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(Agaricales, Tricholomatineae), a new fan shaped
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Journal:	<i>Botany</i>
Manuscript ID	cjb-2015-0172.R3
Manuscript Type:	Article
Date Submitted by the Author:	09-Nov-2015
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Keyword:	Basidiomycota, Clitocybe, Tricholomatoid clade, Agaricomycetes, taxonomy

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Clitolyophyllum akkaabatense gen. nov., sp. nov. (Agaricales, Tricholomatineae), a new fan shaped clitocyboid agaric from Turkey

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Draft

Abstract: *Clitolyophyllum akcaabatense* gen. nov., sp. nov. is described based on both morphological and molecular data from Akcaabat, Trabzon, Turkey. Its characterizing features are a fan shaped pileus, an eccentric and fibrillose stipe, 2-4 spored basidia, smooth, ellipsoid and inamyloid white basidiospores, and growth on the bark of *Picea orientalis*. Phylogeny based on multigene molecular analysis (nrITS, nrLSU, rpb2 datasets) of the Tricholomatoid clade is provided. Colour photographs of fresh basidiomata and of the main micromorphological features are included.

Keywords: Agaricomycetes, Basidiomycota, *Clitocybe*, taxonomy, Tricholomatoid clade

Draft

Introduction

Data on the variety of agaricoid fungi occurring in Turkey are scarce and fragmented (Sesli and Denchev 2008; Sesli and Helfer 2013; Sesli 2014). Some white spored and cortinarioid agarics have been described from the Turkish mycota recently (Liimatainen et al. 2014; Sesli and Moreau, 2015; Sesli et al. 2015a,b; Vizzini et al. 2015).

The aim of the present paper is to describe a new genus for accommodating a new species with a clitocyboid habit (adnate to decurrent lamellae and convex to funnel-shaped pilei, definition from Bas et al. 1998) fruiting on the bark of *Picea orientalis* in Turkey. Based on a multigene phylogenetic study, the genus occupies an isolated evolutionary position within the Tricholomatoid clade sensu Matheny et al. (2006) and Sánchez-García et al. (2014) (= subordo Tricholomatineae Aime, Dentinger & Gaya; Dentinger et al. 2015). Several independent clitocyboid mushroom lineages have been recognized recently in phylogenetic analyses of the Tricholomatoid clade (Matheny et al. 2006; Ammirati et al. 2007; Vizzini et al. 2010, 2011; Vizzini and Ercole 2012; Qin et al. 2014; Sánchez-García et al. 2014, Alvarado et al. 2015; Musumeci and Contu 2015). In accordance with these results, some old genera were resurrected (e.g. *Paralepista* Raithelh., *Singerocybe* Harmaja; Vizzini and Ercole 2012; Qin et al. 2014) and new ones proposed to accommodate genetic lineages deviating from that of the type of the genus *Clitocybe* (Fr.) Staude, *C. nebularis* (Batsch) P. Kumm.: *Cleistocybe* Ammirati, A.D. Parker & Matheny (Ammirati et al. 2007), *Trichocybe* Vizzini (Vizzini et al. 2010), *Musumecia* Vizzini & Contu (Vizzini et al. 2011), *Paralepistopsis* Vizzini (Vizzini and Ercole 2012), *Tephroderma* Musumeci & Contu (Musumeci and Contu 2014), *Atractosporocybe* P. Alvarado, G. Moreno & Vizzini, *Leucocybe* Vizzini, P. Alvarado, G. Moreno & Consiglio, *Rhizocybe* Vizzini, G. Moreno, P. Alvarado & Consiglio (Alvarado et al. 2015), *Pseudolaccaria* Vizzini, Contu & Z.W. Ge (Lavorato et al. 2015).

Materials and Methods

Collecting and microscopical studies

Basidiomata were collected from Hidirnebi-Akcaabat in Trabzon, Turkey, on September 2013 and were photographed with a Canon EOS 600-D camera equipped with macro lens. Features that change over time (taste, smell, texture, colour of basidiomata) were noted in the field. Basidiomata were collected, carried to the laboratory and dried for further microscopical studies. Microscopic studies were performed at the Karadeniz Technical University (Trabzon, Turkey), according to Clmenon (2009). Some molecular studies were performed in ALVALAB, Avda. Bruselas, Oviedo, SPAIN, and the others at the University of Turin, Italy. During the studies in the Karadeniz Technical University, dried basidiomata were sectioned with a razor blade under a Zeiss Stemi 2000-C stereo microscope, and obtained sections were mounted in water or dilute ammonia, stained with Congo red, Melzer's reagent and Cotton blue, separately, and finally examined under a Zeiss Axio Imager A2 trinocular microscope. Basidia have been examined for siderophilous granules according to the methods described in Clmenon (1978; with iron-acetocarmine) and Baroni (1981; with Cotton blue). Colour images were obtained with the Zeiss AxioCam 105 color camera, and measurements were made with Imager Software Programme.

The following abbreviations are used: L= number of lamellae reaching the stipe, l= number of lamellulae between each pair of lamellae, E = quotient of length and width in any one spore, Q = mean of E values. Colour terms in capital letters (e.g., Light Buff, Plate XV) are those of Ridgway (1912). Herbarium acronyms follow Thiers (2015). Author citations follow Index Fungorum (<http://www.indexfungorum.org/authorsoffungalnames.htm>).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from a dry specimen (KATO Fungi 3184) blending a portion of it with the aid of a micropestle in 600 mL CTAB buffer (CTAB 2%, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris-HCl pH 8.0 100 mM). The resulting mixture was incubated 15 min at 65 °C. A similar volume of chloroform: isoamyl alcohol (24:1) was added and mixed with the samples until emulsion, followed by centrifugation for 10 min at 13 000× *g* and the DNA in the supernatant precipitated with an equal volume of isopropanol. After an additional centrifugation step of 15 min at the same speed, the pellet was washed in cold 70% ethanol, centrifuged again for 2 min and dried. It was resuspended in 200 µL ddH₂O. PCR primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) for the nrITS region, LR0R, LR1, LR5 and LR7 (Vilgalys and Hester 1990, Cubeta et al. 1991, van Tuinen et al. 1998) for the 28S nrLSU region, bRPB2-6F, bRPB2-7R, fRPB2-7cR and bRPB2-7R2 for the DNA-directed RNA polymerase II subunit two *rpb2* gene (Liu et al. 1999; Matheny et al. 2005, 2007), were employed for PCR amplification and sequencing purposes. PCR reactions were performed under a program consisting of a hot start at 95 °C for 5 min, followed by 35 cycles at 94 °C, 54 °C and 72 °C (45, 30, 45 s, respectively) and a final 72 °C step for 10 min. PCR products were checked in 1% agarose gels before purification and sequencing. Chromatograms were checked by searching for putative sequencing reading errors and these were corrected. The sequences are deposited in GenBank and their accession numbers are included in Fig. 1 and supplementary figure S1.

Sequence alignment, dataset assembly and phylogenetic analysis

The sequences obtained in this study were checked and assembled with Geneious 5.3 (Drummond et al. 2010) and compared to those available in GenBank database by using the BLASTn algorithm. Then a

general broad combined LSU/*rpb2* dataset (122 collections) with sequences of the Tricholomatoid clade was constructed (following Matheny et al. 2006; Sánchez-García et al. 2014 and Alvarado et al. 2015) in order to narrow down the closest relatives of our sequences. The tree was built using the Sánchez-García et al. (2014) combined dataset retrieved from TreeBase (S16404) and run under RAxML software as described below (supplementary figure S1). Based on these preliminary analyses and taking into account the results of BLAST searches for our sequences, a smaller combined dataset with representatives of the Tricholomatoid clade was constructed and analyzed (Fig. 1, TreeBase S18385). *Tricholoma viridiolivaceum* (JF706316, JF706317, JF706319) was chosen as outgroup taxon.

Alignments were generated for each ITS, LSU, and *rpb2* dataset with MAFFT (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties. Alignments were imported into MEGA 6.06 (Tamura et al. 2013) for manual adjustment. The best-fit substitution model for each alignment was estimated by the Bayesian information criterion (BIC) with jModelTest 2.1.7 (Darriba et al. 2012) to provide a substitution model for the alignment. GTR+G model was chosen for each alignment. Phylogenetic analyses, based on a combined ITS/LSU/*rpb2* dataset, were performed using Bayesian inference (BI) and Maximum likelihood (ML) criteria. BI was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with four incrementally heated simultaneous Monte Carlo Markov chains (MCMC) run for 10 000 000 generations, under the selected evolutionary model. Trees were sampled every 1000 generations, resulting in overall sampling of 10 001 trees; the first 2500 trees (25%) were discarded as burnin. 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 with RAxML 7.3.2 (Stamatakis 2006) with 1000 bootstrap replicates (Felsenstein 1985) with the GTRGAMMA algorithm to perform a tree inference and search for optimal topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the “-fa” option of

RAxML and “-b 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 exceeding 0.75 and MLB over 50% are reported in the resulting tree (Fig. 1). Branch lengths were estimated as mean values over the sampled trees.

Results

Molecular results

Both BI and ML analyses produced the same topology; the BI tree with both BPP and MLB values is shown (Fig. 1). The combined data matrix (2411 bp, 753 for ITS, 930 for LSU and 728 for *rpb2*) comprised 29 collections (including 28 from GenBank).

The newly sequenced collection occupies an independent position in the phylogram where it forms a clade (with low support) together with *Clitocybe subditopoda*, *Atractosporocybe inornata* and *Tephroderma fuscopallens*.

Taxonomy

Clitolyophyllum E. Sesli, Vizzini & Contu, gen. nov.

MycoBank MB 814482

Diagnosis: — It is characterized by a clitocyboid habit, eccentric stipe, hyaline smooth and inamyloid basidiospores, non-siderophilous basidia, by fruiting on dead tree bark and unique ITS/LSU/*rpb2* sequences.

Type species: *Clitolyophyllum akcaabatense* E. Sesli, Vizzini & Contu.

So far, the type species is known only from Akcaabat, Hidirnebi, Trabzon, Turkey, but it is not known whether its occurrence is limited to this area.

Etymology: — *Clitolyophyllum* is a compound name, reflecting the morphological similarity and phylogenetic proximity to *Clitocybe* and *Lyophyllum*.

Clitolyophyllum akcaabatense E. Sesli, Vizzini & Contu, sp. nov. (Figs. 1–4)

MycoBank MB 814483

Diagnosis:—It is distinguished by fruiting on the bark of *Picea orientalis*; fan shaped and umbilicate, thin and striated pileus, whitish and decurrent lamellae; 2–4 spored basidia, smooth and ellipsoid, $(5.5\text{--})6.0\text{--}8.0\text{--}(9) \times (3.3\text{--})3.5\text{--}5\text{--}(6.0) \mu\text{m}$ spores, and unique ITS/LSU/*rpb2* sequences.

Type:—TURKEY, Trabzon, Akcaabat, Hidirnebi, 40°57'07.86'' N - 39°25'27.65'' E, 1481 m alt., 09 September 2013, E. Sesli, KATO Fungi 3184 (Holotype).

Etymology: — “akcaabatense” comes from “Akcaabat”, one of the districts of Trabzon.

Basidiomata clitocyboid. Hyphal system monomitic. Pileus 30–55(–60) mm broad, fan shaped, with a slightly costate to undulating, folded and irregular margin with a small depression resembling a navel and a V-shaped slit where it is connected to the stipe; surface smooth, dull, slightly hygrophanous, translucent-striate toward to margin, gray-beige to beige-brownish, horn-gray or wood colour when moist (Pale Ochraceous-Buff, Ochraceous-Buff, Yellow Ocher, Plate XV; Cinnamon-Buff, Plate XXIX; Chamois, Plate XXX; Avellaneous, Plate XL), slightly darker toward the centre (Orange-Cinnamon, Plate XXIX; Isabella Color, Plate XXX; Wood Brown, Plate XL), pale cream-colored (Light Buff, Plate XV) when dry. Lamellae decurrent, broad towards pileus centre, shallow towards margin, thin, $L = 35\text{--}45$, $I = 1\text{--}4$, interveined at base, at first whitish then light cream or beige (Pale Ochraceous-Salmon, Pale Ochraceous-Buff, Plate XV). Stipe 20–30 $\times$ 4–7 mm, laterally attached to the pileus, cylindrical to flattened, hollow with enlarged apex and tapering towards the base, sometimes twisted, tough, elastic, at

apex smooth and concolorous with pileus surface, 2/3 of the surface towards the base covered with a typical white to creamy, woolly mycelium. Context thin, white to gray-beige. Odour fungoid. Taste indistinct. Spore print white.

Basidiospores $(5.5-6.0-8.0(-9) \times (3.3-3.5-5(-6.0) \mu\text{m}$, on average $7.2 \times 4.2 \mu\text{m}$ ($n=100$), $E=1.50-1.85$, $Q=1.58-1.78$, ellipsoid, ellipsoid-fusoid, sublacrymoid, thin-walled, hyaline, smooth, non-dextrinoid, cyanophilic. Basidia $25-30(-37) \times 6.5-7.5 \mu\text{m}$, 2-4-spored, clavate or subcylindrical, without siderophilous granulation, with basal clamp-connection. Basidioles $15-20 \times 3.5-5.0 \mu\text{m}$, narrowly clavate, subfusoid, subcylindrical. Hymenial cystidia not observed. Hymenophoral trama regular, consisting of cylindrical, subinflated, thin- to slightly thick-walled, non-dextrinoid, $3.5-60(-90) \times (7.5-)15-25 \mu\text{m}$ parallel hyphae. Pileipellis made up of irregularly cylindrical, clavate, thin- to slightly thick-walled, smooth, subinflated, $40-50(-100) \times (5.5-)20-30 \mu\text{m}$ hyphae; some hyphae slightly gelatinized with intracellular pigment; terminal elements adpressed to suberect, sometimes pileocystidia-like. Stipitipellis a cutis of cylindrical, parallel, slightly thick-walled, smooth, non-dextrinoid, $5.5-15 \mu\text{m}$ wide hyphae. Caulocystidia $35-45 \times 4.5-15 \mu\text{m}$, irregular, cylindrical, subfusoid or almost subulate, sometimes branched, thin- to slightly thick-walled. Clamp-connections present in all tissues.

Habit, habitat and distribution: — solitary to gregarious, growing on the dead bark of *Picea orientalis* together with mosses. Fruiting in autumn, so far known only from Turkey.

Discussion

In the combined ITS/LSU/*rpb2* phylogenetic tree (Fig. 1) as well as in the large preliminary LSU/*rpb2* analysis (supplementary figure S1) our new species is not closely related to *Clitocybe nebularis*, the type of the genus *Clitocybe* (Redhead et al. 2002), nor to other clitocyboid taxa or allies, and it represents a

new phyletic line sufficiently different from other taxa within Agaricales to warrant the erection of the new genus.

Morphologically, *Clitolyophyllum akcaabatense* shares the habit (lateral stipe and decurrent lamellae), hyaline, smooth and non-amyloid spores and growth on dead wood with species of *Clitocybe* section *Aberrantissimae* Singer [= section *Candicantes* (Quél.) Konr. & Maubl., subsection *Aberrantissimae* (Singer) Bigelow] as traditionally delimited by Singer (1961, 1978, 1986) and Bigelow (1982, as subsection). This section, however, which, is heterogeneous and probably not monophyletic (an artificial set of disparate and phylogenetically-unrelated taxa, even though molecular data are not available for any species of the complex), is distinguished by the presence of well-developed hymenial cystidia.

Among the *Clitocybe* species of sect. *Aberrantissimae* with morphological similarities to *Clitolyophyllum akcaabatense*, *Clitocybe aberrantissima* Singer, described from Brazil, sharply differs in having white basidiomes, much bigger, ellipsoid-subfusiform basidiospores, $7.5\text{--}10.2 \times 4.8\text{--}5.5 \mu\text{m}$, and incrusted cystidia, $55\text{--}60 \times 8\text{--}11 \mu\text{m}$ (Singer 1953); *Clitocybe subecccetrica* Murrill, described from North America (Florida) is characterized by white basidiomes, basidiospores $6\text{--}8.5 \times 4\text{--}5 \mu\text{m}$, and cylindrical cheilocystidia $47\text{--}54 \times 5.5\text{--}8 \mu\text{m}$ (Bigelow 1982). *Clitocybe peralbida* Murrill also described from North America (Florida), differs in having white basidiomes with a non-striate pileus, bitter taste as well as narrower basidiospores ($\times 3\text{--}3.5 \mu\text{m}$) and hymenial cystidia, $38\text{--}54 \times 6\text{--}11 \mu\text{m}$, according to the type-study by Bigelow (1982).

Clitocybe lentinelloides Miller & Bigelow and *Clitocybe bubalina* Bigelow & Miller from North America, share with *Clitolyophyllum akcaabatense* the buff to pale ochraceous tinges of the pileus, but they differs by growing on hardwood logs (Bigelow 1982); in addition the first shows small basidiospores, $3.5\text{--}5 \times 2\text{--}2.5 \mu\text{m}$, thick-walled hymenial cystidia embedded amid basidia, $18\text{--}35 \times 6\text{--}9$

µm; the second has a non-striate pileus, small basidiospores $4\text{--}6.5 \times 2.5\text{--}3.5$ µm, and small filamentous to narrowly fusiform cheilocystidia.

The genera *Hypsizygus* Singer and *Ossicaulis* Redhead & Ginns might resemble *Clitolyophyllum akcaabatense* based on their lignicolous habit, but the first differs by a centrally-stipitate, symmetrical pileus, a farinaceous odour, globose to subglobose spores, and basidia with the (inconstant) presence of siderophilous granules of the oligo-type s. Cléménçon (1978) (Redhead 1984; Bon 1999; Kalamees 2004; Hofstetter et al. 2014). The second is distinguished by whitish basidiomes, lamellae adnexed, adnate or at most subdecurrent (not decurrent), small ellipsoid basidiospores, presence of narrowly clavate to coralloid cheilocystidia, coralloid hyphae in the epicutis, and basidia with siderophilous granules of the oligo-type (Redhead and Ginns 1985; Holec and Kolařík 2013; Hofstetter et al. 2014).

Among the other clitocyboid/pleurotoid fungi described in literature, *Pleurotus pop-ivanensis* Pilát (*nom. nud.*, no Latin diagnosis provided) from the Eastern Carpathians, Carpatorossia (formerly Czechoslovakia and now part of Ukraine, see Holec 2002), shares with *Clitolyophyllum* the ochraceous tinges of the pileus and the growth on wood (Pilát 1935). This species, suggested by Pilát (1935) to be morphologically close to *Pleurotus lignatilis* Fr. [= *Ossicaulis lignatilis* (Pers.) Redhead & Ginns] has a central stipe, small basidiospores, $5\text{--}6 \times 3\text{--}4$ µm, small basidia, $15\text{--}20 \times 4\text{--}4.5$ µm, and it grows on *Fagus sylvatica* dead wood. Our analysis of the type collection (PRM 20246) confirmed these data [basidiospores $(3.5\text{--}) 4.0\text{--}5.6$ ($\text{--}6.0$) $\times (3.0) 3.2\text{--}3.6$ (4.0) µm, often released in tetrads].

Among the centrally-stipitate clitocyboid taxa, *Clitocybe* subgenus *Pseudolyophyllum* Singer, typified by *C. metachroa* (Fr.) P. Kumm. is also differentiated by having a terricolous basidiome (Kuyper 1982, 1995; Cléménçon 1984; Bon 1999; Raithelhuber 1990, 2004).

Finally, the molecular analysis (Fig. 1) indicated *Clitocybe subditopoda* Peck and the monospecific genera *Atractosporocybe* and *Tephroderma* as the taxa phylogenetically closest to *Clitolyophyllum*.

Clitocybe subditopoda is a species described from North America with a terricolous basidiome, a central stipe, a regular not fan-shaped pileus, a farinaceous odour and taste, subglobose to broadly ellipsoidal basidiospores, $3.0\text{--}5.5\text{--}(6.0) \times 2.5\text{--}3.5\text{--}(4.0) \mu\text{m}$, and growing on coniferous litter under pine or spruce (Bigelow and Hesler 1960; Bigelow 1985). Actually, the morphological analysis of a sequenced collection of *Clitocybe subditopoda* included in the phylogram (AFTOL-ID/PBM2489) revealed spores $5.0\text{--}5.5 \times 3.5 \mu\text{m}$ (Brandon Matheny, pers. comm.).

Atractosporocybe inornata (Sowerby) P. Alvarado, G. Moreno & Vizzini, is distinguished by a centrally-stipitate terricolous basidiome and fusiform basidiospores (Alvarado et al. 2015). *Tephroderma fuscopallens* Musumeci & Contu shows a centrally-stipitate terricolous basidiome, a tenacious elastic-leathery context consisting of thick-walled hyphae, thick lamellae, and a gelatinous hymenophoral trama and pileipellis (Musumeci and Contu 2014).

Acknowledgments

The first author is thankful to the Karadeniz Technical University management for financial support (BAP: 11300). We are grateful to Pierre-Arthur Moreau (University of Lille, France) for his valuable comments, Pablo Alvarado (ALVALAB- Oviedo, SPAIN) for some molecular works, Brandon Matheny (University of Tennessee, Knoxville, USA) for providing morphological information on *Clitocybe subditopoda* collection PBM2489 and Jan Holec (National Museum, Mycological Department, Praha, Czech Republic) for sending the original collection of *Pleurotus pop-ivanensis*.

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Fig. 1. Phylogeny of selected members of the Tricholomatoid clade based on a Bayesian and Maximum Likelihood Inference analysis of a supermatrix of three nuclear gene regions (nrITS, nrLSU and *rpb2*). Bayesian posterior probability (BPP) values (in bold) ≥ 0.75 and Maximum Likelihood bootstrap (MLB) values $\geq 50\%$ are shown on the branches. The newly sequenced collection is in bold.

Fig. 2. *Clitolyophyllum akkaabatense*. (a–c) Basidiomata in situ (holotype). Scale bars = 10 mm. Photos by E. Sesli.

Fig. 3. *Clitolyophyllum akkaabatense*. Microscopic features in ammoniacal Congo red (holotype). (a–b) Lamellar cross sections. (a) General appearance. (b) Hyphae of the hymenophoral trama, basidia and basidiospores. (c) Basidia and basidioles. Scale bars: a–b = 20 μm , c = 10 μm . Photos by E. Sesli.

Fig. 4. *Clitolyophyllum akkaabatense*. Microscopic features (holotype). (a) Elements of the pileipellis. (b) Aerial hyphae on the stipe. (c–f) Basidiospores. (c) in Melzer's reagent; (d) in ammoniacal Congo red; (e) in water; (f) germinating spores in ammoniacal Congo red. Scale bars: a–b = 20 μm , c–f = 10 μm . Photos by E. Sesli.

0.05 expected changes per site

Figure 2

Figure 3

Figure 4