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Ectomycorrhizal morphotypes of naturally grown *Abies firma* seedlings

Yosuke Matsuda* and Naoki Hijii

Laboratory of Forest Protection, School of Agricultural Sciences, Nagoya University, Nagoya 464–8601, Japan

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Ectomycorrhizas of naturally grown Momi fir (*Abies firma*) seedlings were characterized based on morphological features of fungal partners. A total of 128 seedlings were collected over three years (1995–1997) from a 10 × 30 m plot where occurrences of ectomycorrhizal fungal fruitbodies were monitored for the same period. Thirty-seven morphologically distinct ectomycorrhizal types were distinguished based mainly on the color of ectomycorrhizas and the characteristics of fungal mantles. Type 37 was thought to be *Cenococcum geophilum* because of the jet-black mycorrhizas and the characteristic structure of mantle surfaces. For half of the classified morphotypes, fungal partners were inferred to be the genera *Lactarius*, *Russula*, and *Tuber*, and unidentified Basidiomycetes, based on earlier references.

Key Words——*Abies firma*; fir mycorrhizas; morphotypes; *Russula*; seedlings.

Mycorrhizal associations have increasingly been recognized to have significant influences on forest ecosystems by enhancing the nutrient uptake of host trees and improving the adaptation of host trees to various environmental conditions (Brundrett, 1991; Smith and Read, 1997). Earlier studies have revealed that several species of ectomycorrhizal (ECM) fungi are involved in mycorrhizal associations, constituting a fungal community specific to host trees (Molina et al., 1992; Trappe, 1962). The structures of such communities have been investigated based on the occurrence of ECM fruitbodies above or below the ground at various habitats (Arnolds, 1992; Griffiths et al., 1996; Matsuda and Hijii, 1998; Murakami, 1987; Tyler, 1994). The abundance of fruitbodies of an ECM fungus does not always reflect that of the ECM roots nor that of vegetative hyphae of the ECM fungus below the ground (Gardes and Bruns, 1996; Menge and Grand, 1978). In addition, fruitbody surveys have often overlooked ECM fungi which form smaller fruitbodies or hypogeous ones, and imperfect fungi such as the well-known *Cenococcum geophilun* Fr. have totally been ignored by this approach. Thus, characterization and identification of ECM roots and/or hyphae of ECM fungi below the ground are essential for elucidation of the community structure of ECM fungi.

Agerer (1986a) and Ingleby et al. (1990) have established techniques for characterization of ECM roots featuring fungal sheaths, i.e., plan views of the mantle surface, associated hyphae, and rhizomorphs. Using these techniques, morphologies of ectomycorrhizas have been described for several tree species: *Alnus rubra* Bong. (Miller et al., 1991), *Picea abies* (L.) Karst (Agerer, 1986b, 1987, 1988; Brunner et al., 1991), *Picea glehnii* (Fr. Schm.) Masters (Kasuya et al., 1995), *Pinus densiflora* Sieb. et Zucc. (Yamada and Katsuya, 1996) and *Shorea leprosula* Miq. (Lee et al., 1997).

Momi fir (*Abies firma* Sieb. et Zucc.), which forms ectomycorrhizas (Masui, 1926; Nara et al., 1992), is a major tree species in warm-temperate forests in Japan (Oohata, 1994). The fir occasionally forms pure stands, but more often mixes with other tree species (Kitamura and Murata, 1984). Masui (1926) confirmed an ECM association between *A. firma* and *Gomphus floccosus* (Schw.) Sing. by tracing mycelial connections between ECM roots and the fruitbodies. He classified mycorrhizas of *A. firma* into four types, e.g., *G. floccosus* mycorrhizas and an unknown species of flask-shaped cystidia forming mycorrhizas; and Nara et al. (1992) also showed three types of *A. firma* mycorrhizas. However, their criteria were based exclusively on cross sections of the mycorrhizas and attached fungal features of the sections. Thus, little information about the plan view of mycorrhizas has yet been provided for *A. firma*. Moreover, observations of *A. firma* mycorrhizas have so far been conducted only for the medium-aged, ca 30–50-year-old, trees (Masui, 1926; Nara et al., 1992), and hence there are no descriptions of ECM roots on seedlings, which represent an important growth stage in the establishment and survival of the plants.

The objective of this study is to describe mycorrhizas formed on naturally grown *A. firma* seedlings by characterizing the morphological features of fungal partners. The identity of the associated fungus forming each ECM morphotype was also considered based on earlier descriptions (e.g., Agerer, 1987–1996; Ingleby et al., 1990; Goodman et al., 1996).

* Corresponding author.
E-mail: i961206d@mbox.media.nagoya-u.ac.jp

Materials and Methods

Study site The study site was located in Inabu Town, Aichi Prefecture in central Japan (680 m above sea level; 35°11′N, 137°33′E). The site was occupied mostly by a man-made stand of Japanese cedar (*Cryptomeria japonica* D. Don) and Japanese cypress (*Chamaecyparis obtusa* Endl.), both of which are known to form vesicular-arbuscular mycorrhizas (Mizoguchi, 1996), and by a naturally regenerated forest mainly consisting of *A. firma*. Both floors had a sparse understory consisting of *Lindera triloba* Blume. Further description of the vegetation at the site was given by Matsuda (1994). The soil was classified as Bl- or Be-type based on the criteria of Classification of Forest Soil in Japan (Forest Soil Division, 1976). The pH of the soil 10 cm below the litter layer was 4.7. The mean annual precipitation and mean air temperature at the nearest weather station (505 m above sea level; 35°13′N, 137°31′E) from 1979 to 1990 are 1951 mm and 11.1°C, respectively.

Sampling of seedlings A study plot of 10 × 30 m was set on the boundary between the man-made stand and the regenerated fir forest. Current-year or one-year-old seedlings of naturally grown *A. firma* were taken for root samples from the plot for three years, from 1995 to 1997. The seedlings were put in plastic bags for transfer to the laboratory and kept at around 4°C until observation. A total of 128 seedlings were collected from all over the plot. Within a few days after each sampling, the seedlings were washed carefully in running tap water, then soil particles adhering to the root tips were removed with fine forceps under a stereoscopic microscope.

Observation and description of ectomycorrhizas The whole root system of fir seedlings was soaked in a Petri dish filled with tap water. Root tips were observed under the stereoscopic microscope (up to × 160) and categorized by color as black, brown, cream, white, or other color, as described. Since the root systems were not yet ramified at the seedling stage, ramification pattern was not taken into account.

After description of colors, only fresh ectomycorrhizas (Harvey et al., 1976) were used for further microscopic examinations, and apparently wrinkled or shrunk mycorrhizas and non-mycorrhizal roots were excluded. A fresh mycorrhizal tip was placed on a glass slide, and the proximal part of the tip was sliced tangentially with a scalpel to confirm the presence of a Hartig net. The rest of the tip was cut into two parts longitudinally to examine the feature of mantle surfaces. These root fragments were stained with 0.1% (w/v) of aqueous toluidine blue and with 0.1% (w/v) of cotton blue in 10% (v/v) lactophenol/H_2O for about 20 s each according to Ingleby et al. (1990). The slides were examined under a light microscope (up to × 1000 with an object lens of 40 magnification and a drawing apparatus of 1.25 magnification) with respect to the following features: the plan view of the surface layer of fungal mantles, the morphology and diameter of emanating hyphae, presence or absence of clamp connections, presence or absence of cystidia and their shapes. Distinct features of mantle surfaces other than those listed above were also described. The terminology followed Ingleby et al. (1990) for mantle structures.

Results

Classification of ectomycorrhizas Thirty-seven morphologically distinct ectomycorrhizal types were classified based on the macro- and microscopic features (Table 1).

Macroscopic features Four mycorrhizal types showed characteristic colors of the fungal mantle: type 15 was slightly purple, and types 35, 36, and 37 were blackish (Table 1). Besides differences in color, fungal mantles of types 8, 9, and 33 were cottony, while those of types 1 and 10 were grainy in texture. The mycorrhizas of types 2 and 4 were woolly or stringy, and emanating hyphae were occasionally bundled together forming rhizomorphs. Type 14 differed distinctly from other types in its colorless and transparent gelatinous fungal sheath.

Microscopic features Microscopic observation revealed informative characteristics for the classification of mycorrhizas into specific morphotypes. The following five different patterns of outer mantle structures were identified according to Ingleby et al. (1990).

1) A felt-prosenchyma-like hyphal arrangement was confirmed on eight morphotypes: types 1, 3, 6, 9, 12, 17, 23 and 34 (Figs. 1a and 6). Since five of these morphotypes, 1, 3, 6, 9 and 12, were classified as white or cream by color, their microscopic features were important for their discrimination, as shown in Table 1.

2) A net-prosenchyma-like hyphal arrangement was formed on 12 morphotypes: 2, 4, 7, 8, 10, 13, 15, 18, 22, 24, 25 and 26 (Figs. 2, 8a, 10 and 16). Seven of the 12 morphotypes, 4, 10, 13, 15, 22, 24 and 26, showed a net-like arrangement of hyphal bundles (Figs. 2, 8a and 10), similar to that in type A described by Agerer (1995).

3) A net-synenchyma-like hyphal arrangement was formed on three morphotypes: 16, 33 and 37 (Figs. 21 and 23, Table 1). Of these the fungal partner of type 37 (Fig. 23) was thought to be *C. geophilum* because of the jet-black mycorrhizas with isodiametric and thickened fungal cells of mantle surfaces (Agerer, 1987–1996; Chilvers, 1968; Goodman et al., 1996; Ingleby et al., 1990; Yamada and Katsuya, 1996).

4) An irregular-synenchyma-like hyphal arrangement was formed on eight morphotypes: 5, 11, 14, 19, 20, 27, 28 and 29 (Figs. 9, 14a, 17a and 18). Laticiferous hyphae containing granular substances which were similar to that of *Lactarius* spp. mycorrhizas (Agerer, 1986b; Ingleby et al., 1990) were confirmed at the inner layer of fungal mantles of types 11, 19 and 27 (Fig. 17b). In all of these three types, the diameter of laticiferous hyphae was wider than that of emanating hyphae (Table 1). The mantle surface of type 14, for instance, was composed of a distinctive palm-like, interlocking irregular synenchyma of thick-walled fungal cells with a diameter of 5–50 μm (Fig. 9).

5) A regular-synenchyma-like hyphal arrangement

Table 1. Macro- and microscopic features of ectomycorrhizas of fir (*Abies firma*) seedlings.

Type	Macroscopic features	Microscopic features					
	Color	Cys.[a]	Shapes of Cys.[b]	Cl.[c]	Mantle structure[d]	E.h.[e] (μm)	Other specific features
1	white	+	dichotomously branched acicular (24–48 × 1.5–3 μm)		F.P.	rare; 1–2	
2	white			+	N.P.	2–4	fungal rhizomorphs (20–60 μm in diameter) present
3	white with gloss				F.P.	1–2	
4	white with gloss				N.P.	2–4	fungal rhizomorphs (30–40 μm in diameter) present
5	white with gloss				I.S.	2–5	fungal rhizomorphs (10–30 μm in diameter) present
6	cream	+	dichotomously branched acicular (35–80 × 2–3 μm) or flask-shaped (10–25 × 2–4 μm)		F.P.	1–2	
7	cream	+	flask-shaped (10–25(–40) × 2–6 μm)		N.P.	rare; 2–3	
8	cream			+	N.P.	3–4	
9	cream				F.P.	1–2	
10	cream				N.P.	2–3	
11	cream				I.S.	5–8	laticiferous hyphae (6–10 μm in diameter) present at the inner layer of fungal mantles
12	cream with gloss	+	acicular (80–120 × 2–3 μm)		F.P.	1–2	
13	cream with gloss	+	flask-shaped (20–45 × 2–5 μm)		N.P.	2–4	
14	colorless				I.S.	rare; 3–4 or 6–8	
15	purplish			+	N.P.	4–6	
16	purple with gloss				N.S.	2–4	a fungal rhizomorph (18–25 μm in diameter) present
17	brown	+	acicular (35–70 × 2–3 μm), and flask-shaped ((10–)15–30 × 3–6 μm) with or without an apical knob (1–1.5 μm)		F.P.	1–2	
18	brown	+	thick-walled acicular (60–80 × 4–6 μm)		N.P.	2–4	
19	brown	+	oval (15–25 × 5–8 μm)		I.S.	2–4	laticiferous hyphae (4–6 μm in diameter) present at the inner layer of fungal mantles
20	brown	+	thick-walled acicular (80–110 × 4–5 μm)		I.S.	2	
21	brown	+	globular (5–20 × 5–10 μm)		R.S.	1–3	
22	brown			+	N.P.(N.S.)	2–3	fungal rhizomorphs (10–20 μm in diameter) present
23	brown				F.P.	1–2	emanating hyphae stained deep blue with lactophenol cotton blue
24	brown				N.P.	3–4 or 1–1.5	
25	brown				N.P.	1–3	emanating hyphae stained deep blue with lactophenol cotton blue
26	brown				N.P.	1–2	acicular cystidia like (200–300 × 2–3 μm) emanating hyphae were observed
27	brown				I.S.	1–3	laticiferous hyphae (4–8 μm in diameter) present at the inner layer of fungal mantles
28	brown				I.S.	1–2	
29	brown				I.S.	rare; 3–5	
30	brown				R.S.	2–4	fungal cells on mantle surfaces are larger than those of type 31
31	brown				R.S.	1–3	fungal cells on mantle surfaces are less angular and smaller than those of type 30
32	brown with gloss			+	R.S.	2–8	
33	brown with gloss				N.S. (I.S.)	3–10	
34	greyish				F.P.	1–3	
35	black	+	thick-walled acicular (100–250 × 5–8 μm)	+	R.S.	2–8	
36	black			+	R.S.	2–3	
37	black				N.S.	3–6	

a): cystidia; +, presence. b): measurements given are lengths of cystidia by diameters at the bases. c): clamp connections; +, presence. d): F.P., felt prosenchyma; N.P., net prosenchyma; N.S., net synenchyma; I.S., irregular synenchyma; R.S., regular synenchyma. Terminology follows Ingleby et al. (1990). e): diameter of emanating hyphae.

was formed on six morphotypes: 21, 30, 31, 32, 35 and 36 (Figs. 15a, 19, 20 and 22a). Although types 30 and 31 had a similar diameter of emanating hyphae, the mantle surfaces of the former had angular cells similar to type L according to Agerer's classification (Agerer, 1995), whereas the latter had less angular cells (Figs. 19 and 20). These two types could also be differentiated by the diameter of fungal cells: ca 5–25 μm for type 30, and 5–15 μm for type 31. The mantle structure of type 21, comprising isodiametric cells, was highly similar to that of Agerer's type K or type O (Agerer, 1995) in possessing angular cells and bearing heaps of roundish or flattened cells (Figs. 15a and b). This morphotype occurred most frequently among ECM types classified in the present study (Y. Matsuda, unpublished data).

Fungal rhizomorphs were observed on types 2, 4, 5, 16 and 22. Type 4 had undifferentiated rhizomorphs with loosely woven hyphae similar to those of Agerer's type A (Agerer, 1995). The other four types closely resembled type B of Agerer (1995), which formed undifferentiated and compactly arranged rhizomorphs with smooth margins (Fig. 3).

Eleven ECM types had different shapes of cystidia on their mantle surfaces. Types 18 (Fig. 12), 20 (Fig. 14b) and 35 (Fig. 22c) formed thick-walled acicular cystidia which differed in size from each other (Table 1). They were similar to the awl-shaped cystidia that are known to be characteristic of several *Tuber* species (Agerer, 1995; Ingleby et al., 1990). The cystidia on the mantle surface of type 18 (Fig. 12) were similar to type Ik described by Dominik (1969) and to *Tuber* spp.-like mycorrhizas of type 2 described by Yamada and Katsuya (1996). Type 6 formed dichotomously branched acicular or unbranched flask-shaped cystidia (Fig. 4). Cystidia on both types 7 (Fig. 5) and type 13 (Fig. 8b) were flask-shaped. As observed on type De or Df (Dominik, 1969) and type 12 (Yamada and Katsuya, 1996), type 17 (Fig. 11) bore three different shapes of cystidia, unbranched acicular, and unbranched flask-shaped with or without an apical knob at the distal end. The flask-shaped cystidia with or without the an apical knob were similar to those found on *Russula* mycorrhizas (Taylor and Alexander, 1989; Yamada and Katsuya, 1995). The unbranched or dichotomously branched acicular cystidia were also found on mycorrhizas of type 1 (Fig 1b) and type 12 (Fig. 7). On the mantle surfaces, type 19 had oval cystidia (Fig. 13).

Discussion

In this study we did not trace hyphal connections between fruitbodies and ECM roots and thus could not identify the fungal species forming each ECM morphotype. The only exception was type 37, *C. geophilum*. However, we could distinguish ECMs into 37 morphotypes (tentatively 37 fungal species) based on the keys of other well-described ECMs (e.g., Agerer, 1987–1996; Goodman et al., 1996; Ingleby et al., 1990), and our results suggest that diverse ECM fungi were associated with *A. firma* seedlings.

Seven morphotypes (2, 8, 15, 22, 32, 35 and 36) could be colonized by Basidiomycetes, as clamp connections were observed on the emanating hyphae. Laticiferous hyphae were confirmed in types 11, 19 and 27, which were thus likely to be formed by the genus *Lactarius*. Although Nara et al. (1992) described ECMs formed between *L. laeticolorus* (Imai) Imaz. and *A. firma*, they did not mention whether the ECMs had laticiferous hyphae.

One-third of ECM morphotypes bore various shapes of cystidia on their mantle surfaces. Cystidia on *Russula* mycorrhizas are often flask-shaped with or without an apical knob (Agerer, 1995; Taylor and Alexander, 1989; Yamada and Katsuya, 1995), and thus ECM types forming flask-shaped cystidia (types 6, 7, 13 and 17) are probably colonized by species of the genus *Russula*.

Thick-walled acicular cystidia found on types 18, 20 and 35 have often been reported from *Tuber* mycorrhizas (Agerer, 1995; Ingleby et al., 1990). In addition to the cystidia, *Tuber* mycorrhizas have mantle surfaces with regular or irregular synenchyma (Agerer, 1995; Ingleby et al., 1990). Based on these morphological traits, type 20, showing irregular synenchyma-like mantle surfaces, was probably formed by *Tuber* spp. Type 35 was colonized by Basidiomycetes, as indicated above, and therefore there is no reason to assume that this type is colonized by *Tuber* spp. belonging to Ascomycetes. Maia et al. (1996) listed *Abies alba* Mill. as a mycorrhizal host tree for *T. griseum* Pers., referring to earlier literature. This suggests that a species of *Tuber* was responsible for formation of type 20 mycorrhizas.

Because the acicular cystidia found on types 1 and 12 have been reported from several fungal taxa, e.g., *Tuber* spp., *Gomphidius* spp. and *Thelephora terrestris* Pers.: Fr. (Agerer, 1995), we could not specify the fungal

Table 2. Presumed causal fungi of the ectomycorrhizal morphotypes classified in this study.

Type	Causal fungus
2	basidiomycete
6	*Russula* sp. ?
7	*Russula* sp. ?
8	basidiomycete
11	*Lactarius* sp. ?
13	*Russula* sp. ?
15	basidiomycete
17	*Russula* sp. ?
19	*Lactarius* sp. ?
20	*Tuber* sp. ?
22	basidiomycete
27	*Lactarius* sp. ?
32	basidiomycete
35	basidiomycete
36	basidiomycete
37	*Cenococcum geophilum*

partner of these types simply by the cystidia observed.

Fleming (1983, 1984) suggested that seedlings in an undisturbed forest are colonized by ECM rhizomorphs emanating from nearby overstory trees. These hyphal connections between seedlings and mature trees suggest that some ECM fungi can be shared among trees irrespective of their age. Thus, seedlings of *A. firma* are likely to share the same fungi with the mature trees. Some field studies, however, showed that the species composition of associated ECM fungi differed with tree age (Danielson, 1984; Yamada and Katsuya, 1996), and therefore accumulation of ECM descriptions is necessary for various growth stages of *A. firma*.

The present morphotyping of mycorrhizas provided more reliable information on specification of ECM fungal partners of *A. firma* at the genus level, compared with earlier information (Masui, 1926; Nara et al., 1992). However, the fungal partners remain unknown for about 76% of the classified morphotypes (Table 2). Recently, a molecular method employing the polymerase chain reaction (PCR) has been used as a complementary technique for identification of ECM fungi directly from mycorrhizas (Kraigher et al., 1995; Matsuda and Hijii, 1999). Therefore, the description of mycorrhizas combined with molecular analyses of both ECM roots and ECM fruitbodies will provide more information on the ECM fungal partners of *A. firma*.

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Figs. 1–23. Features of ectomycorrhizal types on *Abies firma*. Scale bars=20 μm.

Fig. 1. (a) Plan view of the felt prosenchymatous mantle surface of type 1, (b) Unbranched or dichotomously branched acicular cystidia on the mantle surface.

Fig. 2. Plan view of the net prosenchymatous mantle surface of type 4.

Fig. 3. Rhizomorphs emanating from the mantle surface of type 5.

Fig. 4. Drawing of dichotomously branched acicular or flask-shaped cystidia on the mantle surface of type 6.

Fig. 5. Drawing of flask-shaped cystidia on the mantle surface of type 7.

Fig. 6. Plan view of the felt prosenchymatous mantle surface of type 9.

Fig. 7. Drawing of apical parts of acicular cystidia on the mantle surface of type 12.

Fig. 8. (a) Drawing of plan view of the net prosenchymatous mantle surface of type 13, (b) Drawing of flask-shaped cystidia on the mantle surface.

Fig. 9. Plan view of the irregular synenchymatous mantle surface of type 14, composed of thick-walled, interlocking palm-like cells.

Fig. 10. Plan view of the net prosenchymatous mantle surface of type 15.

Fig. 11. Drawing of flask-shaped cystidia with or without an apical knob and unbranched acicular cystidia on the mantle surface of type 17.

Fig. 12. Drawing of thick-walled acicular cystidia on the mantle surface of type 18.

Fig. 13. Drawing of oval cystidia on the mantle surface of type 19.

Fig. 14. (a) Plan view of the interlocking irregular synenchymatous mantle surface of type 20, (b) Drawing of thick-walled acicular cystidia on the mantle surface.

Fig. 15. (a) Plan view of the regular synenchymatous mantle surface of type 21, (b) Heaps of roundish fungal cells on the mantle surface.

Fig. 16. Plan view of the net prosenchymatous to net synenchymatous mantle surface of type 22.

Fig. 17. (a) Plan view of the irregular synenchymatous mantle surface of type 27, (b) Drawing of laticiferous hyphae containing granular substances found on the inner layer of fungal mantles.

Figs. 18. Drawing of plan view of the irregular synenchymatous mantle surface of type 29 composed of thick-walled roundish cells.

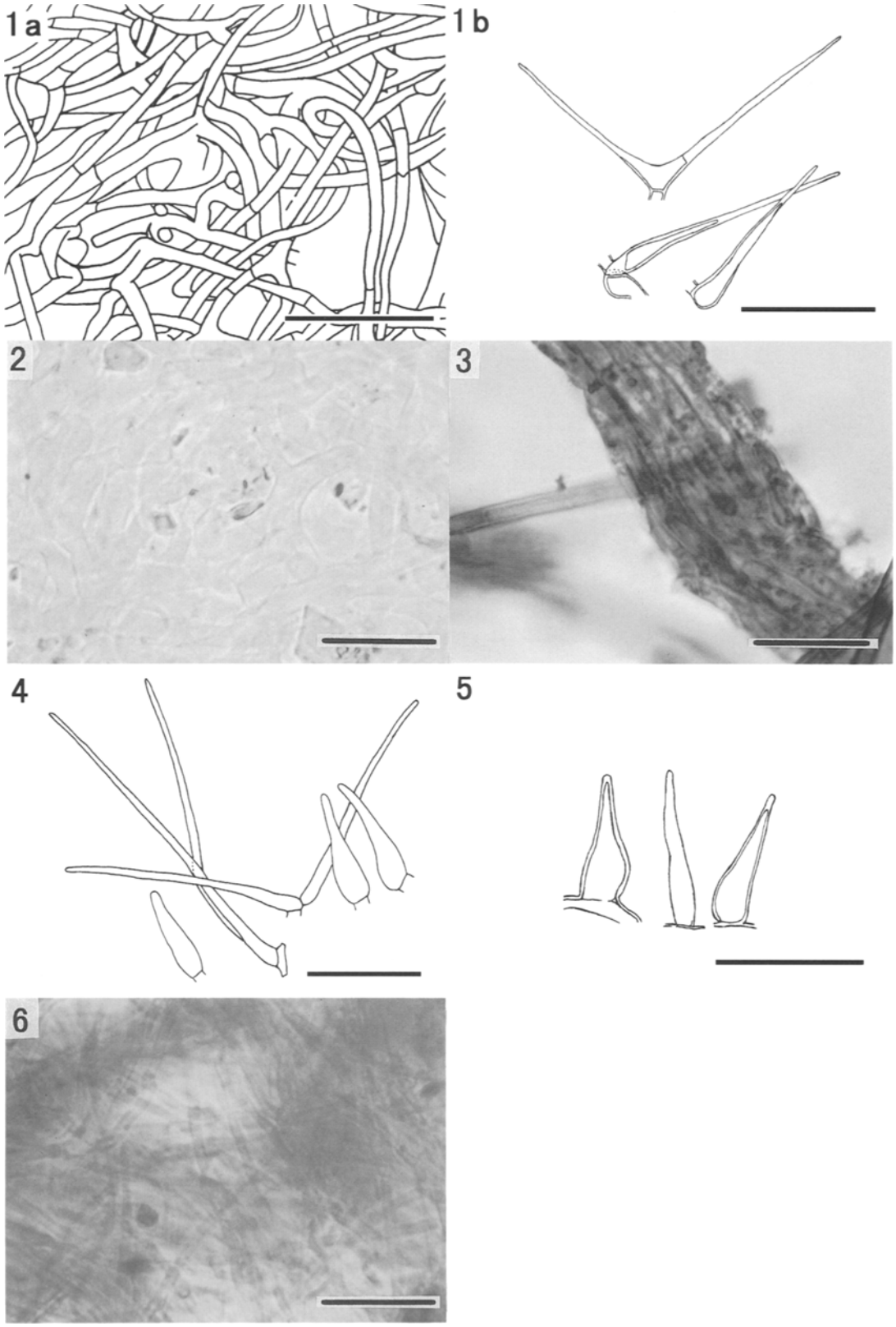
Fig. 19. Plan view of the regular synenchymatous mantle surface of type 30.

Fig. 20. Plan view of the regular synenchymatous mantle surface of type 31.

Fig. 21. Plan view of the net or interlocking irregular synenchymatous mantle surface of type 33.

Fig. 22. (a) Plan view of the regular synenchymatous mantle surface of type 35, composed of thick-walled angular cells, (b) Thick-walled clamped hyphae emanating from the mantle surface, (c) Thick-walled acicular cystidia on the mantle surface.

Fig. 23. Plan view of the net synenchymatous mantle surface of star-like, thickened fungal cells of type 37.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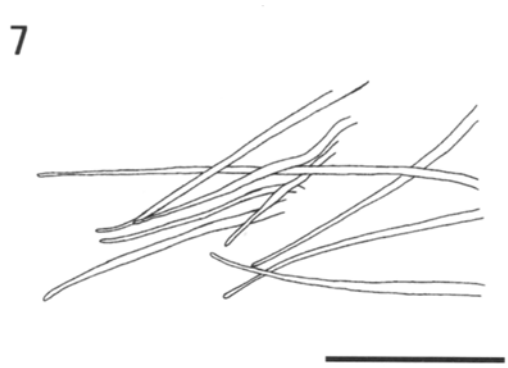
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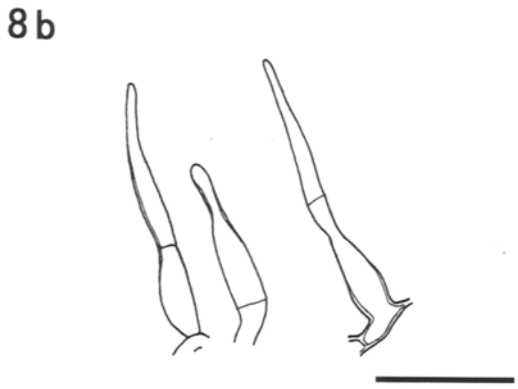
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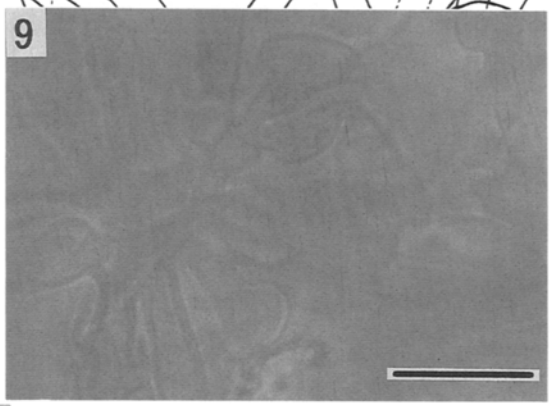
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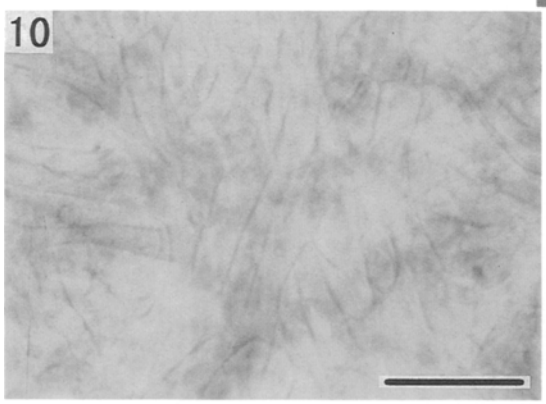
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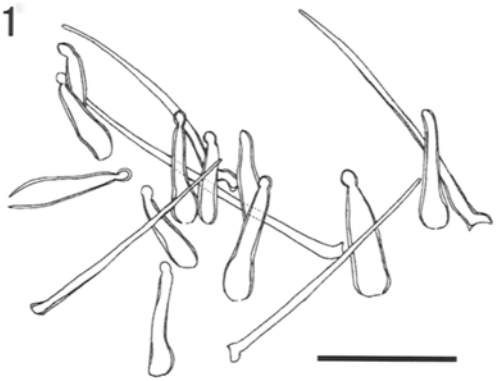
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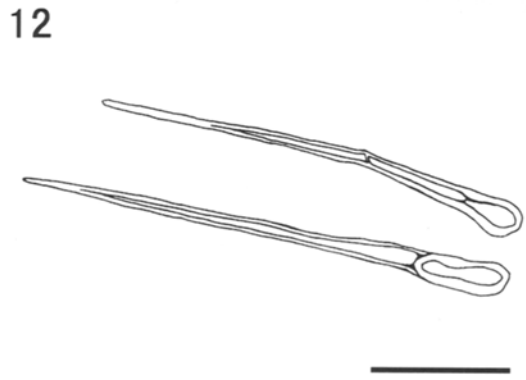
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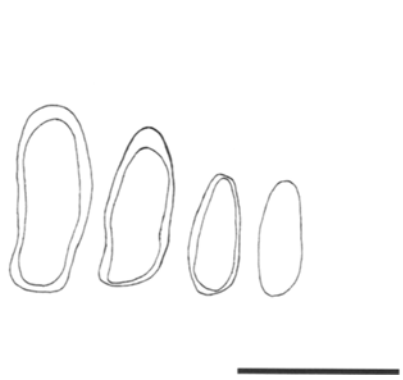
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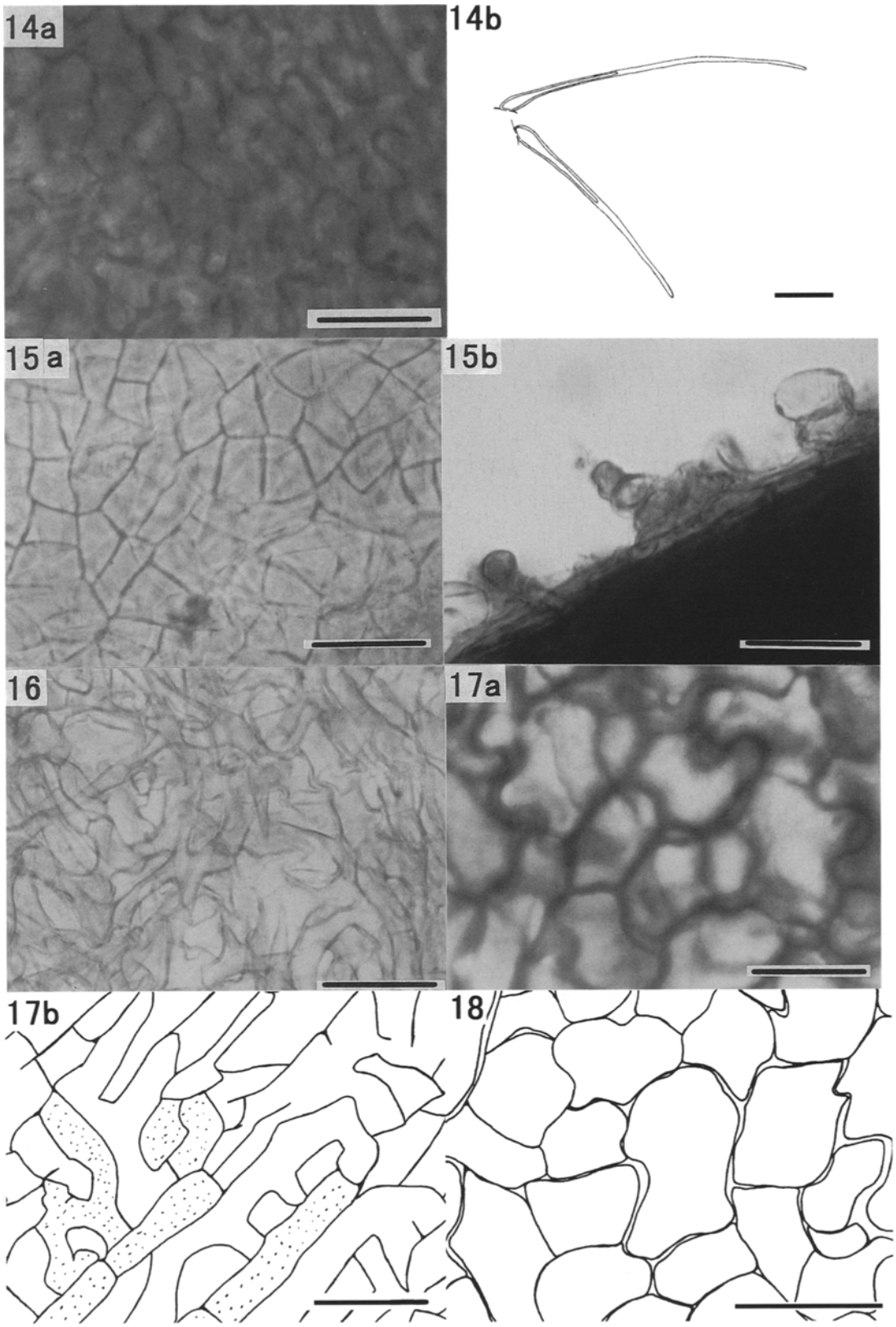
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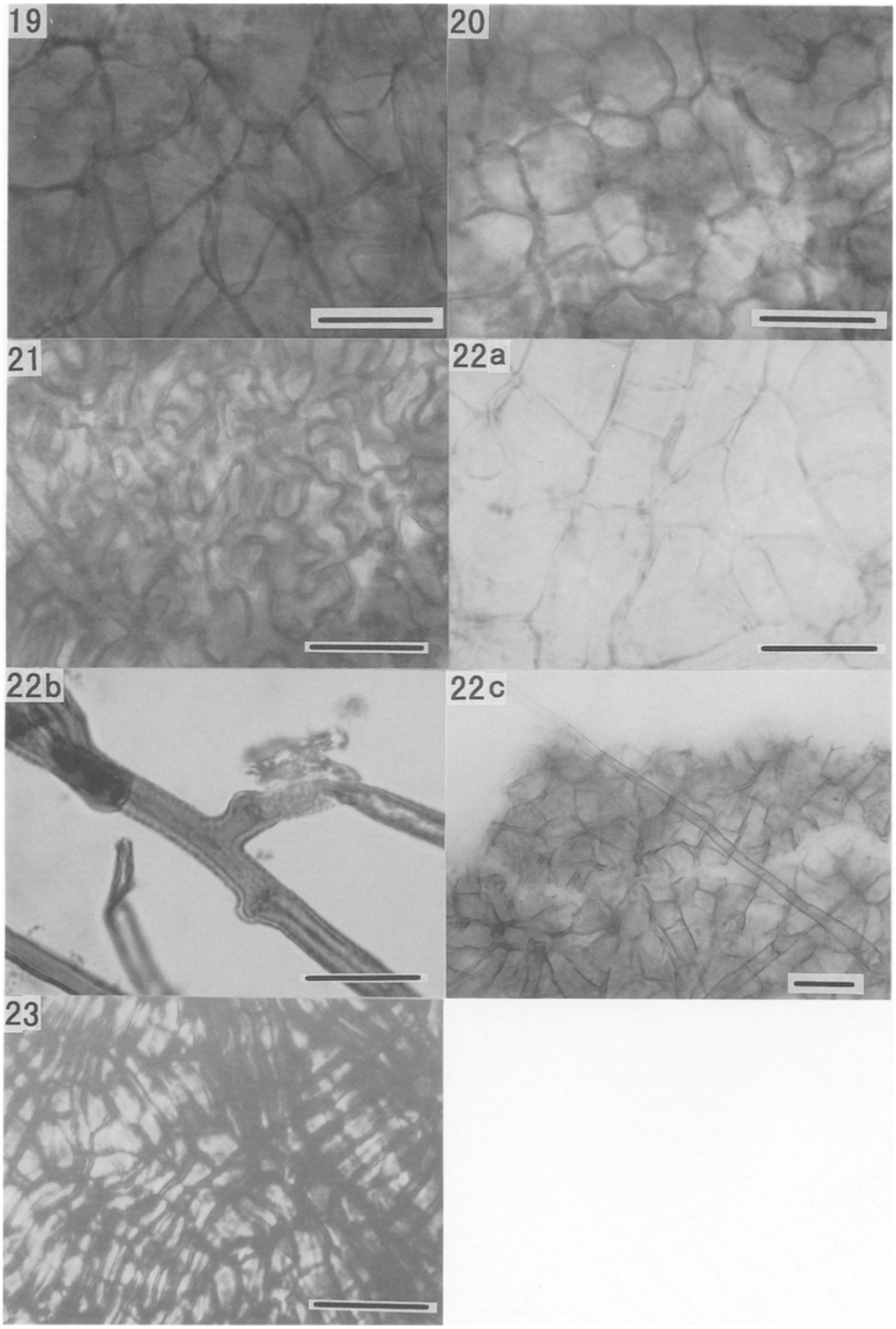
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