

Two new species in the genus *Phaeopezia* (Pezizaceae)

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Ascomycete.org, 14 (1) : 25–33
Mise en ligne le 28/02/2022
doi 10.25664/ART-0344



Abstract: Two new and closely related species *Phaeopezia vittadinii* and *P. calongei* are described, representing the first semi-hypogeous taxa within the genus *Phaeopezia*. Until now, this genus consisted only of the epigeous *P. apiculata* f. *apiculata* and *P. apiculata* f. *alba*.

Keywords: Ascomycota, Pezizales, phylogenetics, taxonomy, 2 new species.

Resumen: Se describen dos nuevas especies fúngicas semi hipogeas del género *Phaeopezia*, *P. vittadinii* y *P. calongei*, hasta ahora sólo constituido por dos taxones epigeos, *P. apiculata* f. *apiculata* y *P. apiculata* f. *alba*.

Palabras clave: Ascomycota, Pezizales, filogenética, taxonomía, 2 nuevas especies.

Introduction

The genus name *Phaeopezia* was invalidly proposed by SACCARDO (1877: 71) while reporting a collection of *Peziza apiculata* Cooke from Italy. The etymology of *Phaeopezia* comes from the ancient Greek, “*phaeo*,” which means dark, and “*pezis*” which means fungus without a stipe. It was later validated by VIDO (1879), who retained *P. apiculata* as the type. Originally, SACCARDO (1877) aimed to segregate from the genus *Peziza* Dill. ex Fr., restricted to white-spored taxa, species with supposedly dark spores. SACCARDO (1889) recognized 14 species. These were a heterogeneous group of taxa but all of them had pezizoid ascumata and dark ascospores, but obviously heterogeneous for many other characters. *Phaeopezia* was included in the genus *Peziza* by ECKBLAD (1968) and RIFAI (1968). VAN VOOREN (2020) reinstated the genus, revealing the phylogenetic singularity of *P. apiculata* within the *Pezizaceae* family, as previously suggested by HANSEN *et al.* (2005), and proposed an emendation of the definition of this genus, including that ascumata could be epigeous or hypogeous (since he was aware of our hypogeous collections).

In December 2016, during a mycological excursion in the “Espais Naturals del Delta del Llobregat”, the ascumata of a hypogeous fungus were found. When performing macroscopic and microscopic studies of the material, we were especially struck by the unique ornamentation of the ascospores, unknown to any of the co-authors of this article. DNA sequencing was carried out, and confirmed that our collection was an undescribed species related to *Peziza apiculata*, and proposed in this work as *Phaeopezia vittadinii*. An earlier collection from 2015 made in France by P. Chautrand turned out to be conspecific with *P. vittadinii*. In 2021, as a result of a molecular analysis carried out by P. Alvarado (ALVALAB), two collections initially determined as *Choiromyces* sp. by J.M. Vidal, from Catalonia and the Balearic Islands, and material described by CALONGE *et al.* (1995) as *Terfezia leptoderma* (Tul. & C. Tul.) Tul. & C. Tul. (MA-fungi 33516) were also shown to belong to the same clade. These collections represent another new species of *Phaeopezia*, which is introduced in this work as *P. calongei*. It must be emphasized that these two hypogeous species of *Phaeopezia* are easily detectable by our dogs specifically trained to look for hypogeous fungi. The trained dogs never locate epigeous fungi, confirming that these *Phaeopezia* produce volatile odors common in hypogeous fungi.

Material and methods

Morphological analysis — The methods used for our investigation and identification of hypogeous fungi are those described in PAZ *et al.* (2012). Microscopical observations of fresh collections were

made in water, Hoyer’s medium, 1% Congo red, and Melzer’s solution. Sections of exsiccatae were rehydrated in chloral hydrate, 10% ammonia, or 3% KOH, before mounting in the same medium. Microscopical observations and photomicrographs were made using two microscopes: a Nikon eclipse E800 coupled to a Nikon D7100 camera with a brightfield, phase contrast and DF device and a Leica DMRB coupled to a Pentax K-20 camera. The images were captured with Helicon Remote software (Helicon soft Ltd), they were then stacked with Helicon Focus (Helicon soft Ltd) and refined with Photoshop (Adobe Systems software Ltd). All measurements were made in water and ascospore measurements included ornamentation. The collections studied were air-dried and deposited in herbarium LIP (University of Lille, France) for the holotypes and in the personal herbarium of A. Paz, L. Sánchez, P. Chautrand, J. Siquier and J.M. Vidal (referred to below as “IC” “LSS”, “PCH”, “JLS” and “JMV”) for iso- and paratypes. Duplicates are being deposited at the University of Barcelona (BCN).

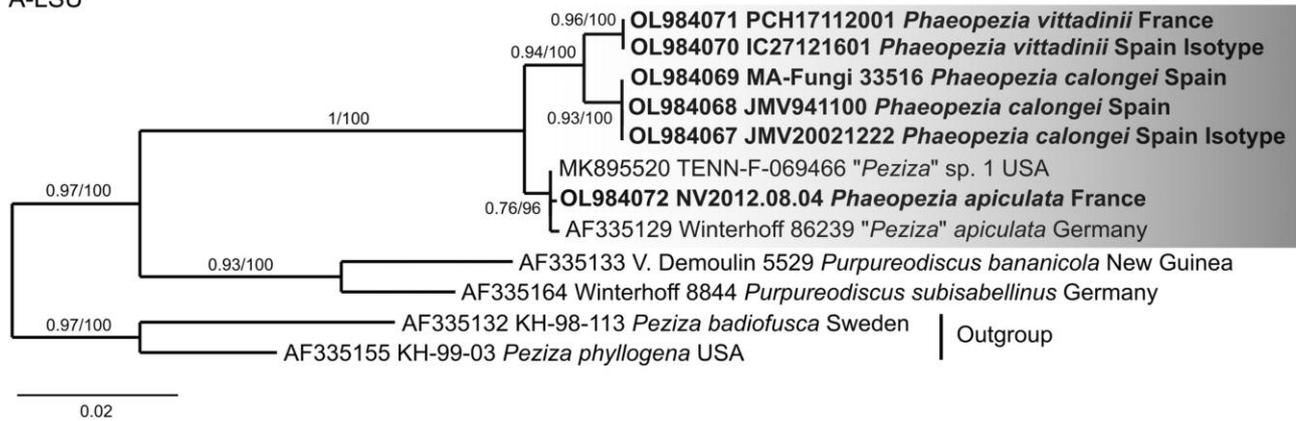
DNA sequencing and phylogenetic analysis — DNA extraction, amplification and sequencing were performed as previously described in ALVARADO *et al.* (2021) or PAZ *et al.* (2017). Nuclear ribosomal ITS and LSU sequences and *rpb2* sequences have been deposited in GenBank under OL984065-OL984066 (*rpb2*), OL984067-OL984072 (LSU) and OM001632-OM001638 (ITS).

Phylogenetic analyses were all conducted online at www.phylogeny.fr (DEREEPER *et al.*, 2008), using MUSCLE v. 3.7 (EDGAR, 2004) for multiple sequence alignments, PhyML v. 3.0 (GUINDON *et al.*, 2010) for Maximum likelihood (ML) analyses and MrBayes v. 3.1.2 (RONQUIST & HUELSENBECK, 2003) for Bayesian inference (BI) of phylogeny. Branch support was assessed by the Shimodaira Hasegawa version of the approximate likelihood-ratio test (SH-aLRT) and the Bayesian posterior probabilities (BPP), respectively for ML and BI analyses. These metrics are considered statistically significant when SH-aLRT > 0.8 (BELLANGER *et al.*, 2015) and BPP ≥ 95%. Phylograms were built using TreeDyn 1.98.3 (CHEVENET *et al.*, 2006) and edited with Inkscape 0.91 (<https://inkscape.org/fr>).

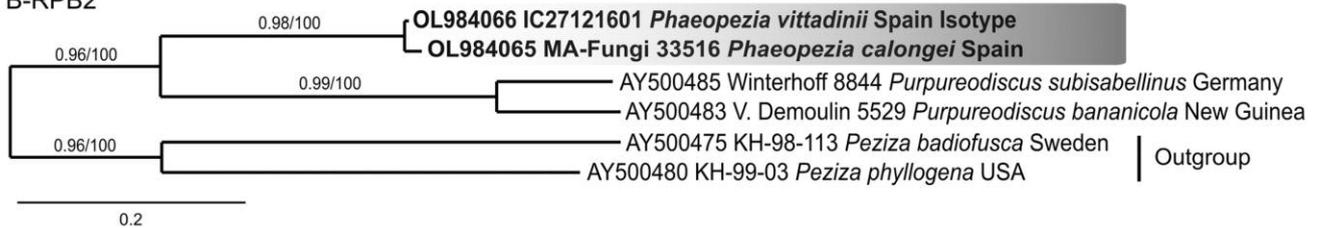
Results

The analysis of the LSU sequences of five of our collections (Fig. 1A) shows that they belong in two strongly supported (0.96/100 and 0.93/100 SH-aLRT/BPP, respectively) and closely related clades, that are described below as *Phaeopezia vittadinii* and *P. calongei* (Taxonomy). These two lineages are sister to *Phaeopezia apiculata*, so far represented in public sequence databases by the two collections TENN-F-069466 (Tennessee, USA) and Winterhoff 86239 (Germany),

A-LSU



B-RPB2



C-ITS

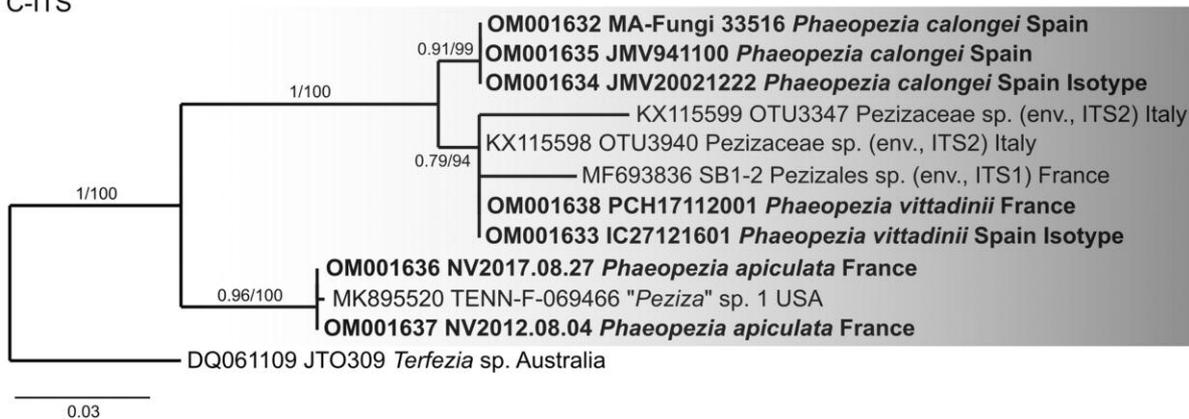


Fig. 1 – Phylogeny of *Phaeopezia*. Maximum likelihood phylograms of *Phaeopezia* LSU (A), *rpb2* (B) and ITS rDNA (C) rDNA sequences and their most closely related known sequences at each locus. Numbers indicate branch support, as SH-aLRT /% BPP values. The thick branches define the boundaries of the genus in its current sequence coverage. The sequences generated for the present study are indicated in bold. "Env." stands for environmental sequence, see text for details.

and here confirmed by two French collections from N. Van Vooren (Figs. 1A and 1C). As previously reported in the multilocus analysis of *Peziza sensu lato* (VAN VOOREN, 2020), the genus *Phaeopezia* (boxed in grey in Fig. 1) is here strongly supported as sister to the genus *Purpureodiscus* (G. Hirsch) Van Vooren (encompassing the couple of species *P. subisabellinus* and *P. bananicola*) in our LSU analysis (Fig. 1A). Such relationships between the two genera are also supported by our *rpb2* analysis (Fig. 1B), although the limited taxon sampling currently available at this locus does not allow definitive conclusions to be drawn.

In order to better estimate the phylogenetic relationships among the taxa with highest similarity at the LSU locus, we generated ITS sequences from our two new species and from two French collections of *P. apiculata*, kindly provided by N. Van Vooren. The two *P. apiculata* sequences are 100% identical and cluster with MK895520 [0](TENN-F-069466), identified as "*Peziza* sp. 1" from a burn site in the Great Smoky Mountain National Park, TN (USA), with

strong support (0.98/100, Fig. 1C). The phylogenetic distance between *P. vittadinii* and *P. calongei* includes 11 substitutions + 1 indel, representing 1.8% sequence divergence at the ITS locus (data not shown). The phylogenetic distance between each species and *P. apiculata* is of 53 to 55 nucleotide positions, representing ~8% of sequence divergence at the ITS locus (data not shown). As seen in the LSU phylogeny, the genus *Phaeopezia* receives here the maximal phylogenetic support (1/100) and its closest published ITS sequence is from an orchid root from Australia, originally annotated "*Terfezia* sp." but clearly not that genus, according to more inclusive phylogenetic analyses (Fig. 1C).

The three sequences of *P. calongei* are 100% identical to each other, as are the two newly generated sequences of *P. vittadinii* to each other, suggesting that the two novel species display limited genetic polymorphism, at least within European populations. Three short sequences from environmental samples found in GenBank likely represent *P. vittadinii*. Indeed, two of them, KX115599 and

MF693836, which included only the 5.8S and ITS2 portions of ITS, differ from our two sequences by six and seven substitutions, respectively, in the ITS2 region. KX115598 and KX115599 have been found in the soil of an orchid-rich Mediterranean grassland in northern Italy, and one of them was also directly recovered from the orchid mycorrhizal (OrM) pellets of *Neotinea tridentina* (VOYRON *et al.*, 2016). MF693836 has also been isolated from plant roots in a truffle plantation of southern France but the “host” was a *Pimpinella* species, which is a non-ectomycorrhizal genus in the *Apiaceae* family (SCHNEIDER-MAUNOURY *et al.*, 2018). Therefore, *P. vittadinii* is evidently an OrM fungus, but perhaps it can establish with plant roots through other types of interactions, such as endophytism (HEALY *et al.*, 2021).

The relatively weak genetic distance between the pezizoid (*P. apiculata*) and the tuberoid (*P. calongei* and *P. vittadinii*) clades did not support the distinction of two genera. However, morphologically it was impossible to predict this close relationship, and no micromorphological trait could be found to explain it afterwards. These two new species illustrate a rare, but not unique situation in *Pezizales*, where pezizoid and tuberoid ascomata are found in the same lineage (e.g. BURDSALL, 1968; LÆSSØE & HANSEN, 2007; SMITH & HEALY, 2009; SMITH, 2014). In the recently revised phylogeny of *Pezizaceae* (VAN VOOREN, 2020), the genus *Paragalactinia* Van Vooren (*P. succosa*-lineage) was also defined by “Ascomata hypogeous or epigeous” and the informally named “violacea-lineage” included the typically tuberoid *Elderia/Mattirolomyces* clade. Previously in the same family *Pezizaceae*, the mainly tuberoid lineage *Pachyphloides* was also shown to include a pezizoid lineage *Scabropezia* (CABERO *et al.*, 2016).

Taxonomy

Phaeopezia vittadinii Paz, P. Chautrand, L. Sánchez, J. Bometón & C. Lavoise, *sp. nov.* –MycoBank MB 843044 – Figs. 2-3.

Studied material: SPAIN, Catalonia, Barcelona, Viladecans, Cal Francès, “Espais Naturals del Delta del Llobregat”, 41° 16' 39.21" N 2° 3' 42.62" E, 1 m elev., between mosses, in settled dunes, with *Spartium junceum*, *Pinus pinea*, *Thymelaea hirsuta*, *Cistus salviifolius*, *Helichrysum stoechas* var. *maritimum*, *Plantago crassifolia*, *Senecio vulgaris*, *Diploxys erucoides*, *Scabiosa atropurpurea* subsp. *maritima*, *Teucrium polium* var. *maritimum*, *Asphodelus fistulosus*, *Hyparrhenia hirta*, with the presence of *Ophrys passionis* and *Aceras anthropophorum*, on calcareous soil (pH 8), 27 December 2016, *leg.* J. Bometón & L. Sánchez, LIP 0002247 (**holotype**)*, herb. pers. A. Paz IC27121601 (isotype), LSS20170315-1; *ibid.* 16 January 2017, *leg.* A. Paz, C. Lavoise & L. Sánchez, IC16021701. FRANCE, Nouvelle-Aquitaine, Charente-Maritime, Corme-Écluse, Les Gripperries, 45° 36' 26.24" N 0° 53' 5.83" W, 20 m elev., in a Mediterranean environment, outside a plantation of *Tuber melanosporum* mycorrhizal with *Quercus ilex*, on calcareous soil, with the presence of orchids and different types of unidentified plants, 12 November 2015, *leg.* P. Chautrand, PCH15111201; *ibid.* 20 November 2017, PCH17112001*; *ibid.* 24 October 2019, PCH19102401.

* Sequenced material

Diagnosis: Ascomata subglobose, strongly lobed, pointed to the base. When young, the surface is creamy white, and reddish brown when mature, with violet tinges due to oxidation. Gleba with a mar-



Fig. 2 – *Phaeopezia vittadinii*. A: Ascomata, holotype LIP 0002247; B, D: Ascomata PCH17112001. Photos: A: L. Sánchez; B–D: P. Chautrand.

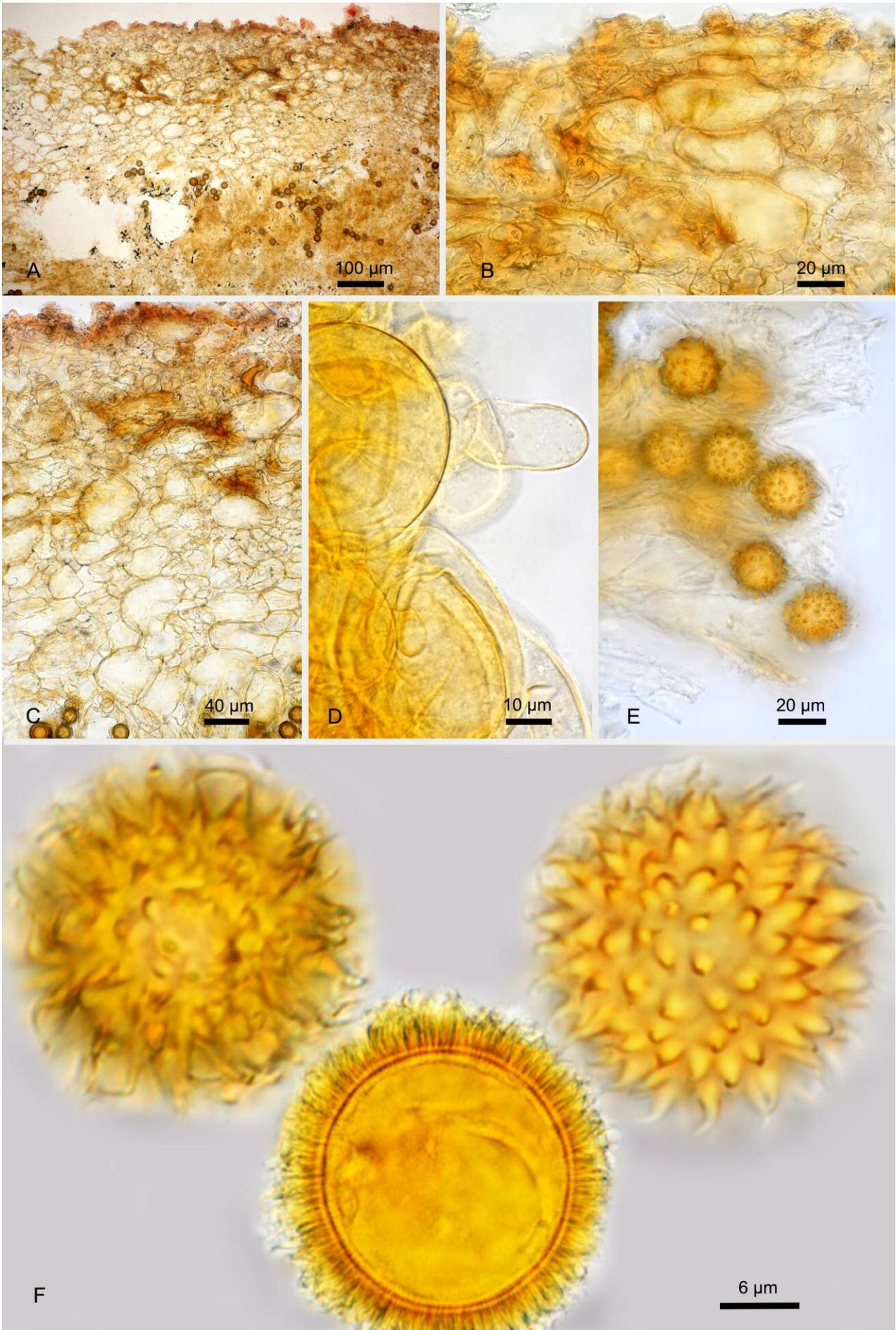


Fig. 3 – *Phaeopezia vittadinii* microscopy. A: Peridium and gleba, in cross section, with the 4x objective; B: Peridium in cross section, with the 20x objective; C: Peridium and gleba in cross section, with the 10x objective; D: Vesicular hyphae, with a 20x objective; E: Asci and ascospores, with the 40x objective; F: Ascospores, with the 100x objective. Photos: A. Paz.

bled appearance, with irregular, elongated fertile areas, directed towards the base. Ascospores spherical, 23–27 µm in diameter, ornamented with thin spines, up to 5.2 µm in height, united to form conical pyramidal warts, with flexuous apices and a thin episporium ≤ 0.75 µm wide.

Etymology: In honor of the great mycologist and truffle expert Carlo Vittadini.

Description: **Ascomata** semi-hypogeous, 25 to 40 mm in diameter, subglobose, lobed to very lobed, initially with dirty white to creamy-yellow tones. When mature, it acquires light brown tones with darker spots and purple tinges on handling. In section view, with an accentuated mycelial base, with the presence of mycelial hairs. **Peridium** almost disappearing at maturity. **Gleba** with a marbled appearance, compact in youth, somewhat labyrinthine in maturity, with irregular fertile areas of greyish-yellowish colour in youth and purplish-brown tones when mature and arranged towards the base, separated by sterile coloured areas, creamy white in youth and with yellowish tones when mature. Pleasant, fungal **odour**.

Peridium thin, 260.5–300.4 µm, the outermost layer 37.1–62.5 µm, is made up of no more than three rows of hyphae (in a cross section). It has enveloping hyphae with brown cytoplasmic content, with thin walls, together with sausage-shaped cells, with septa averaging 52.98 × 14.82 µm, with a mean Q = 3.62, slightly intertwined, all invaded by a dark yellow to brown pigmentation, end mycelial hairs with large septa, bifurcated, with loops and without encrustations. In the inner layer, there is a more globular, irregular texture, with pyriform vesicular hyphae, up to 98 µm in diameter, in which some very widened cylindrical hyphae can be observed, with septa averaging 72.2 × 40.94 µm. These two layers of the peridium, when mature, can disappear and there is a thick layer of globose cells (which already belong to the gleba), regular in shape, but very variable in size, not exceeding 56.6 µm in diameter. These hyphae surround the fertile areas, where a palisade of hymenial elements develops, with cylindrical **asci**, 240–260 × 45–50 µm, inamyloid, with croziers, containing between 6–8 uniseriate ascospores, interspersed with abundant filamentous **paraphyses**, with septa 35–40 × 6–7 µm, widened at the apex, up to 10 µm. The hymenium is protected by large vesicular hyphae, containing an intense yellow cytoplasm. We have observed a diffuse amyloid reaction in the fresh material in the fertile areas, which is apparently due to the cytoplasmic content of these vesicular hyphae. **Ascospores** spherical, 23–27 µm in diameter (including ornamentation), with an exosporium formed by thin spines, up to 5.2 µm in height, which unite to form conical pyramidal warts, with flexuous apices, yellow when young. When mature, the ornamentation acquires reddish-brown tones, and a thin episporium ≤ 0.75 µm wide, with dextrinoid reaction in Melzer's reagent in its internal part. The **sterile part of the gleba** is made up of different layers of filamentous, hyaline, septate and branched hyphae, with septa averaging 48 × 16 µm.

Habitat and distribution: It was collected in Spain, in the community of Catalonia, among moss, in sedimented dunes, with *Spartium junceum*, *Pinus pinea*, and in the west coast of France, in Charente-Maritime. In these two localities the presence of *Ophrys* sp. was noted. The isolates referenced with GenBank KX115598 and KX115599 have also been found in the soil of an orchid-rich Mediterranean prairie in northern Italy, and one of them was also recovered directly from orchid mycorrhizal (OrM) platoon of *Neotinea tridentina*. We hypothesize that their distribution is wider, in the Mediterranean area, on calcareous soils and at low elevations with respect to sea level. The collections were made in the months of October to December.

Observations: The differences between *P. vittadini* and the epigeous *P. apiculata* consist of the following: macroscopically *P. apiculata* is sessile, dark, cupulate to discoid, with a flattened, fleshy,

circular and entire margin (Fig. 6), whereas *P. vittadini* has subglobose ascomata that are highly lobed, have a yellowish-creamy surface and a compact gleba with a marbled appearance. Microscopically, the most notable difference is in the shape and size of the ascospores: *P. apiculata* has narrowly elliptical, 20.3 × 9.8 µm on average (coll. LY NV2012.08.04), whereas *P. vittadini* has globose ascospores, measuring 23–27 µm diam.

P. vittadini differs macroscopically from *P. calongei* by the thickness of the peridium, which disappears in *P. vittadini*, but is persistent and thick in *P. calongei*. The size of the ascospores is similar in these two species, but the exospore in *P. vittadini* is formed by thin spines, up to 5.2 µm in height, that are flexuous at the apices and with a perisporium that is dextrinoid in its internal part. In contrast, *P. calongei* produces ascospores with thick spines ≤ 2.76 µm in height, without flexuous apices and without any reaction in Melzer's reagent.

Phaeopezia calongei Paz, J.M. Vidal, Siquier, G. Mir & C. Lavoise, *sp. nov.* – MycoBank MB 843045 – Figs. 4–5.

Material studied and sequenced: SPAIN, Catalonia, Barcelona, Vilanova i la Geltrú, Ermita de Sant Gervasi, 41° 21' 32.69" N 2° 9' 52.71" E, 15 m elev., under *Pinus halepensis*, on calcareous soil, November 1994, *leg.* J. Marçal, comm. A. Rocabrana, JMV941100*; Balearic Islands, Mallorca, Palma de Mallorca, cementiri de Sa Vileta, 39° 35' 59.49" N 2° 36' 13.73" E, 125 m elev., on calcareous soil, under *Pinus halepensis*, 6 December 1994, *leg.* J. Siquier JLS176P, *det.* F.D. Calonge *sub Terfezia leptoderma*, MA-Fungi 33516*. Mallorca, Santanyi, Cala Mondragó, ED1655, 39° 21' 03.35" N 3° 11' 25.19" E, 15 m elev., under *Pinus halepensis*, with *Pistacia lentiscus*, *Erica multiflora*, *Juniperus oxycedrus*, *Cistus monspeliensis*, *Ampelodesmos mauritanica*, *Asparagus acutifolius*, *Phillyrea angustifolia*, in calcareous soil, 22 December 2002, *leg.* G. Mir, LIP 0002248 (holotype), pers. herb. J.M. Vidal JMV20021222 (isotype)*.

* Sequenced material

Diagnosis: Ascoma subglobose, yellow-cream-white, with permanent thick peridium, gleba with a marbled appearance, compact, with irregular, elongated and sinuous fertile areas. Ascospores spherical, 24–27 µm in diameter, with an exosporium consisting of thick conical warts, up to 2.76 µm in height × 1.47 µm at the base, and a thick perisporium ≤ 2 µm wide.

Etymology: In memory of Professor Francisco De Diego Calonge, pioneer in the study of hypogeous fungi in Spain.

Description: **Ascomata** semi-hypogeous, 23 to 35 mm in diameter, subglobose, little lobed, initially with dirty white to creamy-yellow tones; when mature they acquire greenish-yellow tones with dark brown spots, with a slightly pronounced or non-existent mycelial base. **Peridium** thick, remaining present even at maturity. **Gleba** with a marbled appearance, compact, with irregular, elongated, sinuous fertile areas, giving a labyrinthine appearance, with an intense yellow colour when young and developing yellowish-brown tones when mature, separated by sterile areas of white color in its youth and in shades of yellow-grayish to light brown at maturity. **Smell** weak, similar to that of smoked chorizo according to CALONGE *et al.* (1995).

Peridium thick, 506–658 µm wide, with the outermost layer 158–190 µm, being made up of three to five rows of hyphae (in a cross section); hyphae elongated, bifurcated, with brown cytoplasmic content, thin-walled, with septa averaging 20.94 × 6.41 µm, with a Q = 3.33, slightly intertwined, all marked with a dark yellow to brown pigmentation. In the inner layer, there is a more regular globular texture (horizontal section), formed by cylindrical or polyhedral cells, with septa that measure 46.83 × 35.81 µm on average, interspersed by sausage-shaped cylindrical cells, with septa, averaging 36.33 ×



Fig. 4 – *Phaeopezia calongei*. A: Ascomata, holotype LIP 0002248; B, C: Ascomata, JLS176P (MA-Fungi 33516). Photos: A: J.M. Vidal; B, C: J. Siquier.

15.8 μm , with few pyriform vesicular hyphae, up to 45.2 μm in diameter. Below there is a thick layer of globose hyphae (integrated with the vegetative tissue of the gleba), regular in shape, but variable in size, not exceeding 34.7 μm in diameter, these hyphae surround the fertile areas, where a palisade of hymenial elements develops, with cylindrical **asci**, 220–248 \times 34.2–42.2 μm , inamyloid, with croziers, containing between 6–8 uniseriate ascospores, interspersed by abundant filamentous **paraphyses** with septa of 33–38 \times 6–7 μm , widened at the apex, up to 10 μm . This hymenium is protected by vesicular hyphae containing, an intense yellow cytoplasm. **Ascospores** globose, 24–27 μm in diameter (including ornamentation), with an exosporium consisting of thick conical warts, up to 2.76 μm in height and 1.45 μm at the base, yellow when young, but becoming reddish-brown at maturity, with a thick perisporium \leq 2 μm wide, without reaction in Melzer's reagent. The **sterile part of the gleba** is made up of different layers of filamentous, hyaline, septate, and branched hyphae, with septa averaging 36 \times 14 μm .

Habitat and distribution: only collected in Spain, in the peninsular area, in the community of Catalonia, in the province of

Barcelona, and in the Balearic Islands in Mallorca, although we assume that its distribution is wider, in the Mediterranean area, in calcareous soils and at a low elevation with respect to sea level. The collections were made in the months of November and December.

Observations: See observations of *Phaeopezia vittadinii*. *P. calongei* differs macroscopically from *Terfezia leptoderma* by the shape of the fertile areas of the gleba. In *P. calongei* these areas are irregular, elongated, sinuous, giving a labyrinthine appearance to the gleba, and developing yellowish brown tones when mature, whereas in *T. leptoderma* they are globose and show greenish tones when mature. The asci of *P. calongei* are cylindrical, with uniseriate ascospores, whereas in *T. leptoderma* they are globose and with disordered ascospores. The size of the ascospores is 24–27 μm diam. for *P. calongei* ornamented with thick conical spines, up to 2.75 μm high and in *T. leptoderma* 16–20 μm diam., ornamented with sub-cylindrical spines, up to 4 μm high.

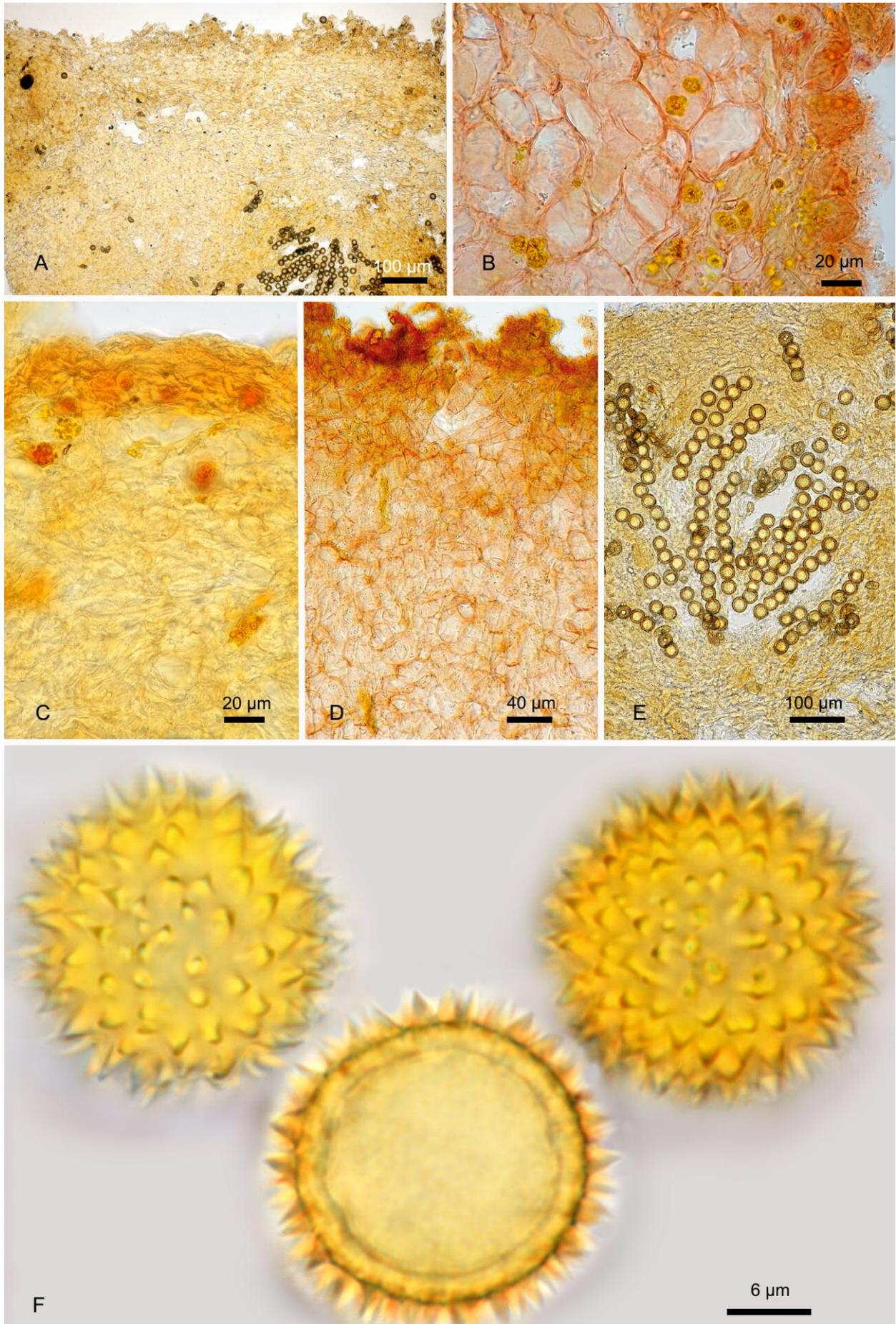


Fig. 5 – *Phaeopezia calongei* microscopy. A: Peridium and gleba in cross section, with the 4x objective; B: Peridium and gleba in cross section, in Congo red, with the 20x objective; C: Peridium and gleba in cross section, with the 10x objective; D: Peridium and gleba in cross section, in Congo red, with a 10x objective; E: Hymenium, with the 10x objective; F: Ascospores, with the 100x objective. Photos: A. Paz.



Fig. 6 – *Phaeopezia apiculata*. A: Ascomata in situ, LY NV 2012.08.04; B: Ascospores in water. Photos: N. Van Vooren.

Acknowledgements

We thank the Consortium for the Protection and Management of the “Espais Naturals del Delta del Llobregat” for providing us permits to collect samples with our dogs. To Pablo Alvarado for the molecular analysis of the Spanish collections of both *Phaeopezia*. To Nicolas Van Vooren for the donation of the exsiccatae of *P. apiculata* f. *apiculata* and *P. apiculata* f. *alba* to perform the DNA sequences. Finally, we thank Rosanne Healy and Matthew Smith (University of Florida, USA) for their valuable review.

Authors' contributions

Aurelia Paz was responsible for the study conception and design, associated to P.-A. Moreau. Morphological analyses were performed by A. Paz. Molecular analyses were performed by J.-M. Bellanger, as well as the registration in GenBank. Morphological analyses of the studied collections were made by P. Chautrand and J.M. Vidal, eventually revised by the first author. The first draft of the manuscript was written by A. Paz and was subsequently updated by all authors. All plates have been designed by A. Paz. All authors read and approved the final manuscript.

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