**Barssia hellenica** sp. nov. (Ascomycota, Pezizales), a new hypogeous species from Greece

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**Introduction**

In 2013, an unknown hypogeous fungus was collected in Greece, and we soon realized we probably discovered a not yet described species. In 2015 we were able to collect again several specimens, to bring to completion the morphological study and to conduct a phylogenetic study with the two known collections to confirm our hypothesis. The constant and laborious field work with hypogeous fungi, often under really peculiar conditions, is sometimes rewarded: *Barssia hellenica* is another demonstration of the special fungal diversity of Greece, encouraging us to continue researching to enhance our knowledge.

**Materials and methods**

**Morphological study**

The macroscopic study was conducted on fresh and dried specimens. Micromorphological observations were performed under three different trinocular microscopes with plan achromatic lenses (4×, 10×, 40× and 100× oil immersion). Micromorphological features were studied and measured in water mounts. The negative amyloid reaction was checked with Melzer’s reagent. Cotton blue was used to highlight spore ornamentations, and Congo red was employed to stain cell walls of different elements. Finally the dimensions of the ascospores were obtained by measuring at least 50 randomly selected ascospores.

**Phylogenetic analysis**

**DNA extraction, amplification and sequencing.** — Total DNA was extracted from dry specimens blending a portion of them with the aid of a microprobe in 600 μl CTAB buffer (CTAB 2%, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris–HCl pH 8.0 100 mM). The resulting mixture was incubated for 15 min. at 65°C. A similar volume of chloroform: isoamyl alcohol (24:1) was added and carefully mixed with the samples until their emulsion. It was then centrifuged for 10 min at 13,000 g, and the DNA in the supernatant was precipitated with a volume of isopropanol. After a new centrifugation of 15 min at the same speed, the pellet was washed in cold ethanol 70%, centrifuged again for 2 min and dried. It was finally resuspended in 200 μl of dH2O. PCR amplification was performed with the primers ITS1F and ITS4 for ITS region (White et al., 1990; Gardes & Bruns, 1993), while LR0R and LR5 were used to amplify the 28S nLSU region (Vilgalys & Hester, 1990). PCR reactions were performed under a program consisting of a hot start at 95°C for 5 min, followed by 35 cycles at 94°C, 54°C and 72°C (45, 30 and 45 s respectively) and a final 72°C step 10 min. PCR products were checked in 1% agarose gels, and positive reactions were sequenced with primer ITS4 or LR0R. Chromatograms were checked searching for putative reading errors, and these were corrected.

**Phylogenetic analyses.** — BLAST was used to select the most closely related sequences from INSD public databases. Sequences came mainly from Cardone et al. (2013). 28S nLSU sequences were first aligned in MEGA 5.0 (Tamura et al., 2011) with the closest matches in public databases (O’Donnell et al., 1997; Bonito et al., 2013; Nguyen et al., 2013; Crous et al., 2014). The aligned loci were loaded in PAUP* 4.0b10 (Swofford, 2001) and subjected to MrModeltest 2.3 (Nylander, 2004). Model GTR+I+I was selected and implemented in MrBayes 3.1 (Ronquist & Huelsenbeck, 2003), where a Bayesian analysis was performed (data partitioned, two simultaneous runs, six chains, temperature set to 0.2, sampling every 100th generation) until convergence parameters were met after about 60,000 generations, standard deviation having fell below 0.01. Significance threshold was set above 0.90 posterior probability (PP).

**Taxonomy**

*Barssia hellenica* Kaounas, Agnello, P. Alvarado & Slavova sp. nov. — MycoBank 813811

**Diagnosis:** Differes from other *Barssia* spp. by ovoid ascospores (>70%), irregularly clavate or broadly ellipsoid asci and the habitat under Abies cephalonica.

**Typification:** Holotypos here selected MCVE 28663

**Etymology:** hellenica from Hellas (Greece), the country where the species was discovered.

**Description**

*Ascocarps* hypogeous, 0.5–2.7 × 0.5–2.0 cm in diam., spherical to subspherical, elongated and compressed, creating an irregular shape, more or less lobed, usually with an irregular apical orifice, sometimes creating folds on the inside, reddish orange to brownish red or blackish brown. The surface is covered by irregular polygonal shapes, more or less lobed, usually with an irregular apical orifice, sometimes creating folds on the inside, reddish orange to brownish red or blackish brown. The surface is covered by irregular polygonal warts, densely packed together when young, and more isolated with age, sometimes giving the appearance of a network. *Peridium* 260–420 μm, composed by a pseudoparenchymatous hyphal structure with polygonal cells measuring 20–41.5 × 15–31.0 μm, amber to brownish in color and thick walled (2–5 μm) in the outer layer, colorless and with thinner walls in the internal layer. Presence of rare, yellowish, light brown hairs, simple or branched, septate, thick-walled emerging from the terminal cells of the peridium with two different external surfaces: the first smooth and/or few incrusted, the
second verrucose and/or incrusted, measuring up to 100 × 9 μm. **Gleba** mostly compact, whitish, sometimes looking a bit cottony, with irregular sinuous, labyrinth-like and vertical straight veins, whitish or yellowish, gelatinous; sterile part formed by interwoven septate hyphae, 2–5 μm in diam. **Paraphyses**, hyaline, cylindrical, simple or forked, septate, longer than the asci, 3–6(–8) μm wide. **Asci** irregularly clavate or broadly ellipsoid, with a protruding hump at the apex and a pleurotyphous base, inamyloid, 8-spored, measuring 110–185 × 36–45 μm. **Ascospores** ovoid, rarely ellipsoid, smooth, hyaline, irregularly arranged inside the asci, measuring 21–27 × 16.5–20.5 μm (23.5 × 18.1 μm on average), Q (L/I) = 1.18–1.43 (Qm= 1.30), usually containing one or two large oil drops and several smaller droplets.

**Habitat:** Ascomata are found growing solitary, hypogeous, under *Abies cephalonica*, 1040 m alt.

**Studied collections:** GREECE, 13.III.2013, Mainalon Arkadia, under *Abies cephalonica*, leg. G. Proutzopoulos, MCVE 28664, Genbank LSU KT350939, ITS KT350942; 04.VI.2015, Parnitha Attica, under *Abies cephalonica*, leg. V. Kaounas, MCVE 28663 (holotype), Genbank LSU KT350940, ITS KT350941.

**Discussion**

The genus *Barssia* was first proposed by Gilkey (1925) describing the species *B. oregonensis* collected in Oregon (USA) by H.P. Barss. This species has red-orange asccarps with irregular protrusions, an opening at the top, a solid gleba, and ellipsoid, smooth, hyaline ascospores (15 × 26 μm), lacking a mycelium tuft at the base of the ascoma. Gilkey (1925) classified the genus *Barssia* within the family *Tuberaceae* Dumort., but it was later transferred by Trappe (1979) to the family *Balsamiaceae* E. Fisch. However, after the ultrastructural study of Kimbrough et al. (1996) and phylogenetic inference analyses conducted by O’Donnell et al. (1997) and Lassab & Hansen (2007), *Balsamia* and *Barssia* were shown to represent a sister clade to other genera in the family *Helvellaceae* Fr., and hence considered a part of this family.

In the protologue of the *B. oregonensis*, it is said to be associated with *Rhamnus purshiana* (literally “Cascara Sagrada”), but it has always been found later associated with *Pseudotsuga menziesii* in North-Western USA, finally considered as abundant especially in young plants (Trappe et al., 2007; Trappe et al., 2009). There is a single European collection identified as *B. oregonensis* (Lawrynowicz & Skirgiel, 1984) found on calcareous soil of the Carpathian Moun-

Plate 1 — *Barssia hellenica*

ABCF: MCVE 28663 (holotype); DE: MCVE 28664. Photos V. Kaounas
Plate 2 — *Barssia hellenica*. Microscopic characters
Plate 3 — *Barssia hellenica*. Microscopic characters
tains in Poland, under *Picea abies*, but the special ecological niche suggests a closer investigation before claiming that this taxon is present also in Europe.

A second species, *B. yezomontana* Kobayasi, was described in Japan in the year 1937 (as *B. yezo-montana*): the only known collection of this species was lost during the bombing of WWII (Gilkey, 1961). The globose ascospores described in the protologue suggest it should be considered a species of uncertain placement. Recently, a third species, *B. maroccana* G. Moreno, Manjón, Carlavilla & P. Alvarado, has been described from the Moroccan Atlas *Cedrus atlantica* forests. It differs by its larger, ellipsoid ascospores measuring 29–36 × (16–) 18–22 μm.

Mattirolo (1936) described *Stephensia peyronelii* from Piedmont (Italy) under *Larix decidua* (alt. 1400 m). Only Ceruti (1960) evoked this species, based on the original description because he could not locate any existing herbarium material. Later, Ceruti (1961) suggested that it might be a synonym of *Barssia oregonensis*. In our opinion, its placement in the family Helvellaceae, probably in the genus *Barssia*, seems appropriate after Mattirolo’s description (smooth, ellipsoid ascospores measuring 26–29 × 15–16 μm).

From a molecular point of view, the new species is most closely related to the recently proposed Mediterranean species *B. maroccana*. Both are related to the American type species of *Barssia, B. oregonensis*, but both lineages seem to be too divergent from each other. The discovery of additional taxa in both lineages could help to resolve the most suitable taxonomic status of the whole group. The subtle differences between the closely related *Barssia* and *Balsamia* (Montecchi & Sarasini, 2000) could suggest their synonymization. However, *B. hellenica* displays a conspicuous apical depression, one of the most important features used to discriminate between *Balsamia* and *Barssia* (Gilkey, 1925), which is however not evident in *B. maroccana*, thus supporting the generic split.

**Conclusion**

*Barrassia hellenica* can be easily discriminated from other species due to its particular morphological, ecological and genetic features. Morphologically it differs by its ovoid ascospores (> 70%) of intermediate size, shape and size of the asci, and the presence of trichomes (although scarce) on the peridium. The habitat seems also to play an important role:

- *B. oregonensis* → *Pseudotsuga menziesii*
- *B. maroccana* → *Cedrus atlantica*
- *B. hellenica* → *Abies cephalonica*.

**Legend of Plate 2.** A: Section with peridium and gleba in water; BC: Peridium in water; DE: Peridium inside; F: Gleba in Congo red; G-O: Hairs in water and Congo red. Black scale bars =100 μm; red scale bars = 25 μm. Photos ABDFM: V. Kaounas; CEGIO: M. Slavova; HLN: C. Agnello.

**Legend of Plate 3.** AB: Gleba in Congo red; CDF: Ascospores in water; E: Ascus and ascospores in water; G: Pleurorynchous base of ascus; H: Ascus in water. Scale bars = 25 μm. Photos ABH: M. Slavova; CF: C. Agnello; EG: V. Kaounas.

**Fig. 1** — *Barssia hellenica*. Microscopic characters
A: Peridium; B: Asci; C: Ascospores. Drawing: C. Agnello
The obvious host-specific association with conifers suggests that the already mentioned specimens found under *Picea abies* and *Larix decidua* might well represent additional independent *Barssia* species.

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**References**


Key of the known species of Barssia based on morphological and ecological characters

1. Globose ascospores ................................................................................................................................. B. yezomontana
2. Ellipsoid ascospores ................................................................................................................................. 4
3. Ovoid spores.................................................................................................................................................. 5

4a. Ascospores 24.0–32.0 x 12.0–17.0 μm, with cylindrical asci, under Pseudotsuga menziesii ......................... B. oregonensis
4b. Larger ascospores, on average 29.0–36.0 x 16.0–22.0 μm, with ellipsoid/clavate asci, apical depression not evident, under Cedrus atlantica.......................................................... B. maroccana
5. Ascospores 21.0–27.0 x 16.0–20.5 μm, with irregularly clavate or broadly ellipsoid asci, under Abies cephalonica .... B. hellenica

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