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# ***Lepiota sanguineofracta* (Basidiomycota, Agaricales), a new species with a hymeniform pileus covering from Italy**

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## **Abstract**

*Lepiota sanguineofracta* sp. nov., a taxon with a hymeniform pileus covering, from Italy, is here described. A full description, colour pictures of basidiomata, line drawings of microscopic features, and ITS phylogenetic analysis are provided. It is morphologically and molecularly close (sister) to *Lepiota coloratipes* from which it differs mainly by olivaceous tinges on the pileus surface, basidiome surfaces and context strongly reddening on handling, a sweetish smell of withered rose and binucleate, not metachromatic spores.

## **Keywords**

*Agaricomycetes Agaricaceae Lepiota* Hymeniform pileus covering ITS sequences Taxonomy

## **Introduction**

Species of *Lepiota* (Pers.) Gray with a hymeniform pileus covering correspond to the morphology-based section *Cristatae* (Kühner ex Wasser) Bon as delimited by Kühner ([1936](#)) and Singer ([1986](#)) or section *Lilaceae sensu* Vellinga and Huijser ([1999](#)) and Vellinga ([2001](#)). Bon ([1993](#)) distributed these species over sections *Cristatae* (subg. *Lepiotula* (Mre.) Locquin ex Horak), *Integrellae* (Kühner ex Bon) Bon and *Lilaceae* Bon (subg. *Paralepiotula* M. Bon) as a whole, mainly based on different spore shapes (either ellipsoid or spurred) and spore nuclear number (mononucleate vs binucleate). According to recent molecular analyses, the species with a hymeniform pileus covering do not form a monophyletic lineage (Vellinga [2001](#), [2003](#), [2010a](#); Vizzini et al. [2013](#)), even though most of them (with different spore shapes and nuclear number) fall in a clade (named clade 3 in Vellinga [2003](#)) which includes also taxa (*L. cystophoroides* Joss., *L. luteophylla* Sundb. and *L. scaberula* Vellinga) with a hymeniform pileus covering giving rise to loose globose elements (a transition between hymeniderm and epithelium, see Vellinga [1998](#)).

During a survey of macrofungi in the Botanical Garden of Turin (Piedmont, western Italy), collections of a remarkable *Lepiota* with a hymeniform pileus covering were recorded in flowerbeds.

A thorough literature search including monographic treatments and papers on world lepiotoid fungi (e.g., Morgan [1906](#); Murrill [1914](#); Kauffman [1924](#); Beeli [1932](#), [1936](#); Dennis [1952](#), [1970](#); Smith [1954](#), [1966](#); Aberdeen [1962](#); Pegler [1968](#), [1972](#), [1975](#), [1977](#), [1983](#), [1986](#), [1987a](#), [b](#), [1990](#); Pegler and Rayner [1969](#); Wasser [1980](#); Natarajan and Manjula [1982](#); Enderle and Krieglsteiner [1989](#); Sundberg [1989](#); Candusso and Lanzoni [1990](#); Guzmán and Guzmán-Dávalos [1992](#); Bizio et al. [1993](#); Bon [1993](#); Akers [1997](#); Vellinga and Huijser [1999](#); Vellinga [2001](#), [2010a](#); Montoya and Bandala [2005](#); Wang and Yang [2005](#); Zelený [2006](#); Kosakyan et al. [2008](#); Kumar and Manimohan [2009](#); Albuquerque et al. [2010](#); Gierczyk et al. [2011](#); Liang and Yang [2011](#); Ferreira and Cortez [2012](#); Razaq et al. [2012](#); Kaur et al. [2013](#); and Nawaz et al. [2013](#)), highlighted the unique nature of this taxon: its features do not fit with the description of any published species. The aim of the present contribution is to describe this *Lepiota* as a new species, providing full morphological and molecular data (ITS sequences analysis) and a comparison with allied taxa.

## Materials and methods

### Morphology

Macroscopic description was based from detailed field notes of fresh basidiomes. Colour terms in capital letters (e.g., Mars Orange, Plate II) are those of Ridgway ([1912](#)). Micromorphologic features were observed on dried material; sections were rehydrated in 5 % KOH, and then mounted in ammoniacal Congo Red, Cotton Blue, Cresyl Blue, Methyl Blue and Melzer's reagent, separately. Measurements were made at  $\times 1000$  magnification with a calibrated ocular micrometer (Wild M11 optical light microscope). The width of each basidium was measured at the widest part, and the length was measured from the apex (sterigmata excluded) to the basal septum. The DNA fluorescent dye 4',6-diamidino-2-phenyl-indoldihydrochloride (DAPI) was used to stain nuclei in spores following Horton ([2006](#)). The number of nuclei in spores were then determined using a Leica TCS-SP2 confocal microscope. Samples were excited with 405 nm light and fluorescence was recorded at 440–500 nm.

The following abbreviations are used: L = number of lamellae reaching the stipe, l = number of lamellulae between each pair of lamellae; the notation [X, Y, Z] indicates that measurements were made on X spores, in Y samples from Z collections; Q = the spore quotient (length/width ratio); Q<sub>av</sub> = the average spore quotient. Terminology for descriptive terms is according to Vellinga ([2001](#)). Herbarium abbreviations follow Thiers ([2013](#), continuously updated). The holotype collection is kept in TO. Author citations follow the Index Fungorum –Authors of Fungal Names (<http://www.indexfungorum.org/authorsoffungalnames.htm>).

### DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was isolated from 25 mg of two herbarium specimens (TO-HG2916 and TO-HG2917) using the DNeasy Plant Mini Kit (Qiagen, Milan Italy). Universal primers ITS1F/ITS4 were used for the ITS region amplification (White et al. [1990](#); Gardes and Bruns [1993](#)). Amplification reactions were performed in a PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25  $\mu$ l reaction mixture using the following final concentrations or total amounts: 10 ng DNA, 1  $\times$  PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 0.4  $\mu$ M of each primer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 0.5 units of Taq polymerase (Promega). The PCR

program was as follows: 3 min at 95 °C for one cycle; 30 s at 94 °C, 45 s at 50 °C, 2 min at 72 °C for 35 cycles, and 10 min at 72 °C for one 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](#)). The sequences are deposited in GenBank under the accession numbers given in Fig. [1](#).

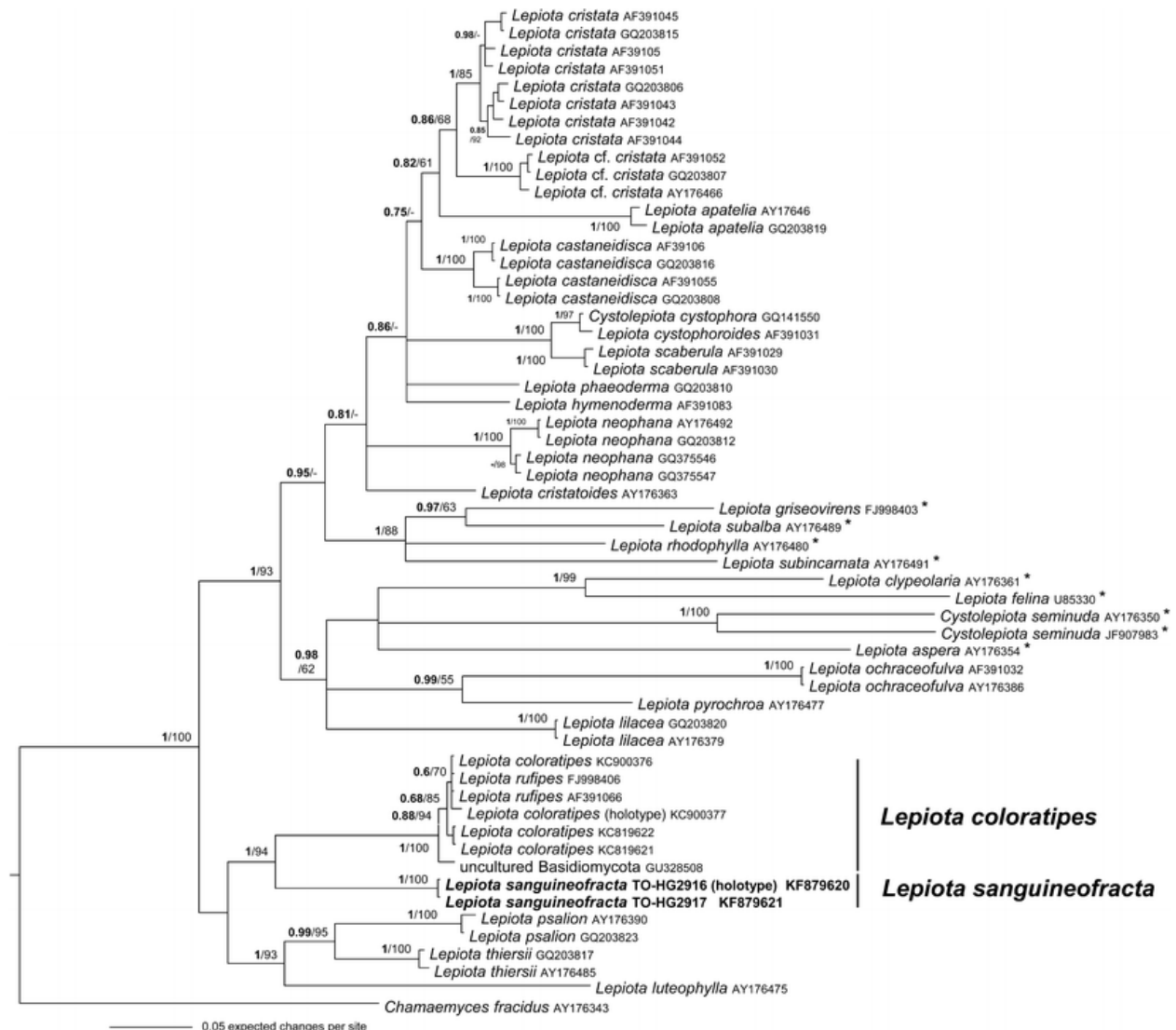


Fig. 1

Bayesian phylogram obtained from the general nrITS sequence alignment of *Lepiota* spp. Here there are included *Lepiota* species with a hymeniform pileus covering, eight species representative of the major clades in *Lepiota* (indicated by \*), and *Chamaemyces fracidus* as an outgroup taxon. Support values in either the Bayesian (Posterior Probabilities values [BPP]) or Maximum likelihood (ML Bootstrap percentage [MLB]) analyses are indicated. Only BPP values over 0.70 (in bold) and MLB values over 50 % are given above clade branches. Newly sequenced collections are in bold

## Sequence alignment and phylogenetic analysis

The sequences obtained in this study were compared to those available in the GenBank database (<http://www.ncbi.nlm.nih.gov/>) by using the BLASTn algorithm. Based on the BLASTn results (sequences were selected based on the greatest similarity) and outcomes of recent phylogenetic

studies focused on *Lepiota* with a hymeniform pileus covering (Vellinga [2003](#), [2010a](#); Vizzini et al. [2013](#)) sequences were retrieved from GenBank for the comparative phylogenetic analysis. Besides *Lepiota* species with a hymeniform pileus covering, eight species (indicated by an asterisk in Fig. [1](#)) representative of the major clades in *Lepiota* as delimited by Vellinga ([2003](#)) were chosen for comparison.

Alignments were generated using MAFFT (Katoh et al. [2002](#)) with default conditions for gap openings and gap extension penalties. The sequence alignments were then imported into MEGA 5.10 (Tamura et al. [2011](#)) for manual adjustment. *Chamaemyces fracidus* (Fr.) Donk (AY176343) was used as an outgroup taxon because it is basal in the *Agaricaceae* (Vellinga [2004](#), [2010a](#)). Best-fit models were estimated by both the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) with jModelTest 0.1.1 (Posada [2008](#)) to provide a substitution model for the alignment. Phylogenetic analyses were performed using the Bayesian Inference (BI) and Maximum likelihood (ML) approaches. The BI was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist [2001](#)) with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run over 10 million generations, under the GTR +  $\Gamma$  evolutionary model. Trees were sampled every 1,000 generations resulting in an overall sampling of 10,001 trees; the first 2,500 trees were discarded as “burn-in” (25 %). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP).

ML estimation was performed through RAxML v.7.3.2 (Stamatakis [2006](#)) with 1,000 bootstrap replicates (Felsenstein [1985](#)) using the GTRGAMMA algorithm to perform a tree inference and search for a good topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the “-f a” option of RAxML and “-x 12345” as a random seed to invoke the novel rapid bootstrapping algorithm.

BI and ML analyses were run on the CIPRES Science Gateway web server (Miller et al. [2010](#)). Only BPP values over 0.70 and MLB over 50 % are reported in the resulting tree (Fig. [1](#)). Branch lengths were estimated as mean values over the sampled trees.

Pairwise % identity values of ITS sequences were calculated using MEGA 5.10 (Tamura et al. [2011](#)).

## Results

### Molecular analysis

Both Bayesian and Maximum likelihood analyses produced the same topology; therefore, only the Bayesian tree with both BPP and MLB values is shown (Fig. [1](#)). The ITS data matrix comprised a total of 57 sequences (including 55 from GenBank). The alignment comprised 803 characters, and contains 395 variable sites.

In the obtained Bayesian phylogram (Fig. [1](#)), our two *Lepiota sanguineofracta* sequences clustered sister (BPP = 1; MLB = 94) to seven sequences of *L. coloratipes*. The pairwise % identity value between the *L. sanguineofracta* and *L. coloratipes* clades is 94.1.

### Taxonomy

*Lepiota sanguineofracta* Vizzini, sp. nov. (Figs. [2](#), [3](#), and [4](#))



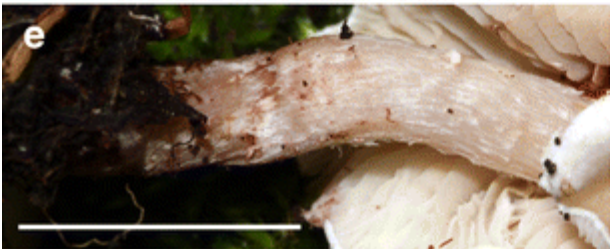




Fig. 2

*Lepiota sanguineofracta*. **a** Basidiomata; **b** pilei with green tinges; **c** reddening of context and basidiome surfaces; **d**, **e** details of veil remnants on stipe and pileus margins; **f** detail of the reddish stipe base. Photos by Alfredo Vizzini (all from the holotype). Scale bars **a–d**, **f** = 10 mm; **e** = 5 mm

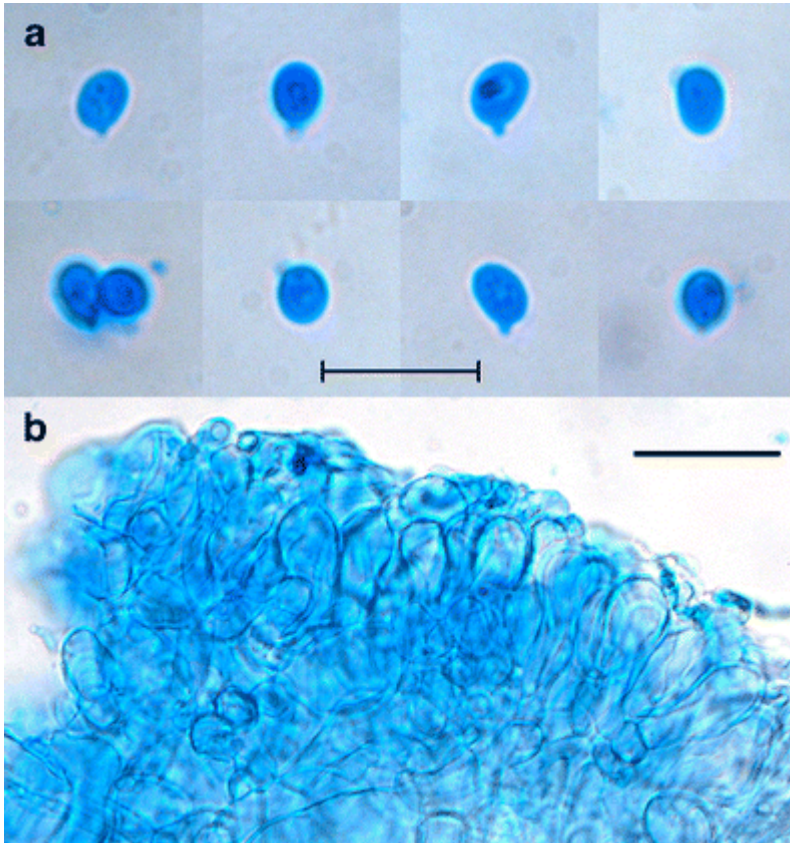


Fig. 3

*Lepiota sanguineofracta*. Microscopic characters (in Cresyl blue, from the holotype). **a** Spores; **b** pileus covering elements. Scale bars **a** = 10  $\mu$ m; **b** = 40  $\mu$ m (Photos by Alfredo Vizzini)



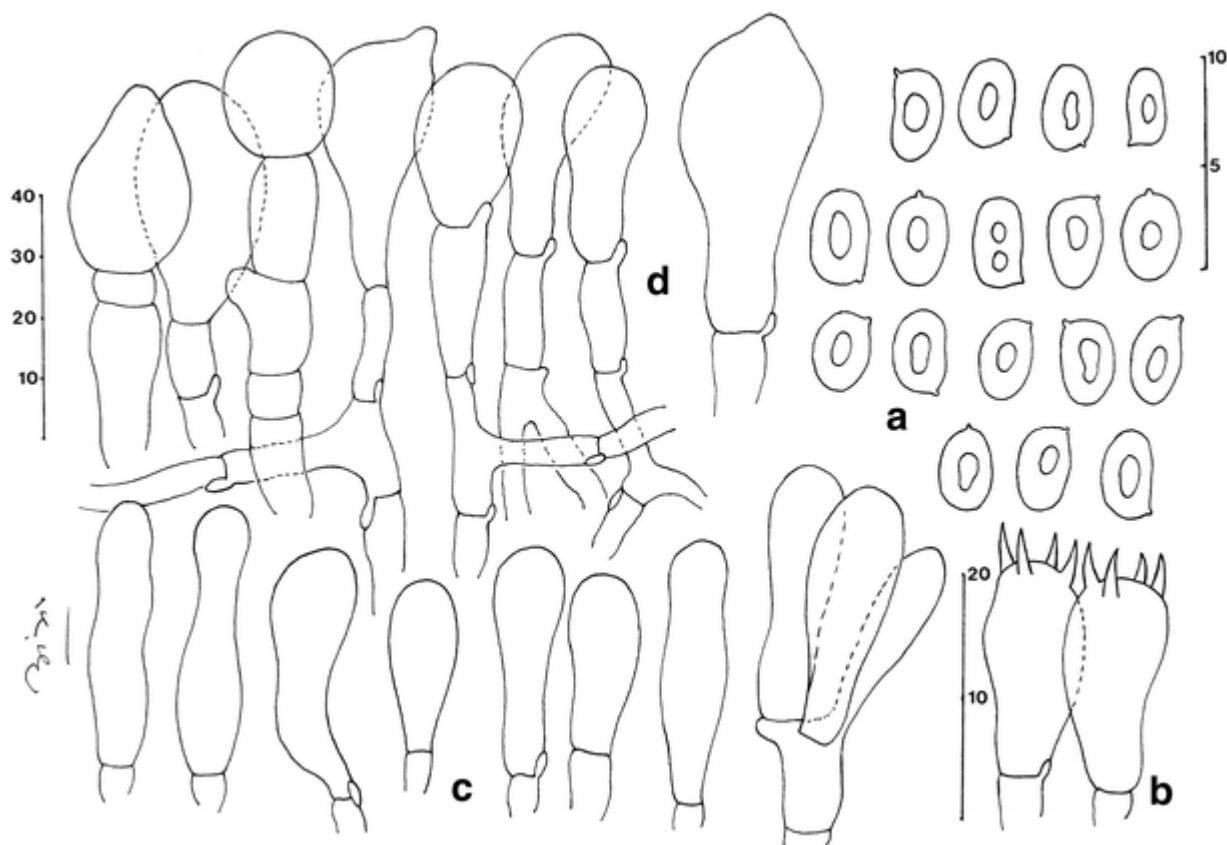


Fig. 4

*Lepiota sanguineofracta*. Microscopic characters (from the holotype). **a** Spores; **b** basidia; **c** cheilocystidia; **d** pileus covering elements. Scale bars **a** = 10  $\mu$ m; **b-c** = 20  $\mu$ m; **d** = 40  $\mu$ m (Drawings by Enrico Bizio)

Mycobank MB 807073

*Etymology* the specific epithet from the Latin words *sanguineus* (meaning blood red coloured) and *fractus* (broken) refers to the basidiome surfaces and context which turn blood-red on bruising.

*Macrocharacters* Pileus 4–22 mm wide, at first slightly obtusely campanulate, hemispherical or broadly conical, later plano-convex to applanate-expanded, subumbonate, with a shallow umbo; not hygrophanous; margin not striated when wet and dry, slightly exceeding the lamellae when young, sinuous-undulate, lobate, entire or slightly fringed with age; surface dry, at first smooth, micaceous, later rugulose-wrinkled, sometimes irregularly cracking in concentric zones towards the margin; variable in colour, the pileus is at first completely white, soon becoming brownish-orange (Mars Orange, Orange Rufous, Plate II; Xathine Orange, Mars Yellow, Plate III; Ferruginous, Cinnamon-Rufous, Plate XIV; Vinaceous-Tawny, Plate XXVIII) spotted in the centre, when mature turns light brown (5D4, 5D5), pale ivory (Pale Pinkish Buff, Plate XXIX), remaining paler towards the margin; often with large green spots (Oriental Green, Rinnemann's Green, Plate XVIII) in the centre and near margin. Stipe 15–35  $\times$  1.0–1.8(–2.4) mm, central, cylindrical, usually regular, but sometimes also slightly flexuous, hollow; shiny, at first white, soon becoming reddish-brown (Acajou Red, Pompeian Red, Madder Brown, Plate XIII) overall starting from the base and progressing upward; minutely silky fibrillose along all length; stipe and pileus margin connected initially with whitish fibrils of veil, forming an indistinct ring zone on the upper part of the stipe and soon disappearing. Lamellae 2–3(4) mm wide, L = 33–42, l = 1–3(4), subfree to adnexed, at first white, soon with evident pinkish tints (Pale Salmon Color, Seashell Pink, Plate XIV); edge finely

granulose. Context elastic, in pileus dull white, in stipe concolorous with its surface, but shiny white in central cavity; without specific taste, smell strong, sweetish, reminiscent of dried rose petals as in *Lepiota helveola*/L. *josserandii*. Pileus and stipe surface, lamellae edge and context strongly reddening (Scarlet-Red, Plate I; Acajou Red, Dragon's-blood Red, Plate XIII) on bruising or with age. Spore print pale cream.

**Microcharacters** Spores [60, 2, 2] (3–)3.5–4.5(–5) × (2.5–)2.9–3.3(–3.5) µm, on average 4.23 × 2.94 µm, Q = (1.29–)1.3–1.55(–1.66), Q<sub>av</sub> = 1.43, ellipsoid, rarely subcylindrical, hyaline, thin-walled, smooth, not verruculose in Melzer's reagent, not metachromatic in Cresyl Blue, non-amyloid, non-dextrinoid, cyanophilic in Cotton Blue and Methyl blue, congophilic, binucleate. Basidia 4-spored, 15–22 × 4–6 µm, clavate, hyaline, thin-walled; sterigmata up to 3–4 µm long. Cheilocystidia (10–)15–20 × 5–8(–9) µm, numerous and crowded, hyaline, thin-walled, various in shape, mostly clavate to subutriform, occasionally subfusiform, subcapitulate, lecythiform. Pleurocystidia absent. Pileus covering (pileipellis) a hymeniderm with transition to an epithelium, with up to 3(4) colourless elements on top of each other; terminal elements tightly packed, (12–)18–30(–50) × (10–)15–20(–25) µm, vesiculose, sphaeropedunculate to clavate-pyriform, sometimes with a short and obtuse apical mucro; slightly thick-walled (walls ca. 0.5 µm), with walls embedded in gelatinous matter; olivaceous tinges, when present, as intracellular pigment; subpellis composed of densely arranged and branching cylindrical hyphae, up to 5 µm wide. Hymenophoral trama subregular, consisting of hyphae 3–15 µm wide; trama of pileus and stipe similar to that in lamellae. Caulocystidia present only at apex of stipe, absent towards the base, very various in shape, 10–20 × 5–8 µm, hyaline, thin-walled, cylindrical, clavate, subglobose, sometimes sinuose, moniliform; occasionally in the form of simply lateral projections (diverticula) of the stipe hyphae. Clamp connections present and abundant in all tissues. Extracellular oil droplets absent in all tissues and on spore surface.

**Habitat and distribution** in small groups, terrestrial, on loamy or sandy humus-rich soils, growing at the edges of and in flowerbeds in Turin Botanical Garden. Fruiting in September. So far it is known only from the type locality.

**Collections examined** ITALY, Piedmont, Turin, Turin Botanical Garden, in flowerbeds of *Lonicera* spp. and *Viburnum* spp., 07 Sept 2011, leg. A. Vizzini (TO-HG2916, Holotype; GenBank acc. n. KF879620); ibidem, 13 Sept 2012, leg. A. Vizzini (TO-HG2917; GenBank acc. n. KF879621).

## Discussion

Among all the described species of *Lepiota* with a hymeniform pileus covering, *L. sanguineofracta* is clearly circumscribed on the basis of a distinctive set of features such as a micaceous and not squamulose pileus surface with evident green tinges when mature, a fugacious partial veil not forming an annulus but leaving fibrillose remnants on the stipe surface, a stipe with reddish tinges towards the base, context smelling of dried rose petals, basidiome surfaces and context strongly reddening on handling, with the hymeniform pileus covering consisting of a tightly arranged palisade of clavate to sphaeropedunculate elements, binucleate spores, versiform cheilocystidia, and a unique ITS sequence. The reddening of the context is striking and quick, reminding one of *L. maculans* Peck (sect. *Lepiota*, Birkebak et al. [2011](#)) and several species of *Leucoagaricus* Locq. ex Singer sect. *Piloselli* Singer (Vellinga [2010b](#)).

According to both morphological and molecular data (Fig. [1](#)), *Lepiota coloratipes* is the most closely related species to *L. sanguineofracta*. This recently described species (Vizzini et al. [2013](#)), only so far known from Europe, China and North America, was established for the taxon usually referred to as *L. rufipes* in Europe, non-ss. orig. (= *Cystolepiota seminuda* according to Vellinga

[2010a](#)). It shares with *L. sanguineofracta* the reddish stipe base, the usually not diffracted pileus surface, the micaceous pileus surface due to the tightly packed claviform elements of pileipellis, the versiform cheilocystidia and the absence of a well-formed annulus, but differs in having an unchanging (not reddening) context and surfaces, a not distinctive smell, lamellae without cream-pink hues, slightly smaller (on average  $3.4 \times 2.5 \mu\text{m}$ ) and mononucleate spores, that are metachromatic when mature and usually verruculose when observed in Melzer, and minute oil droplets in all tissues (Kühner and Maire [1937](#); Smith ([1954](#)); Josserand ([1955](#)); Lanzoni [1986](#); Candusso and Lanzoni [1990](#); Vellinga and Huijser [1999](#); Vellinga [2001](#), [2010a](#); Mohr [2008](#); Lange [2012](#); Vizzini et al. [2013](#)). Greenish tinges may be present on its pileus surface, but very faint and limited (Vizzini et al. [2013](#)).

The other species of *Lepiota* with hymeniform pileus covering and not squamulose-diffracted pileus surface, all without colour changes when bruised or with age, are also clearly distinguished from the new species: *L. neophana* Morgan (including var. *europaea* Bizio & Migl. and f. *papillata* Migl. & L. Perrone) shows a buff to dark brown and umbonate pileus centre, a well-formed annulus, very rare clamp-connections in the pileus trama and no cheilocystidia (Anonymous [1992](#); Bizio et al. [1993](#); Vellinga and Huijser [1999](#); Vellinga [2010a](#)). *L. psalion* Huijser & Vellinga is characterised by a distinct annulus, a looser pileipellis structure and clavate cheilocystidia (Vellinga and Huijser [1999](#); Vellinga [2001](#)) and, finally, *L. pyrochroa* Malenç. has an orange-red pileus and stipe, oil droplets in tissues, rough spores when studied in Melzer's reagent and clavate cheilocystidia (Malençon and Bertault [1970](#); Rioussset and Josserand [1976](#); Bon and Rioussset [1992](#); Bizio et al. [1993](#); Antonín and Vágner [1998](#); Vellinga and Huijser [1999](#)).

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