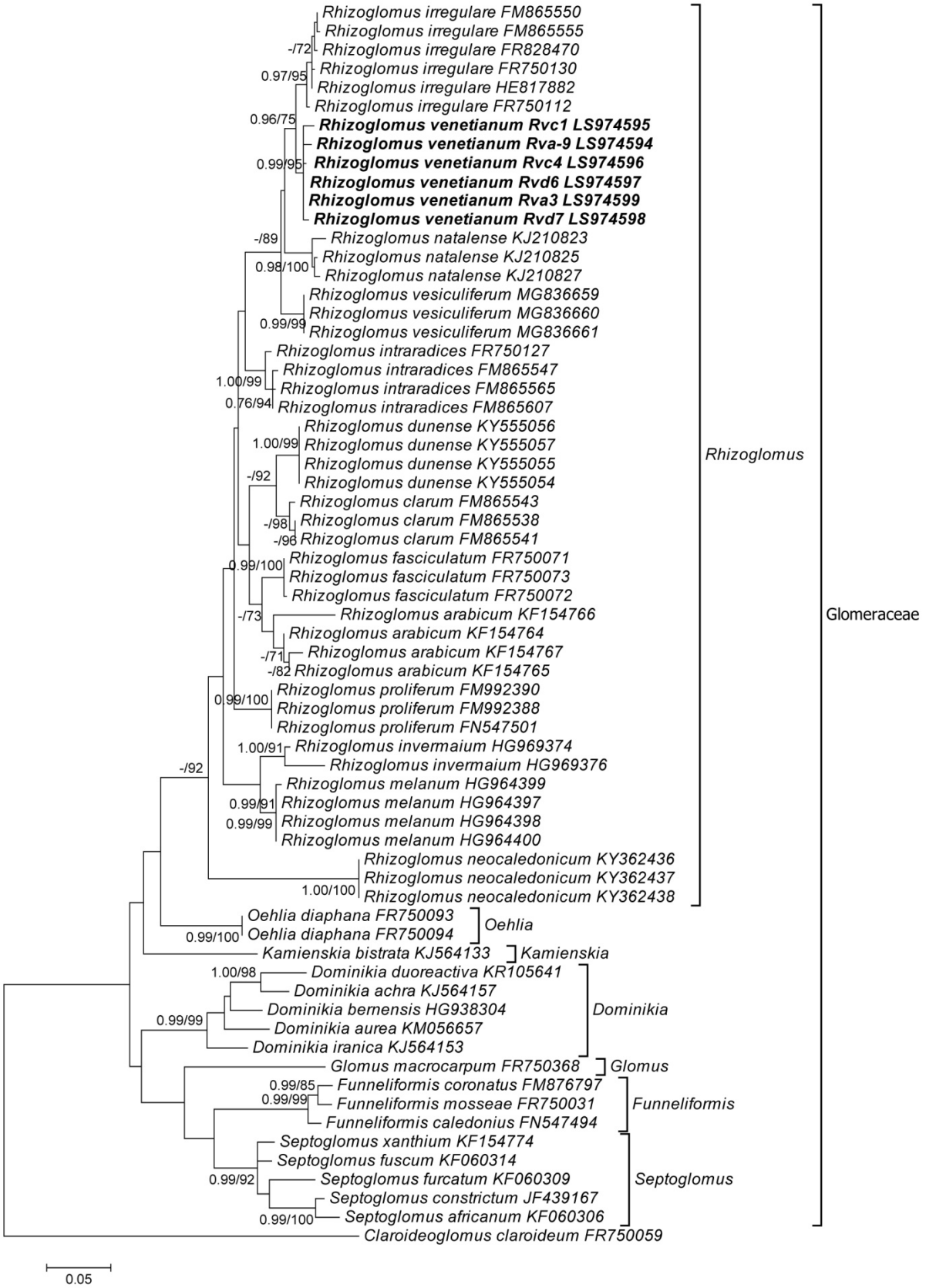


Fig. 19

Maximum likelihood phylogenetic tree of glomeromycotan sequences obtained using the GTR+G model. The analysis is based on partial SSU, ITS, and partial LSU region of the nuclear rDNA sequences (1763 characters;

606 SSUmCf3-LSUmBr1 fragment) and involved 66 nucleotide sequences. The Bayesian posterior probabilities and
607 ML bootstrap values are shown near the branches, respectively, when they exceed 0.50 and 70% (1000
608 replications). Sequences of the new species obtained in the present study are shown in bold, and their accession
609 numbers are prefixed with clone identifiers. Different genera and families are indicated in brackets. The tree is
610 rooted by a sequence of *Claroideoglomus claroideum*.
611
612 **Author contributions**
613 The work was conceived by M.G. and A.T. A.T. isolated and carried out the trap cultures. M.S. and A.T. carried
614 out the molecular and phylogenetic analysis. F.O. performed the morphological description. A.T., F.O and M.G.
615 carried out the manuscript preparation for submission. All authors commented on the final draft of the
616 manuscript.
617