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Research article

Three new species found in the Bracciano-Martignano Regional Natural Park in Lazio, Italy

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Abstract
We report the finding of three recently described species in late autumn and winter (October to December) in the Bracciano-Martignano Regional Natural Park in Lazio (Italy). The species are *Amanita calida*, *Lepiota elseae* and *Xerocomellus sarnarii*. This is the first record of *Lepiota elseae* for Italy. The collection site, a *Quercus* thicket, is located on the slopes of San Bernardino del Malpasso in Trevignano Romano, between Monte Rocca Romana (Sabatini mountains) and the nearby Bracciano Lake. A description of the specimens is given, and a comparison is made with the few descriptions of the ambient available for previous records of the respective species.

Keywords
Amanita calida, Lepiota elseae, Xerocomellus sarnarii, Quercus forest, Natural Parks

Introduction

Three species, described as new during the last decade, belonging to the genus *Amanita*, *Lepiota* (Agaricales) and *Xerocomellus* (Boletales), were found in the same area near Rome in Lazio. The species are *Amanita calida* Plaza & Illescas (2022), *Lepiota elseae* A. Caball., Vizzini, G. Muñoz & Contu (2015) and *Xerocomellus sarnarii* Simonini, Vizzini & U. Eberh. (2015) respectively.

Amanita calida is inserted in the section *Vaginatae* (Tulloss 1994; Tulloss and Yang, 2022) and was recently described from Spain (Illescas and Plaza, 2022) based on collections obtained in Andalusia and Catalonia, associated with *Quercus*, in spring-summer and in October. Macroscopically, this species is characterized by a whitish, beige to grayish cuticle, with a prominent darker brown umbo, a widely striate margin of the pileus, and a very elongated stipe. This *Amanita* occurs in Spain (OK316924, ITS of holotype) and in Italy with two sequences present in the International Nucleotide Sequence Database (INSD) with temporary codes, later confirmed as *A.*

calida (MT073006, MW013162) from collections made in Central Italy (Terni, Umbria), in woods of *Quercus ilex* L. and *Fagus*, between the beginning of July and the beginning of August of 2018. (Tulloss and Yang, 2022). One sequence obtained from a soil sample (UDB0153760) in Estonia also coincided with that of the holotype.

Lepiota elseae was found and described in Spain in 2015 (Caballero et al., 2015). This species belongs to the section *Lepiota* and is known from Mediterranean areas in Spain in association with *Q. ilex* and determined as a new species on both morphological and molecular characteristics (analysis of the ITS region of the ribosomal RNA gene sequences). No evidence of previous findings of fruitbodies in Italy has been reported. A soil sample from Italy (UDB02883342) and two samples from Bulgaria (UDB02886835, UDB02886840) contained DNA from *L. elseae*.

Regarding *X. sarnarii*, findings in Sardinia and Tuscany have been recorded. Based on Simonini et al. (2016) some specimens of this species have also been found in France. It is described as a rare Boletales species, so far known from Italy, France, some middle eastern region of Europe and in Cyprus (Ariyawansa et al., 2015; Loizides et al., 2019). Its habitat are the Mediterranean areas, especially under *Quercus suber* L. and *Q. ilex* trees. The main fruiting period is late autumn (November). It is reported that this taxon seems to prefer climatically moderate zones and undisturbed habitats. For this species, eDNA from soil samples have been recorded in Georgia (UDB07503546), Greece (UDB07503362) and Italy (UDB07502840).

Our initial tentative attributions of the three species found in the *Quercus* wood zone in the Bracciano-Martignano Regional Natural Park, based on the morphological characteristics, indicated an *Amanita* of the section *Vaginatae* with characteristics between *Amanita vaginata* (Bull.) Lam. and *Amanita battarrae* (Boud.) Bon, the other two species as *Lepiota clypeolaria* (Bull.) P. Kumm., and *Xerocomellus chrysenteron* (Bull.) Šutara or *Xerocomellus porosporus* (Imler ex Watling) Šutara. The correct identifications of specimens were obtained by molecular analysis of the rDNA ITS barcode.

Materials and Methods

Area of study and sample

The small wood area is part of the Bracciano-Martignano Regional Natural Park in Lazio and located on the volcanic slopes of San Bernardino del Malpasso. The total area measures somewhat less than 5,000 m² with a maximum length of 160 m, a maximum width of 45 m at about 200 amsl (Fig. 1), in Trevignano Romano between Monte Rocca Romana (Sabatini mountains) and the nearby shores of the Bracciano Lake at 42°09'19" N 12°16'17" E. This thicket is crossed by an almost permanently dry ditch and is located close to uncultivated fields and vineyards and inhabitations too (Via delle Fossette). It displays the peculiar morphology of fertile volcanic soil. In the Sabatino volcanic system, mainly pyroclastic soils, andosols and acid brown soils born on beds of volcanic scoria and ash are found. The volcanic soils examined in the territory have a low content of calcium carbonate and a subacid to neutral reaction (pH = 5.3–7.8) and in general are fertile but fragile pedoenvironments (Various authors, 2009). The soils in the area facing the lake tend to take on the typical characteristics of arid environments which adapt well to the coenoses of the xerophilous Mediterranean vegetation. The Bracciano-Martignano area figures as a transition zone from a more distinctly Mediterranean climate to a temperate climate, typical of the inland areas of the Lazio

Apennines (Various authors, 2009). Differently from the other side of the Monte Rocca Romana (Knijn et al., 2021), this wooded *Quercus* area overlooking the lake does not show a humid climate. The rainfall in the autumn and early winter period is given by light rains, the month with the highest rainfall is November (30%) while it drops to 10% in June. As for the temperature, in this part of the Bracciano Lake in the autumn months it varies from 23 °C, perceived as 25 °C, to 15 °C during the day and in December it averages around 13 °C, while in June the maximum temperatures reach an average of 26 °C. The thicket vegetation, characteristic of the Mediterranean woods, mainly consists of various types of oaks, such as downy oaks (*Quercus pubescens* Willd.) and Turkey oaks (*Quercus cerris* L.) as deciduous plants and holm oaks (*Q. ilex*) as evergreens. The undergrowth is made up of butcher's broom (*Ruscus aculeatus* L.), *Asparagus acutifolius* L. for which the area is very popular, ivy (*Hedera helix* L.) and cyclamen.

The presence of the various fungal species was noted in several specimens for *A. calida* in autumn-winter period from 2018 to 2022, as well as for *X. sarnarii*, the discovery of *L. elseae* instead took place only in October 2020. The specimens of *A. calida* and *X. sarnarii* morphologically and molecularly characterized in this study were harvested in October 2022. The analysed specimens are conserved as a part of The Mycological Collections of the Natural History Museum and Botanical Garden of The University of Tartu, Estonia.

Fig. 1. – Aerial photograph of the ticket where the three species were found. The site measures about 160 × 45 m (area < 5,000 m^2) at 200 amsl and is located alongside Via delle Fossette at 42°09'19" N 12°16'17" E. Maxar (Vivid) imagery captured on 2 Sep 2022. Esri Community Maps Contributors, Esri, HERE, Garmin, Foursquare, GeoTechnologies, Inc, METI/NASA, USGS | Maxar, Microsoft.

Microscopic analyses

Photographs and notes were taken of fresh basidiomata. Microscopic investigations of dried basidiomata were carried out using a light microscope Leica DM750 with Leica Application Suite v4.13.0 at ×1000. Measurements of basidiospores were made in 3% KOH and stained in Congo Red. Melzer's reagent was used to test amyloid or dextrinoid reaction.

Molecular analyses

Ribosomal DNA-based analysis was performed on the specimens in the frame of the UNITE project (Kõljalg et al., 2013). DNA extraction, PCR amplification of SSU partial, ITS1, 5.8S, ITS2, LSU partial regions and sequencing were performed as in Voitk et al. (2018). Initial molecular analyses consisted in BLAST 2.13.0+ (Camacho et al., 2009) alignment of the obtained sequences against the Full UNITE+INSD v9.0 dataset for Fungi (Abarenkov et al., 2022).

In the case of the *Xerocomellus* specimen, the result of this analysis was used to select six closely related sequences from *X. sarnarii* for phylogenetic analyses together with two sequences from the sister species *X. chrysenteron* as outgroup. These sequences were aligned using the L-INS-I method of the MAFFT v7.520 algorithm (Katoh and Standley, 2013) in Aliview 1.27 (Larsson, 2014) and trimmed to include only nucleotides from the ITS1, the 5.8S ribosomal RNA gene and the ITS2 as determined by ITSx v1.1.3 software (Bengtsson-Palme et al., 2013) resulting in an alignment of 642 nucleotide sites.

Maximum Likelihood and Bayesian inference were engaged with HKY+G4 as the model for nucleotide evolution as suggested by AIC, BIC and AICc criteria applied using ModelTest-NG v0.1.7 (Darriba et al., 2020; Flouri et al., 2014). In PhyML 3.1 (Guindon et al., 2010), Nearest Neighbour Interchange was set up as topology research, initial trees obtained applying BIONJ and Neighbour-Joining to a matrix of pairwise distances and 100 replicates for non-parametric bootstrap analysis. In MrBayes 3.2.6 (Ronquist et al., 2012), the Markow Chain Monte Carlo algorithm was run using four chains (three hot, one cold), a temperature of 0.1 and iterations for 2×10^6 generations on two trees with a diagnose frequency every 500 generations and a 25% burn-in. The final mean standard deviation of the separated frequencies was 0.002. The phylogenetic tree of the sequences combined with their Single-Nucleotide Variants (SNVs) were visualised using FigTree v1.4.4 (Rambaut et al., 2018) and snipit v1.1 softwares (O'Toole and Tomkins-Tinch, 2021) and annotated manually. The sequences of the three specimens are available in the UNITE database at https://unite.ut.ee/ (*Amanita calida*: UDB07673016; *Lepiota elseae*: UDB0799853; *Xerocomellus sarnarii*: UDB07673020).

Results

In the autumn-winter periods of the various years, the following fungi typically grew in this quercous wooded area of the park, among others: *Amanita pantherina* (DC.) Krombh., *Amanita rubescens* Pers., *Cantharellus alborufescens* (Malençon) Papetti & S. Alberti, *Helvella crispa* (Scop.) Fr., while *Helvella panormitana* Inzenga (UDB0799868) under downy oaks further ahead. *Hydnum ibericum* Olariaga, Liimat. & Niskanen and *Hydnum ovoideisporum* Olariaga, Grebenc, Salcedo & M.P. Martín, such as the basidiomata described from the chestnut forest of Monte Rocca Romana (Knijn et al., 2021), *Psathyrella bipellis* (Quél.) A.H. Sm., *Russula rubroalba* (Singer) Romagn., sometimes in proximity with *Xerocomellus sarnarii*, *Russula globispora* (J. Blum.) Bon (UDB0799051) and

Russula violeipes Quél. (UDB07673018), various *Tricholoma* species. *Boletaceae* fungi such as *Hemileccinum impolitum* (Fr.) Šutara (UDB0799854), *Leccinellum crocipodium* (Letell.) Della Magg. & Trassin., *Suillellus queletii* (Schulzer) Vizzini, Simonini & Gelardi (UDB07673017). Lignicolous fungi: *Lentinellus castoreus* (Fr.) Kühner & Maire (UDB0754099) and *Lentinus arcularius* (Batsch) Zmitr. (UDB07672111).

Fig. 2 – *Amanita calida* (a) Specimen photographed in December 2018; (b,c) Specimens found in October 2022 and environment; (d,e,f) Specimen of October 2022 corresponding to TUF137142.

Morphological and molecular characteristics of the three species found in Lazio:
Amanita calida (Fig. 2): pileus at first conical to convex, then plane, with a prominent umbo; light beige, light greyish towards brown, usually obviously marked with concentric colour bands, umbo darker as well as the band towards the edge of the cap; margin broadly striated to sulcate; whitish patches of veil remnants rarely present, mostly disappeared. Lamellae rather crowded, free, white; lamellulae of different lengths which are truncated at ninety degrees. Stipe cylindrical, very long (much longer than pileus diameter), smooth; ring absent, almost rooting when buried in the ground; the colour varies from whitish to light ochre, especially in the specimen with the beige-brown cap, in this case the colour is darker in the centre of the stem and lightens towards the portion under the cap and towards the base; sac-like, membranous, fragmented, whitish volva brown-spotted. Pileus and stipe surface may stain darker when handled. Context whitish. Smell indistinct. Associated with *Quercus* trees.

Microscopy (Fig. 3): Basidiospores subglobose to broadly ellipsoid, rarely globose or ellipsoid with an apiculum, hyaline, inamyloid, with large lipid droplets inside; (n = 20) 10.4–16.5 × 7.9–12.2 µm, average 11.6 × 9.8 µm, Q = 1.1–1.4, Qav = 1.2. Claviform basidia, with small and large lipid droplets inside, 4-spored, 43–55 × 13–14 µm. Molecular analysis of a specimen resulted in a 100% match of the ITS barcode with that of the *A. calida* type specimen JA-CUSSTA 9324 (OK316924).

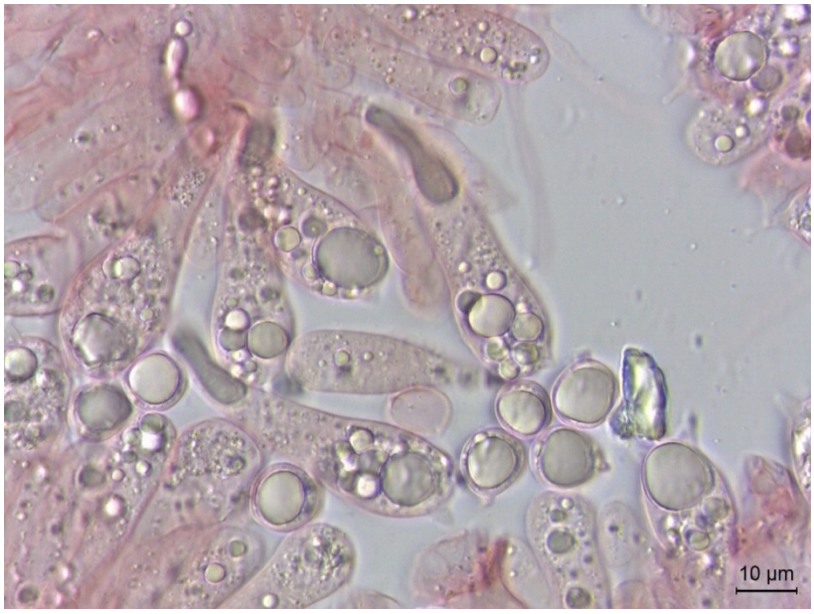

Fig. 3 – *Amanita calida* basidia and basidiospores in Congo Red.

Lepiota elseae (Fig. 4): pileus convex, about 6 cm in diameter, whitish to brownish, covered by dark brown, appressed felty squamules or scales, particularly in the centre of the cap; margin appendiculate for velar remnants. Lamellae crowded, free, white. Stipe cylindrical, widens a little at the base, fragile, white, felted, smooth above ring zone; ring silky-floccose, evanescent, white; below ring zone whitish or brownish floccose-scaly. Context white, thin. Smell indistinct. Saprobic on *Quercus* litter.

Microscopy (Fig. 4): basidiospores (n = 20) 11.5–16.3 × 5.2–6.3 µm, average 14.2 × 5.6 µm, Q = 2.1–3.0, Qav = 2.6, fusiform, amygdaliform, hyaline, dextrinoid. Claviform basidia, 4-spored, 32–35 × 8.5–9.6 µm. Molecular analysis of a specimen gave a 100% match with the ITS barcode of the *L. elseae* type specimen AH-40487 (KP640556).

Xerocomellus sarnarii (Fig. 5): Pileus convex to plane, felty/tomentose, pruinose cuticle cracking, revealing the whitish or very pale yellowish context with pinkish outlines, reddish spots can be seen when dry; bronze, brown or reddish brown. Tubes long, lemon yellowish, yellowish-ochre, bluing when bruised, sometimes decurrent on the stem. Pores same colour as tubes, at first narrow then become wide, angular, staining slowly dark blue when touched. Stipe cylindrical, wider at the base, fistulous, ribbed to form a very large gride (lattice), fibrillose, whitish, beige background, sometimes with intense yellow colours upper part and even dark purple red at the base. Context whitish, yellowish, soft in pileus, where we have not noticed a change to dark blue even after a certain time but possibly a slight change to light blue, at the base of the stipe the darkening of the red. Smell indistinct. Sometimes clusters of three specimens were observed. Associated with *Quercus* trees.

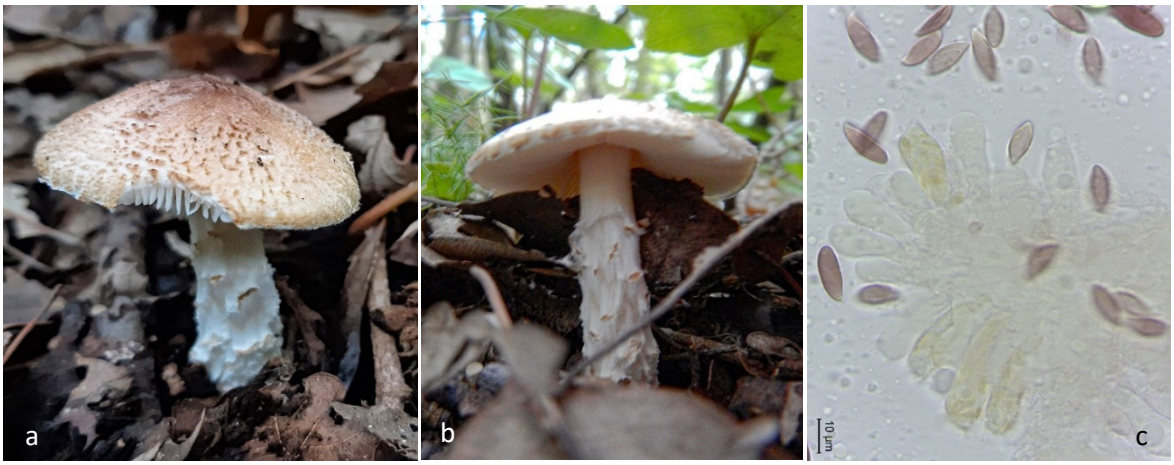

Fig. 4 – *Lepiota elseae*: (a,b) Basidiomata; (c) Basidiospores (TUF105926) in Melzer's reagent.

Fig. 5 – *Xerocomellus sarnarii*: (a) specimen photographed in December 2020; (b) specimen photographed in November 2021; (c,d) specimen photographed in October 2022 corresponding to Accession TUF137146; (e) basidiospores in 3% KOH.

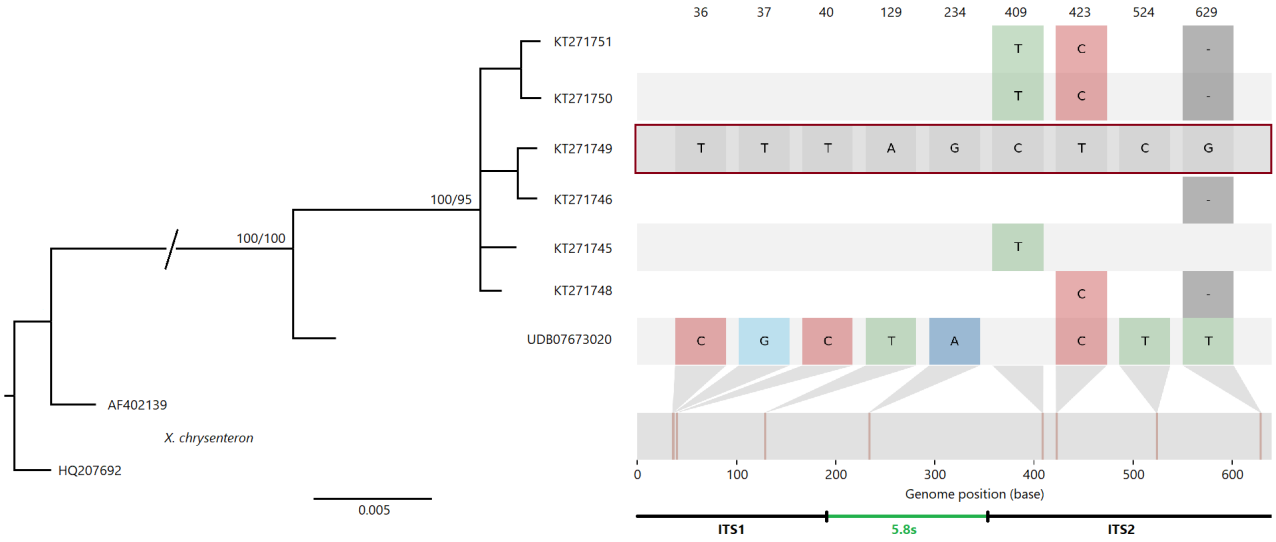

Fig. 6 – On the left, a Bayesian MCMC inferred phylogenetic tree of the ITS1-5.8S-ITS2 sequences of six specimens of *Xerocomellus sarnarii* indicated by their INSD accession together with the UNITE sequence UDB07673020 of the specimen under study. Accession KT271749 is a sequence from the species' holotype MCVE 28577. AF402139 and HQ207692 are sequences from the sister species *X. chrysenteron* used as outgroup. Posterior probabilities (left) and maximum likelihood bootstrap (right) values of the nodes are indicated. On the right, Single-Nucleotide Variants of the same sequences without the two sequences from the outgroup are shown with respect to the holotype sequence (highlighted in the red box). The barcode regions are annotated at the bottom.

Microscopy (Fig. 5): basidiospores (n = 20) 11.4–14.0 × 4.8–6.8 µm, average 12.7 × 5.7 µm, Q = 2.0–2.6, Qav = 2.2, fusiform, olive brown with lipid droplets inside. Molecular alignment of the ITS barcode of a specimen resulted in a close 98.9% match with that of the holotype of *X. sarnarii* (MCVE28577; KT271749). Including six sequences from the same species, a monophyletic tree was obtained (Fig. 6). Observing the Single-Nucleotide Variants (SNVs) associated with these clades, grouped SNVs seem to exist as a basis of these clades. The specimen found in this study displays an SNV at locus position 234 in the 5.8S rRNA region, together with four mutations in the ITS1 region and three in the ITS2 region (Fig. 6). Inclusion of 34 sequences from global soil samples (Tedersoo et al., 2014; Supplementary Table S1) revealed the existence of various clades in this region but did not allow to assign the sequence UDB07673020 to a specific phylospecies. UDB07673020 had all seven SNVs in the ITS1 and ITS2 regions in common with most of these soil sequences with respect to the sequences included in Fig. 6 but the SNV at locus gatAaag@234 was unique, preventing UDB07673020 to cluster with other sequences (Supplementary Fig. S1).

Discussion

Several of our findings in the zones surrounding the Bracciano Lake within the Bracciano-Martignano Regional Natural Park in Lazio (Italy), show similarities with findings in Spain (Knijn et al., 2021). The small wood area under investigation in this study is located on the slopes of San Bernardino del Malpasso in Trevignano Romano, between Monte Rocca Romana (Monti Sabatini) and the nearby Bracciano Lake. The Mediterranean vegetation mainly consists in downy oaks (*Q. pubescens*), Turkey oaks (*Q. cerris*) as deciduous plants and holm oaks (*Q. ilex*). The three different fungal species discussed here, were found in this area growing in close proximity to each other, in the same environment with homogeneous ecology.

The volcanic soil of these areas is subacid to neutral, probably not dissimilar to that described for the zone of finding of *A. calida* in Spain (Illescas and Plaza, 2022). In fact, the authors indicate that *A. calida* shows a preference for associating with *Quercus* in acidic or neutral soils. The temperatures in the Bracciano Lake areas are relatively lower than those reported for the spring-summer harvest areas in Spain. We have found *A. calida* in October and December of different years from 2018 on but not in the summer (June-September), when the climate is drier.

Based on the microscopical analyses it is possible to note the presence of numerous lipid droplets in *A. calida* basidia and spores which sometimes completely occupy the internal space. Lipid droplets act as intracellular storage organelles for neutral lipids and sterols, in most types of cells and are principally involved in energy homeostasis and lipid metabolism. In fungi, lipid bodies are also the storage organelle for carotenoid-type pigments which are produced by several species belonging to all major phyla and, in this case, they assume a yellowish colour (Weber et al., 2002). The significance of the presence of lipid bodies in fungi is yet under study. For example, some reports of studies performed on a fungal system like *Schizosaccharomyces pombe* Lindner propose a novel stress adaptation mechanism in which heat-induced triacylglycerol synthesis contributes to membrane rigidisation and reveal that the metabolic crosstalk between membrane and storage lipids facilitates homeostatic maintenance of the membrane physical/chemical state that resists negative effects on cell growth and viability in response to heat stress (Péter et al., 2017). Besides, there is increasing evidence that lipid droplets are involved in cellular detoxification. In fact, trapping of endogenous toxins and absorbing external lipophilic toxins by lipid droplets are resistance mechanisms to reduce the production of reactive oxygen species (Chang et al., 2015).

Lepiota elseae was described from Mediterranean areas in Spain fund in October and associated with *Q. ilex* (Caballero et al., 2015). We report the first finding of *L. elseae* fruitbodies for Italy, apart from the soil sample there are no data about this finding in the Italian territory, perhaps due to the similarities of this species with *L. clypeolaria*. The finding of *L. elseae* on litter of mixed oaks wood only occurred once in October 2020, in five years of monitoring the area. We could suggest that because the area is near inhabitations, rather anthropized and crowded, the area was less visited in 2020 due to the COVID pandemic constraints and remained more unaffected. There are no relevant differences in the morphology between the *A. calida* and *L. elseae* findings of the *Quercus* wood zone of the Bracciano-Martignano Regional Natural Park and the specimens described in the literature.

On the contrary, although it has been reported that the *X. sarnarii* species is quite rare (Simonini et al., 2016), we have evidenced the presence in the same site of several specimens in the autumn of all the years we have visited the mixed *Quercus* thicket. Some Boletales genera contain compounds arising from enzyme-controlled dimerization of p-hydroxyphenillpiruvic acid, derived by oxidation of aromatic amino acids. The derived compounds that turn blue when the fungi are cut or bruised are quinones (Bertrand, 1901; Bertrand, 1902). In 1968 Edwards demonstrated that the bluing is caused by enzymatic oxidation of the more hydroxylated pulvinic acid derivatives: variegatic acid (Beaumont et al., 1968) and the closely related xerocomic acid (Gill et al., 1987). However, many of the species containing variegatic and xerocomic acid do not turn blue, probably due to loss of the oxidase enzymes activities that produces the blue chinone methides (Nelsen, 2010). We have not noticed, in the *X. sarnarii*, the described change to dark blue in the whitish, yellowish context as occurs instead in the tubes, even after a certain time but only a slight change to light blue. This means the specimen could be defined as a morphospecies of *X. sarnarii* as well as a phylospecies which

molecular analysis seem to indicate. However, more extensive research is needed to permit the definition of a new intermediate species between the two sister species *X. sarnarii* and *X. chrysenteron*.

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