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Agaricus megalosporus: a new species in section *Minores*

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Résumé – Une description, des dessins, et des photographies en couleurs d'*Agaricus megalosporus*, une espèce nouvelle, sont présentés. La nouveauté de cette espèce est supportée à la fois par sa morphologie et par une analyse phylogénétique. *A. megalosporus* se caractérise par ses sporophores de taille moyenne ou grande, un stipe fortement fibrillo-squamuleux et des basidiospores relativement grandes. L'analyse de séquences ITS par maximum de vraisemblance, maximum de parcimonie non pondérée et analyse Bayesienne démontre que *A. megalosporus* appartient à la section *Minores*.

Agaricaceae / ITS / Phylogénie / Taxinomie / Thailand

Abstract – In this paper *Agaricus megalosporus* sp. nov. is introduced with a description, line drawings, color photographs. Its novelty is supported both by its morphology and phylogenetic analysis. *A. megalosporus* is characterized by medium to large fruiting bodies, a heavily fibrillose squamulose stipe and relatively large basidiospores. This new species is compared with some morphologically similar species. ITS sequence data analysis by maximum likelihood, unweighted parsimony and Bayesian analysis shows that *A. megalosporus* should be placed in section *Minores*.

Agaricaceae / ITS / Phylogeny / Taxonomy / Thailand

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INTRODUCTION

Agaricus L. Fr. comprises numerous species of high medicinal and edible interests. One of the most notable medicinal species is *A. subrufescens* Peck (Angeli *et al.* 2006, Bernarshaw *et al.* 2007), while *A. bisporus* (J.E. Lange) Pilát is one of the most widely consumed edible species (Adams *et al.* 2008). *Agaricus* has a long history of study and there are many systems to classify species in different sections (such as Cappelli 1984; Heinemann 1978; Parra 2008; Singer 1986). Section *Minores* has been treated as a subsection of section *Arvenses* by Heinemann (1978). Recent studies have shown that section *Minores* should be redefined to include species being characterized by a positive orange or red Schäffer's reaction and strongly positive KOH reaction. *Minores* have a strong odor of anise or almond and the annulus is simple, thin and not floccose; and cheilocystidia are simple (not catenulate) (Nauta, 2001; Parra 2008). This arrangement has been accepted by most mycologists.

Mushroom research in Northern Thailand has resulted in several publications (Desjardin *et al.* 2009; Kerekes and Desjardin 2009; Van de Putte *et al.* 2010; Wannathes *et al.* 2009; Zhao *et al.* 2010) and the present study is a continuation of this work. Recent phylogenetic studies on tropical *Agaricus* species support the existing classical sections including section *Minores*. Furthermore, three completely new main clades have been revealed (Zhao *et al.* 2011). Although some species of section *Minores* from Europe and the subantarctic have been described and sequenced (e.g. *A. campbellensis* Geml, Laursen & D. Lee Taylor, *A. pseudolutosus* (G. Moreno, Esteve-Rav., Illana & Heykoop) G. Moreno, Parra, Esteve-Rav., & Heykoop, *A. heinemannianus* Esteve-Rav.; Esteve-Raventós 1998; Geml *et al.* 2007; Moreno *et al.* 1999), related research in the tropics is lacking. In this paper a new species of *Agaricus* in section *Minores* is introduced.

MATERIALS AND METHODS

Morphological analysis

Collections of *Agaricus* and related genera were made in northern Thailand between 2004 and 2010 (Zhao *et al.* 2010). Photographs were taken *in situ* and the odor and color change on bruising were noticed in the field. Collections were gathered and wrapped in foil or kept separately in a box in order to avoid mixing or crushing. The macrocharacters, including chemical testing and further photography of fresh samples were carried out as soon as possible after return from the field following the methodology described by Largent (1986). Colour terms follow those of Kornerup and Wanscher (1978) or Online Auction Color Chart^{tm} (www.OnlineAuctionColorChart.com). Then specimens were dried completely using a food drier, sealed in plastic bags, and deposited in the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU). Duplicate specimens are deposited in the BIOTEC Bangkok Herbarium (BBH) and the H.D. Thiers Herbarium at San Francisco State University (SFSU),

Herbarium acronyms are those of Holmgren and Holmgren (1998). Strains isolated from sporocarps or issued from spores of some collections are kept in the culture collection of Mae Fah Luang University.

Micromorphological features were examined from dried specimens following the protocols of Largent (1986) including of anatomy of lamellae, pileipellis, stipitipellis and partial veil, and features of basidiospores, basidia and cystidia. Measurements of anatomical features (spores, basidia and cheilocystidia) are presented based on at least 20 measurements, and include x, the mean of length by width ± SD; Q, the quotient of basidiospore length to width, and Q_m, the mean of Q-values ± SD.

Molecular analysis

DNA extraction, PCR and sequencing:

DNA extraction from dried fungal specimens was realized with a commercial DNA extraction kit (E. Z. N. A. Forensic Kit, D3591-01, Omega Bio-Tek). Protocols for PCR and sequencing generally followed those of White *et al.* (1990) with some modifications, and were performed using primers ITS4/ITS5 or ITS1F/ITS4B (Zhao *et al.* 2010).

Sequence alignment and phylogenetic analyses:

The original sequences which produced from this work plus the sequences retrieved from GenBank were involved in analyses. Sequences were initially aligned using Clustal X with default settings (Thomson *et al.* 1997), then manually adjusted in BioEdit v. 7.0.4 (Hall 2007), and gaps was not removed from the alignment. The alignment has been submitted to TreeBase (submission ID 11913).

Unweighed maximum parsimony (UP) analysis, was performed using PAUP* 4.0b10 (Swofford 2004), by heuristic searches with unordered characters, random addition of sequences, gaps treated as missing data, and the tree bisection-reconnection (TBR) branch swapping. Maximum likelihood (ML) was also performed in PAUP* 4.0b10 (Swofford 2004). The best nucleotide substitution model for maximum likelihood was chosen by using MrModeltest 2.2 (Nylander 2004). Bootstrap values (BS) were obtained from 1000 replicates. Bayesian inference was performed with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001, Ronquist and Heulsenbeck 2003). One million generations were run for four Markov chains and sampled every 100^{th} generation resulting in 10,000 trees. The first 2000 trees were discarded as part of the burn-in phase, and the remaining 8000 trees were used to build a 50% majority rule consensus tree and calculate posterior probabilities. Trees were viewed in TreeView and exported to graphics programs (Page 1996).

RESULTS

Phylogenetic analysis

The ITS dataset included 32 sequences representing 29 *Agaricus* species, with *A.* aff. *trisuphuratus* as the outgroup (Table 1). The alignment contained 693 characters, of which 457 were constant, 81 were parsimony-uninformative and 155 were parsimony-informative characters. Gaps were treated as missing data.

Table 1. Voucher table. * Original sequence already used in previous papers (Zhao et al. 2011; 2012). All sequences, except for the specimens in bold, retrieved from GenBank. [a] BBH: Biotec Bandkok Herbarium, National Science and Technology Development Agency, Klong Luang, Pathumthani, Thailand; CGAB: Collection du germoplasme des agarics à Bordeaux, INRA, Bordeaux, France; MFLU: Mae Fah Luang University, Chiang Rai Prov., Thailand.

Taxon	*Country*	*Collection Number*	*Herbarium[a] Number*	*GenBank Accession Number*
A. aff *trisulphuratus*	Thailand	ZRL2128	BBH19508	JN664955*
A. albolutescens	USA			AY484675
A. aridicola	France		CGAB CA101	JF797195*
A. arvensis	France		CGAB CA640	JF797194*
A. augustus	USA			AY484672
A. bisporus	USA			DQ404388
A. bitorquis	USA			AF432898
A. bresadolanus	France			DQ185572
A. brunneolus	France		CGAB CA377	JF797203*
A. campbellensis ISOTYPE	New Zealand			DQ232644
A. campestris	USA			AF432877
A. comtulus	France			JF715065
A. cupreobrunneus	France			DQ182532
A. endoxanthus	Spain			DQ182511
A. excellens	USA			AY484682
A. fissuratus	Denmark			AY484683
A. flocculosipes HOLOTYPE	Thailand	ZRL3028	BBH19544	JN664954*
A. fuscovelatus	USA			AY484677
A. gennadii	Israel			AJ884633
A. heinemannianus	Spain			JF797182
A. lilaceps	USA			AY484676
A. litoralis	France		CGAB CA120	JN204436*
A. macrocarpus	USA			AY484686
A. megalosporus HOLOTYPE	Thailand	LD030*	MFLU100774	JF514521*
A. megalosporus	**Thailand**	**LD2011026**	**MFLU111309**	**JQ359015**
A. megalosporus	**Thailand**	**ZRL2124**	**BBH19503**	**JN664951**
A. megalosporus	**Thailand**	**ZRL2125**	**BBH19505**	**JN664952**
A. megalosporus	**Thailand**	**NTS106**	**MFLU100666**	**JN664953**
A. pseudolutosus	Spain			JF727868
A. sylvaticus	France		CGAB CA358	JN204434*
A. subrufescens	USA			AY818651
A. subrutilescens	USA			AY943973
A. xanthodermus	England			DQ182534

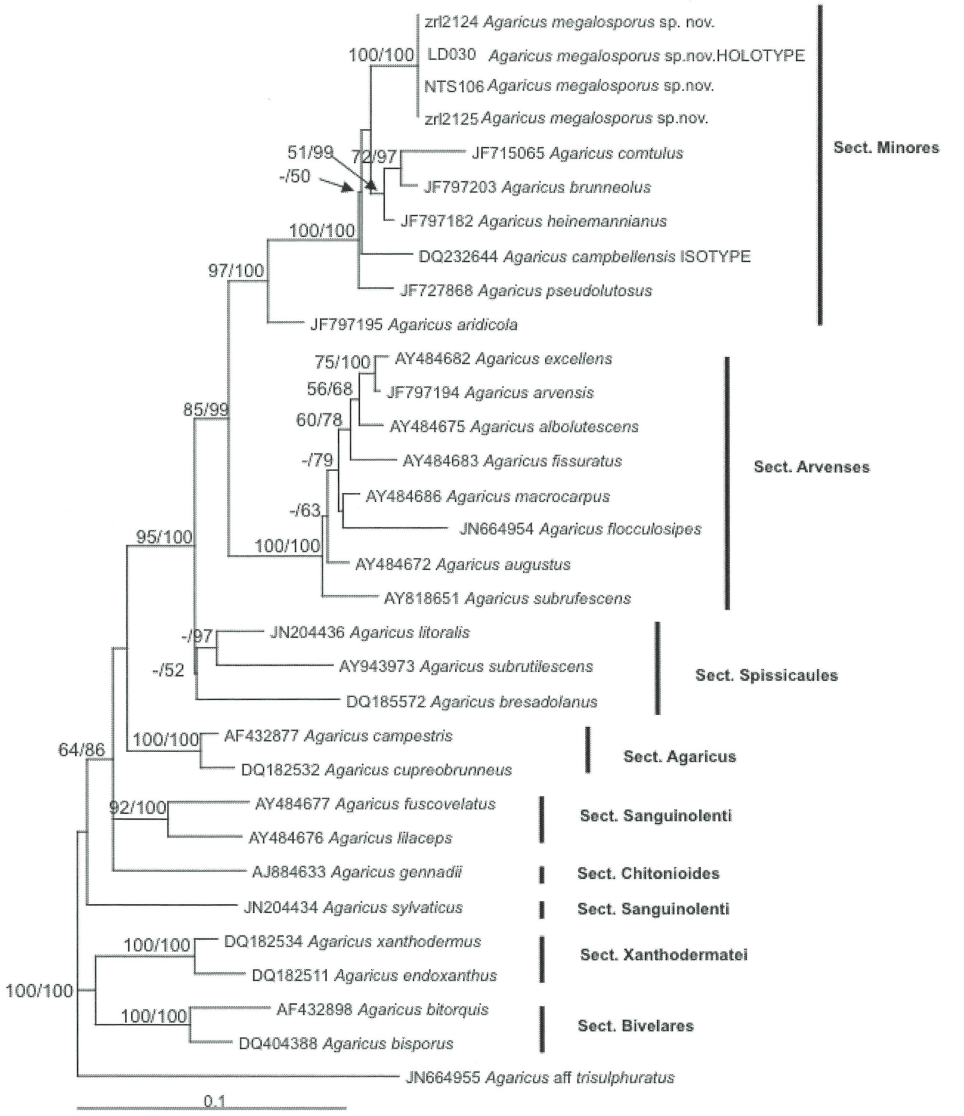


Fig. 1. Phylogeny of *Agaricus* generated from Maximum Likelihood analysis of ITS sequences, rooted with *A.* aff *trisulphuratus*. Parsimony bootstrap support values >50% (BS) and Bayesian posterior probabilities (PP) are given at the internodes (BS/PP).

The phylogenetic trees produced by UP, ML and MrBayes methods are almost identical, and the ML tree is shown in Fig. 1. This tree was obtained after 11134 rearrangements and the score of the best tree was 3712.79031. The section Minores is nearly fully supported by BS and PP values (BS = 97%, PP = 100%) as a monophyletic lineage. This clade comprises *A. aridicola, A. brunneolus*, *A. campbellensis, A. comtulus, A. megalosporus*, *A. heinemannianus* and *A. pseudolutosus*. The four specimens of the new species group together with

almost identical sequences, and sister to the subclade of *A. brunneolus*, *A. comtulus* and *A. heinemannianus.*

The ITS1+2 sequences of the four specimens of the new species used in the phylogenetic analysis are similar and do not exhibit any heteromorphism. A fifth specimen, LD2011026 recently collected, has a similar sequence but with a heteromorphism (C/T) instead of T at position 111 of ITS1 (..gtatYgagg..). The ITS1+2 sequence of the new species is less variable than those of many species of the genus.

Taxonomy

Agaricus megalosporus J. Chen, R.L. Zhao, Karunarathna & K. D. Hyde, **sp. nov.** **Figs 2, 3**

MycoBank: MB563067

Etymology: refers to the large spores compared to other species in section *Minores.*

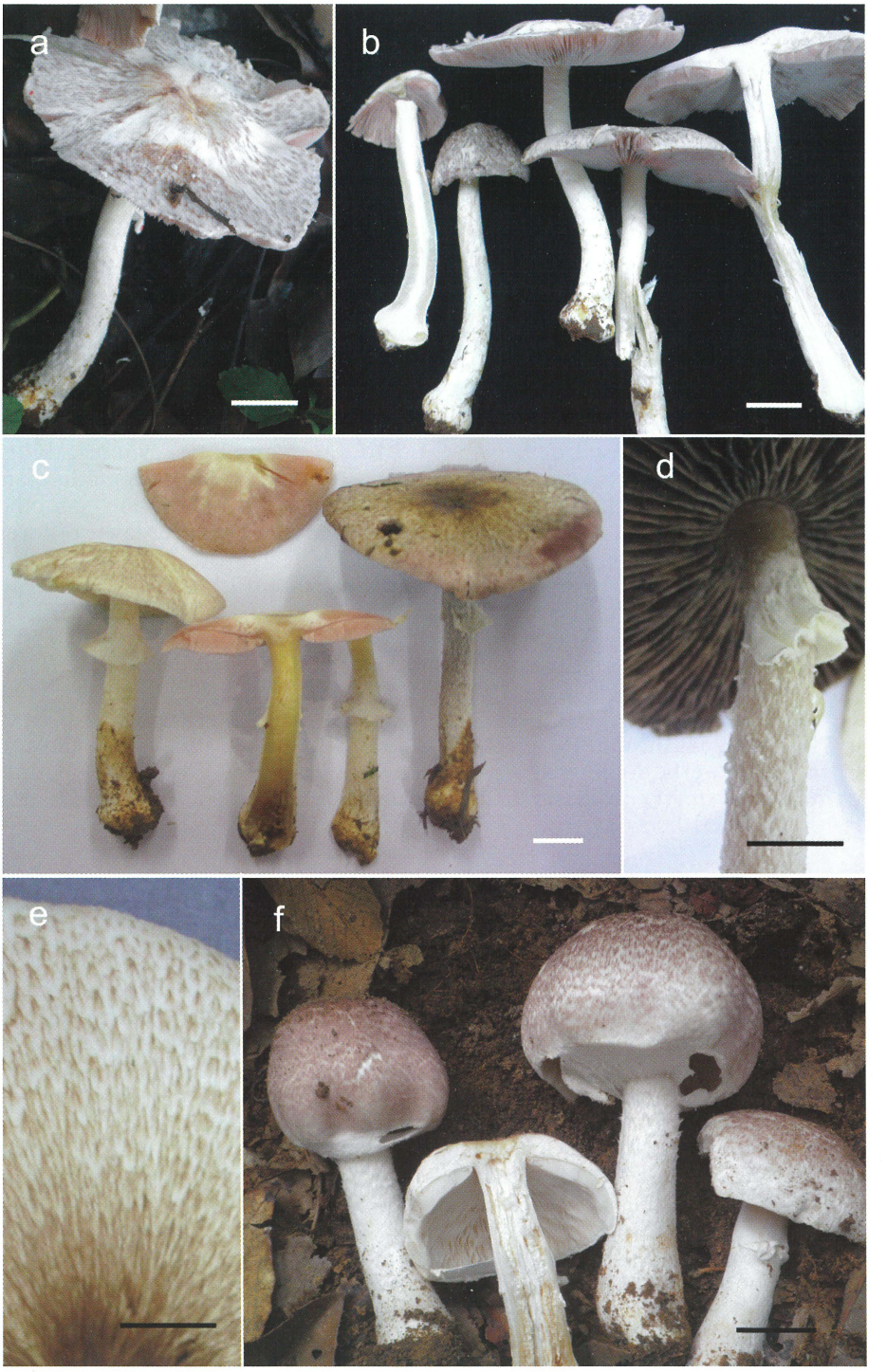
Pileus 35-110 mm diam., hemisphaericus, convexus ad plano-convexus, squamosus, purpureo-brunneus. Lamellae liberae, roseae, dein brunneae. Stipes 50-120 × 8-11 mm (basi 10-17 mm diam), cylindraceus, bulbosus, fistulosus, albus, annulatus, squamosus. Sporae 5.5-7.5 × 3-4 µm, ellipsoideae, brunneae. Cheilocystidia 20-43 × 10-15 µm, clavulata, piriformia. Pleurocystidia absentia. Pileipellis cutis, ex hyphis 5-10 µm diam. cylindraceis.

Pileus 35-110 mm diam., circular in top view, parabolic and truncate at disc, then convex to plano-convex, flat or slightly depressed at the disc when mature; aspect of margin straight, inflexed, or reflexed; shape of margin crenulate, eroded; surface dry, heavily fibrillose when young, purplish-brown to brown (oac639), then disrupted into triangle-shaped fibrillose squamules, dense at the disc, and more scattered towards margin, squamules easily rubbed by rain, light brown (7D4) to brown (7D7, oac669) against white background, with pink tone when wet. *Context* 5-9 mm broad, firm, white. *Lamellae* free, crowded, lamellulae with 3-5 series, 4-7 mm broad, very young, white, then pink, pale red (7A3), light brown (6D4), brown, to finally dark brown with age. *Stipe* 50-120 × 8-11 (base 10-17) mm, cylindrical-bulbous, surface smooth above annulus, heavily fibrillose, recurved fibrillose-squamulose when young, squamulose floccose or rubbed off when mature below annulus, white, narrowly hollow. *Annulus* membranous, pendant, single, superior, upper side smooth and lower side fibrillose, fugacious, white, up to 10 mm broad. *Smell* almond. Staining light yellow to yellow on stipe (pileus not distinct) on touching and cutting.

Macrochemical reactions: KOH reaction yellow. Schäffer's reaction orange.

Spores 5.5-6.5-7(-7.5) × 3-4 µm, [x = 6 ± 1 × 3.5 ± 0.6, Q = 1.45-2.3, Q_m = 1.7 ± 0.6, n = 20], ellipsoid to oblong, rarely cylindric, smooth, reddish brown, thick-walled. *Basidia* 15-25 × 6-9 µm, clavate, hyaline, smooth, 4-spored, but in some case looks like 2-spored because of the overlapping of sterigma in side view.

Fig. 2. Slightly depressed at the disc (LD030); **b.** type specimens (LD030); **c.** the overall appearance and the pileus with pink tone when wet (ZRL2125); **d.** squamulose floccose stipe and annulus (ZRL2125); **e.** triangle-liked fibrillose squamose on the pileus (ZRL2124); **f.** the overall appearance (LD2011026) Scale bar **a**, **b**, **c** and **f** = 20 mm; **d** and **e** = 10 mm. ▶


a
b
c
d
e
f

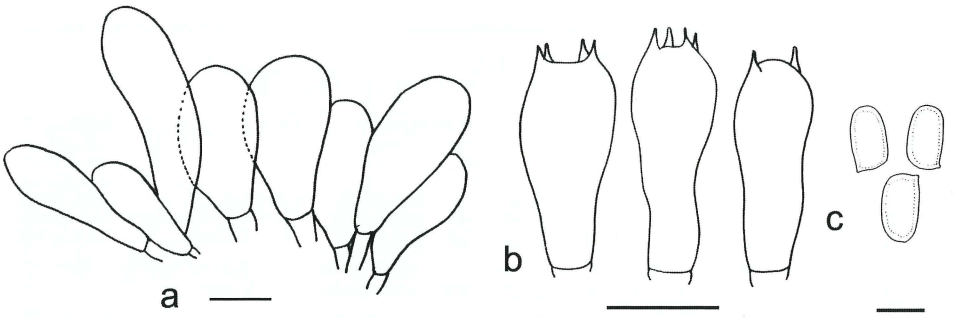


Fig. 3. **a.** Cheilocystidia; **b.** Basidia; **c.** Spores. Scale bars **a** and **b** = 10 μm; **c** = 5 μm.

Cheilocystidia 20-43 × 10-15 μm, abundant or lacking in old samples, broadly clavate to pyriform with cylindrical base, hyaline, smooth. *Pleurocystidia* absent. *Pileipellis* a cutis composed of hyphae of 5-10 μm diam., long, cylindrical, light brown, smooth, slightly constricted at the septa. *Annulus* hyphae 5-8 μm in diam., apex inflated to 10-13 μm in diam., cylindrical to long clavate, hyaline, smooth.

Habit: scattered or gregarious in the opened areas or litter layer of forest.

Material examined: THAILAND, Chiang Rai Prov., Mae Fah Luang University, 3 Aug. 2010, collector Jie Chen, LD030 (MFLU10 0774, **holotype**); Chiang Rai Prov., Doi Pui, 1 Sep. 2011, collector Jie Chen, LD2011026 (MFLU11 1309); Chiang Mai Prov., Mae Taeng Dist., Ban Pha Deng village, N 19°17.123' E 98°44. 009', elev. 900 m., 12 August 2005, collected by Kevin D Hyde, ZRL2124 (BBH 19503); same location, 13 August 2005, collected by Kevin D Hyde, ZRL2125 (BBH 19505); THAILAND, Chiang Mai Province, Mae Taeng District, Ban Pha Deng, Mushroom Research Centre, N 19°17.123' E 98°44.009', elevation 900 m, rainforest dominated by *Castanopsis armata*, *Erythrina* sp., and *Dipterocarpus* sp., 11 August 2010, collected by Samantha C. Karunarathna, NTS106 (MFLU10 0666).

DISCUSSION

Some recent new species and combinations in section *Minores*, such as *A. pseudolutosus, A. campbellensis* and *A. heinemannianus*, have been published from Europe and the subantarctic, (Esteve-Raventós 1998; Moreno. *et al.* 1999; Geml *et al.* 2007). *A. campbellensis* is easily differentiated from *A. megalosporus* as it has larger spores (7-8.8 × 4-4.5 μm) and lacks cheilocystidia. *A. heinemannianus* and *A. pseudolutosus* have a similar spore size to our new species, but the pileus of *A. heinemannianus* is covered by purplish-brown radially arranged fibres which never disrupt into triangular squamules; in *A. pseudolutosus* the base of the stipe tapers, which is different from the cylindrical-bulbous stipe in *A. megalosporus*. *A. aridicola* Geml, Geiser & Royse is a secotioid species (syn. *Gyrophragmium dunalii* (Fr.) Zeller) that morphologically differs from all the species of the section *Minores*. In fact, the phylogenetic position of this species is uncertain and appears, according to Zhao *et al.* (2011), more closely related to the tropical clades allied to the section *Minores* than to the section *Minores sensu stricto*.

On the basis of spore size, *A. megalosporus* can be separated from other species of section *Minores*, i.e., *A. xantholepis* (F.H. Møller) F.H. Møller (4-5.6 × 3-3.5 µm, Heinemann 1990), *A. diminutivus* Peck (4.5-5.3 × 3.3-3.9 µm, Heinemann 1990), *A. meijeri* Heinem. (4.2-5.5 × 3.3-4.1 µm, Heinemann 1993), *A. comtulus* Fr. (4-5 × 3-3.5 µm, Cappelli 1984; Heinemann 1986; Kerrigan 1986); *A. lutosus* (F.H. Møller) F.H. Møller (4-5.5 × 3-3.5 µm, Cappilli 1984) and *A. niveolutescens* Fr. (4-5.5 × 3-3.5 µm, Cappelli 1984)

There are some species in section *Minores* with spore size close to or overlapping with *A. megalosporus*. For instance, *A. dulcidulus* S. Schulz (often known as *A. semotus* or *A. purpurellus*) (Nauta 2001) is a common species of section *Minores*, which is distributed throughout Europe, Southeast Asia, South America and East Africa (Cappelli 1984; Heinemann 1980; 1986; 1990; Pegler 1977). *A. dulcidulus* differs from *A. megalosporus* by its often purple or pinkish brown pileus, smaller spores (5.1-5.6 × 3.4-3.7 µm) and cheilocystidia which occur in short chains of globose to quadrangular elements with clavate or globose terminal elements. *A. megalosporus* differs from *A. brunneolus* (J.E. Lange) Pilát, *A. nothofagorum* Fr., *A. singeri* Heinem., *A. viridopurpurascens* Heinem. and *A. goossensiae* Heinem. (Heinemann, 1956; 1974; 1980; 1990) by its cheilocystidial characters.

The species morphologically most similar to *A. megalosporus* are *A. johnstonii* Murr, *A. martinicensis* Pegler and *A. luteomaculatus* (F.H. Møller) F.H. Møller, because they share a similar spore size and cheilocystidia characters. However *A. johnstonii* has a cylindrical stipe, non-squamulose cap and no characteristic smell, whereas *A. megalosporus* has a bulbous stipe, typical squamulose cap and almond smell (Pegler 1983). *A. martinicensis* was treated as a member of subsection *Flavescentes* when it was first described, and it differs from *A. megalosporus* by its purplish vinaceous pileus and the characters of stipe which is cylindrical and slightly thickened at the base, and the surface of which is covered with granular squamules (Pegler 1983). *A. luteomaculatus* is a species originally described from Europe, but it is also distributed in the tropics (Pegler 1977; Heinemann 1956; Cappelli 1984). This species has slightly smaller, 4.5-6 × 3.2-4 µm, spores, a pale yellow pileus with a brownish centre and bright yellow staining on bruising. The hyphae of the pileipellis have vacuolar pigments and those are different from *A. megalosporus* (5.5-6-7.5 × 3.5-4 µm, pileus lacking a yellow tone and with no color change in bruising, membranous pigments in pileipellis).

Based on the morphological examination and molecular analysis, we introduce *A. megalosporus* as a new species in section *Minores*. This species is characterized by middle to large-sized fruiting bodies, a heavily fibrillose squamulose stipe and relatively large spores.

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