# *Paxillus involutus* f. *eburneus* f. nov. (*Agaricomycetes*, *Boletales*), a molecularly confirmed infraspecific taxon in the *P. involutus* complex from Italy

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According to a phylogenetic analysis of ITS rDNA sequences, *Paxillus involutus* f. *eburneus* f. nov. is described on the basis of several collections from a birch-oak woodland standing in the sulphureous bog of Caldara di Manziana, an interesting natural habitat near Rome (Latium region, Italy). A detailed description of this remarkable albinotic form is provided, along with a colour picture of fresh basidiomata in situ and a line-drawing of the most relevant anatomical features.

**Key words** – *Paxillaceae* – *Paxillus albidulus* – ITS sequences – taxonomy

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## Introduction

An extensive field investigation of a mixed birch-oak woodland standing in the sulphureous bog of Caldara di Manziana, (Rome, Latium region, Italy), led to the recent discovery of several interesting fungal species. In the present contribution, based on morphological and molecular (ITS) analyses, the authors describe *Paxillus involutus* f. *eburneus* f. nov., an albinotic form of *P. involutus* (Batsch) Fr. s. stricto occurring in such unique and hitherto understudied ecological niche. The new taxon, differing from typical *P. involutus* on account of the overall ivory-beige colour of basidiomata as well as for other minor differences, seems to be restricted to this particular hygrophilous habitat, as it has not been observed elsewhere in Italy.

## Methods

Morphology – The macroscopic description was based upon detailed field notes of fresh basidiomata. Micro-morphologic features were observed on dried material; free-hand made sections of this latter were revived in 5% potassium hydroxide (KOH) or in Congo red in an ammonia solution. The observation of the hymenial elements and stipitipellis structure was performed in ammoniacal Congo red, as well as the measurement of the spores and the pileipellis terminal elements, while the colour and the intensity of pigmentation was described after examination in 5% KOH. Metachromatic and amyloid reactions were also tested colouring the spores in Brilliant Cresyl blue and Melzer's reagent, respectively. Spores and pileipellis terminal elements dimensions are given as (minimum) average ± standard deviation (maximum), 1/w= average quotient (length/width ratio) ± standard deviation, while average spore volume was approximately estimated as a rotation ellipsoid {v = 4/3 × (length/2) × [(width/2) ×width] × π /2 ± standard deviation}. The notation [249,7,7] indicates that measurements were made on 249 spores in seven samples from seven collec-

tions. The type collection of the new form has been deposited in TO (Herbarium generale del Dipartimento di Biologia Vegetale, Università degli Studi di Torino, Italy). Herbarium acronyms follow Thiers (2011) except "MG" that refers to the personal herbarium of Matteo Gelardi.

**DNA extraction, PCR amplification, and DNA sequencing**

Genomic DNA was isolated from 1 mg of a herbarium specimen by using the DNeasy Plant Mini Kit (Qiagen, Milan Italy) following the manufacturer's instructions. Universal primers ITS1f/ITS4 were used for the ITS region amplification (White et al. 1990, Gardes & Bruns 1993). Amplification reactions were performed in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 µl reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1× PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 unit of *Taq* polymerase (Promega). The PCR program was as follows: 3 min at 95°C for 1 cycle; 30 s at 94°C, 45 s at 50°C, 2 min at 72°C for 35 cycles, 10 min at 72°C for 1 cycle. PCR products were resolved on a 1.0% agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced by DiNAMYCODE srl (Turin, Italy). Sequence assembly and editing were performed using Geneious v5.3 (Drummond et al. 2010).

**Sequence alignment and phylogenetic analysis**

Sequences included in the phylogenetic analysis were either generated in this study (*P. involutus* f. *eburneus*) or retrieved from GenBank according to recent studies on *Paxillus* (Hedh et al. 2008). Alignment of the ITS dataset was generated using MAFFT v6.814b (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties. The alignment was slightly edited using MEGA 5.0 (Tamura et al. 2011). The phylogenetic analysis was performed through the Maximum Likelihood (ML) approach with RAxML (Stamatakis 2006) under GTRGAMMA model, and using thorough bootstrap with 20 runs and 1000 replicates. *Paxillus rubicundulus* (Genbank accession EU084667) was chosen as outgroup. Bootstrap values (MLB) over 50% are reported in the resulting tree (Fig. 1). The ITS sequence of *Paxillus involutus* f. *eburneus* was submitted to GenBank under the accession number JN673368.

**Results**

***Phylogenetic analysis***

Maximum likelihood inference was performed on a total of 23 samples, including 22 sequences available from GenBank. Final alignment length was 682 bp. The four phylogenetic European taxa that were recognized by Hedh et al (2008) in the *P. involutus* complex are also recovered in our analysis, viz. *P. involutus* s. stricto (= *P. involutus* clade I), *P.* sp. (= *P. involutus* clade II), *P. obscurosporus* C. Hahn, and *P. validus* C. Hahn (Fig. 1). According to Hedh et al (2008), the sequences within the *P. involutus* clade II most likely represent a not yet described species which is closely related to *P. involutus* s. stricto.

In our analysis the newly generated sequence clusters within the *Paxillus involutus* I clade with high ML bootstrap percentage (99%). Taking into account the robustness of the clade, the nine sequences can be considered conspecific.

***Paxillus involutus*** f. ***eburneus*** Gelardi, Segneri & Vizzini, **f. nov.** Figs 2–3
MycoBank MB 563275.

Etymology – the epithet derived from the Latin adjective *eburnĕus*, *eburnĕa*, *eburnĕum* (ivory) and refers to the overall ivory colour of the basidiomata.

A typo differt colore pilei, stipitis et mycelii basali albido vel eburneo.

Habitat – solitarius vel gregarius ad terram in nemoribus frondosis (*Betula pendula*, *Quercus cerris*), in humo sulphurata acĭdaque.

Holotypus – Italia, Latium, in loco Caldara di Manziana dicto, prope Romam, 27/11/2010 lectus, legit. G. Segneri, in Herbario TO BP05 conservatus est. (Fig. 4)

Basidiomata – large, fleshy, putrescent.

Pileus – 5.7–10.4 (13.1) cm broad, convex at first but soon applanate, in mature specimens with a moderately depressed morphology toward the discal zone, becoming

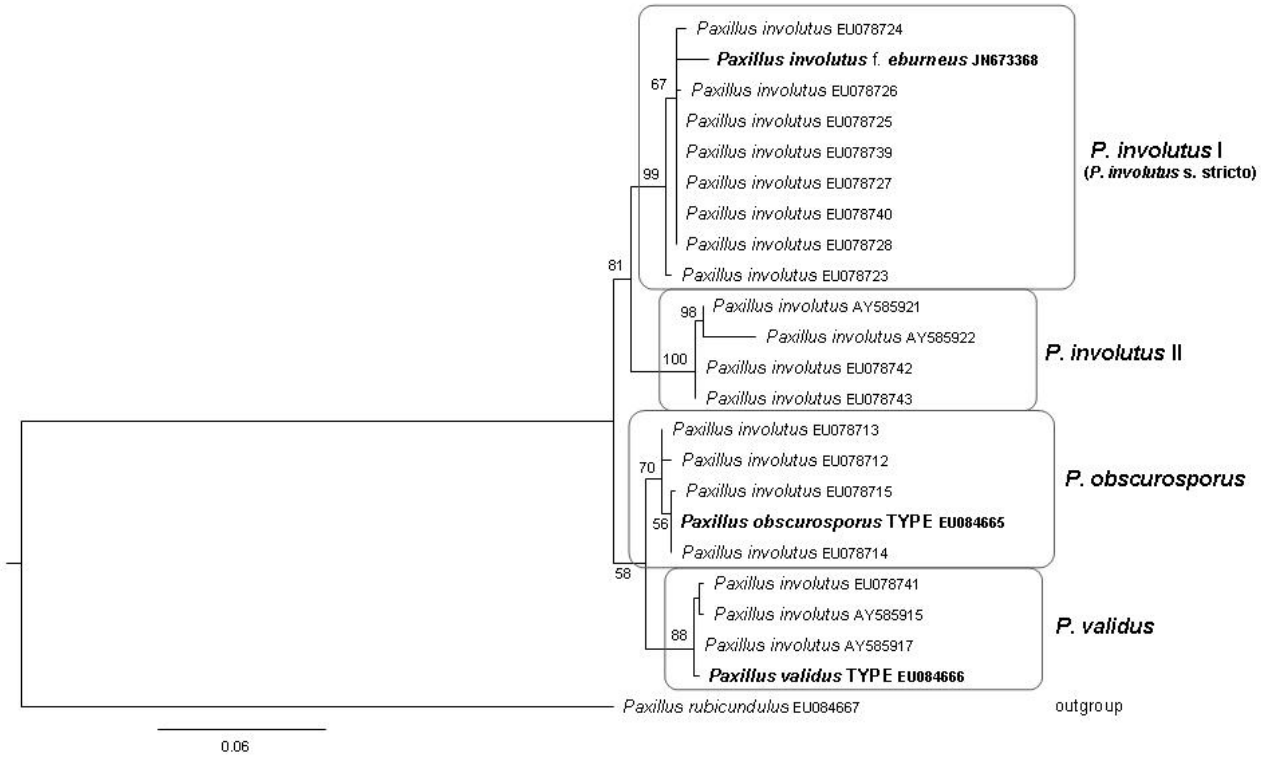


**Fig 1** – Maximum likelihood tree of the *Paxillus* sequences; MLB values are indicated above branches. The bar indicates number of substitutions per site.

progressively deeper in old specimens, sometimes with a central, shallow and broad umbo, tending to disappear at maturity, fleshy, firm at the beginning then more tender and finally soft; margin involute and folded on itself, remaining, at least in part, broadly enrolled even into maturity, faintly ribbed at the rim but only in young basidiomata, then becoming completely smooth or almost so, constantly lobed and rippled, not or only slightly exceeding beyond the lamellae; surface matt, dry but sticky to decidedly slimy with humid weather, finely velvety at first but quickly glabrescent starting from the centre (albeit long lasting retaining a tomentose texture under the enrolled margin), not or hardly cracked at margin (as in collection MG178a); cuticle entirely separable, evenly ivory-whitish, ivory-beige to pale beige-ochre, becoming ochraceous to very pale brownish only with very drought weather and limited to old specimens, but even in this case remaining bleached at the extreme margin, in depressions or wherever it is hidden by dry leaves or grass, always without olive shades; rapidly and strongly changing to rusty-brown on handling or when injured, especially in young specimens; subcuticular layer beige.

Hymenophore – lamellate, with lamellae deeply arcuate-decurrent and easily detachable from the pileus context above, crowded, shorter than pileus context thickness, somewhat broad, intermixed by several lamellulae, having an even to slightly undulate edge, moderately to strongly furcated or interveined especially towards the stipe; pale beige-fleshy to ochreous-brown starting from the stipe and finally fading to dark brown owing to the ripening of the spores but always paler at margin, changing to dark rusty-brown on bruising or cutting.

Stipe – (2.1) 2.7–5.4 (7.9) × 1.0–2.4 (2.9) cm, as long as the pileus diameter in young specimens but always shorter than pileus broadness at maturity, usually squat, central to slightly off-centre, solid, firm, dry, straight or faintly curved, particularly downwards, cylindrical or progressively tapering from the apex to the base, not rooting; surface smooth, very finely pruinose in the upper part, glabrous

**Fig 2** – *P. involutus* f. *eburneus*. Young to mature basidiomata in habitat (collection MG307a). – Bar = 2 cm. This picture is copyright of Matteo Gelardi.

elsewhere; initially ivory-beige throughout, soon with fleshy-brown shades at the base, discolouring to rusty-brown on pressure; basal mycelium and rhizomorphs ivory, more rarely ivory-rose.

Context – firm, later soft textured in the pileus, more fibrous in the stipe, beige to cream in the pileus and upper half of the stipe, gradually brownish downwards, quickly turning deep flesh pink on exposure, mainly in young specimens, finally fading to drab ochreous-rose; exsiccate ochreous-brown to pale fleshy-brown coloured. Smell somewhat strong and pleasant, fruity. Taste mild to hardly bitter.

Spore print – dark brown.

Macrochemical reactions – (tested upon all collections):

- 25 % ammonium hydroxide: reddish-brown on cuticle and stipe surface (but pink flesh on viscid specimens in case of wet weather), brown on lamellae, none to pale pinkish on context.

- 20 and 30% KOH: brownish-pink (20%) to dark purplish-red wine (30 %) everywhere.

- $FeSO_4$ (iron sulphate): olivaceous throughout then sordid brown on cuticle and olive-brown on stipe.

- Phenol (tested only upon collection 161SG10): citrine yellow on context.

- Aniline (tested only upon collection 161SG10): orange on cuticle and context.

- Sulphobenzaldehyde (tested only upon collection 161SG10): returning to the original colour on context after oxidation phenomenon.

- Guaiac: none.

- Tl4: none.

- Melzer's reagent: inamyloid (macroscopically reddish-black on stipe, lamellae and context, none on pileus).

Spores – [249,7,7] (6.9) 8.7 ± 0.90 (13.0) × (4.4) 5.4 ± 0.34 (6.1) µm, 1/w= 1.60 ± 0.16, v= 135 ± 26 µm³ (Tab. 1) (n= 249, mostly taken from the spore print, the remaining from the hymenophore of mature specimens), broadly elliptical to subovoid in side view, ovoid to amygdaliform in face view, smooth, with a short apiculus and without supra-apicular depression, moderately thick-walled (0.5–0.7 µm), straw-yellow coloured in water and 5% KOH, almost exclusively with one central oil drop, rarely with two or three guttules when mature, inamyloid and showing a clear metachromatic reaction (Fig. 3A) (Table 1).

Basidia – (32.0) 35.0-53.9 (64.0) × (7.0) 7.5–14.9 (15.7) × 3.0–8.5 µm (n= 46), cylindrical-clavate, predominantly 4-spored but also 2-spored, having rather prominent sterigmata, hyaline to pale straw-yellow in water, usually with bright yellow oil guttules and often showing basal clamps (Fig. 3B).

Cheilocystidia – (38.1) 48.5–96.1 (105.5) × (8.0) 8.2–19.7 (20.3) µm (n= 43), uncommon, decidedly slender, straight but sometimes

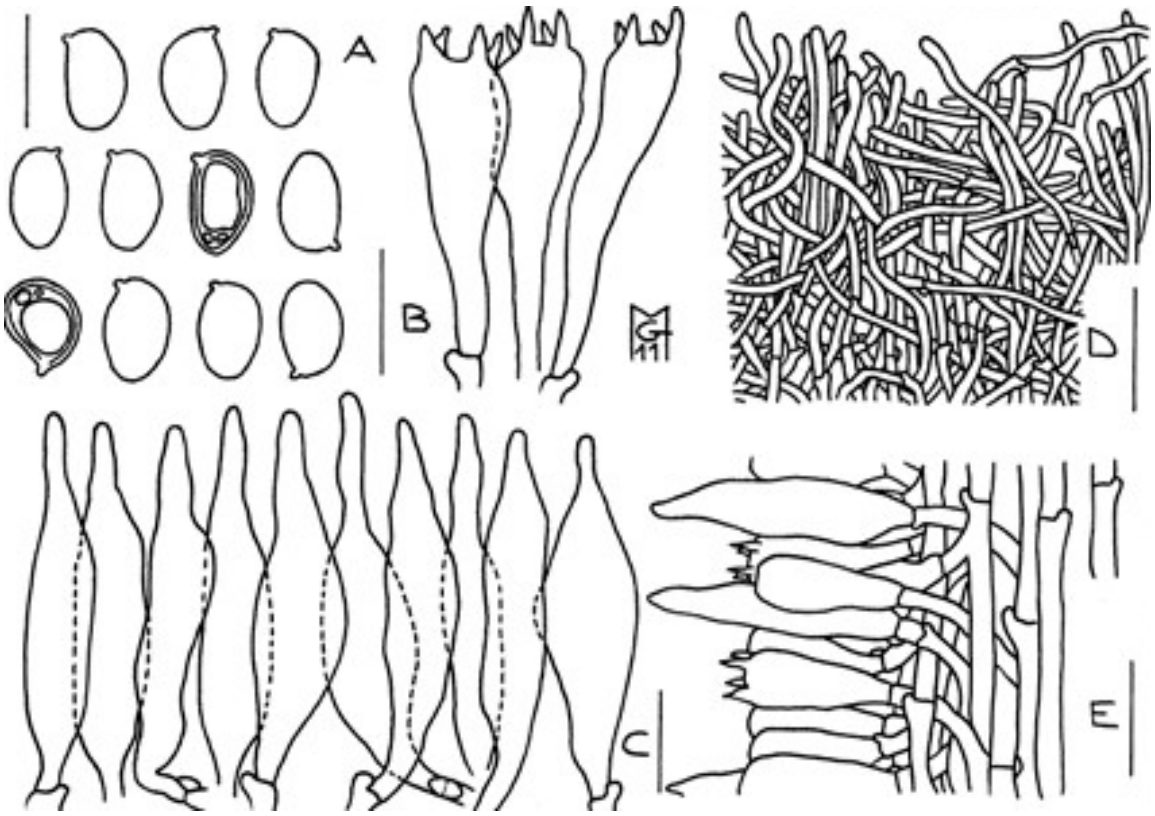


**Fig 3** – *Paxillus involutus* f. *eburneus*, microscopic features (from holotype) A Spores; B Basidia; C Cheilo- and pleurocystidia; D Pileipellis; E Stipitipellis at stipe apex. – Bars: A= 10 μm; B= 20 μm; C= 20 μm; D= 40 μm; E= 30 μm. Drawn by M. Gelardi

flexuous, cylindrical-fusiform or ventricose-fusiform to rarely lanceolate, with a relatively long, tapering neck and rounded apex, smooth, hyaline to yellow-ochre and showing crystal encrustation at the tip in water (such encrustations completely melt and disappear in ammoniacal or alkaline solutions) (Fig. 3C).

Pleurocystidia – similar in shape, colour and size but less frequent than cheilocystidia (Fig. 3C).

Pileal surface – (pileipellis) a trichoderm becoming an ixotrichoderm in case of humid weather, consisting of erected and strongly interwoven, filamentous, sinuous, rarely branched, thin-walled, septate and clamped hyphae, tending over time to repent and thus turning into a cutis partially embedded in a gelatinous matter; terminal elements generally long and slender, cylindrical, with rounded apex, (2.5) 3–7.5 (9) μm broad and up to 166 μm long, hyaline to pale yellow in water and 5% KOH; subterminal elements similar in shape, size and colour with terminal ones; terminal and subterminal elements smooth to very slightly encrusted by a granular, extremely pale pigment (Fig. 3D).

Stipital surface – (stipitipellis) a texture of slender, parallel arranged, adpressed hyphae, (2.5) 4–10 (13) μm broad, yellowish in water, sometimes encrusted by a granular pigment. The stipe apex is covered by a well developed caulohymenial layer, with caulobasidioles, scattered caulocystidia and fertile caulobasidia (these latter extremely rare in collction MG 178a), similar in shape, colour and size with the hymenial ones (Fig. 3E); elsewhere the stipe surface exhibits sterile cystidioid elements. Lateral stipe stratum under caulohymenium absent. Stipe trama made up by longitudinal and densely arranged, subparallel to loosely intermingled, smooth hyphae, 10–22 μm broad, inamyloid.

Hymenophoral trama – subparallel to bilateral-divergent, with slightly divergent and densely arranged, not gelatinous hyphae (lateral strata hyphae in transversal section touching each other). Mediostratum consisting of a tightly adpressed, not gelatinous hyphae,

**Table 1** Spore size of examined *Paxillus involutus* f. *eburneus* collections.

| Collection | Length (µm) | Width (µm) | Average volume (µm³) | Average quotient (length/width) | n |
|---|---|---|---|---|---|
| MG178a | (7.8) **8.7** ± 0.62 (10.5) | (4.9) **5.4** ± 0.33 (6.1) | **137** ± 23 | **1.59** ± 0.11 | 34 |
| MG302a | (7.0) **8.9** ± 1.13 (12.4) | (4.4) **5.1** ± 0.32 (5.9) | **126** ± 30 | **1.73** ± 0.14 | 34 |
| MG303a | (6.9) **8.5** ± 0.86 (10.8) | (4.8) **5.3** ± 0.37 (6.0) | **128** ± 27 | **1.61** ± 0.13 | 34 |
| MG307a | (7.6) **8.5** ± 0.78 (11.7) | (5.1) **5.5** ± 0.29 (6.1) | **138** ± 25 | **1.54** ± 0.11 | 34 |
| MG398a | (7.0) **8.6** ± 0.78 (10.8) | (5.0) **5.3** ± 0.26 (5.9) | **130** ± 23 | **1.60** ± 0.10 | 34 |
| MG399a | (7.2) **9.1** ± 1.25 (13.0) | (4.8) **5.4** ± 0.32 (6.0) | **142** ± 25 | **1.66** ± 0.26 | 34 |
| TO BP05 (holotype) | (7.3) **8.6** ± 0.71 (10.5) | (5.0) **5.6** ± 0.29 (6.1) | **145** ± 23 | **1.53** ± 0.12 | 45 |
| TOTAL | (6.9) **8.7** ± 0.90 (13.0) | (4.4) **5.4** ± 0.34 (6.1) | **135** ± 26 | **1.60** ± 0.16 | 249 |

**Fig 4** – The unusual habitat of Caldara di Manziana (Latium region, Rome, Italy). This picture is copyright of Matteo Gelardi.

8–16 µm broad; in Congo red the mediostratum is substantially concolorous with lateral strata.

Rhizomorphs – consisting of strongly interwoven, smooth and clamped hyphae, 2–8 µm diam., hyaline in water and 5% KOH.

Clamp connections – present and abundant in all tissues.

Hyphal system – monomitic.

Habitat – gregarious or solitary on very humid and acid soil, amongst dry leaves or on rotting birch-wood debris, in mixed deciduous woodland with predominance of *Betula pendula* Roth and presence of *Quercus cerris* L., standing close to sulphureous fountainheads and a large bog. Specimens usually appearing over a long period, from late spring or early summer to late autumn or even during the winter, albeit only in relatively mild weather conditions. Other species found in the same habitat (not necessarily in the same period of growth): *Amanita fulva* Pers., *A. muscaria* (L.) Lam., *A. junquillea* Quél., *A. rubescens* Pers., *A. pantherina* (DC.) Krombh., *Boletus edulis* Bull., *B. edulis* f. *albus* (Pers.) J.A. Muñoz, *B. aestivalis* (Paulet) Fr., *Fomes fomentarius* (L.) Fr., *Gymnopilus junonius* (Fr.) P.D. Orton, *Gymnopus erythropus* (Pers.) Antonín, Halling & Noordel., *Hypholoma sublateritium* (Fr.) Quél., *Lactarius helvus* (Fr.) Fr., *Leccinum scabrum* (Bull.) Gray, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Russula heterophylla* (Fr.) Fr., *R. medullata* Romagn., *Schizophyllum commune* Fr., *Scleroderma citrinum* Pers., *Xerocomus badius* (Fr.) E.-J. Gilbert, etc.

Known distribution – up to now known only from the type locality (Caldara di Manziana, Rome, Italy).

Material examined – Italy, Latium region, Caldara di Manziana (Rome), 320 m a.s.l., 8 December 2008, legit. M. Gelardi, exsicc. number MG178a; loc. cit., 2 May 2010, legit. M. Gelardi & G. Segneri, exsicc. number MG302a; loc. cit., 2 May 2010, legit. G. Fanelli, exsicc. number MG303a; loc. cit., 22 May 2010, legit. M. Gelardi, exsicc. number MG307a (Fig. 2); loc. cit., 25 November 2010, legit. M. Gelardi, exsicc. number MG398a; loc. cit., 25 November 2010, legit. M. Gelardi, exsicc. number MG399a; loc. cit., 27 November 2010, legit. G. Segneri, exsicc. number TO BP05 (holotype).

**Discussion**

Our molecular analysis clearly showed that the newly sequenced *Paxillus* collection is conspecific to *P. involutus*. However, its macro- and micromorphological features are sufficiently different from those of *P. involutus* to warrant the erection of the new form *eburneus* (Fig. 1).

This outstanding albinotic form is easy to recognize in the field due to the striking ivory colour of the entire basidiome at least till maturity, when it may sometimes reach, especially with very drought weather conditions, a pale ochreous-brownish tint, but in any case always without olivaceous shades. The absence of the dark brownish-olive pigmentation of the typical *P. involutus* might have been determined by the occurrence in an unusual environment, characterized by the growth on humid and acid soil with the presence of bogs and sulphureous fountainheads in the neighborhood.

Difference in colour and habitat apart, the other discriminating morphological characters between this new form and *P. involutus* s. stricto can be summarized as follows:

- pileal margin faintly ribbed at the rim but only in young basidiomata, then becoming completely smooth or almost so;

- lamellae more markedly furcated and anastomosing towards the stipe connection zone;

- basal mycelium ivory coloured;

- spores straw-yellow and showing no shallow depression on abaxial surface opposite to the side of the apiculus (this latter observed, by contrast, in typical *P. involutus*, Hahn & Agerer 1999 and M. Gelardi, personal observations);

- cystidia slightly larger;

- pileipellis terminal elements shorter and somewhat narrower in diameter, hyaline to pale yellow in water and 5% KOH; hymenophoral trama subparallel to bilateral-divergent, with slightly divergent and densely arranged, not gelatinous hyphae and mediostratum concolorous with lateral strata (neatly bilateral-divergent, with loosely arranged, gelatinous hyphae and mediostratum darker than lateral strata in typical *P. involutus*).

With the exception of the above-mentioned minor differences, the remaining macro- and micro-morphological characters of the new taxon perfectly fit those of the typical *P. involutus* (Szczepka 1987, Šutara 1992, Hahn & Agerer 1999, Hahn 2000, Henrici 2004, Watling & Hills 2005, Knudsen & Vesterholt 2008) and the first author's personal observations.

The question now remains as to whether the taxon illustrated in the present contribution is identical to another related species earlier described in the former Czechoslovakia; in fact, one would be tempted to equate this albinotic form with the chromatically close *P. albidulus*, a critical and not yet confirmed species described by Šutara (1991). Effectively, macro- and micro-morphological phenotypic features of our collections are quite similar to those of Šutara's species. However this species has been reported only from Czech Republic and found just twice, in 1981 and 1984 and not reported again (Šutara in litt.). In addition it has not been possible to amplify any ITS sequences from herbarium material (Hedh et al. 2008). For this reasons and since it is at the moment impossible to establish any trustworthy relationships between the Italian and the Czech collections, we disregard the use of the name "*albidulus*" at any taxonomical rank for the collections reported in the present paper, preferring to assign them to a newly described name defined at the rank of form.

*P. involutus* f. *subrubicundulus* Bon seems to be a sort of semi-xanthoid form of *P.*

*involutus*, differing from *P. involutus* f. *eburneus* in having a throughout yellowish colouration (Bon & Van Haluwyn 1981).

The extensive occurrence of *Paxillus* species across Europe clearly requires a better understanding, however, the discovery of this new chromatic form clearly indicates that intensive field work can uncover previously unknown taxa from particular and poorly explored habitats.

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