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Morphological Discretion of Basidiospores of the Puffball Mushroom *Calostoma* by Electron and Atomic Force Microscopy

KIM, MISUN[1], KI WOO KIM[2], AND HACK SUNG JUNG[1]*

[1]*School of Biological Sciences, Seoul National University, Seoul 151-742, Korea*
[2]*National Instrumentation Center for Environmental Management, Seoul National University, Seoul 151-921, Korea*

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Abstract Comparative morphology among species of the genus *Calostoma*, including *C. cinnabarina*, *C. ravenelii*, and *C. japonicum*, was investigated by scanning electron microscopy and atomic force microscopy. Spore morphology of *C. cinnabarina* and *C. ravenelii* showed no dramatic differences by light microcopy and scanning electron microscopy. To differentiate these species, atomic force microscopy was employed. Quantitative analysis of the surface roughness of basidiospores revealed subtle differences in height fluctuation at the nanometer scale between the species of *Calostoma*. Basidiospores of *C. cinnabarina* had a relatively rougher surface than those of *C. ravenelii* at 2.0×2.0 μm^2 scan areas.

Keywords: Morphology, ornamentation, scanning electron microscopy, atomic force microscopy, quantitative analysis

The genus *Calostoma* means beautiful mouth, because their features appear like a colored lip. In Korea, they are called "Yonji," which means one's rouge cheeks. *Calostoma* species have lost their spore discharge mechanism and possess enclosed spore-bearing structures [24]. The genus *Calostoma* has more refined basidiocarp structures than other gasteroid fungi; and pitted-spore reticulations are especially elaborated. Reticulated basidiospores are interwoven with nurse cells and highly scaled capillitial hyphae, and therefore, spores are prevented from being blown away all at once [11, 21]. Morphological and developmental studies of the genus remain controversial in resolving the taxonomic relationships within the taxon [20]. *Calostoma* and *Scleroderma* are maintained in the order *Sclerodermatales* [23], in which the arrangement of basidia in the gleba is emphasized. A previous molecular phylogenic study did not support their emphasis on the basidium and the stipe [8]. Currently, the genus *Calostoma* is classified in the *Tulostomatales* with other stalked puffballs [9]. The genus *Calostoma* shows an unusually large number of inferred nucleotide substitutions relative to other *Boletales* [16], indicating its extreme morphological divergence [10]. Castro-Mendoza *et al.* [5] considered the spore ultrastructure of *Calostoma* to be sufficient for moving the genus from the *Tulostomatales* to the *Sclerodermatales*. The absence or presence of the capillitium is an another character of the order *Sclerodermatacea*.

Ecological variations make it more difficult to identify. The exoperidium of the species in *Calostoma* develops a thicker and more gelatinous layer under the humid conditions of the deciduous forest [17]. As the viscid, lacunose, gelatinous stalk expands, the exoperidium sloughs off, exposing the endoperidium and raised, delimited peristome. Since these taxa may fruit in late fall or winter, the exoperidium may serve to protect the maturing gleba from the changes of weather as well as insect attack. Although molecular studies have provided insights into the phylogeny of *Calostoma* [3], it is necessary to evaluate spore ornamentations or reticulum morphology for discretion of species within the genus [12].

Atomic force microscopy (AFM) is a family of microscopy that forms surface images using a physical probe that scans the specimen. Since its inception in the mid-1980s, AFM has played a unique and pivotal role in disciplines ranging from physics to microbiology [1, 6, 14, 18, 22]. The images are generated not by using an incident beam such as visible light and laser, but by measuring physical interactions between the tip and the specimen. It might be analogous to sensing surface features by passing a finger over the surface, so it can be regarded as lensless imaging together with scanning tunneling microscopy [19]. One of the advantages of AFM is that the topographic data obtained by AFM contain the x, y, and z-coordinates of the specimen, which allows surface profiles to be quantitatively analyzed [2].

The fine structure of *Calostoma* basidiospores by electron microscopy has earlier been compared between species of *Calostoma*; however, quantitative aspects of spore

*Corresponding author
Phone: 82-2-880-6708; Fax: 82-2-872-1993;
E-mail: minervas@snu.ac.kr

ornamentations could not be obtained in the previous study [5, 10]. In spite of the importance of surface feature or ornamentation in fungal taxonomy, only limited information is available on the quantitative measurement of the surface structure of fungi [13, 15]. AFM can be a suitable method for this purpose, since it can provide quantitative information on surface topography and its morphometry, as emphasizing on the surface reticulations of fungal spores. Here, we report a comparative morphological study of *Calostoma* species by complementary microscopy for species discretion.

Three species of *Calostoma* were used for comparative morphology in this study. Two species were collected in Korea as follows: (i) *C. ravenelii* (Berk.) Massee (SFC040825-62) sampled from Mt. Deogyu in 2004 and (ii) *C. cinnabarina* Desv. (SFC030828-81) sampled from Mt. Jiri in 2003. *C. japonicum* Henn. (TMI EN05-108NR) was provided from the Tottori Mycological Institute in Japan. Identification was verified by comparing with a previous taxonomical study [5, 10], and complementary 18S nuclear ribosomal gene sequence (rDNA) was compared with downloaded sequences of *Calostoma* species from GenBank (data not shown). Each species inquired three specimens out of several isolates, and the isolates were stored in paper envelopes (10×22 cm^2) at room temperature and subjected to specimen preparation for microscopy and morphological analyses.

Fungal materials were mounted on a metal stub using two-sided adhesive carbon tape. The specimens were then coated with gold and examined with a scanning electron

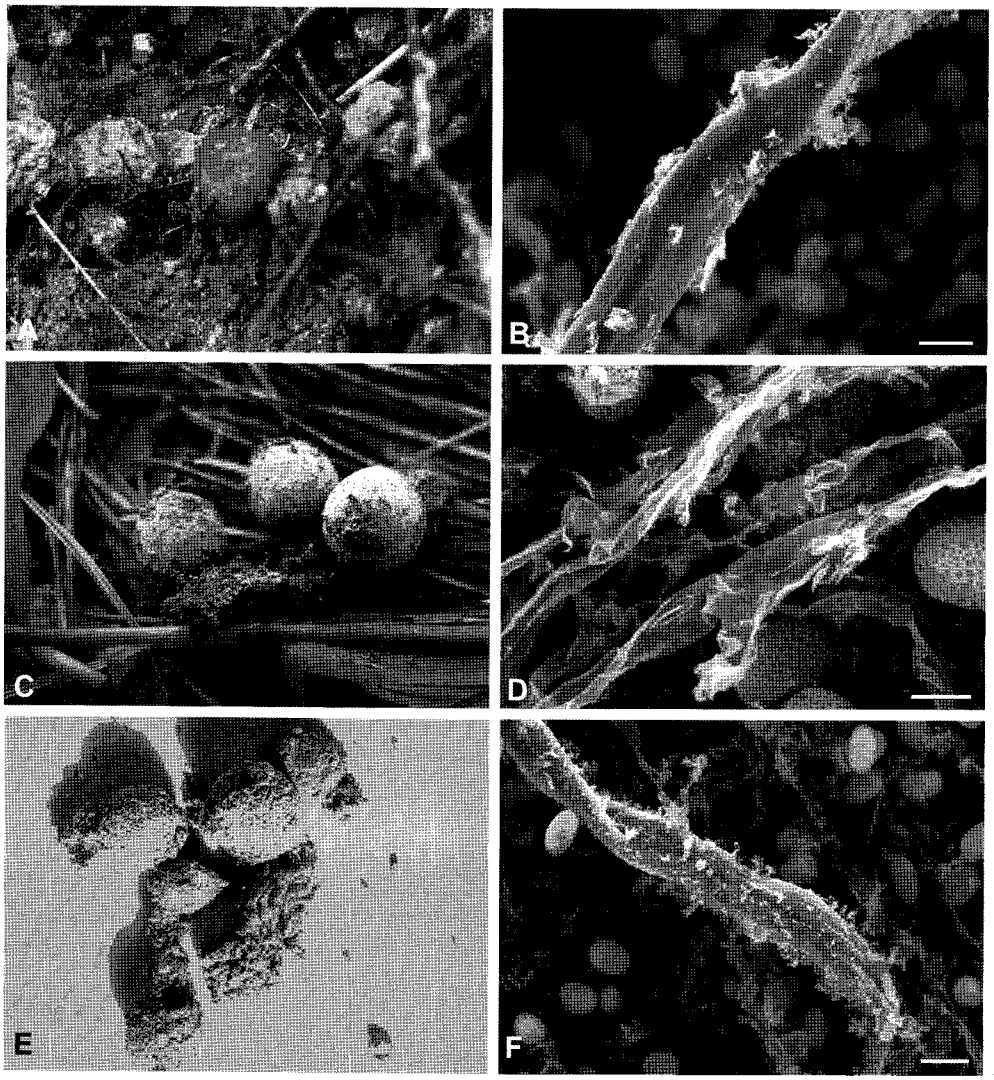

Fig. 1. Morphological characteristics of *Calostoma* species.
A. Fruitbodies of *C. cinnabarina* in nature. Basidiocarps 8–10 mm thick, almost entirely dark orange colored. **B.** Capillitial thread of *C. cinnabarina*, Bar=10 μm. **C.** Fruitbodies of *C. ravenelii* in nature. Basidiocarps 5–10 mm thick, creamy colored with red ostiole. **D.** Capillitial thread of *C. ravenelii*, Bar=5 μm. **E.** Naturally dried fruitbodies of *C. japonicum*, 5–9 mm thick, creamy colored with red ostiole. **F.** Capillitial thread of *C. japonicum*, Bar=10 μm.

microscope (JSM-5410LV; JEOL, Tokyo, Japan) at an accelerating voltage of 20 kV.

The two Korean species of *Calostoma* were subjected to AFM analysis. Basidiospores of the two species were mounted on a steel disc using two-sided adhesive tape, respectively. The steel disc was then magnetically fixed to the piezoscanner (maximum x, y-scan range of 20 μm) of the AFM (SPA-400; Seiko Instruments, Tokyo, Japan). Non-contact AFM was performed to acquire topographic images under ambient conditions using standard cone-shaped silicon cantilevers (PPP-NCHR; Nanosensors, Neuchatel, Switzerland) with a specified spring constant of 42 N/m. Topographic images over 2.0×2.0 μm^2 scan areas were recorded from at least three basidiospores of each species selected randomly on the disc. To quantify surface profiles of basidiospores from acquired topographic images (region analysis), the conventional statistical surface parameters including mean height, root-mean-squared (RMS) roughness (standard deviation of all height values within the scanned area), and surface area were analyzed and processed using a software package provided with the microscope. Moreover, from at least 20 line profiles of each species (line analysis), the maximum peak-to-valley height and the angle between the lines connecting the peak and valley of the basidiospore reticulations were obtained and analyzed as described in the region analysis.

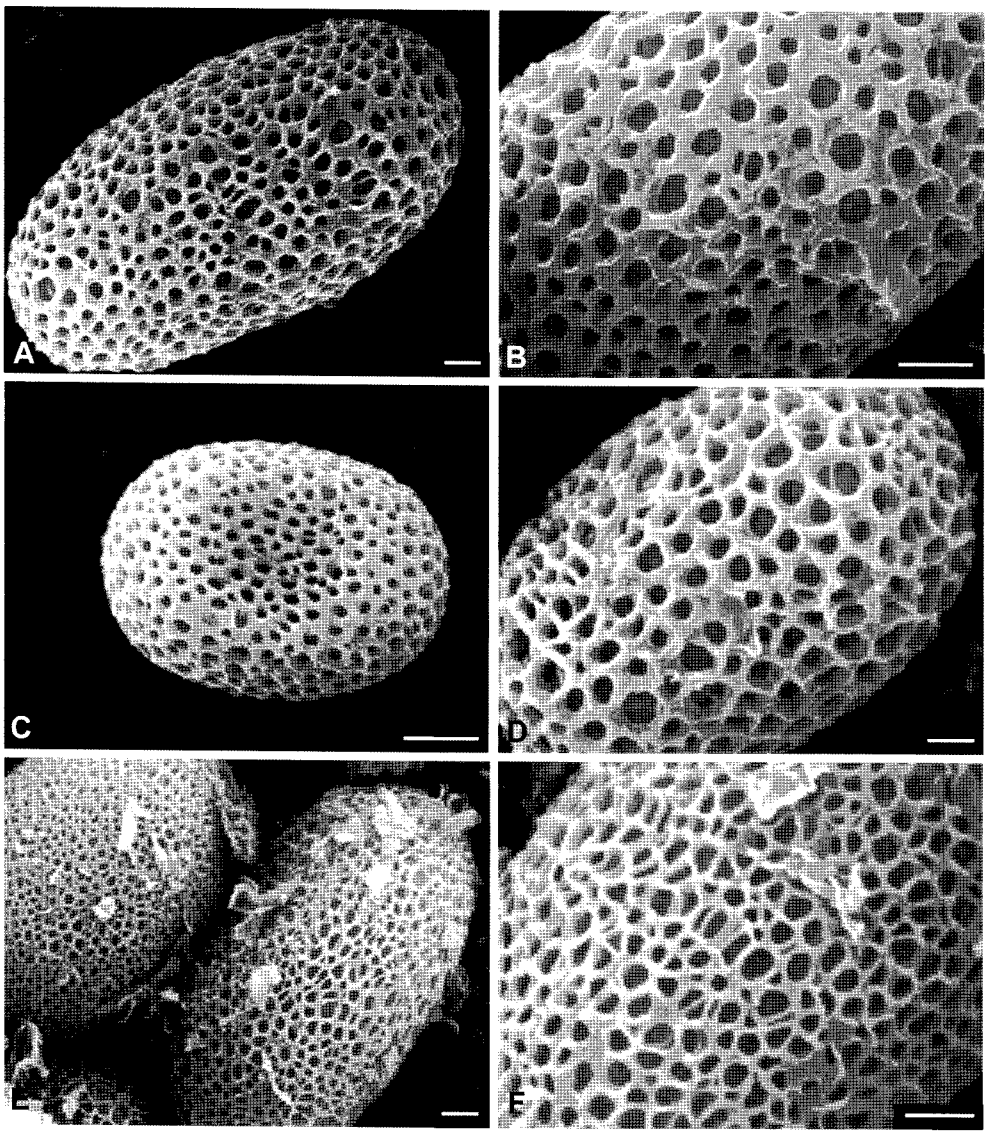

Fig. 2. Scanning electron micrographs of basidiospores of *Calostoma* species.
A. A whole basidiospore of *C. cinnabarina*, highly reticulated cylindrical basidiospores, Bar=1 μm. **B.** *C. cinnabarina* has no feeder cells or nurse cells, Bar=1 μm. **C.** A whole basidiospore of *C. ravenelii*, cylindrical white to cream color, Bar=2 μm. **D.** *C. ravenelii* has no feeder cells or nurse cells, Bar=1 μm. **E.** Whole basidiospores of *C. japonicum*, oblong to broadly ellipsoidal basidiospores, Bar=1 μm. **F.** *C. japonicum* has many scales around the more reticulated spores and has many debris, Bar=1 μm.

In this study, we provided microscopic data of two species of *Calostoma* by light microscopy and SEM. There were some morphological differences between Korean and Japanese *Calostoma*, which may partially be due to geographical variations. Fruitbodies of *C. cinnabarina* were 8–10 mm thick, almost entirely dark orange colored, and globose to subglobose shaped. The exoperidium was thick, hyaline, falling off at maturity; the mesoperidium was hard, orange, mostly falling off with the exoperidium; basidia were not seen; hyaline to yellow in Melzer's reagent (Fig. 1A). The hyphal system of *C. cinnabarina* was monomitic, thick-walled, nonbranched; basidiospores were intermingled with capillitial threads (Fig. 1B). Fruitbodies of *C. ravenelii* were 5–10 mm in size, attached tightly to the substrate; surface whitish to brownish to the substrate and distinctly bounded with rhizoid, shaped globose with small warts of exoperidial material. It was entirely orange colored; however, it was sometimes decolored at maturity without ostiole. The exoperidium was hard and dry, persisting at maturity, yellow in Melzer's reagent; the endoperidium was hard, whitish grey, remaining as a globose head; abundant clamp connections (Fig. 1C). Thick-walled hyphae of *C. ravenelii* were usually nonbranched and its scales were abundant (Fig. 1D). Fruitbodies of *C. japonicum* had well-developed red ostiole; the rhizoid was prominent and 5–9 mm in size, globose, almost sessile (Fig. 1E). *C. japonicum* had many scaled capillitial hyphae around more reticulated spores and had many debris of capillitial hyphae, which are called feeder cells or nurse cells. The capillitial thread was fragile, 5–7 (up to 9) μm wide (Fig. 1F). The two Korean isolates had white to creamy colored spores in the gleba, and the fruitbodies were larger than the Japanese species. Electron microscopic study clearly revealed morphological differences between the Korean and Japanese species, but differences of basidiospores within the Korean species were relatively small (Fig. 2). Basidiospores of *C. cinnabarina* were mostly oblong to elliptical in shape, measured approximately

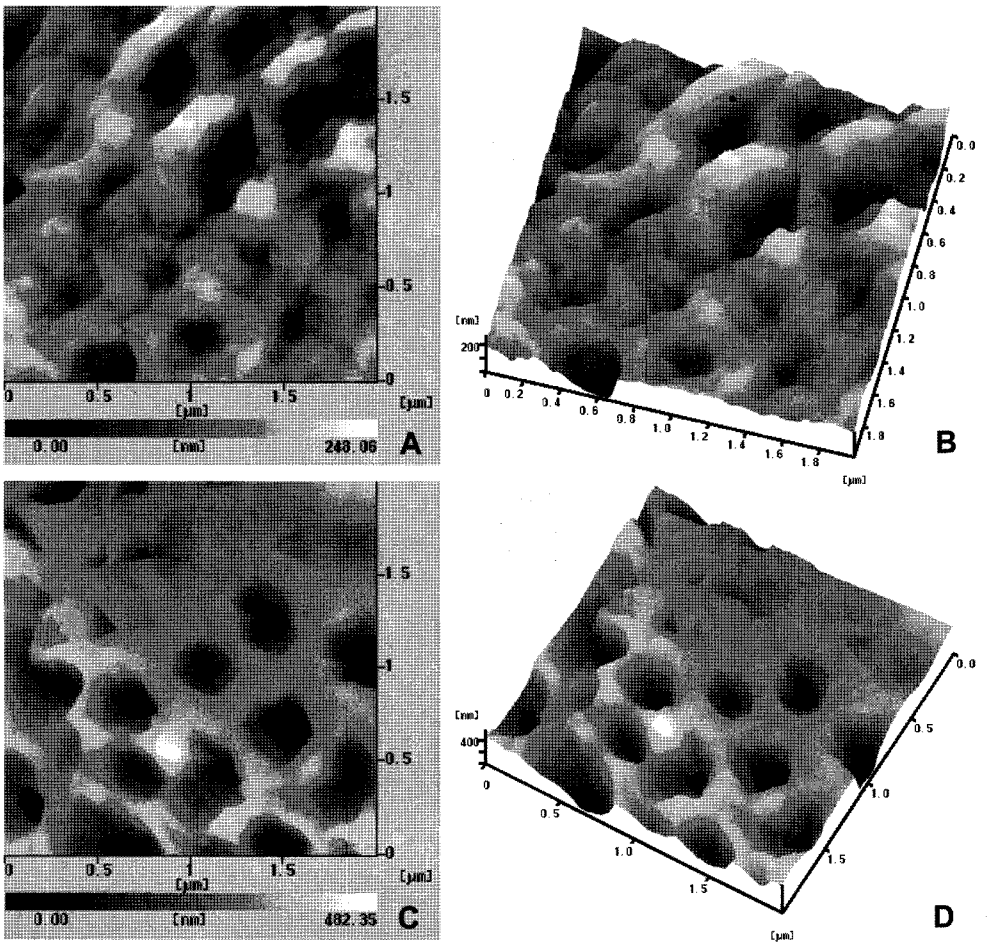

Fig. 3. Atomic force micrographs of *Calostoma* species.
A. Two-dimensional image of *C. ravenelii*. The height of spore surface was up to approximately 250 nm. Brightness corresponds to vertical amplitude in the color scale format where higher points are brighter and lower points are darker. **B.** Three-dimensional rendering of Fig. 4A. **C.** Two-dimensional image of *C. cinnabarina*. The height of spore surface was up to approximately 480 nm. **D.** Three-dimensional rendering of Fig. 4C.

5–8×10–20 μm in length (Fig. 2A), and had an elaborate recticulum, which pored 2–3 per 1 μm (Fig. 2B). Basidiospores of *C. ravenelii* were mostly ovoid in shape, measured approximately 5–6×10–12 μm in length (Fig. 2C), and its shape of reticulum was similar to those of *C. cinnabarina* (Fig. 2D). Basidiospores of *C. japonicum* were oblong to broadly ellipsoidal in shape and mostly 12–14.5×6.6–7 μm in size and yellowish colored (Fig. 2E), and the recticulum of *C. japonicum* was more elaborate, which pored 3–4 per 1 μm (Fig. 2F).

AFM exhibited surface topographic images of basidiospores under ambient conditions (Fig. 3). Prominent reticulations were readily observed on the spore surface of the two species. The maximum peak-to-valley height of *C. ravenelii* basidiospores was approximately 250 nm, measured from the highest point on the adjacent peak by the color scale of the topographic images (Fig. 3A). Reticulations were not uniform in size and arranged with a lateral diameter of 200 to 500 nm. Surface fluctuations were more apparent in three-dimensional renderings than two-dimensional images (Fig. 3B). Moreover, *C. cinnabarina* basidiospores showed deep valleys of reticulations throughout the scan areas (Fig. 3C and 3D). The height was up to approximately 480 nm. Furthermore, the line profile analysis unraveled the height differences of reticulations between the two *Calostoma* species at the nanometer scale. Based on the acquired topographic images (Fig. 4A and 4B), each line profile of the two species revealed surface fluctuations (Fig. 4C and 4D). The peak-to-valley height of *C. ravenelii* basidiospores was less than 250 nm, whereas that of *C. cinnabarina* basidiospores amounted to approximately 400 nm.

Subtle differences in height fluctuation of the two species of *Calostoma* could be resolved at the nanometer scale by quantifying surface profiles of the acquired topographic images. Numerical analysis of the surface parameters over 2.0×2.0 μm^2 scan areas revealed a relatively rougher surface of *C. cinnabarina* basidiospores than *C. ravenelii* (Table 1). Results from the numerical analysis of surface parameters of the region analysis were consistent with those of the line profile analysis. The mean height of *C. cinnabarina* basidiospores in the scan areas was greater than that of *C. ravenelii*. Moreover, the results from RMS roughness and surface area measurements supported

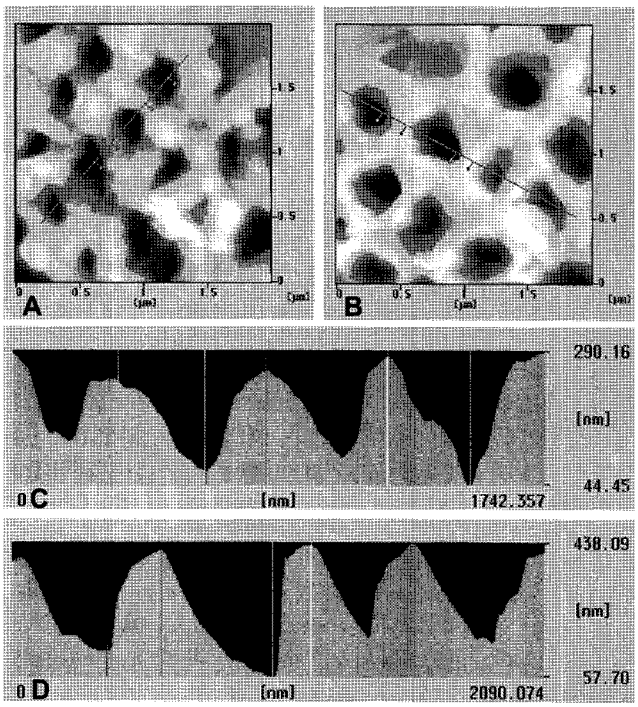

Fig. 4. Atomic force micrographs of *Calostoma* species.
A. Two-dimensional image of *C. ravenelii*. **B**. Two-dimensional image of *C. cinnabarina*. **C**. Height profile of a straight line in 4A. The height of the spore surface was up to approximately 290 nm. Vertical lines in the height profile are positioned at peaks and valleys along the straight line. **D**. Height profile of a straight line in 4B. The height of the spore surface was up to approximately 440 nm.

a rougher surface of *C. cinnabarina* basidiospores than *C. ravenelii* basidiospores. In particular, the RMS roughness and peak-to-valley height of *C. cinnabarina* basidiospores were approximately one and a half times greater than *C. ravenelii* basidiospores, as shown in the line profile analysis. This study demonstrated the application of AFM for imaging and quantitative surface analysis of reticulate basidiospores of *Calostoma* for species discretion. For surface observation, the SEM and AFM that had been employed in this study provided different types of information on the surface morphology. One key advantage of SEM is its long depth of field and small electron beam size that made it possible to image reticulate structures far below the top layer of *Calostoma* basidiospores. Nevertheless, conventional SEM

Table 1. Comparison of surface parameters of two species of *Calostoma* basidiospores.

Fungal species	Region analysis[a]			Line analysis[b]	
	Mean height (10^1 nm)	RMS roughness (10^1 nm)	Surface area (10^6 nm^2)	Peak-to-valley height (nm)	Angle (degree)
C. ravenelii	4.12±0.77	5.25±0.97	5.28±0.22	182.95±53.50	38.51±12.61
C. cinnabarina	6.37±0.51	8.15±0.80	6.57±0.80	309.98±65.90	53.23±12.64

[a]The values in columns indicate the arithmetic means and standard deviations of each parameter from scan areas (2.0×2.0 μm^2) in each specimen.
[b]The values in columns indicate the arithmetic means and standard deviations of each parameter from line profiles in acquired scan images.

could not obtain quantitative information on the surface parameters of the spores. On the other hand, AFM is intrinsically a nondestructive three-dimensional imaging tool that is of great importance when elaborate surface ornamentations have to be morphometrically analyzed. In this study, some spore surfaces of *Calostoma* were found to be highly rough and exceeded the maximal vertical performance range of the AFM equipment (data not shown). Despite the limitation, it was evident that AFM could provide unique information on "untreated" surface features that is beyond the capability of conventional microscopic techniques.

In conclusion, this study advanced our knowledge on the surface characteristics of *Calostoma* by AFM. To the best of our knowledge, this is the first report on the imaging and nanoscale morphometry of the puffball mushroom using AFM. However, the versatility of AFM on diverse isolates and allied genera should be validated in future to confirm its broad-spectrum efficacy and to establish standardized analysis procedures for routine applications. To make a new concept of polyphasic species of *Calostoma*, we should have special criteria for resolving spore ornamentation between closely related taxa, and nanotechnology such as AFM analysis can be used to meet the needs in mycology.

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