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Viscospora peruviscosa, a new fungus in the Glomeraceae from a plantation of *Theobroma cacao* in Peru

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Summary

A new fungus, *Viscospora peruviscosa*, was detected in a *Theobroma cacao* plantation in the Huallaga province of San Martín State in Peru. The fungus was propagated in the greenhouse on *Sorghum vulgare* and *Brachiaria brizantha*. The fungus is similar to *V. viscosa* as it has two spore wall layers and also a viscose outer spore surface, but its spores are smaller ((30-)44-56(-65) × (25-)44-54 µm) and the subtending hyphae generally are more pronounced funnel-shaped. Also, the walls of the spores and subtending hyphae are thinner than in *V. viscosa*. Phylogenetically, both species form two well separated sister clades in the genus *Viscospora*. Based on the partial nrDNA gene, the two species have 90-91% maximum identity (MI). So far, the fungus is only known from the cacao plantation in Huallaga. No environmental sequences in the public data bases suggest that the fungus has already been found elsewhere in the neotropics or worldwide. This is the second species in the genus *Viscospora* (Glomeraceae) described, hence *Viscospora* is no longer monospecific.

Key words: Glomeromycetes, Glomerales, Mycorrhiza, Taxonomy, Tropical agriculture.

Introduction

The monospecific genus *Viscospora with V. viscosa* (T.H. Nicolson) Sieverd. Oehl & G.A. Silva was originally described in Oehl et al. (2011a), and then included in the Entrophosporaceae (Oehl et al., 2011b), based on morphological characteristics and sequences that had been published in public databases (e.g., Schwarzott et al. 2001). Redecker et al. (2013) rejected the erection of *Viscospora* and proposed that *V. viscosa (basionym G. viscosum*; Walker et al., 1995*)* is a species of *Septoglomus* Sieverd., G.A. Silva & Oehl. However, spores of *V. viscosa* are morphologically totally different to all other species of *Septoglomus*, and indeed all now available molecular genetic analyses revealed that *Viscospora* is an own genus, and *V. viscosa* does not belong to *Septoglomus* (Silva et al., 2023). The hitherto single species attributed to the genus is characterized by rather small, hyaline spores (44-97 × 46-94 µm; mean 60 × 58 µm), and by a viscose outer spore surface, which can capture soil debris during degradation of the evanescent outermost spore wall layer. Hitherto, *Viscospora viscosa* has been known from at least three continents and from several countries, such as Italy, France, USA, Japan and China (e.g., Pivato et al., 2007; Torrecillas et al., 2012; Berruti et al., 2017; Redecker et al., 2013; Yamato et al., 2012; Zeng et al., 2014).

During studies of arbuscular mycorrhizal fungi (AMF) in cocoa, coffee and inka nut plantations in San Martín State of Peru, a new, small spored fungus was found in a cocoa plantation in the province Huallaga. The spores were generally smaller than those of *V. viscosa*, but showed a similar viscose spore surface, where the evanescent outer spore wall layer still was present on the spores. Thus, the objective of this study was to verify, if this species might belong to the genus *Viscospora* and if the species is indeed 'new to science' and should be described hereafter.

Material and methods

Study site and soil sampling

Soil samples (0-25 cm depth) were taken in an agricultural field site (approximately 1 ha) planted with cocoa at San Andres (6°53'32.56" S, 76°44'41.19" W, 386 m.a.s.l) in the province Huallaga of the department San Martín in the Peruvian part of Western Amazonia. Before the establishment of the cocoa (Clone CCN51), the area was a coffee plantation. At the time of soil sampling, the cocoa tree plantation was already ten years old. The cocoa plantation had a tree coverage of 10% and a herbaceous cover of 60%, calculated according to the methodology proposed by Scaccabarozzi et al. (2020). The cocoa trees were associated with *Inga* sp. trees for shade, and were cultivated and maintained without any addition of chemical fertilizers and pesticides. Mean annual temperatures at the study site are about 24-28 °C, with variation between 18.7-31.4 °C throughout the year. Mean annual precipitation is approximately 700 mm.

For soil sampling five trees were selected initially, with a minimum distance of 15 m between the single trees. Directly after collection, the soil samples were air-dried and stored in the laboratory of the Molecular Biology and Genetics Laboratory, Faculty of Agricultural Sciences, National University of San Martín-Tarapoto, in Peru until inoculation and establishment of the bait cultures (see below), about six weeks after sampling. The soil had a clayey-loamy texture. Soil pH (H_2O) was 6.5, and available P ('Olsen-P', Olsen et al., 1954) was 17.1 mg P kg^{-1}.

AM fungal bait cultures

Five bait cultures were established in the greenhouse of the Facultad de Ciencias Agrarias, Universidad Nacional de San Martín-Tarapoto under ambient temperature conditions in cylindrical 3 L pots with 3 kg of substrate, corresponding to the five soil samples taken in the cocoa plantation. The substrates consisted of a mixture of collected soil and of coarse river sand and vermiculite (2:1:1/v:v:v, Corazon-Guivin et al., 2022). The substrate mixtures were autoclaved at 121 °C for 60 min, 4 weeks before establishment of the bait cultures. At bait culture establishment, the pots were first filled to 75% with the autoclaved substrate. Thereafter 100 g of rhizosphere soils were added to the substrate surface, and seeds of *Sorghum vulgare* Pers.

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and *Brachiaria brizantha* (A.Rich.) Stapf were sown in order to establish the mycorrhizal association and reproduce spores of the new fungal species together with the native AMF species communities. The seeds were surface sterilized before seeding, using the methodology proposed by Corazon-Guivin et al. (2019a). Finally, the seeds were covered with the remaining 25% of the autoclaved substrate. The cultures were maintained in the greenhouse of the Facultad de Ciencias Agrarias, Universidad Nacional de San Martín-Tarapoto for 10 months, with 22.5 °C, 30 °C, and 38.0 °C as minimum, mean, and maximum air temperatures, respectively. The relative air humidity varied between 54 to 88% between March 2018 and December 2018 in the greenhouse. The plants were irrigated every other day, and a Long Ashton nutrient solution was added every two weeks, with reduced P contents (60% reduction), (Hewitt, 1966). In three of the five pot cultures, the new fungus was detected after ten months of propagation.

Morphological analyses

The fungal spores and spore clusters were separated from the soil samples by using standard isolation techniques, i.e., wet sieving followed by sugar gradient-centrifugation, spore washing and decantation into Petri dishes (Sieverding, 1991). The description of the morphological spore characteristics and their subcellular structures are based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske and Tessier, 1983), Melzer's reagent, a mixture of PVLG and Melzer's reagent (Brundrett et al., 1994), a mixture of lactic acid to water at 1:1, and water (Spain, 1990). Terminology of the spore structure follows Oehl et al. (2011b), Błaszkowski (2012) and Oehl et. al. (2011a) for Glomeraceae species. Photographs were taken with a digital camera (Leica DFC 295) on a compound microscope (Leitz Laborlux S), using Leica Application Suite version 4.1 software (Leica Microsystems, GmbH, Bochum, Germany). Specimens mounted in PVLG and a (1:1) mixture of PVLG and Melzer's reagent were deposited at Z+ZT, the joint mycological herbarium of the University of Zurich and the Federal Institute of Technology (ETH) in Zurich, Switzerland.

Molecular analyses

Intact, healthy spores were isolated from the bait culture pots, and cleaned by friction on fine filter paper (Corazon-Guivin et al., 2019a, b). Spores were surface-sterilized (Mosse, 1962) using a solution of chloramine T (2%), streptomycin (0.02%) and Tween 20 (2-5 drops in 25 mL final volume), for 20 min and rinsed five times in milli-Q water. One independent group of sterile spores, containing 20-30 spores, were selected under a laminar flow hood and individually transferred into Eppendorf PCR tubes. Crude extract was obtained by crushing the spores with a sterile disposable fine-tipped pilon in three µL milli-Q water under the observation at 5× magnification using a Carl Zeiss stereoscope (Corazon-Guivin et al., 2021). Direct PCR of these crude extracts was performed in an automated thermal cycler (Eppendorf Mastercycler nexus, Germany) with a Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions with 0.4 µM concentration of each primer. A two-step PCR was conducted to amplify the ribosomal fragment consisting of partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA using the primers SSUmAf/LSUmAr and SSUmCf/LSUmBr, consecutively, according to Krüger et al. (2009). PCR products from the second round of amplifications (~1500 bp) were separated electrophoretically on 1.2% agarose gels, stained with Diamond™ Nucleic Acid Dye (Promega) and viewed by UV illumination. The band of the expected size was excised with a scalpel and isolated from the gel with the GFX™ PCR DNA and Gel Band Purification Kit (Sigma-Aldrich) following the manufacturer's protocol, cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and transformed into One Shot® TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Sixteen recombinant colonies were selected by blue/white screening and the presence of inserts detected by PCR amplification with KOD DNA Polymerase (Sigma-Aldrich) using universal forward and reverse M13 vector primers. After isolation from transformed cells, plasmids were sequenced on both strands with M13F/M13R primers using the BigDye Terminator kit 3.1v (Applied Biosystems). The products were analyzed on an automated DNA sequencer (ABI 3730XL DNA analyzer-Macrogen Inc).

Phylogenetic analyses

The AM fungal sequences (partial SSU, ITS region, and partial LSU rDNA) obtained were aligned with other Glomeraceae sequences from GenBank in MAFFT (Katoh et al., 2019), using the default parameters. For some isolates, just sequences from the partial LSU rDNA were used. Those isolates are indicated in the phylogenetic tree generated. Only DNA fragments with more than 600 bp were used in the analyses. *Entrophospora etunicata* (W.N. Becker & Gerd.) Błaszk., Niezgoda, B.T. Goto & Magurno was included as outgroup. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al., 2004). Bayesian (two runs over 5×10^6 generations, with a sample frequency of 500 and a burnin value of 25%) and maximum likelihood (1,000 bootstrap) analyses were performed, respectively, in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and PhyML (Guindon and Gascuel, 2003), launched from Topali 2.5, using the GTR + G model.

Results

Molecular and phylogenetic analyses

The phylogenetic analyses from the partial SSU, ITS region, and partial LSU nrDNA sequences placed the new fungus in a separated clade close to *Viscospora viscosa* (Fig. 1). The support values for the clade of the new species were 100 % in all analyses. Querying the BLASTn on NCBI (Johnson et al., 2008), with ITS and LSU nrDNA fragments from the new species, no environmental sequences with ≥ 97% of maximum identity (MI) were found. The species sequences (considering the partial SSU, ITS region and partial LSU nrDNA together) with closest match to the new fungus are from *V. viscosa* (90.5%).

Taxonomy

Viscospora peruviscosa Corazon-Guivin, G.A. Silva & Oehl, sp. nov., Figs. 2-11
MycoBank MB 850127

Diagnosis: Differing from *Viscospora viscosa* (with spore size of 44-97 × 46-94 µm; mean 60 × 58 µm diam.) in having smaller spores ((30-)44-56(-65) × (25-)44-54 µm; mean 48 × 46 µm diam.) and thinner spore and subtending hyphae walls (SH walls 1.0-1.9 instead of 3.0-4.2 µm), and by the related but distinct phylogenetic position.

Etymology: *peruviscosa*, referring to the country Peru, from which the new fungus was firstly reported, and to the viscose nature of the evanescent outer spore wall layer.

Holotype: accession ZT Myc 66938, deposited at Z+ZT, derived from a bait culture established on *Sorghum vulgare* and *Brachiaria brizantha* in the greenhouse of the Molecular Biology and Genetics Laboratory, Faculty of Agricultural Sciences, National University of San Martín-Tarapoto, Peru. Fungal inoculum for the culture originated from a cocoa (*Theobroma cacao*) plantation of San

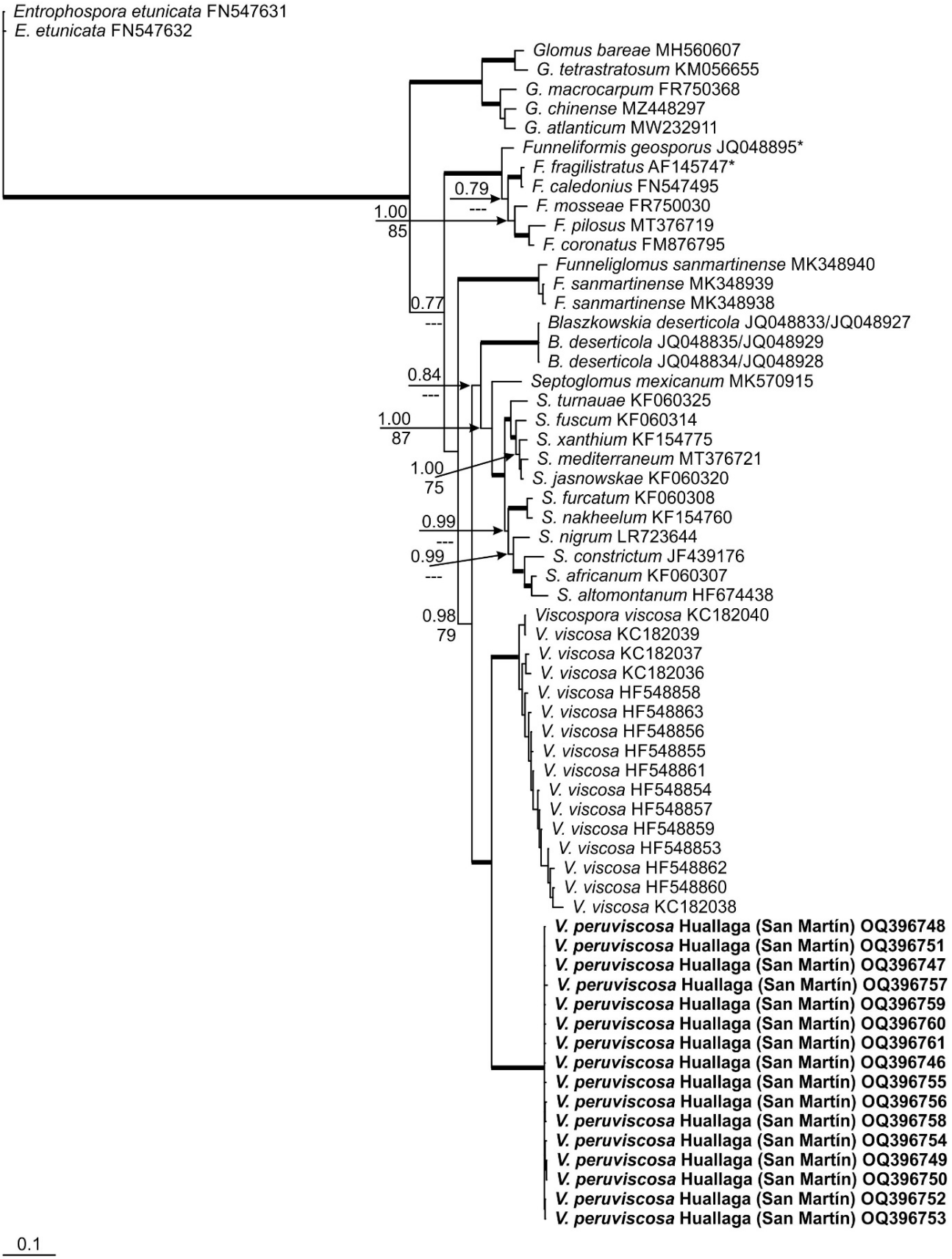
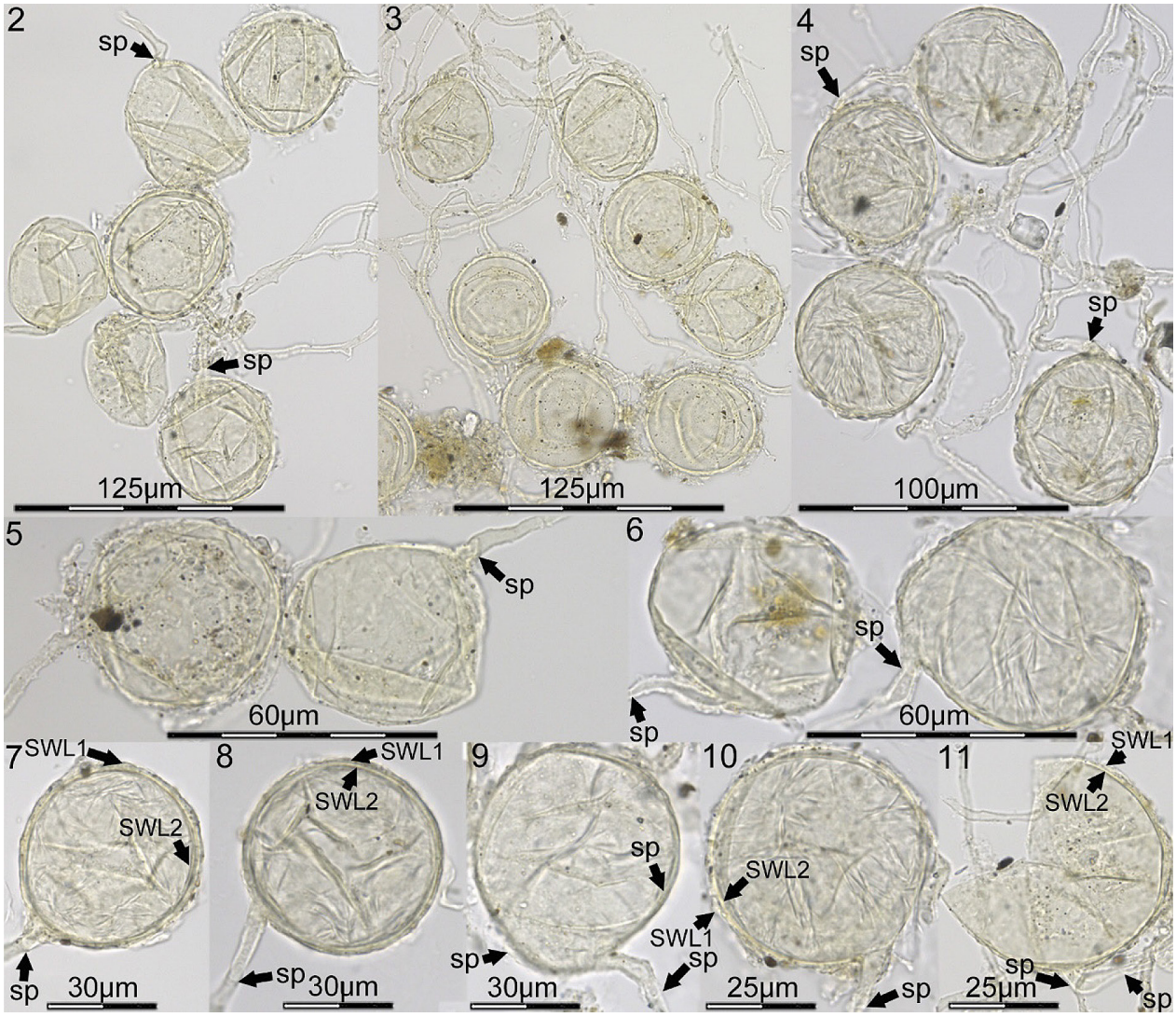


Fig. 1: Phylogenetic tree obtained by analysis from partial SSU, ITS region, and partial LSU nrDNA sequences of different Glomeraceae spp. Sequences are labeled with their database accession numbers. Support values (from top) are from Bayesian inference (BI) and maximum likelihood (ML), respectively. Sequences obtained in this study are in boldface. Only support values of at least 75% are shown. Thick branches represent clades with more than 90% of support in all analyses. The tree was rooted by *Entrophospora etunicata*. Sequences with only the partial LSU nrDNA are indicated by *.



Figs. 2-11: *Viscospora peruviscosa*. **2-4.** Small spore clusters comprising 4-8 hyaline spores, which have attracted a certain amount of soil debris during the degradation of the viscose outer spore wall layer. Wherever visible already under low magnification, pore of the subtending hyphae (SH) is closed by a rather long septum (sp) in a short distance to the spore base. **5-11.** Spores are bi-layered (SWL1-2). SH generally funnel-shaped to rarely cylindrical, slightly constricted to inflated, straight or slightly flared. Septa in irregular distances to the spore base within the SH. The viscose nature of SWL1 is most clearly visible in Figs. 5-7 and Figs. 9-11. Spores had been mounted in PVLG + Melzer's reagent (Figs 2-3; 11) or in PVLG alone (all other figures). SWL2 might stain weak yellowish in Melzer's.

Andres (6°53'32.56" S, 76°44'41.19" W, 386 m.a.s.l) in the province Huallaga of the Department San Martín in the Peruvian part of Western Amazonia. In the plantation, the cocoa trees were associated with *Inga* sp. trees. Collector was Mike Anderson Corazon Guivin and collection date was 18.01.2018. Isotypes from the bait culture (ZT Myc 66937) were also deposited at Z+ZT. Living cultures of the fungus are currently maintained at the Universidad Nacional de San Martín-Tarapoto.

Description: The fungus differentiates hyaline spores terminally or intercalary on pronounced funnel-shaped to slightly funnel-shaped to rarely cylindrical or inflated subtending hyphae, singly or in loose clusters with up to ca. 50 spores per cluster, which are up to 350 × 350 µm in diam. The spores are bi-layered, (30-)44-56(-65) × (28-)44-54 µm (mean 48 × 46 µm) in diam., or rarely ovoid to oblong. Regularly, one to several straight to curved septa are formed in the subtending hyphae close to the spore base or in a distance of up to 10-60 µm to the spore base.

Spore wall (SW) bi-layered, hyaline. Outer layer SWL1 is evanescent, 0.7-1.0 µm thick, viscose, as long as present on the spores. SWL2 is persistent and unite, 1.1-1.6(-2.3) µm thick and might stain whitish yellow to yellowish, when exposed to Melzer's reagent.

Subtending hyphae (SH) of spores straight or recurved, sometimes crestfallen to rarely flared, generally pronounced funnel-shaped, to slightly funnel-shaped to rarely cylindrical or inflated. SH 4.0-12.0 (-15.0) µm broad at spore base, usually tapering to 4.0-6.5 µm within a short distance of 10-20 µm from the spore base. Both spore wall layers continue in the subtending hyphae, with similar to slightly smaller thickness towards the mycelia hyphae: SHWL1 is 0.5-0.7 µm and SHWL2 is 0.5-1.2 µm broad at spore base, tapering to 0.8-1.1 µm in the mycelial hyphae, on which generally only the inner of the two wall layers is visible. Pore is approximately 2.8-10.0 µm wide at spore base, generally closed by one to several, straight to curved septa within the SH, about 1.0-2.0 µm thick. Mycelia hyphae 4-6 µm are in diameter.

Mycorrhiza formation not known so far from single species cultures.

Specimen examined: Holotype (ZT Myc 66938), isotype specimen (ZT My 66937), and two paratype specimen (ZT Myc 66936; ZT Myc 66940). The paratypes originated from additional pot cultures with *S. vulgare* and *Brachiaria brizantha*, initially inoculated with two soil samples, taken from the rhizosphere of different cocoa trees within the same plantation.

Distribution: So far, the fungus was found in one agroforestry site (cocoa plantation of San Andres) in the Province of Huallaga, Department of San Martín. Soil pH at the site was 6.5, and available P (P-Olsen) was 17.1 mg P kg^{-1}.

Discussion

The new fungus phylogenetically belongs to the genus *Viscospora*, a sister genus of *Septoglomus* (Silva et al., 2023). By the discovery of *V. peruviscosa,* the genus *Viscospora* is no longer monospecific. Like *V. viscosa*, also *V. peruviscosa* forms colorless, hyaline to white spores with a viscose spore surface, thus hitherto these features are assumed to be common within the genus. The two *Viscospora* species can be differentiated by spore size, since *V. viscosa* generally forms bigger spores than *V. peruviscosa*. Additionally, also the spore wall is generally thicker in *V. viscosa* (2.5-3.0-4.5 µm) than in *V. peruviscosa* (1.8-2.6(-3.3) µm), as well as the wall of the subtending hyphae, which is 3.0-4.2 µm in *V. viscosa* and 1.0-1.9 µm in the new species. Finally, for *V. viscosa* an internal thickening of the subtending hyphae was described, which was not detected by us in *V. peruviscosa*.
Viscospra viscosa is known from several continents and habitats, such as from croplands, orchards, vineyards and coastal areas in Western and Southern Europe (e.g., Pivato et al., 2007; Alguacil et al., 2012; Cesaro et al., 2008; Torrecillas et al., 2012; Berruti et al., 2017; De Castro et al., 2018), coastal areas and croplands in Eastern Asia (e.g., Yamato et al., 2012; Cheng et al., 2013; Zeng et al., 2014), and from croplands, switch grass plantations, and natural environments of the perennial herb *Asclepias speciosa* in the USA (e.g., Moebius-Clune et al., 2013; Hahn et al., 2018; Dirks and Randall, 2020). The new fungus, *V. peruviscosa*, is so far only known from the isolation site, the cocoa plantation in San Andres of the province Huallaga in San Martín State of Peru. Only the future will reveal, if this fungus is restricted to a specific habitat, vegetation, soil or climate type in Western Amazonia, or might have also a wider distribution, as known for many AMF species.

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Conflicts of interest
No potential conflict of interest was reported by the authors.

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