Fine structure in ascosporogenesis of freeze-substituted *Arthroderma simii*

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Summary We applied the freeze-substituion method to the detailed observation of the ascosporogenesis in *Arthroderma simii*. The ascospore formation of *A. simii* underwent the same processes as did that of other dermatophytes, namely an enveloping membrane system (EMS) composed of two invaginated unit membranes that delimit the daughter nuclei in the cytoplasm of the ascus. The inner leaflet of the EMS was found to have changed into an ascospore plasma membrane, whereas the outer leaflet and intercisternal space became the ascospore cell wall.
In the freeze-substituted asci, we could not observe the lomasomal structure thought to be the origin of the EMS in dermatophytes.

Key words Ascosporogenesis, *Arthroderma simii*, Lomasome, Freeze-substitution method, Ultrastructure

As previously reported [1], we have investigated the process of ascosporogenesis of *Arthroderma simii*, which has been considered to be closely related to members of the *Trichophyton mentagrophytes* complex [2], using scanning and transmission electron microscopy by conventional chemical fixation method. Freeze-substitution method has recently been applied to fungal cells as a preparation technique for electron microscopy and is generally considered to be a superior method of cell structure preservation, diminishing artifacts associated with chemical fixation [3].

In the present study, we applied the freeze-substitution method to the detailed observation of the ascosporogenesis in *A. simii*. The results were compared with those materials obtained by the conventional chemical fixation procedure.

Fungi and culture. Both mating types of *A. simii*, CBS 448.65 "+" and CBS 520.75 "-" were inoculated on the plates of Sabouraud dextrose agar diluted 1/10 with salts as described by Takashio [4] and incubated at 27°C. After several weeks, gymnothecia developed on the marginal zone between the two inocula.

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Details of the sample preparation for SEM and conventional electron microscopy of *A. simii* have been described previously [1]. For freeze-substitution, initial immobilization of gymnothecia was achieved in liquid propane. The specimens were transferred to substitution fluid consisting of 2% osmium tetroxide in anhydrous acetone and were substituted at -79°C in dry-ice acetone for 3 days, -20°C for 3 hours, -4°C for 3 hours. Following substitution, the specimens were embedded in Spurr's resin. Ultra-thin sections were double stained as previously reported [1].

Scanning electron microscopy. A gymnothecium, resembling a ball of wool, produced after 4 weeks of incubation, was shown (Figure 1a). The gymnothecium was attached to the culture medium with hyphae. The inside of the gymnothecium at this stage is shown (Figure 1b). Many smooth-surfaced, egg-shaped asci were observed. The walls of some asci were disrupted, and developing ascospores were observed.

Transmission electron microscopy. Figures 2a, 3a, 4a and 5a were obtained using transmission electron microscopy by conventional chemical fixation method. Figures 2b, 3b, 4b and 5b were obtained at each corresponding stage by the freeze-substitution method. In the freeze-substitute specimen, the ascus plasma membrane is characteristically smooth in profile. In double membrane-bounded organelles, e.g., nuclei and mitochondria, the two membranes generally exhibit a parallel and smooth profile.

Comparison of conventional chemical fixation method and freeze-substitution method in TEM. At an early stage there were asci containing membranous structures composed of two unit membranes at the peripheral zone of the ascus (Figure 2a). The membranous structure was considered to be an eveloping membrane system, which had been observed in previous studies of ascomycetous fungi. Glycogen granules were seen clustered in

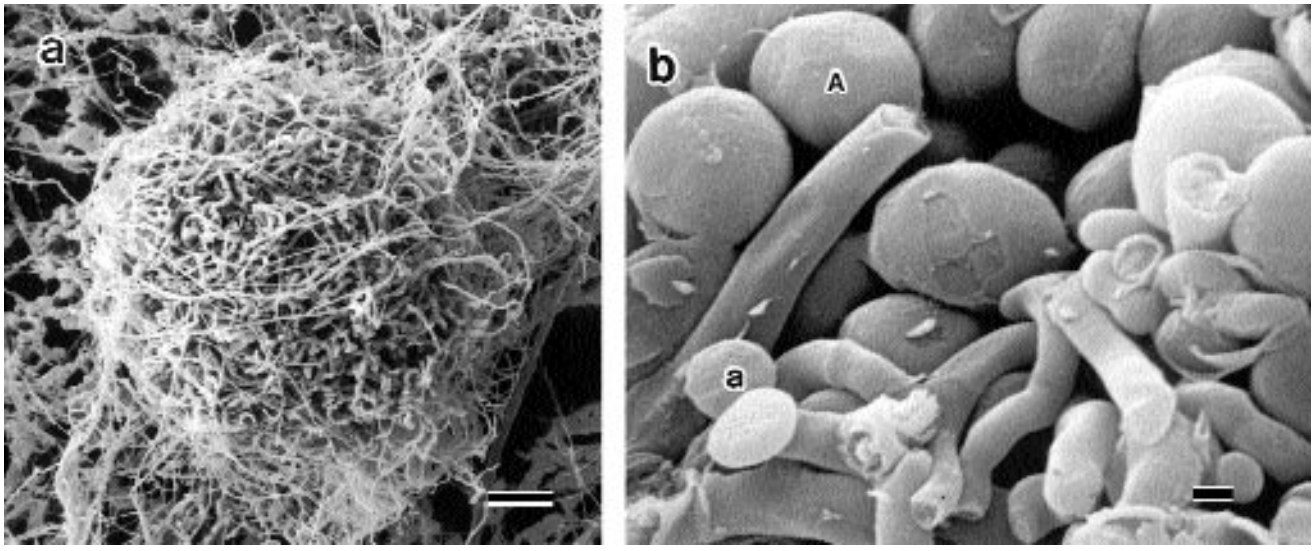


Figure 1. a: A gymnotecium observed after 4 weeks of inoculation. The gymnotecium is attached to the surface of the medium by developed hyphae; Bar = 50 µm.

b: Asci observed inside the gymnotecium obtained after 4 weeks of inoculation. Developing ascospores were observed through the disrupted cell wall of an ascus. Ascus (A) and ascospore (a) are marked; Bar = 1µm.

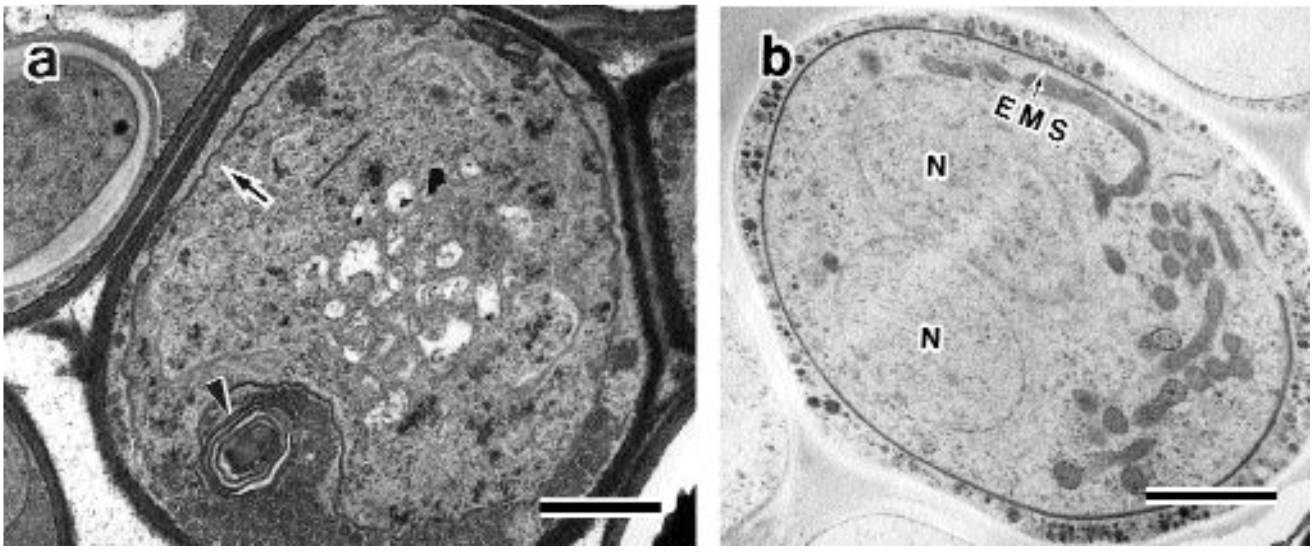


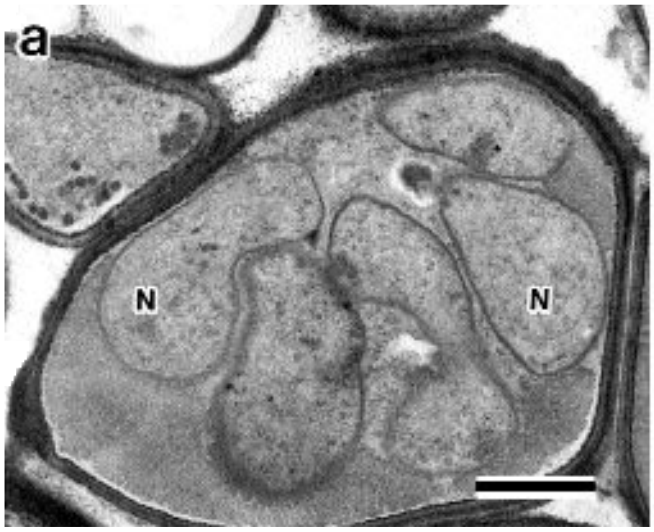
Figure 2. a: In the ascus, near the ascus plasma membrane, there were two unit membranes (arrow), which were considered to be an enveloping membrane system. The concentric membrane system is marked (arrow head), g: glycoprotein granules;

b: At the same stage observation by the freeze-substitution me-thod, the myelinated membranes could not be seen. The enveloping membrane system (EMS) and nucleus (N) are marked; Bars = 1µm.

the space between the enveloping membrane system and the ascus plasma membrane. Other membrane structures were also seen in the space between the enveloping membrane system and the ascus plasma membrane. The structure consisted of multiple membranes with a myelinated appearance and amorphous material located in the central area. It was considered to be a structure similar to the concentric membrane system [6] observed in *Arthroderma vanbreuseghemii*. At the same stage in the freeze-substitution method, the enveloping membrane system was observed, while the myelinated membrane, corresponding to the concentric membrane system, could not be seen (Figure 2b). At the stage of ascus development, the enveloping membrane system invaginated, and delimited daughter nuclei were seen (Figure 3a, b). At the more advanced stage in the ascus, the space between the two unit membranes of the ascospore initially became wider and the primary ascospore cell wall materials were deposited in the inter-membranous space. There were no apparent morphological differences between outer and inner membranes at this stage (Figure 4a, b). Subsequently, electron-dense materials aggregated outside the primary ascospore cell wall. As a result, secondary wall formation of the ascospore was completed (Figure 5a, b).

Beckett (1981) has noted that there was more or less universal agreement that during subcellular events in the ascus two mechanisms were at work [7]: i) nucleate portions of cytoplasm were delimited by an envelope of two unit membranes, ii) ascospore wall material was deposited between these two membranes which separate as the spore matures.

These processes have been well documented in some dermatophytes i.e. *A. vanbreuseghemii* [8], *A. gypsea* [9], and *A. benhamiae* [10]. We have also observed the similar processes in the ascosporogenesis of *A. simii*, a species closely related to members of the *Trichophyton mentagrophytes* complex, using scanning and transmission electron microscopy by conventional chemical fixation method [1]. In the present study, we applied the freeze-



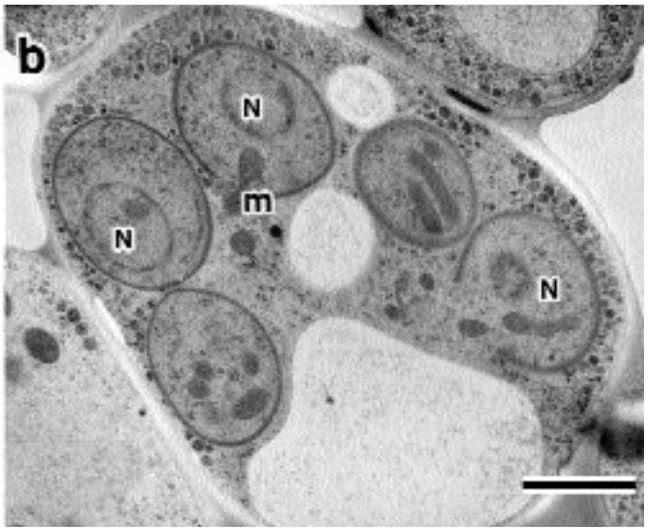
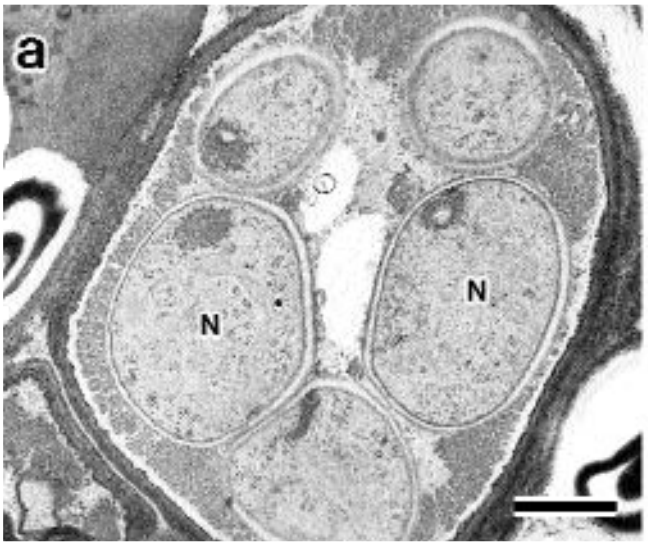


Figure 3. a: At the next stage in the ascus, the enveloping membrane system invaginated, delimiting daughter nuclei; N: nucleus

b: Observation by the freeze-substitution method at the same stage; Nucleus (N) and mitochondria (m) are marked; Bars = 1μm.



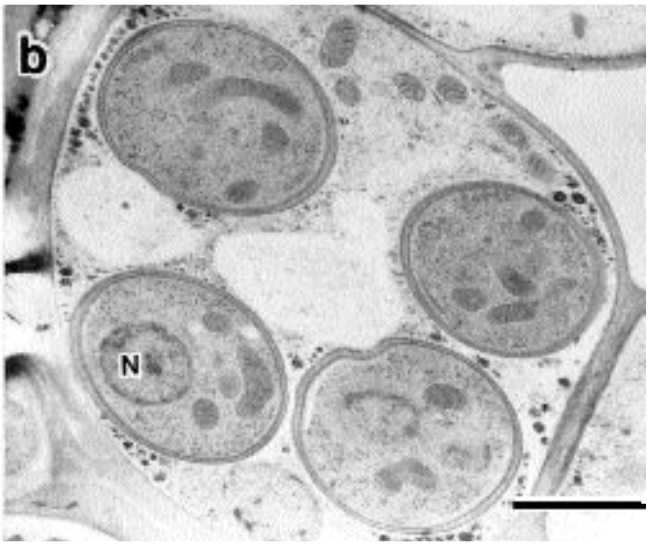
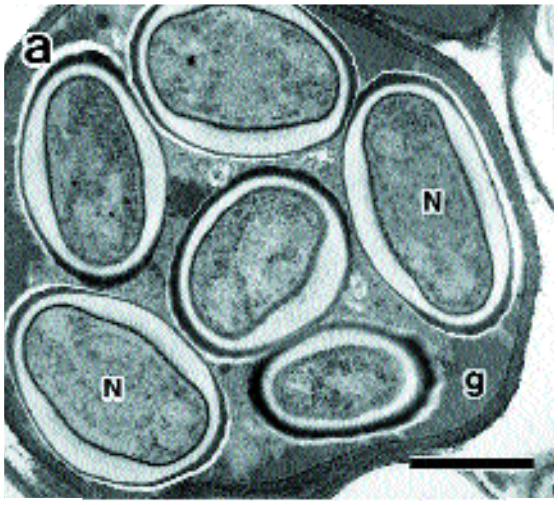


Figure 4. a: The intertcisternal space of the enveloping membrane system became widespread and a primary ascospore cell wall was deposited. No morphological difference between outer and inner membrane had been found at this stage; N: nucleus

b: Observation by the freeze-substitution method at the same stage; N: nucleus; Bars = 1μm.



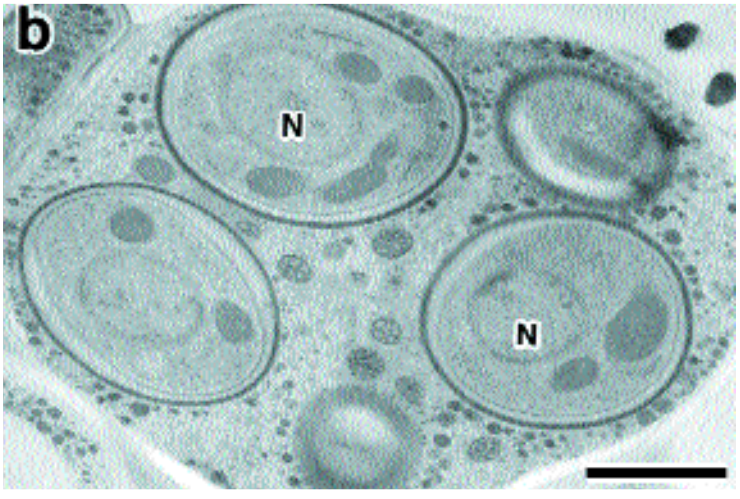


Figure 5. a: The electron-density of the secondary ascospore cell wall increased markedly ;Nucleus (N) and glycogen granules (g) are marked;

b: Observation by the freeze-substitution method at the same stage; the surface of the ascospores was smooth and the fold was not seen; N: nucleus; Bars = 1μm.

substitution method to the detailed observation of the ascosporogenesis. In the freeze-substituted asci of *A. simii*, we could not observe the lomasomal structures, i.e., myelin figures [9], mesosomes [11], which had been thought to be the origin of the enveloping membrane system in dermatophytes. These lomasomal structures, however, are regarded as fixation artifacts by many researchers at present [12]. Our results also supported that consideration. Recently, two studies using freeze-substituion have shown that the membranes of the enveloping membrane system in filamentous ascomycetes originate from the ascus plasma membrane [13,14]. However, the continuity between the enveloping membrane system and the ascus plasma membrane could not be found in *A. simii*. Though the enveloping membrane system was suggested to concern Golgi apparatus in *A. vanbreuseghemii* closely related to *A. simii* [15], further studies are needed to elucidate the origin of the enveloping membrane system.

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