Morphological and molecular identification confirms the occurrence of the rare macromycete *Phaeolepiota aurea* in Greece

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Zusammenfassung: Es wird über ein griechisches Vorkommen des seltenen Makromyzeten *Phaeolepiota aurea* im Fraktos-Urwald der zentralen Rhodopen, der sich am nordöstlichen Ende der Präfektur Drama befindet, berichtet. *Phaeolepiota aurea* gilt in Europa als selten, obwohl sie in mehreren europäischen Ländern weit verbreitet ist. Der Großpilz wurde anhand seiner morphologischen (makro- und mikroskopischen) Merkmale sowie molekulargenetischer Daten aus der Analyse eines partiellen 28S nuclear ribosomal large subunit (nrLSU) rDNA-Barcodes identifiziert. Nach unserem Wissen ist dies der erste Bericht von *P. aurea* in Griechenland. Vorläufige phylogenetische Analysen unter Verwendung der partiellen nrLSU-Sequenz unterstützen frühere Ergebnisse, die zeigten, dass *P. aurea* enger mit Arten der Gattung *Cystoderma* verwandt ist. Dennoch bleibt die Frage, ob *P. aurea* zur Gattung *Cystoderma* gehört oder ob es sich um eng verwandte Schwestergruppen handelt, weiterhin ungeklärt.

Abstract: This work reports the occurrence of the rare macromycete *Phaeolepiota aurea* in Fraktos Virgin Forest of the central Rhodope, which is situated at the northeast end of the Prefecture of Drama Greece. *P. aurea* is considered to be rare in Europe despite its widespread occurrence in several European countries. The macromycete was identified based on its morphological (macro- and microscopic) characters, as well as molecular data derived from the analysis of a partial 28S nuclear ribosomal large subunit (nrLSU) rDNA barcode. To our knowledge this is the first report of *P. aurea* in Greece. Preliminary phylogenetic analysis using the partial nrLSU sequence further supports previous findings that showed *P. aurea* to be more closely related to species of the genus *Cystoderma*. Nevertheless, whether *P. aurea* belongs to the *Cystoderma* genus or they are closely related sister groups still remains inconclusive.

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Macromycetes constitute a large group within the kingdom of fungi. Taxonomically they are classified into two main phyla, *Ascomycota* and *Basidiomycota*, and they are commonly known as wild mushrooms due to the formation of visible fruiting bodies that emerge from the soil. Over the last 30 years scientific research on macromycete diversity in Greece has shown that the country is particularly rich in macromycete species and over 3000 species have been sporadically recorded in the county (TSAKIRIS & STARA 2016). Nevertheless, most of those data are fragmentary and the idententification of the species is based only on the morphological characteristics of the described macromycete.

The clade of *Agaricales*, which is one of the largest monophyletic groups of the *Basidiomycota*, includes widespread and diverse species ranging from desert, grasslands, forests, temperate and other habitats (MATHENY & al. 2007). Within the *Agaricales*, the golden cap mushroom *Phaeolepiota aurea* (MATT.) MRE. of the monotypic genus *Phaeolepiota* is found throughout North America, Europe and Asia (VASAS 2004, PHILLIPS 2005). It is considered to be rare, despite its widespread distribution in Europe. To date, it has been reported to occur in UK, Ireland, Croatia, Czech Republic, Finland, France, Italy, Germany, Netherlands, Poland, Romania, Russia, Slovakia, Hungary, Ukraine, Bulgaria (VASAS 2004, BOCCARDO & al. 2008, JORDAN & al. 2017). It is common in the Alps, e.g. in Austria, Switzerland, and known from Sweden, Norway, Denmark and Portugal (GBIF 2020, https://www.gbif.org/occurrence/search?continent=EUROPE&taxon_key=2535253). In some of these countries the species is included in their Red-Lists (e.g. Poland, Hungary, Ukraine) and its status is stated either as vulnerable or endangered. In other countries (e.g. UK) it was removed from the national Red-List despite the fact that it is assessed as widespread but rare (JORDAN & al. 2017).

Phaeolepiota aurea is saprobic, prefers nutrient rich loamy soils and commonly grows in pastures under hedges, ruderal places (e.g. roads, roadsides), in woods and parks beneath beech and alder and often associated with nettles. It occurs from September to November and although it was initially listed as an edible mushroom, its consumption is not considered safe anymore, due to its high HCN content (WELLS & KEMPTON 1965).

Molecular genetic studies have contributed significantly in our interpretation and classification of macromycetes and their relatives (MATHENY & al. 2006). *Phaeolepiota aurea* was initially described as *Agaricus aureus* in 1779 and later placed in several genera, e.g. *Pholiota, Togaria* and also *Cystoderma.* In addition, it has several heterotypic synonyms (www.speciesfungorum.org). According to phylogenetic and taxonomic studies it is considered to represent either an unsupported sister group or member of *Cystoderma* (SAAR & al. 2009). Herein, we report for the first time the occurrence of *P. aurea* in Greece and provide comprehensive identification in terms of morphological characteristics and barcoding data derived from part of the 28S nuclear large subunit (*nrLSU*) rDNA region.

Materials and methods

Sampling: In November 2019, a colony of approx. 15 basidiomata was spotted in Fraktos Virgin Forest of the central Rhodope, near the road, at the location with coordinates 24.483°, 41.520°and 1440 m s. m. The colony was located in a particularly dump place, beneath beech trees (*Fagus*) and among *Petasites*. Notes on macromorphological features and ecology and photographs were done in the field. Specimens were collected and brought to the lab for further studies and for genetic analysis.

Morphological character examination: The macroscopic characteristics were described based on the observation of fresh specimens. For microscopic analyses, fresh and frozen (–9 °C) materials were

used. Identification and descriptive terminology follows, e.g. PHILLIPS (2005). Morphological observations were carried out with a CARL ZEISS Stemi-2000-C stereomicroscope, while microscopic features were observed with a Carl ZEISS Axio-Imager A1 microscope, coupled with and Axion Vision camera. Nomenclature follows Index Fungorum (2020) and MycoBank (2020). The specimens were deposited in the herbarium collection of the Forest Research Institute (FRI) of Thessaloniki, Greece.

Tab. 1. BLAST analysis of the *Phaeolepiota aurea nrLSU* sequence with accessions from GenBank.

Identified species name	Accession number	Score (bits)	e-value	Identity (%)
Phaeolepiota aurea	MH876401	1489	0	100.00
Phaeolepiota aurea	DQ071704	1489	0	100.00
Cystoderma superbum	AM946443	1378	0	97.52
Cystoderma superbum	AM946442	1378	0	97.52
Galerina triscopa	MH828261	1365	0	97.28
Galerina pumila	AY207204	1360	0	97.15
Galerina vexans	MH828269	1360	0	97.15
Cortinarius teraturgus	AF388755	1354	0	97.03
Galerina vittiformis	MH828283	1349	0	96.91
Hebeloma affine	FJ436324	1349	0	96.91
Hebeloma collariatum	KT591560	1349	0	96.91
Hebeloma crustuliniforme	MK880546	1349	0	96.91
Cortinarius cramesinus	KT875178	1349	0	96.90
Cortinarius laquellus	MH108355	1347	0	96.90
Cortinarius orixanthus	KT875185	1347	0	96.90
Cortinarius promethenus	MK277834	1349	0	96.90
Cortinarius armiae	MH108406	1343	0	96.78
Cortinarius chrysma	MK358064	1343	0	96.78
Cortinarius diaphorus	MN492672	1343	0	96.78
Cortinarius gymnocephalus	NG_064342	1343	0	96.78
Cortinarius mycenarum	KT875188	1343	0	96.78
Cortinarius rufus	MF489798	1343	0	96.78
Cortinarius viscostriatus	MW263587	1343	0	96.78
Galerina pseudocamerina	KP100540	1343	0	96.78
Hebeloma alpinum	JN939952	1343	0	96.78
Hebeloma eburneum	JN939973	1343	0	96.78
Hebeloma flaccidum	MK278125	1343	0	96.78
Hebeloma lutense	JN939964	1343	0	96.78
Hebeloma minus	JN939959	1343	0	96.78
Hebeloma pusillum	JN939968	1343	0	96.78
Hebeloma velatum	MK278132	1343	0	96.78
Hebeloma velutipes	MK880559	1343	0	96.78

DNA extraction, PCR and sequencing: DNA was extracted from 0.2 g of lyophilized basidioma tissue with a modified version of the CetylTrimethylAmmonium Bromide (CTAB) protocol (DOYLE & DOYLE 1987). Part of the *nrLSU* region was amplified with the praimer pair LR0R (REHNER & SAMUELS 1994) and LR5 (VILGALYS & HESTER 1990). Polymerase Chain Reaction (PCR) was carried out with 20 ng genomic DNA, 1× PCR buffer, 0.5 μM forward and reverse primers, 0.2 mM dNTPs, and 1 U Kapa Taq DNA polymerase (Kapa Biosystems, USA), supplemented with H_2O to 20 μl total reaction volume. The PCR conditions were: initial denaturation at 95 ºC for 3 min, followed by 35 cycles of 94 °C for 30 sec, 54 ºC for 30 sec, and 72 °C for 90 sec. The amplicons were sequenced in two directions with the Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) in an ABI 3730

sequencer (PE Applied Biosystems). Sequences were manually curated for sequencing ambiguities. The generated *nrLSU* sequence of the *P. aurea* specimen was deposited in GenBank with the accession number MW147712.

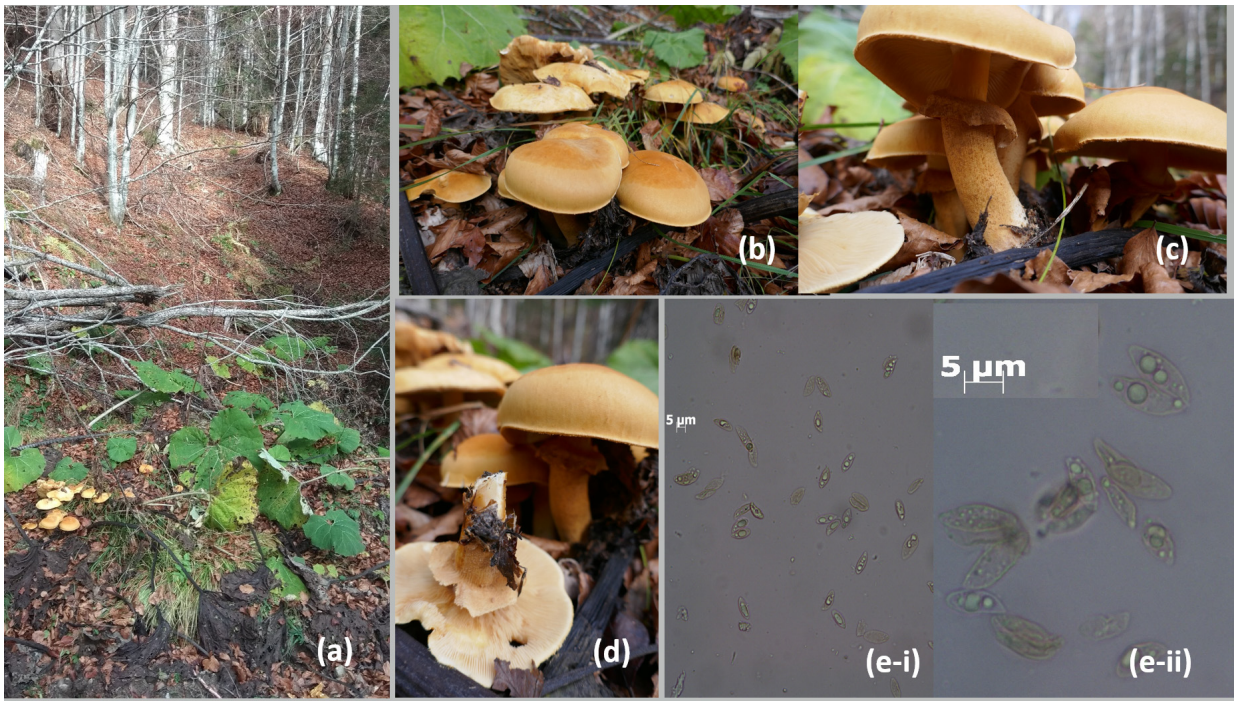

Fig. 1. *Phaeolepiota aurea, a-d* basidiomata in situ, *e-i, e-ii* spores.

BLAST analysis, alignments and phylogenetic analysis: Specimen identity was verified based on DNA sequence similarity with reference sequences from the GenBank repository, using the default search parameters. The Basic Local Alignment Search Tool (BLAST) hits with the highest score (bits) and percent identity values (threshold set at 95 % identity) were considered as candidate species. Duplicate species entries were removed from the analysis. Multiple sequence alignment of the *nrLSU* query sequence with representative reference sequences from Genbank was carried out with the Molecular Evolutionary Genetics Analysis X (MEGA X) version 10.05 software (KUMAR & al. 2018) using the MUSCLE algorithm. Bayesian Information Criterion (BIC) scores were also calculated for the *nrLSU* alignment using MEGA X, and the model with the lowest BIC value was chosen for the phylogenetic tree reconstruction. The Maximum Likelihood (ML) tree was generated using the corresponding substitution model with 1000 bootstrap replicates. *Russula paludosa* was used as an outgroup to root the tree.

Results

Morphological analysis

Morphological description of the examined basidiomata in field and in the laboratory matched the available descriptions of *P. aurea* (STANA 1995, VASAS 2004, PHILLIPS 2005).

Pileus 8–20 cm in diam., orange-tan to golden brown, with a whitish to pale yellow flesh, covered by flaky, powdery veil, often rubbing off and remaining as appendiculate veil remnants at the margin. Gills adnate to free, broad and closely attached, pale yellow to orange-brown. Stipe relatively tall, 8–15 cm, expanded towards the base (2–3 cm thick); colour more or less similar to the pileus with a conspicuous ring. Spores smooth to finely roughened, yellowish to light brown, 10–14 × 5–6 µm (Fig. 1).

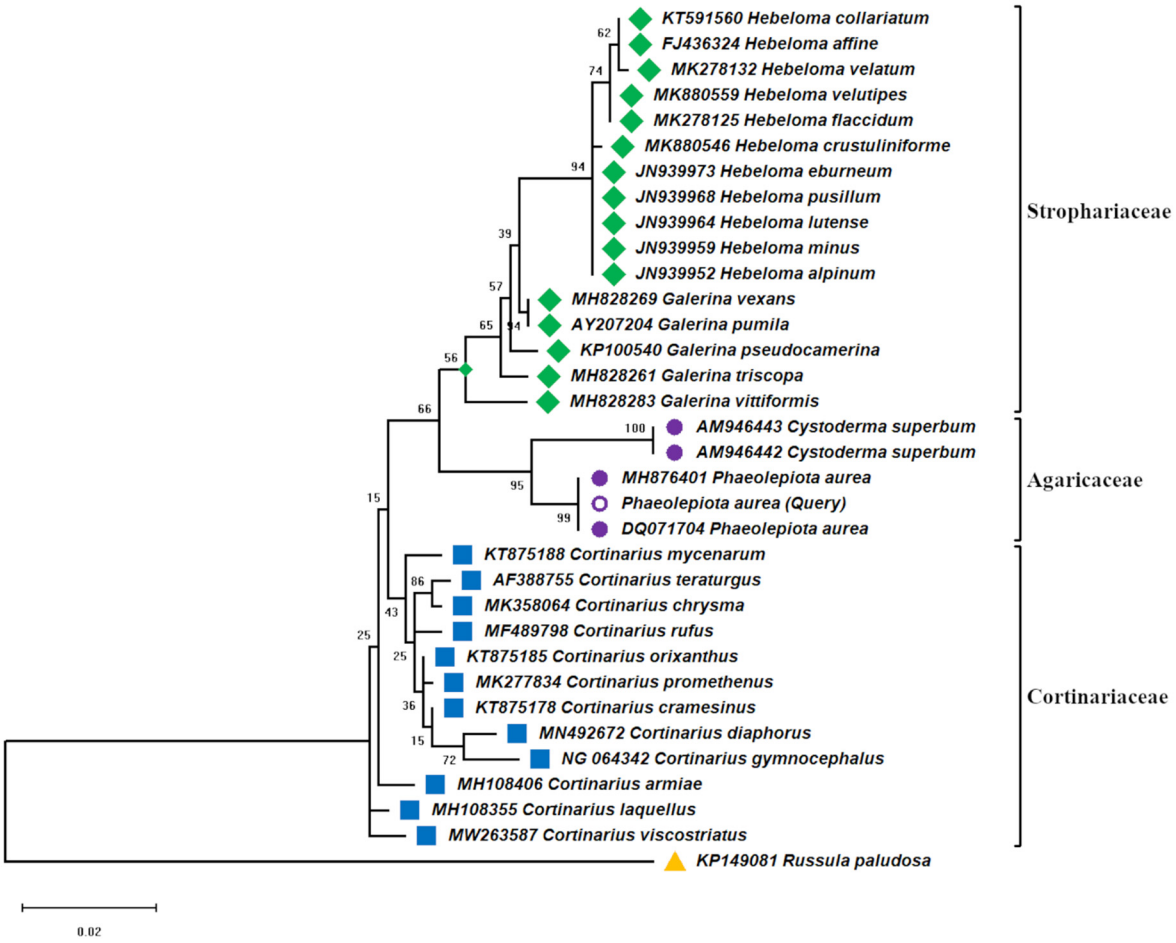

Fig. 2. *nrLSU* Maximum Likelihood phylogenetic tree generated for the *Phaeolepiota aurea* query sequence with reference sequences from GenBank. The tree with the highest log likelihood (-2099.61) is shown. The percentage of replicate trees in which the corresponding taxa clustered (1000 bootstrap replicates) is shown next to the respective branches. *Russula paludosa* was used as an outgroup to root the tree. The annotated scale bar represents the number of substitutions per site.

Molecular analysis

Molecular genetic analysis of the *nrLSU* region was carried out to confirm morphological identification. The use of the *nrLSU* region in molecular phylogentic analyses has considerably enhanced identification accuracies for several macromycete clades (TAYLOR & MCCORMICK 2008, GEML & al. 2009, PARK & al. 2014). In BLAST analysis of the *nrLSU* sequence (Tab. 1) the *P. aurea* species accessions (MH876401 and DQ071704) showed 100 % similarity with the query sequence, which further verifies the phenotypic identification of the species.

Furthermore, multiple sequence alignment and phylogenetic tree inference were performed for the partial *nrLSU* query sequence with representative reference species sequences (Tab. 1). The *nrLSU* alignment generated a matrix of 816 sites in total, with 689 conserved, 119 variable, and 58 parsimony-informative sites (Suppl. Fig. 1). For the construction of the ML phylogenetic tree, BIC score analysis was carried out for the *nrLSU* alignment using the default MEGA X parameters for model assessments (Suppl. Tab. 1). Based on the BIC values, the Kimura 2-parameter model with discrete Gama distribution

(K2+G) was used as the best fit for the *nrLSU* data. The *nrLSU* phylogenetic tree shows that the query species is more closely related to the reference *P. aurea* sequences with high bootstrap value (99 %) within the *Agaricaceae* clade (Fig. 2), than other species of the *Agaricales* that showed high similarity in the BLAST analysis (Tab. 1). Furthermore, clustering of the *Phaeolepiota* clade with the closely related sister group *Cystoderma* is also supported by high bootstrap values (above 95 %), in contrast to other clades on the tree that had lower bootstrap support (Fig. 2).

Collectively, the morphological characterization, as well as the BLAST and phylogenetic analyses presented herein indicate that the Greek macromycete specimen of this study is *Phaeolepiota aurea*.

Discussion

Phaeolepiota aurea, growing near the road and beneath beech trees, was identified with morphological and molecular genetic analysis. To our knowledge this is the first report of *P. aurea* in Greece. This species is considered as rare and in some countries included in national red lists. A significant number of papers recording macromycetes has been published in Greece during the last decades, e.g. ZERVAKIS & al. (1998, 2004), DIAMANDIS (2000a), but besides of the first Catalogue of fungi in Greece, published in 1973 by M. PANTIDOU, there is no other official list for the occurrence of macromycetes in the country. Therefore, comparisons on species distribution and/or occurrence is difficult (DIAMANDIS 2000b). Despite the fact that macromycetes are key players in ecosystem processes and have high utilitarian value for their nutritional and medicinal properties, their distribution, ecology and conservation status have been largely neglected in most European countries (SENN-IRLET & al. 2007). During the last decade most European countries have initiated an evaluation of the status of macromycete diversity, in order to produce national fungal Red-Lists and take actions regarding their conservation status (SENN-IRLET & al. 2007). However, an official Red-List of threatened species has not been produced at national level for Greece and as such there are no past records to compare data of rare fungi. Only an unofficial list with approximately 150 species which are considered threatened or rare in Greece was published by DIAMANDIS in 2000 (DIAMANDIS 2000b) and *P. aurea* is not included among them.

Obviously, *P. aurea* is uncommon in Mediterranean countries. So far it is reported from Italy where it is considered as a rare species (BOCARDO & al. 2008). However, these Italian records come from the Alps in North Italy, e.g. Bergamo and Trentino (personally checked records in iNaturalist.org). Most reports of *P. aurea* occurrence are coming from Central and North Europe, where it is reported to have large distribution from submeridional to the boreal zones (VASA 2004). The spotted colony was recorded in the Virgin Forest of Fraktos, which is regarded as a forest with a high ecological interest which has been declared as "Natural Monument". The climate in such a height (1440 m s.m.) is characterized as humid continental and differs significantly from the Mediterranean climate of the plains at lower elevation. The climate on the sampling site resembles the mid-European climate and therefore, the occurrence of *P. aurea* in this site is not surprising. However, based on this unique observation of *P. aurea* in Fraktos Virgin Forest, it seems that Greece does not belong to the main distribution centre of the species. However, more observations are needed in order to verify the distribution of *P. aurea* in the country.

According to the BLAST analysis, both the *P. aurea* query and reference sequences are highly similar, as expected, and differ considerably from other closely related species in the *Agaricales* (Tab. 1). Preliminary phylogenetic inference using representative *nrLSU* reference sequences for the corresponding taxa revealed that the *P. aurea* clade is clearly separated from the other taxa with high bootstrap support (Fig. 2). It is also noteworthy that *P. aurea* seems to be more closely related to *Cystoderma* species (Fig. 2), which further supports the result by GARNICA & al. (2007) showing a close relationship between the genera *Cystoderma* and *Phaeolepiota*. Although sufficient phylogenetic resolution was achieved for many *Agaricales* lineages using multi-locus phylogenies (MATHENY & al. 2006, SAAR & al. 2009), whether *P. aurea* is a closely related sister group or belongs to the genus *Cystoderma* still remains inconclusive. A more extensive analysis with the examination of more specimens and multiple genetic loci should be included in future work in order to explore the relationship of *P. aurea* with related clades.

By combining morphological with DNA-based identification we were able to verify the presence of *P. aurea* in Greece for the first time. Species identification based on combined morphological and molecular genetic analysis is an important step towards understanding macromycete diversity, ecology and phylogeny.

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