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Molecular phylogeny and morphology of *Pseudobaeospora cyanea*

Dario Gisotti[1], Fabrizio Boccardo[2], Mirca Zotti[1], Simone Di Piazza[1]

1 *Laboratory of Mycology, Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132 Genoa, Italy* **2** *Via Filippo Bettini 14/11, 16162 Genoa, Italy*

Corresponding author: Simone Di Piazza (simone.dipiazza@unige.it)

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Abstract

Pseudobaeospora cyanea, a rare basidiomycete belonging to an underinvestigated genus, is currently recorded from only three localities of the Iberian Peninsula. Moreover, to date no sequences of this rare species have been deposited in GenBank. In this paper a new collection from NW Italy is reported with detailed morphological descriptions and iconography. The first ITS and LSU sequences for the species are provided and uploaded to GenBank, and the taxonomic placement of *P. cyanea* within the genus is discussed.

Keywords

Basidiomycota, distribution, *Pseudobaeospora*, taxonomy

Introduction

The genus *Pseudobaeospora* Singer, circumscribed in 1942 to accommodate the type species *Pseudobaeospora oligophylla* (Singer) Singer, currently includes 30 species (http://www.mycobank.org/ accessed February 2021) of small white-spored agarics characterized by small thick-walled dextrinoid basidiospores (Bas 2002). The ecology is unclear, although suggested to be presumably saprotroph (Arauzo 2011). Its current accommodation in the family Tricholomataceae, with *Leucopaxillus* as a sister taxon, is supported by Desjardin et al. (2014) and by Sánchez-Garcia and Matheny (2017), although this placement is questioned by Wu et al. (2017). The genus *Pseudobaeospora* has been overlooked by taxonomists for a long time and until 1995 only two species were known in Europe (Arnolds et

al. 2003), which were synonymized a few years later (Roniker and Moreau 2007). Several new European species have been described since then, mostly on a macro and micro-morphological basis (Bas 2002), leading to more than 20 species being currently known for Europe (Adamčík and Jančovičová 2011), most of which are rare and only known from very few locations. The coverage of genus *Pseudobaeospora* in GenBank and UNITE is currently insufficient, with only seven species identified (about 20% of the total of described species) having available sequences in the database (https://www.ncbi.nlm.nih.gov/genbank/ and https://unite.ut.ee/search.php#fndtn-panel1 accessed February 2021).

Pseudobaeospora cyanea Arnolds, Tabarés & Rocabruna is a species described in Spain, based on macro- and micro-morphological analyses of a collection from Vidreres, Catalonia. The holotype specimens were collected in early November on Mediterranean hills (200 m a.s.l.) with *Pinus pinaster* Aiton, *Arbutus unedo* L., and *Erica arborea* L. (Arnolds et al. 2003). The species has been reported again in late October 2007 from two localities of the Basque Country (N Spain), around 600 m a.s.l., in the litter of *Chamaecyparis lawsoniana* (Murray) Parl. plantations (Arauzo 2011).

In the present study, a new collection of *P. cyanea* from a locality near Genoa (NW Italy) is reported. Morphological and molecular analyses of this collection are carried out, with the aim of increasing the knowledge about distribution, genetics, and phylogenetic relationships in this poorly known genus.

Material and methods

Morphological analysis

The specimens were identified through macro-morphological observations and evaluation of micro-morphological features. The herbarium specimens were prepared with a dryer and deposited in the mycological herbarium of the "Giacomo Doria Civic Museum of Natural History" (GDOR M3986).

The dried specimens were rehydrated in pure water, and the microscope slides were mounted with Congo red. More slides were prepared with Melzer's reagent and cotton blue, to observe the dextrinoid and cyanophylic reactions of the basidiospores. The slides were observed at 100× magnification with a Leica DM 500 binocular optical microscope. For basidiospores and other structures, at least 20 individuals were measured.

DNA extraction and sequencing

Genomic DNA was extracted from 100 mg of dried specimens by a modified CTAB method (Doyle and Doyle 1987). The sample was disrupted by high-speed shaking (18 Hz) for 1 min using a TissueLyser (Retsch GmbH, Haan, Germany), and incubated for 1 hour at 65 °C in 350 ml CTAB extraction buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA; 1.4 M NaCl; 2% PVP; 2% CTAB) with 5 µg of proteinase K (Sigma-Aldrich, St Louis, MO, USA) and 15 µg of RNase A (Sigma-Aldrich). After the incubation the

sample was centrifuged (10 min × 5900 g), the aqueous layer was mixed (in proportion 1:1) in a new tube with phenol, chloroform, isoamyl alcohol mixture (25:24:1) (Sigma-Aldrich). The previous step was repeated mixing the supernatant with chloroform (in proportion 1:1) to clear the supernatant, which was then incubated (15 min 4 °C) in a new tube with 2-propanol (in proportion 3:2). After the incubation the tube was centrifuged (10 min × 9200 g), the aqueous layer was discarded; the DNA pellet was washed in ethanol, air-dried and resuspended in 100 µl of Milli-Q water. DNA extracts were stored at -20 °C. Universal primers ITS1F/ITS4 were used for the ITS region amplification (White et al. 1990), and LR0R/LR5 for the LSU (Vilgalys and Hester 1990). The PCR reaction contained 24 µl mix (15.875 µl Milli-Q water, 1.5 µl 50 mM $MgCl_2$, 5 µl 5× Green GoTaq Buffer, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM of each primer, 0.125 GoTaq 5 U/µl) and 1 µl of DNA template. The PCR program was: 2 min 95 °C, 35 × (45 sec 95 °C, 45 sec 55 °C, 2 min 72 °C), 5 min 72 °C, 10 °C for ∞. PCR products were purified and sequenced using BMR Genomics (Padua, Italy).

Sequence alignment and phylogenetic analysis

The BLASTN algorithm was used to compare the sequence obtained in the present work against the GenBank database. The sequence was then aligned with the other *Pseudobaeospora* sequences currently available on GenBank and UNITE, with the addition of *Xerula pudens* (Pers) Singer (Physalacriaceae) for rooting purposes, using the MUSCLE tool in the MEGA 7 software (Pennsylvania State University, PA, USA). Then a phylogenetic tree was inferred by maximum likelihood with 500 bootstrap replicates, using MEGA 7.

Results

Habitat and ecology

The specimens were collected on 6 December 2016, *D. Gisotti & F. Boccardo* (GDOR M3986) in the locality of Pegli, Genova, 44°25'53.4"N, 8°48'34.2"E, at an elevation of 95 m, in an area of shrub-like Mediterranean vegetation with *Pinus pinaster*, *Arbutus unedo*, *Erica arborea*, *Cistus salvifolius* L., and *Quercus ilex* L., on poor acidic soil with serpentine bedrock. The basidiomata are gregarious, growing in the needle litter of *P. pinaster*. The species is reported to be presumably saprotrophic (Arnolds et al. 2003).

Macro-morphological observations

Small collybioid basidiomata, with pileus 10–30 mm broad, campanulate to planoconvex, finally flattened, in some specimens vaguely umbonate, with somewhat undulate-revolute margin in mature specimens, dry and velvety, from blue to purple with a paler margin (Fig. 1A, B, D). Dried pileipellis showing a bluish-green reaction with KOH. Gills adnexed and quite crowded, rather thick, ventricose, 3–4 mm broad,

Figure 1. *Pseudobaeospora cyanea* **A, D** basidiomata **B** gills **C** pileus. Scale bars: 1 cm. (photos D. Gisotti).

cream to beige (Fig. 1B). Stipe 28–40 mm × 2–3 mm, purplish brown, sparsely covered by silky whitish fibrils, with long whitish strigose hair at the base (Fig. 1A, D). Basidiospore print white.

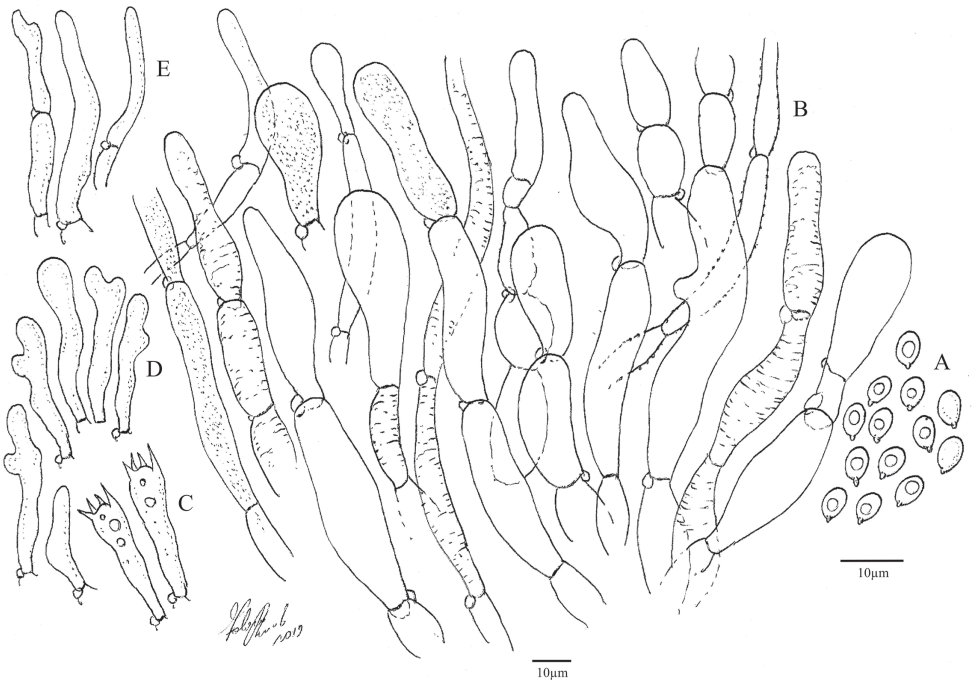

Figure 2. Micro-morphological features of *Pseudobaeospora cyanea* **A** basidiospores **B** pileipellis **C** basidia **D** cheilocystidia **E** caulocystidia (drawing F. Boccardo).

Micro-morphological observations

Basidiospores 4.5–5.3 × 3–4 µm (Q_{av} = 1.47, n = 30), from ellipsoid to ovoid, more or less oblong, smooth and hyaline, visibly apiculate, with central oil-drop, cyanophylic, thick-walled and dextrinoid at maturity (Fig. 2A). Basidia 20–25 × 5–6.5 µm, clavate, mostly tetrasporic (Fig. 2C), with some bisporic elements. Cheilocystidia 15–25 × 3.5–6 µm, irregularly cylindrical, lobed, often with a rounded bifurcated apex (Fig. 2D). Caulocystidia 15–70 × 3.5–7 µm, filiform and often septate, sometimes irregularly lobed (Fig. 2E). Pleurocystidia absent. Clamp connections present in all tissues (Fig. 2). Pileipellis trichoderma type, with ascending pluriseptate elements of subcylindrical shape, often with clavate or subglobose apex (Fig. 2C). Presence of encrusting extracellular pigments and bluish intracellular pigments

Sequencing and phylogenetic analysis

The sequences obtained from the specimen were uploaded with accession number MT271829 for ITS, and MT889638 for LSU, representing the first entry for this species in GenBank. The BLAST comparison of the ITS and LSU sequences obtained from our specimens did not show high similarity against any of the sequences contained in GenBank. In particular, the comparison of the ITS sequence showed the percent identity against other *Pseudobaeospora* sequences to be very low; the closest

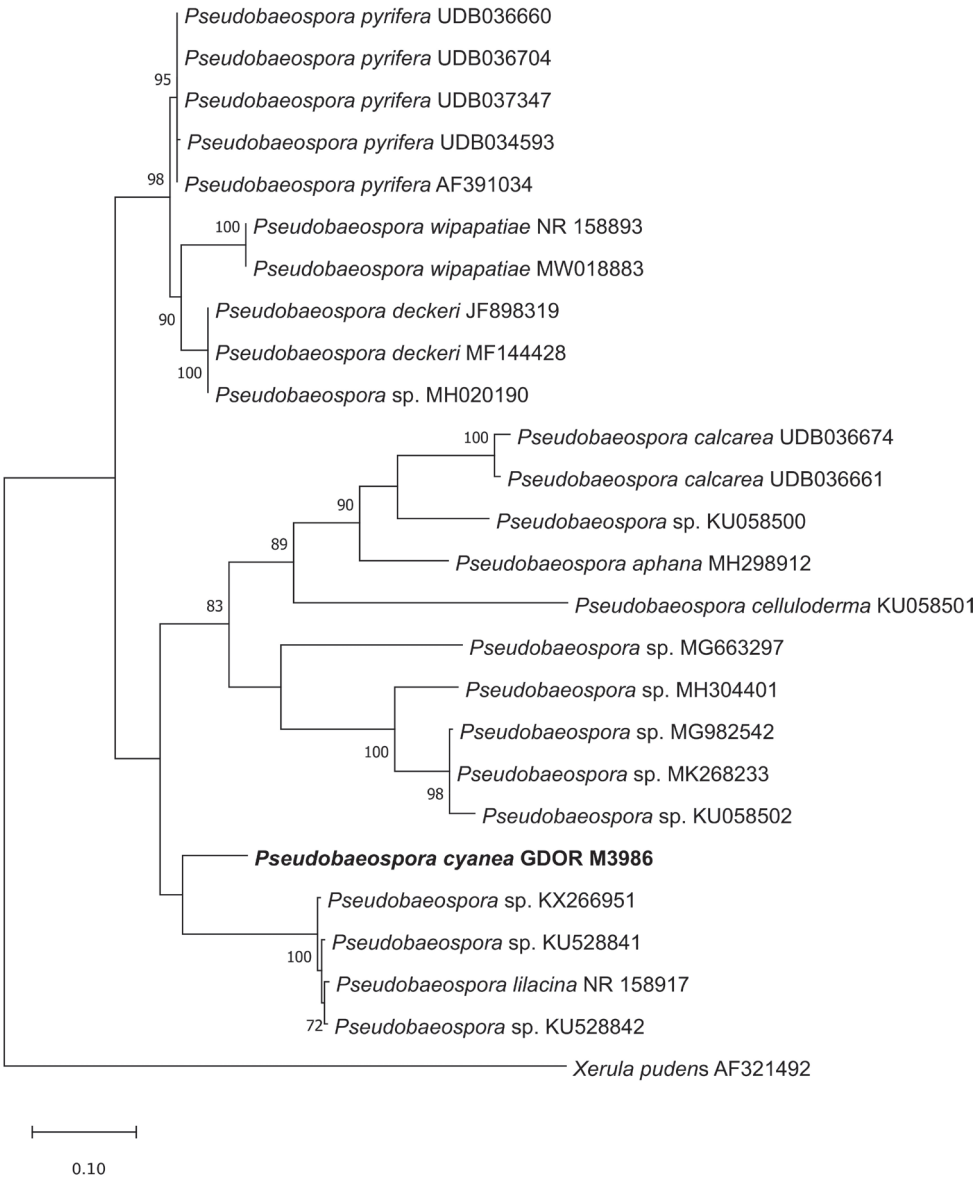

Figure 3. Maximum-likelihood phylogenetic tree with 500 bootstrap replicates, obtained from the ITS sequence alignment of *Pseudobaeospora* sequences in GenBank. *Xerula pudens* was used as outgroup taxon.

species is *P. pyrifera* with 85.23% of identity. The similarity search with LSU sequence retrieved *Pseudobaeospora lilacina* X.D. Yu, Ming Zhang & S.Y. Wu (95.99%), *P. wipapatiae* Desjardin, Hemmes & B.A. Perry (95.94%) and *P. pyrifera* Bas & L.G. Krieglst. (95.28%) as closest species. Figures 3 and 4 show the phylogenetic trees based on ITS and LSU sequences available in GenBank and UNITE.

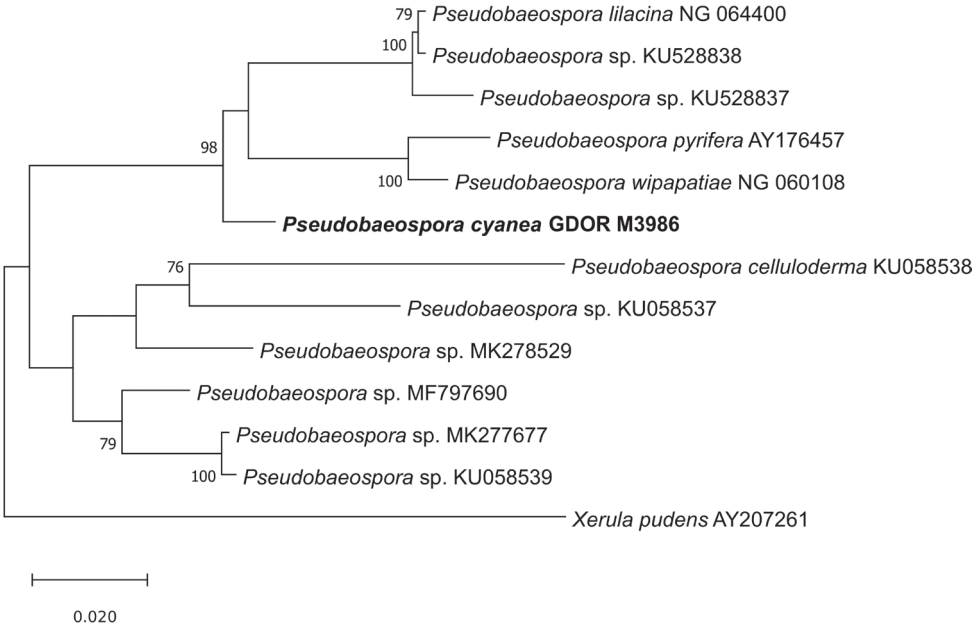

Figure 4. Maximum-likelihood phylogenetic tree with 500 bootstrap replicates, obtained from the LSU sequence alignment of *Pseudobaeospora* sequences in GenBank. *Xerula pudens* was used as outgroup taxon.

Discussion

The species is highly distinctive in terms of both macro- and micro-morphological features: the combination of pale gills, vivid bluish-purple pileus, green reaction of the pileipellis to KOH and the presence of cheilocystidia readily separates *P. cyanea* from other European species (Arnolds et al. 2003). Our observations are fully consistent with the original description, allowing for a confident identification of our collection. All three previous findings were in Spain (Arnolds et al. 2003; Arauzo 2011); the type specimens were collected in November on Mediterranean hills with *Pinus pinaster*, *Arbutus unedo* and *Erica arborea* (Arnolds et al. 2003). This Italian report, similar in habitat and season of growth, widens remarkably the known area of occurrence of this species, raising questions about its possible presence in other areas of Western Europe in which suitable habitats are present.

The original identification of the species is based on macro- and micro-morphological features, and no genetic data are available yet from the holotype specimens and from the material of the Basque collections. Since there are very few sequences of *Pseudobaeospora* available it is difficult to establish the taxonomic position of this species within the genus. The phylogenetic tree based on ITS (Fig. 3) suggests that *P. cyanea* is closely related with *P. lilacina*; while the LSU tree (Fig. 4) suggests that *P. cyanea* is part of a clade that also comprises *Pseudobaeospora lilacina*, *P. pyrifera*, and

P. wipapatiae. The difference between the two trees is due to the fact that the deposited ITS and LSU sequences do not always come from the same samples. Other taxa that show morphological affinity with the species, such as *P. dichroa* Bas, *P. pallidifolia* Bas, A. Gennari & Robich, *P. jamonii* Bas, Lalli & Lonati, and *P. laguncularis* Bas, could not be included in the phylogenetic tree because no sequences are currently available on the public database for any of them.

The species mentioned above share with *P. cyanea* several morphological features, like the coloured basidiomata, the greenish to lilac reaction of the pileipellis to KOH (except *P. lilacina*), the non hymenidermoid nature of the pileipellis (except *P. wipapatiae*) and the presence of clamp connections (Bas 2003; Schwarz 2012; Desjardin et al. 2014; Wu et al. 2017). Considering the intra-generic morphogroups proposed by Bas, based on basidioma coloration and micro-morphological features, *P. cyanea* fits in the "Pyrifera group", that includes *P. pyrifera*, *P. jamonii*, and *P. laguncularis*, grouped by the presence of cheilocystidia (Bas 2003). The species characterized by the absence of cheilocystidia, like *P. lilacina*, *P. deckeri*, *P. dichroa*, and *P. pallidifolia*, can be placed in the similar "group" (Bas 2003). Although these groups are deemed probably artificial by Bas himself, our molecular investigation indeed supports a rather close relationship between *P. cyanea* and *P. pyrifera*. This is in contrast with Voto's intra-generic arrangement, which separates these two species. Indeed, Voto (2009) placed *P. cyanea* in sect. *Anistoderma* Voto and *P. pyrifera* in sect. *Pseudobaeospora* Singer, based exclusively on the differences in the structure of the pileipellis.

The genus *Pseudobaeospora* includes several new species described in the recent past with a controversial position within the Tricholomatoid clade of Agaricales (Desjardin et al. 2014; Wu et al. 2017). Many unanswered questions also remain on its ecology, the distribution of its species, and their phylogenetic relationships. The scarcity of species with available genetic data is a liability to the definition of the phylogeny and intra-generic arrangement of *Pseudobaeospora*, and it is advisable to promote the sequencing of more species in the future.

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