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Research article

A new species of *Agaricus* (section *Sanguinolenti*) from Rome, Italy

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Abstract

We report the finding of an *Agaricus* specimen in the section *Sanguinolenti* and describe it as a new species *Agaricus hortusdamarum*. Based on ITS data, the species is distinct and well separated from all known sequenced species within this section. The type specimen grew in late autumn, under *Cupressus sempervirens*, near a *Pinus pinea* alley in the Villa Borghese Gardens in Rome, in Italy. The new species is phylogenetically close to *Agaricus iranicus*, a rare species of temperate climate, which is so far described only in the Guilan province in Iran. Its morphological characters and phylogenetic pattern are described and divergence from *A. iranicus* is discussed.

Keywords

Pseudochitonia, single-nucleotide variants, urban park, Villa Borghese

Introduction

Agaricus (Basidiomycota, Agaricales, Agaricaceae) specimens are well-defined and straightforwardly assigned to its genus but the internal taxonomy of the genus *in toto* has been revolutionised during the last 25 years with the advent of molecular phylogenetic studies (Bunyard et al., 1996; Geml et al., 2004; Kerrigan et al., 2005; Zhao et al., 2011; Chen et al. 2015; Parra et al., 2015; Zhao et al., 2016; Chen et al. 2017). Currently, a revised taxonomic system has been proposed, identifying 6 subgenera and 24 sections in the genus, based on a combination of morphological characters with phylogenetic analyses and estimation of divergence time (Zhao et al., 2016; He et al., 2018; Jaichaliaw et al.; 2021).

Currently, a total of over 500 species are recognised (Wang et al., 2015; Callac and Chen, 2018) and new species of this genus are still found frequently, as for instance *Agaricus iranicus* Mahdizadeh, Safaie, Goltapeh, L.A. Parra & Callac, the epithet meaning “from Iran”, the country where the holotype specimen was collected (Mahdizadeh et al., 2018). This species belongs to the section *Sanguinolenti* within the subgenus *Pseudochitonia* (Zhao et al., 2016).

The section *Sanguinolenti* has been redimensioned since several species have been shifted to other sections according to the new phylogenetic analyses (Zhao et al., 2011; Zhao et al., 2016). It is considered together with sections *Bohusia* and *Nigrobrunnescentes* among those that contain fungal species that grow in a temperate environment (Mahdizadeh et al., 2018). The section now includes just six species: the best-known *A. sylvaticus* Schaeff. and *A. benesii* (Pilát) Pilát together with *A.*

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hupohanae Kerrigan, *A. cordillerensis* Kerrigan, *A. thujae* Kerrigan and the before mentioned *A. iranicus* (Mahdizadeh et al., 2018). *Agaricus iranicus* is therefore inserted among the species that prefer a temperate climate, and its presence has been reported only in the Guilan province, in the South of the Caspian Sea, in Iran (Mahdizadeh et al., 2018; Ghobad-Nejad et al., 2020).

The species found in Rome is described here by morphological characters as a new species belonging to *Agaricus* section *Sanguinolenti* through its phylogenetic pattern inferred from a combined dataset of internal transcribed spacer (ITS) and partial nuclear 28S rDNA (LSU) sequence data. Divergence of this new species from *A. iranicus* is discussed.

Material and Methods

Area of study and sample

A first specimen was found in November 2020 and, more recently, a further five in December 2021 in the area called 'Parco dei Daini' (Fallow Deer Park) of the Villa Borghese Gardens, the most popular urban park in Rome, Italy. The basidiomata grew terricolous on litter of pine and cypress needles and grass, under *Cupressus sempervirens* L. trees, in an alley formed by *Cupressus* and *Pinus pinea* L. and close to a *Cedrus libani* A. Rich. tree, at 50 m a.s.l. (Fig. 1). The collected specimens (Fig. 2) are conserved as part of the Mycological Collections of the Natural History Museum and Botanical Garden of the University of Tartu, Estonia.

Fig. 1 - "Parco dei Daini" (Fallow Deer Park) in the Villa Borghese Gardens: occurrence site of the new *Agaricus* species.

Morphological analyses

Microscopic images were obtained with a digital camera AmScope (Irvine CA, USA) MU 500 mounted on an AmScope B490 microscope, operating at ×800 magnification. Fresh lamella fragments were mounted in distilled water to determine the presence of cheilocystidia. The observation of these elements must be carried out on fresh material because in most *Agaricus* species they are no longer observable after drying (Basso, 2012).

Microscopic images on the dried specimen were obtained using a light microscope Leica DM750 with Leica Application Suite v4.13.0 [Leica Microsystems (Schweiz) AG] at magnification ×1000. Dried lamella fragments were mounted in 3% KOH.

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Molecular analyses

Ribosomal DNA-based analysis was performed on the holotype specimen in the frame of the UNITE project (Kõljalg et al., 2013; https://unite.ut.ee/). DNA extraction, PCR amplification of SSU partial, ITS1, 5.8S, ITS2, LSU partial regions and sequencing were performed as in Voitk et al. (2018). BLAST 2.8.1+ (Camacho et al., 2009) was used to select 17 sequences for phylogenetic analyses. These sequences were aligned using the MUSCLE v3.8.425 algorithm (Edgar, 2004) in Aliview 1.26 (Larsson, 2014) and adjusted manually. Maximum Likelihood and Bayesian inference were applied. 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. 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 5×10^3 generations and a 25% burn-in. The phylogenetic tree was visualised using FigTree v1.4.3. A further alignment was performed on 657 overlapping bases of the 8 sequences of *A. iranicus* clade species only and their Single-Nucleotide Variants were highlighted by the snipit software (O'Toole and Tomkins-Tinch, 2021). The ITS sequence (UDB0799866) is available in the UNITE database.

Fig. 2 - *Agaricus hortusdamarum* a) holotype on litter of conifers needles; b) bulbous stipe and evanishing annulus; c) appendiculate cap; d) holotype ex situ; e) holotype scraped stem slowly coloured reddish brown.

Results

Molecular analyses

Phylogenetic analysis of the 17 most similar *Agaricus* ITS sequences from the GenBank databases (Fig. 3) revealed the genetic similarity of the new specimen with the *A. iranicus* clade. Because the specimen did not fit perfectly within the clade (PP=100%), a Single-Nucleotide Variants (SNV) analysis was performed on the 8 sequences of this clade only to further investigate intraspecies differences (Fig. 4). As could already be seen from the phylogenetic tree, SNV analysis confirmed that the sequences from the specimens collected by V. Mahdizadeh (accessions KY474551- KY474556) in December 2013 in Iran are identical between themselves.

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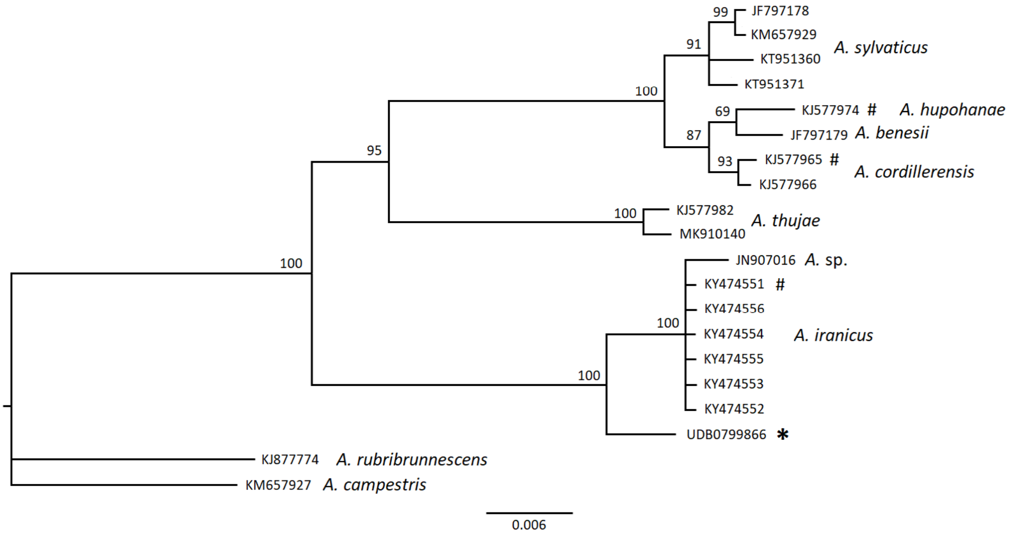
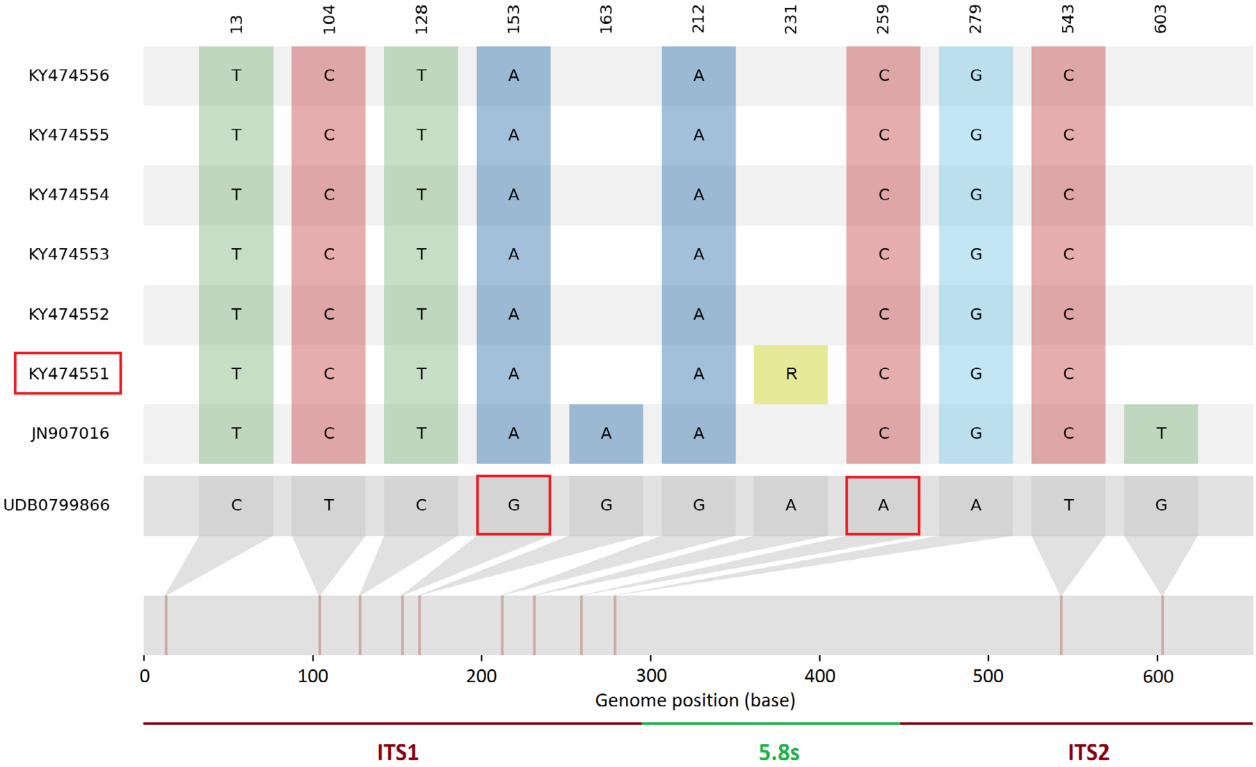


Fig. 3 - Phylogenetic tree generated from Bayesian analysis of ITS sequences of *Agaricus* section *Sanguinolenti*, identified by their UNITE/GenBank accession numbers and species. The sequences KM657927 from *A. campestris* and KJ877774 from *Agaricus rubribrunnescens* Murrill were used as outgroup. The sequence from the new sample described in the article (accession UDB0799866) is indicated by an asterisk and the sequences from the *Agaricus* holotypes by a hash symbol. Bayesian posterior probabilities are reported on the main branches. The final mean standard deviation of the separated frequencies was < 0.007.



	13	104	128	153	163	212	231	259	279	543	603
KY474556	T	C	T	A		A		C	G	C	
KY474555	T	C	T	A		A		C	G	C	
KY474554	T	C	T	A		A		C	G	C	
KY474553	T	C	T	A		A		C	G	C	
KY474552	T	C	T	A		A		C	G	C	
KY474551	T	C	T	A		A	R	C	G	C	
JN907016	T	C	T	A	A	A		C	G	C	T
UDB0799866	C	T	C	G	G	G	A	A	A	T	G

Fig. 4 - Alignment of the overlapping 657 nucleotides of 8 most similar ITS sequences, indicated by their UNITE/GenBank accession. Accession KY474551 is a sequence from the *Agaricus iranicus* holotype specimen VM035. Single-Nucleotide Variants are indicated with respect to the UDB0799866 sequence. The nucleotides at positions 153 and 259 are considered species-specific ITS markers for *A. iranicus*. The barcode regions are annotated at the bottom.

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A single sequence from a specimen found on the Xianshan Mountain in the Beijing region by Xu et al. (accession JN907016) is very similar to the *A. iranicus* holotype sequence with only two nucleotide variations (position 163 and 603). The sequence of the new Italian specimen (accession UDB0799866) on the other hand, has seven nucleotide variants between the 13th and 279th nucleotide positions (ITS1 region) and one at the 543rd nucleotide position (ITS2 region) with respect to the *A. iranicus* holotype.

Taxonomy

Agaricus hortusdamarum A. Ferretti, I. Saar & A. Knijn, *sp. nov.*

Index Fungorum IF559484

Typification – Holotype: Italy, Rome, Parco dei Daini, urban park, under *C. sempervirens*, 41.916111°N, 12.493055°E, 14 Nov 2020, leg. Arnold Knijn, Amalia Ferretti (TUF105944, ITS sequence UDB0799866).

Etymology – hortusdamarum is a compound noun created from the Latin words for park/garden (hortus) of red deer (damarum, genitive plural), a translation of Parco dei Daini, where the holotype was collected.

Macromorphology – Pileus convex then plane and depressed, diameter up to 9 cm, reddish-brown by the squamules that became darker with age, white trama exposed under the scales. The pileus margin slightly exceeded the lamellae, with appendiculate remains of the white universal veil along the entire circumference (Fig. 2c). Lamellae free, crowded, intercalated with lamellule, ventricular, at first pale, with age brown to dark brown. Stipe about 4–7 cm, sometimes shorter than the diameter of the cap, cylindrical, central, fistulose, fibrillose, greyish-white, with an evanescent disappearing greyish-white membranous annulus. The bulbous base covered with white mycelium and soil, some mycelial cords are present (Fig. 2b). Stipe trama whitish, turning slowly reddish brown when handled or cut (Fig. 2e). Odour: persistent marked good fungal smell.

Micromorphology – Basidiospores (3 basidioma, n = 46) smooth, ellipsoid, the major part with a brown colouration, few of them with apiculum, lipid droplets and some granules were evident inside (Fig. 5a). Basidiospores are inamyloid, not cyanophilous, not metachromatic. Their average size was 6.3 x 4.7 µm [(7.7)–6.3–(5.2) x (5.5)–4.7–(3.9) µm], Qm=1.3 Q=[(1,5)–1.3–(1.2)]. Basidia bisporic and trisporic (Fig. 5b), 18–27 x 7–8 µm, with sterigmata up to 3.8 µm long (average 3.4 µm). Cheilocystidia abundant, hyaline, septate, cylindrical and clavate (Fig. 5a,c), single septated elements measuring 24–48 µm x 11–17 µm (holotype) and 31–44 µm x 7–17 µm (December 2021). Such cheilocystidia origin from the lamellar trama, as was described for *A. iranicus*. Velar remnant of the pileus margin composed by cylindrical hyphae 5-11 µm wide (Fig. 5e). Pileipellis composed by cylindrical hyphae up to 15 µm wide and up to 10 µm wide near the septa (Fig. 5d).

Habitat – On needle debris of pine and cypress, in urban park, autumn.

Distribution – So far only known from Italy.

Comments – Most similar to the closely related species *A. iranicus*. *Agaricus hortusdamarum* differs in an evident although slow reddish-brown discoloration when touched, scraped and cut the stem surface and in cap with darker brown squamules. Microscopic evidence of larger cheilocystidia supports the distinction between the two species. Both species grow in temperate environments but *A. iranicus* presence is reported only in Asia and *A. hortusdamarum* is found in southern Europe. ITS barcode: eight single-nucleotide variations occur between both species, two of which identified as species-specific markers for *A. iranicus*.

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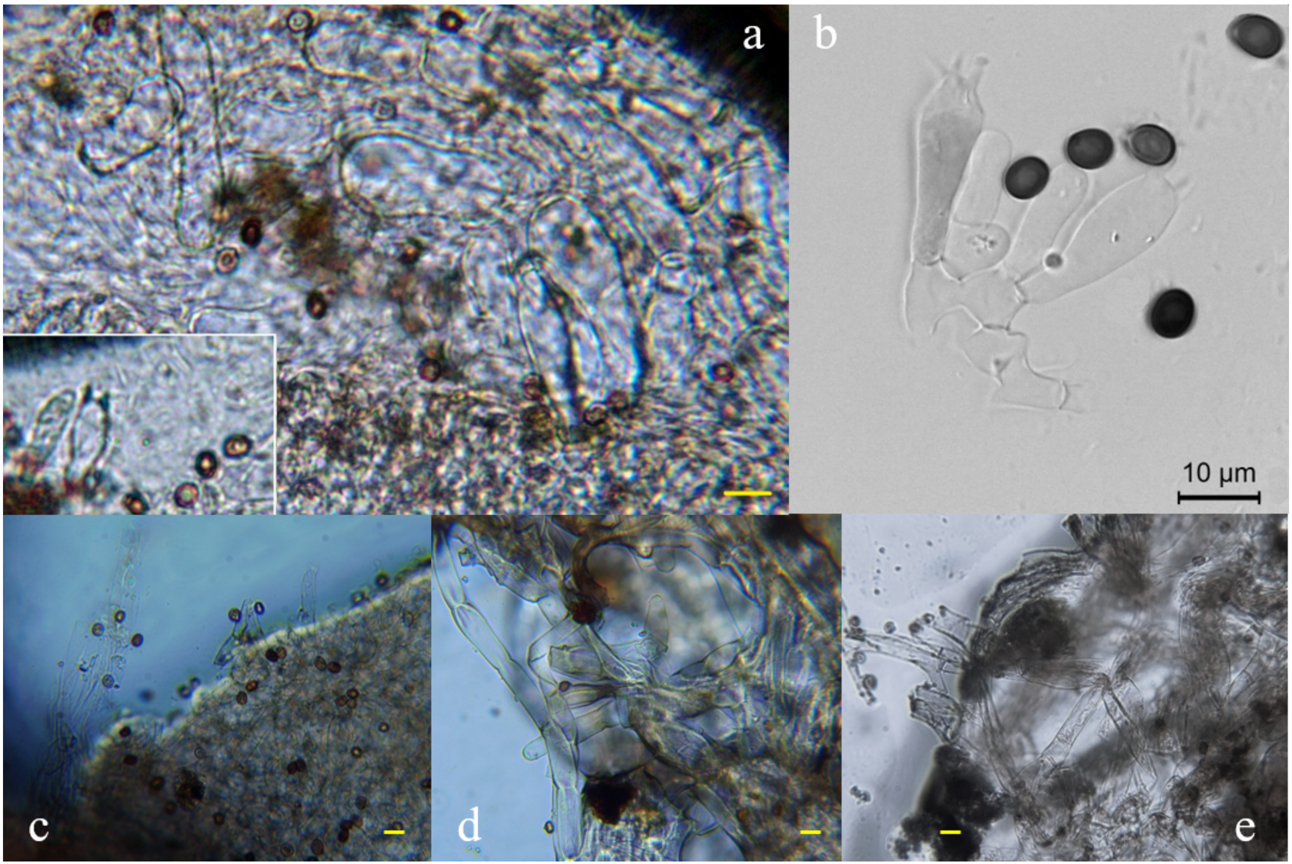

Fig. 5 - Microcharacters of *Agaricus hortusdamarum*. a) fresh lamella fragments showing the large cheilocystidia and the basidia (in the insert) for comparison. b) dried lamella fragments mounted in KOH, basidia and basidiospores. c) fresh lamella fragments; d) cuticula fragments; e) velar fragments; Bar = 10 µm

Discussion

We report the finding of an *Agaricus* fungus growing in autumn and winter in the Villa Borghese Gardens in Rome (Italy), that we describe as a new species belonging to the section *Sanguinolenti*. This *Agaricus* species is well defined from all known sequenced species within the section *Sanguinolenti* and has a phylogenetically definite position. In fact, the new species is phylogenetically very close to but distinct from *A. iranicus*, another species included in section *Sanguinolenti*. *Agaricus* section *Sanguinolenti*, with the other two groups *Bohusia* and *Nigrobrunnescentes* are considered among those containing fungal species growing in a temperate environment, 75% of the species in the first two groups show a preferential coniferous habitat and it was suggested that it might reflect an ancient association (Mahdizadeh, 2018).

The genus *Agaricus* from Iran has been well studied from a taxonomical and phylogenetical point of view (Mahdizadeh et al., 2016; Mahdizadeh et al., 2018; Ghobad-Nejad et al., 2020), and *A. iranicus* is reported to grow in some sites of the Guilan province, in particular under *Cupressus* trees. The new Italian species confirms the preferential association of the species in section *Sanguinolenti* with conifers, in this case with *C. sempervirens* also in the presence of *P. pinea* and *C. libani*.

Fungi of the genus *Agaricus* present a saprophytic behaviour, but some studies (La Chiusa, 2013; AMINT, 2015), report that many species of *Agaricus,* after a first phase of development of the mycelium in which they are saprophytic, tend to establish a relationship with arboreal such as with herbaceous plants.

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The Villa Borghese Gardens in Rome and the 'Parco dei Daini' were created in the 17th century, at the time of the construction of the Casino Nobile. The back of the Villa overlooked a large square, from which one could enter in an area called second enclosure, surrounded by walls that separated it from the surrounding countryside. The zone today corresponds to Parco dei Daini (*Hortus Damarum* in Latin), so called because there were fallow deer and gazelles that were left free in the holm oak grove. Afterwards, the gardens underwent changes until the first years of 1900, but the present *P. pinea* and *Cupressus* trees in the Gardens are centenary. During the second world war, the area was used for the cultivation of horticultural crops ("orti di guerra", so-called war gardens) as a response to the food crisis (Campitelli, 2005). If the hypothesis of a symbiotic-like association between *Agaricus* and coniferous plants is possible, the presence of the new species in this part of the Villa Borghese Gardens would be due to a non-recent interaction.

The macroscopic morphology of the sample from Rome does not show strong differences from *A. iranicus*, the most relevant is the presence of squamules on the cap that seems to be darker brown. Microscopic analyses showed a similar size of basidiospores and basidia within biological variability, however, in this new studied specimen the abundant presence of very large cheilocystidia is evident, with about twice the size of those found in lamellae of *A. iranicus*. This notable difference probably reflects the divergences found in the molecular phylogeny between the aforementioned different species.

Phylogenetic analysis indicates the new specimen's affinity with the *A. iranicus* clade, however, also highlights some significant distinctions. In fact, among the eight single-nucleotide variations with respect to the holotype, two in particular seem significant (nucleotide positions 153 and 259) since they have been identified as part of the eight species-specific ITS markers for *A. iranicus* (Mahdizadeh et al., 2018), corresponding respectively to tgagAactt@153 and gtctCtaca@259 mentioned in the article.

Another trait that could have phylogenetical significance is discoloration. The studied specimen showed a slow but evident reddish-brown discoloration when touched, and a slow reddish-brown discoloration scraping and cutting the stem surface. In the specimens from Iran discoloration was not observed if not a late reddish-brown discoloration at stipe base (Mahdizadeh et al., 2018). In any case, the behaviour is rather different from that of other species in section *Sanguinolenti*, because *A. sylvaticus* Schaeff., *A. benesii* (Pilát) Pilát and *A. thujae* Kerrigan all turn pinkish-red when injured and strongly turn reddish when cut.

It is reported that the browning reactions in some *Agaricus* fungi are due to either mechanical damage or bacterial activity in tissue (Naveen and Beelman, 2006). Enzymatic browning is catalysed by the enzyme tyrosinase (polyphenol oxidase) (Weijn et al., 2013). Tyrosinase naturally occurs at high levels in the surface tissues (Soler-Rivas et al., 2000). It has been hypothesised that tyrosinase and its substrates are located on separate subcellular compartments in intact fungi and when the fungi are bruised the cellular membrane is damaged and rapid browning is observed. The loss of membrane integrity provides greater access of tyrosinase to its phenolic substrates such as γ-L-glutaminyl-4-hydroxybenzene (GHB), allowing for the activated enzyme to oxidise the compounds into the melanin products with associated brown discoloration of *Agaricus* fungi (Boekelheide et al., 1979; Burton, 1986; Jolivet et al., 1995). It has been suggested that tyrosinase and its metabolites such as GHB, which is also the precursor of spore wall melanin, play a role in defence systems and in the resistance response to stress in fungi (Jolivet et al., 1998). Besides, it was reported that the variation of bruising sensitivity observed in different *A. bisporus* strains correlated with the presence of phenolic compounds (GHB) in the fungus' skin (Weijn et al., 2013). The article suggests that the enzymatic conversion of *p*-aminophenol to GHB by the enzyme γ-glutamyltransferase (GGT), present in high levels in gills, pileipellis and stipe base (Jolivet et al., 1998), is the rate-limiting step of the reactions in the different strains and might be caused by genetical mutations in the expression or in altering the activity of the enzyme (Weijn et al., 2013). Therefore, variations in the production of pigments or in the lack of discolouration in genus *Agaricus* could be due to alterations in the mechanisms involved in the regulation of the enzymatic conversions also consequent to genetic mutations.

This study reports further evidence of the presence of particular and/or rare fungi in the urban Parks of Rome, such as the (sub-)tropical *Punctularia atropurpurascens* (Berkeley & Broome) Petch 1916 (Knijn and Ferretti, 2018). The findings and the surprising variety of fungi are probably related with the botanical biodiversity of both autochthonous and allochthonous trees present in the various ancient Parks of the metropolis and confirm the importance to maintain and preserve the public green areas of the city.

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