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Morphological classification of ectomycorrhizas of *Pinus densiflora*

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Morphological classification of ectomycorrhizas of *Pinus densiflora* was conducted. Fifty soil samples containing pine ectomycorrhizas, and 40 pine seedlings were collected randomly in two separate reforested stands of *P. densiflora* (45 yr old) from May 1992 to October 1994. Fifty-six types of ectomycorrhizas could be classified based upon microscopically observable morphological characteristics. Fifty percent of the types showed cystidia or other specific characteristics such as laticiferous hyphae in the fungal sheaths, verrucose emanating hyphae and a positive hyphal reaction to UV irradiation. Four mycorrhizal types were confirmed to be formed by the fungi *Russula delica*, *R. mariae*, *R. nigricans*, and *Cenococcum geophilum*, respectively. Although the other 52 types were unidentified mycobionts at species level, it was inferred that they were formed by the fungi *Hebeloma*, *Lactarius*, *Russula* and *Tuber*. There was a slight difference in the observed mycorrhizal types between the tree ages.

Key Words——ectomycorrhizas; morphological classification; mycorrhizal fungi; *Pinus densiflora*; *Russula*.

Since the last decade, morphological classification and identification of ectomycorrhizas have generally been based upon synecological studies. The integrated morphological information is based on comparable studies both in the laboratory (Zak, 1976; Alexander, 1981; Molina and Trappe, 1982; Godbout and Fortin, 1985) and in the field (Chilvers, 1968; Dominik, 1969; Zak, 1973; Agerer, 1987–1994; Ingleby et al., 1990; Agerer, 1991).

Ectomycorrhizas of the genus *Pinus* have been studied most extensively in the world (Mikola, 1973; Zak, 1973; Molina and Trappe, 1982), and the diversity of their association is being elucidated (Bruns, 1995). On *P. densiflora* Sieb. et Zucc., which is widely distributed in Japan, Korea and northeast China (Satake et al., 1989), ectomycorrhizas have been confirmed involving 17 fungal species: *Tricholoma robustum* (Alb. et Schw.: Fr.) Ricken s. Imazeki, *T. matsutake* (S. Ito et Imai) Sing., *Suillus bovinus* (L.: Fr.) O. Kuntze, *Dermocybe cinnamomea* (L.: Fr.) Wunsche, *Gomphus floccosus* (Imai) Parmasto, *Sarcodon* sp., *Boletopsis leucomelas* (Pers.: Fr.) Fayod, *Scleroderma cepa* Pers. and *Lyophyllum shimeji* (Kawam.) Hongo (Masui, 1927), and *Laccaria bicolor* (Maire) P. D. Orton, *Hebeloma* sp., *Suillus granulatus* (L.: Fr.) O. Kuntze, *Russula mariae* Peck, *R. nigricans* (Bull.) Fr., *Lactarius chrysorrheus* Fr., *Scleroderma areolatum* Ehrenb, and *Cenococcum geophilum* Fr. (Yamada and Katsuya, 1995). However, floristic data of epigeous macrofungi (including putative ectomycorrhizal fungi) (Imazeki and Hongo, 1987, 1989) suggest the presence of more diverse fungal associations with the pine.

The objective of the present study was to classify the mycorrhizas on *P. densiflora* morphologically at the microscopic level. Mycorrhizal sampling was conducted in a restricted stand area for reproducible and comparable examination. Accordingly, either the taxonomic relationship of the causal fungi of the classified mycorrhizas, or the state of mycorrhizal association with the host plant was discussed.

Materials and Methods

Study site Climatological and vegetational descriptions of the study sites were previously reported by Yamada and Katsuya (1995). Two plots, A and B (25×25 m each), were established in two different reforested stands of *P. densiflora* (age: 45 yr old) at the campus of the University of Tsukuba. Bushes on the stand floors had previously been cut every few years.

Sampling and observation of ectomycorrhizas Soil block samples (ca. 10×10×10 cm) containing mycorrhizal roots of mature pine trees were collected randomly at 50 points in the plots. Forty pine seedlings (emerged in the current yr) were also collected randomly by picking up the whole root system. The soil samples were separately taken to the laboratory and dissected, and pine roots were picked up and washed with tap water.

Under a stereomicroscope, sound mycorrhizal tips were roughly separated based on the external color, branching system, and conditions of emanating hyphae. Then, the mycorrhizal tips were observed under a light microscope according to the method of Yamada and Katsuya (1995) and classified morphologically into mycor-

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Table 1. Morphological characteristics used for mycorrhizal classification.

Characters	Characteristics
(under stereomicroscope)	
1. external color of the fungal sheath and emanating hyphae	pale color or white, light to dark brown, or black
(under microscope)	
1. cystidia on the surface of the fungal sheath present or absent	acicular, obclavate, ventricose, etc.[a]
2. texture of surface layer of the fungal sheath	felt prosenchyma to regular synenchyma[b]
Characters	characteris tics
3. emanating hyphae; clamp connections	present or absent
diameter	1.0 μm to more than 10.0 μm
4. other characters	ornamentation on any surface, specialized emanating hyphae, reaction to UV irradiation, etc.

a) Morphological descriptions follow the terminology of Hawksworth et al. (1983).
b) Descriptions follow terminology of Ingleby et al. (1990).

rhizal types (Table 1). Almost all of the mycorrhizas used for the classification were preserved as preparations in the laboratory.

Results

Morphological classification All the pine mycorrhizas examined were ectomycorrhizas, because they showed both fungal sheaths and Hartig nets but no regular intracellular penetration (Harley and Smith, 1983). Fifty-six types of ectomycorrhizas were classified as shown in Table 2. Mycorrhizal type 17 was confirmed to be connected with a sporocarp of *Russula delica*. Moreover, the same mycorrhizal type formed on the roots of *Cedrus deodra* Loud. at another site was connected with a sporocarp of *R. delica* (data not shown). This type is characterized by the presence of relatively long and slender obclavate cystidia (Fig. 16). Types 19 and 21 have been shown to be formed by *R. nigricans* (Bull.) Fr. and *R. mariae* Peck, respectively (Yamada and Katsuya, 1995). Type 54 was identical with mycorrhizas formed by *Cenococcum geophilum* Fr. (Trappe, 1964; Pigott, 1982; Agerer, 1987-1994; Ingleby et al.; 1990, Yamada and Katsuya, 1995) based on the jet-black emanating hyphae, star-like arrangement of the fungal sheath (Fig. 25), and infrequent presence of black sclerotia.

About 50% of the other 52 types showed cystidia or other specific characteristics. Type 2 was similar to the mycorrhizas formed by *Tuber* spp. (Fontana and Centrella, 1967; Palenzona et al., 1972; Agerer, 1987-1994; Ingleby et al., 1990), showing distinct acicular cystidia (Fig. 2A) and an irregular synenchymatous hyphal arrangement (Fig. 2B) on the surface of the fungal sheath. Type 11 showed characteristic thick-walled, and relatively enlarged and closely arranged obclavate cystidia (Fig. 10), which were similar to those on the

Table 2. Ectomycorrhizal types and their characteristics.

Type	Color[a]	Cystidia on the surface of the sheath[b]	H. a.[c]	E. h.[d]	
				C. c.	Diam (μm)
1	PW	acicular (like emanating hyphae but thick wall), 2.0-4.0 × 40-250 μm	IS	+	2.0-2.5
2	PW	acicular (distinct) and thick wall, 50-105 μm	IS	+	1.5-10.0
3	PW	club-like, sometimes rather short	IS	+	2.5-3.0
4	PW	ventricose to acicular, 20-35 μm, rarely globular	IS	+	1.5-4.0
5	PW	globular, club-like or ventricose	IS	+	1.5-2.5
6	PW	irregularly radiated and rather thick wall	IS	−	6.0-12.0
7	PW	club-like, often irregularly branching	IS	−	3.0-6.0
8	PW	club-like, often dichotomous	IS	−	2.0-2.5
9	PW	bulb-like to ampulliform	IS	−	1.5-2.0
10	PW	acicular to hypha-like, 2.0 × 70-120 μm	NS	+	2.0-2.5
11	PW	obclavate (less than 20 μm)	NS	−	1.5-2.0
12	PW	acicular to sublate, or obclavate (dimorphic)	NS	−	1.5-2.0
13	PW	acicular, branching to 2 to 4, basal part enlarged	NS	−	2.0-2.5
14	PW	acicular to hypha-like (thick wall, more than 100 μm in length), no regular septum on emanating hyphae	NS	−	1.5-2.0

15	PW	hypha-like (fine, thick wall and often irregularly curved), no regular septum on the emanating hyphae	NS	−	1.0–4.0
16	PW	obclavate to club-like (25–30 μm), adhering to a small globoid (1.0–1.5 μm in diam) center at the apex	NP	−	2.0
17	PW	obclavate to club-like (slender, 25–65 μm)	NP	−	2.0–2.5
18	PW	obclavate (less than 25 μm)	NP	−	2.0
19	PW	obclavate (20–30 μm), adhering to 1 or 2 small globoid (2.0–3.0 μm in diam) structures at side or center of the apex	NP	−	1.5–2.5
20	PW	obclavate (20–25 μm), adhering to 1 to 3 small globoid (1.0–2.0 μm in diam) structures at the apex	NP	−	1.5–2.5
21	PW	acicular, sometimes dichotomous	NP	−	1.5-2.0
22	PW	no cystidia	RS	+	2.0–3.0
23	PW	no cystidia, surface layer cells of the fungal sheath; regularly arranged (triangular to tetragonal)	RS	−	2.0–2.5
24	PW	no cystidia, surface layer cells of the fungal sheath; irregularly arranged	RS	−	2.0–4.0
25	PW	no cystidia, surface layer cells of the fungal sheath; rather small	RS	−	1.5–2.0
26	PW	no cystidia	IS	+	1.5–2.5
27	PW	no cystidia	IS	+	2.0–4.0
28	PW	no cystidia, laticiferous hyphae present in the fungal sheath	IS	−	1.5–2.0
29	PW	no cystidia, sheath layer has globular cells up to 20 μm in diam	IS	−	2.0–5.5
30	PW	no cystidia, surface layer of the sheath consists mainly of an interlocking irregular synenchyma	IS	−	5.0–12.0
31	PW	no cystidia, surface layer of the fungal sheath lacks interlocking irregular synenchyma	IS	−	2.5–5.0
32	PW	no cystidia, sheath consists mainly of an interlocking irregular synenchyma, and shows cells with an irregular shape which are conspicuously stained with blue dyes and are positive to UV irradiation	IS	−	2.0–2.5
33	PW	no cystidia	NS	+	2.5–3.5
34	PW	no cystidia, emanating hyphae are verrucose	NS	−	3.0–7.5
35	PW	no cystidia	NS	−	2.0–4.0
36	PW	no cystidia	NP	+	2.0–2.5
37	PW	no cystidia	NP	−	2.0–3.5
38	PW	no cystidia, laticiferous hyphae present	NP	−	2.0
39	PW	no cystidia, emanating hyphae are verrucose, and cells with an irregular shape which are positive to UV irradiation are present in the fungal sheath	NP	−	2.5–5.0
40	PW	no cystidia, emanating hyphae are verrucose and positive to UV irradiation	FP	+	1.5–2.5
41	PW	no cystidia	FP	−	1.5–2.0
42	PW	no cystidia, sheaths are covered with a gelatinous layer	FP	−	2.0–3.0
43	PW	no cystidia	FP	−	2.5–3.0
44	PW	no cystidia, verrucose emanating hyphae are more than 10 μm in diam	FP	−	2.5–10.0
45	L-DB	club-like, irregular form, or globose 7.5–9.0 × 10–12 μm	RS	+	2.5–6.0
46	L-DB	short club-like to globose	IS	+	2.5–3.5
47	L-DB	no cystidia, surface layer of the fungal sheath; bearing mounds of flattened cells	RS	+	4.0–4.5
48	L-DB	no cystidia, emanating hyphae verrucose, surface layer of the fungal sheath consists mainly of an interlocking irregular synenchyma	IS	+	2.5–7.5
49	L-DB	no cystidia	IS	−	2.0–4.5
50	L-DB	no cystidia, emanating hyphae often irregularly curved	IS	−	3.0–5.0
51	L-DB	no cystidia, emanating hyphae are verrucose	IS	−	3.5–7.5
52	BL	bulbous-shape	RS	+	4.5–5.0
53	BL	no cystidia	RS	+	1.5–6.0
54	BL	no cystidia, emanating hyphae are verrucose, surface layer of the fungal sheath shows stellar arrangement	RS	−	2.5–4.5
55	BL	no cystidia, emanating hyphae are verrucose	IS	+	2.5–4.5
56	BL	no cystidia, emanating hyphae and surface of the fungal sheath are covered with black secretions or deposits like scale	IS	−	3.5–5.0

a) PW: pale color or white; L-DB: light to dark brown; BL: black. b) Morphological descriptions follow the terminology of Hawksworth et al. (1983). c) H. a.: hyphal arrangement of surface of the fungal sheath; RS: regular synenchyma; IS: irregular synenchyma; NS: net synenchyma, NP: net prosenchyma; FP: felt prosenchyma. Descriptions follow the terminology of Ingleby et al. (1990). d) E. h.: emanating hyphae which disperse from the surface of the fungal sheath. C. c.: clamp connection.

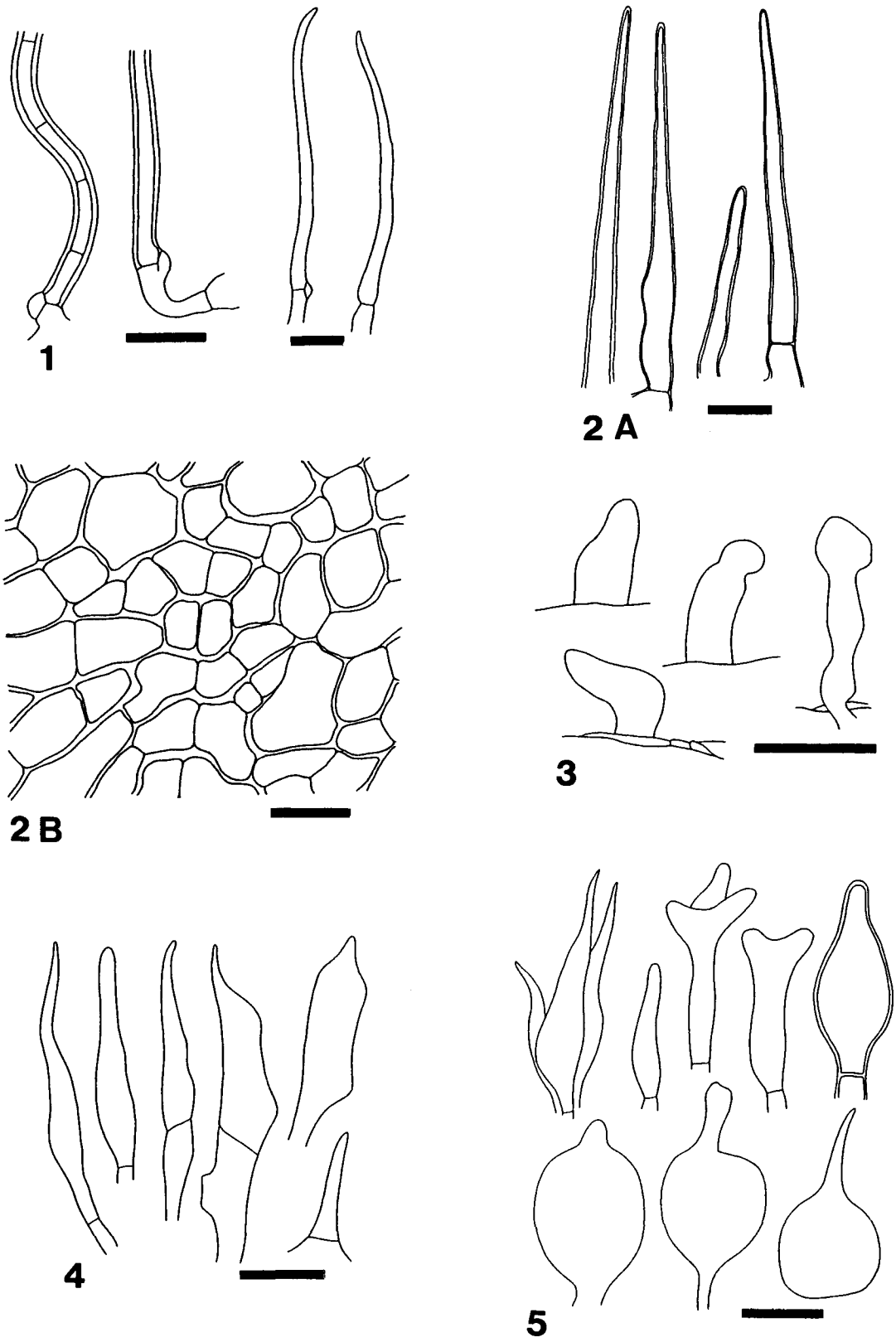
mycorrhizas formed by *Russula illota* Romagn. on *Fagus sylvatica* L. (Agerer, 1987-1994). Types 12 (Fig. 11), 16 (Fig. 15), 18 (Fig. 17) and 19 (Fig. 18) also had characteristic cystidia (obclavate) which have been observed on the mycorrhizas formed by *Russula* spp. (Agerer, 1987-1994; Taylor and Alexander, 1989; Yamada and Katsuya, 1995). These cystidia could be clearly distinguished from each other by their size and form. On type 12, a large number of acicular cystidia (often dichotomous) was also observed. The dimorphism of acicular-obclavate cystidia was similar to Dominik's mycorrhizal types De and Df (Dominik, 1969). Type 13 had characteristic branching acicular cystidia (Fig. 12), which were similar to those on the mycorrhizas formed by *R. papludosa* or an unidentified mycorrhizal type on *Salix* (Agerer, 1995). Branching cystidia of type 13 were often similar to those on type 12, although the basal part of the former cystidia was enlarged. Types 28 and 38 (Fig. 19) both had laticiferous hyphae, which were characteristic of the mycorrhizas of *Lactarius* spp. (Agerer, 1987-1994; Ingleby et al., 1990). Laticiferous hyphae showed a positive reaction to UV irradiation. The emanating hyphae of type 40 were verrucose and showed a positive reaction to UV irradiation (Fig. 20), which was similar to that of mycorrhiza formed by *Hebeloma mesophaeum* (Pers.) Quél. (Ingleby et al., 1990) and *Hebeloma* sp. (Yamada and Katsuya, 1995). This mycorrhizal type was also externally similar to the mycorrhizas formed by *Hebeloma edurum* Mert. (Agerer, 1987-1994). Types 14 (Fig. 13) and 15 (Fig. 14), in which distinct regular septa on the emanating hyphae could not be confirmed, may be formed by Zygomycetes known as *Endogone* spp., but neither palmate hyphae (Walker, 1985) nor terminal vesicles (Fassi, 1965) were observed. They resembled each other in the hypha-like cystidia and emanating hyphae, but the cell wall of the former type was relatively thick and stained densely with blue dyes (cotton blue and toluidin blue). The cystidia of types 14 and 15 were also similar, but the former were straight and slightly curved and the latter irregularly curved and enlarged. Type 52 had bulbous-shaped cystidia (Fig. 24) similar to those of Agerer's mycorrhizal type, *Fagirhiza spinulosa* (Agerer, 1987-1994) on *F. sylvatica*. Type 47 had mounds of roundish cells (Fig. 23) as in the Agerer's mycorrhizal type, *Piceirhiza nigra* on *Picea abies* L. Karst. (Agerer, 1987-1994). Types 7 (Fig. 7) and 30 had rather thick emanating hyphae, and the cells of the surface layer of the fungal sheath were relatively big as reported in some Ascomycetes (Agerer, 1987-1994; Ingleby et al., 1990). Other types which show characteristic cystidia, such as acicular cystidia (type 1, Fig. 1, and type 10), radial-form cystidia (type 6, Fig. 6), ventricose cystidia (types 4 and 5, Figs. 4 and 5, respectively), and club-like, bulb-like or globular cystidia (types 3, 7-9, 45 and 46, Figs. 3, 7-9, 21 and 22, respectively), or are covered with secreted or deposited materials like scale (type 56, Fig. 26), were also recognized, but they could not be assigned to a specific taxonomic state.

Mycorrhizal association with host plant Of the 56 mycorrhizal types, 52 were recorded on roots of mature

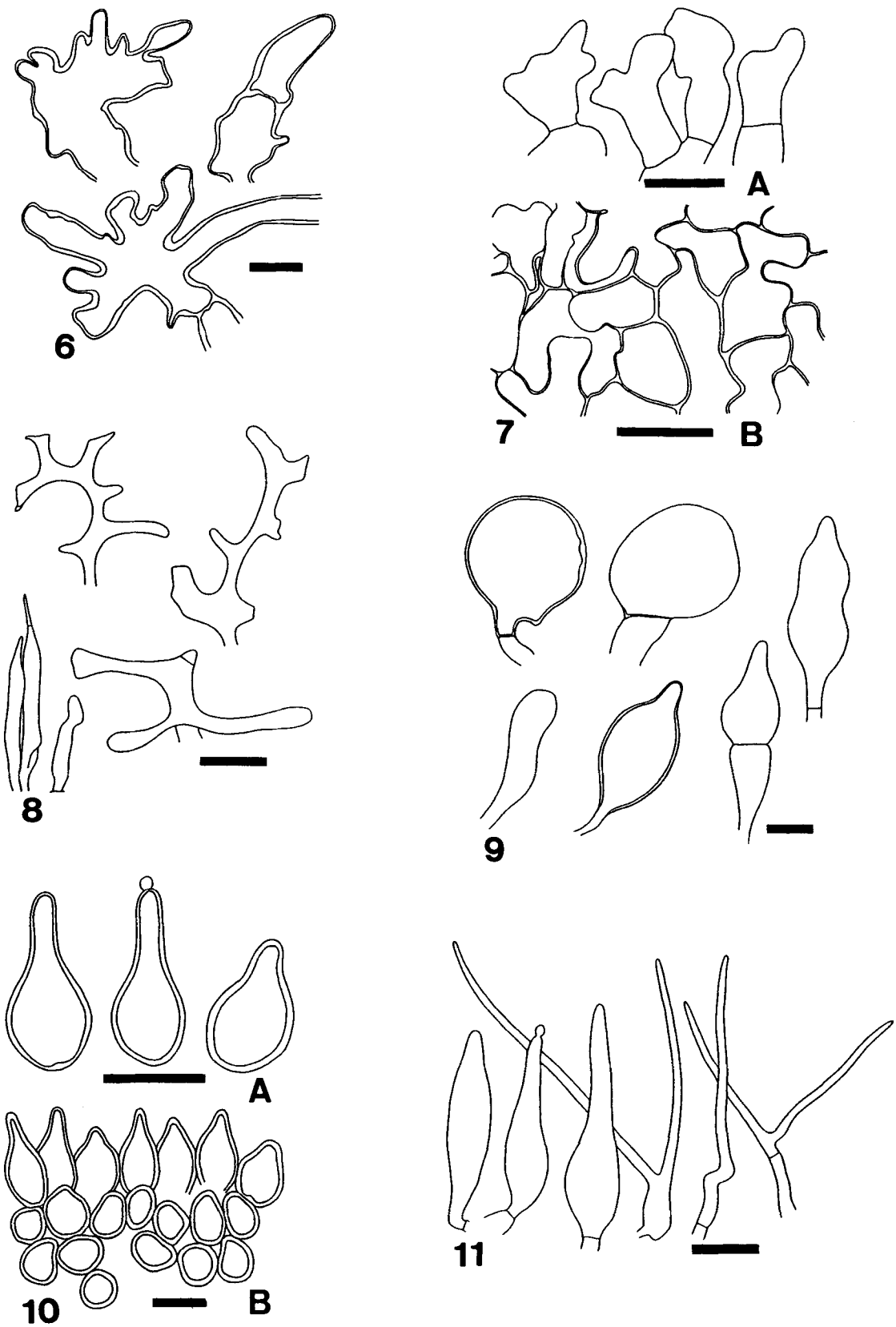
Table 3. Number of observations of mycorrhizal types.

Type	Plot A		Plot B	Total	Causal fungus
	m[a)]	s[b)]	m[a)]		
1	3		1	4	
2	1			1	*Tuber* sp.?
3		1		1	
4	2			2	
5	1			1	
6	3	1	1	5	
7			1	1	
8	2		1	3	
9	2	1		3	
10	2			2	
11	1			1	*Russula* sp.?
12	7			7	*Russula* sp.?
13	2	1		3	
14	1	1		2	
15	2	2		4	
16	1	1		2	*Russula* sp.?
17	1			1	*R. delica*
18	1			1	*Russula* sp.?
19	3			3	*R. nigricans*
20		1		1	*Russula* sp.?
21	1			1	*R. mariae*
22	1			1	
23	1			1	
24	1			1	
25	1			1	
26	3	3	1	7	
27	1			1	
28	1			1	*Lactarius* sp.?
29	1			1	
30	1			1	
31	1			1	
32	1	1		2	
33	1			1	
34	1	1		2	
35	1			1	
36	2		2	4	
37	2			2	
38		1	2	3	
39	1			1	
40	2		1	3	*Hebeloma* sp.?
41	1		1	2	
42			1	1	
43		2		2	*Lactarius* sp.?
44	1			1	
45			1	1	
46			1	1	
47	1			1	
48	1			1	
49	1		1	2	
50	1			1	
51	1			1	
52			1	1	
53	1	2		3	
54	1		2	3	*C. geophilum*
55	1			1	
56	5	1		6	

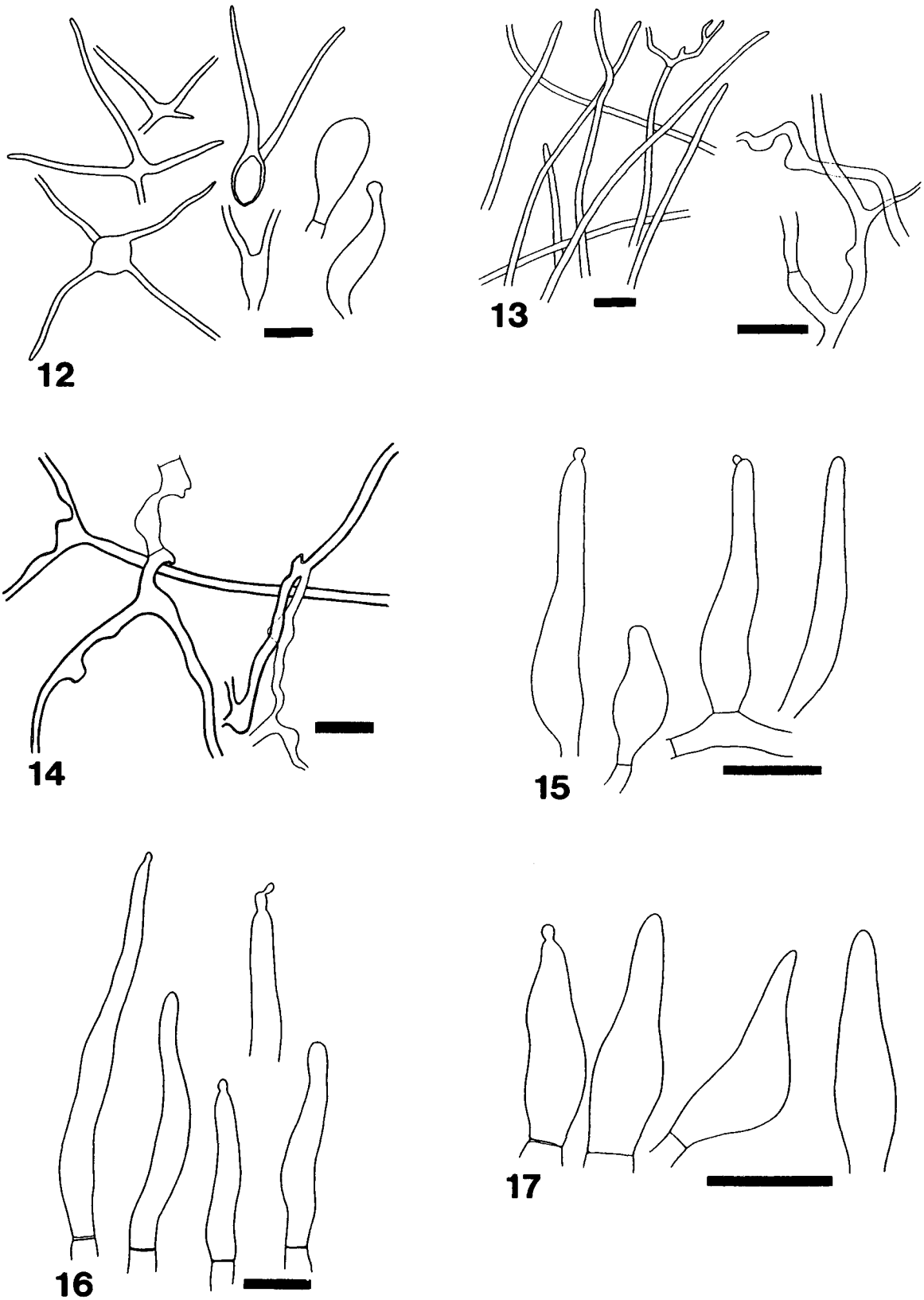
a) Mature pine tree. b) Pine seedling.



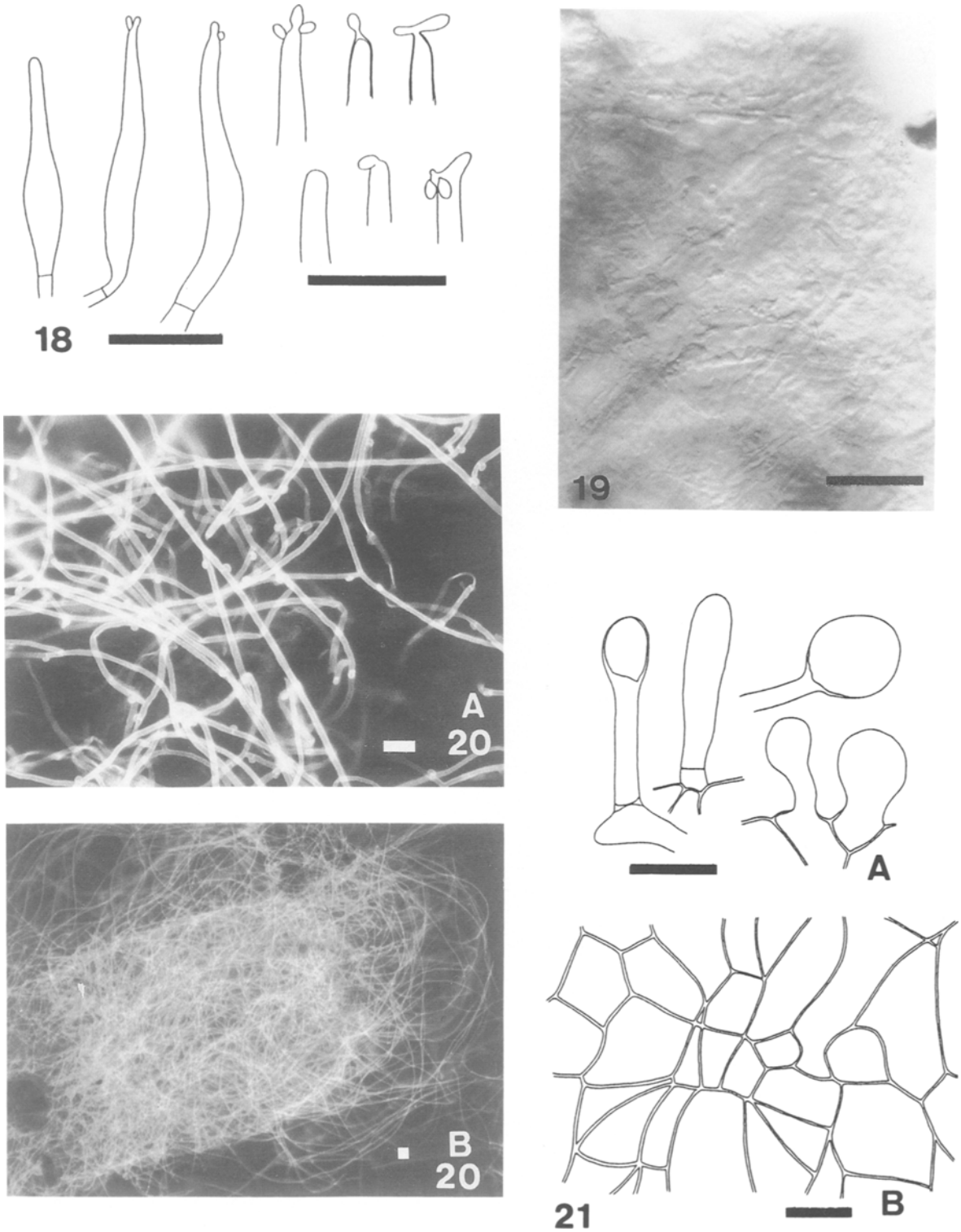
Figs. 1-5. Morphological characteristics of the ectomycorrhizas I.
1. Cystidia of mycorrhiza type 1. 2. Cystidia (A) and surface view (B) of the fungal sheath of mycorrhizal type 2. 3. Cystidia of type 3. 4. Cystidia of type 4. 5. Cystidia of type 5. Bar = 10 μm.



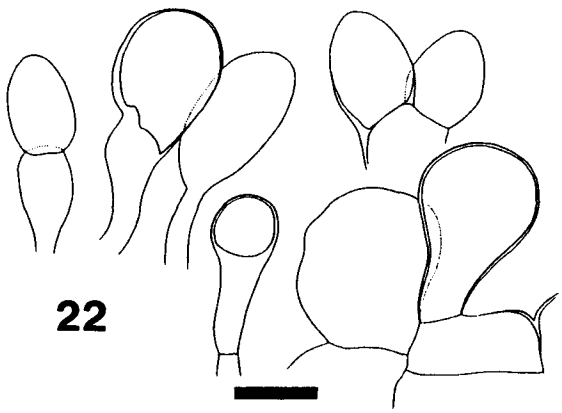
Figs. 6-11. Morphological characteristics of the ectomycorrhizas II.
6. Cystidia of type 6. 7. Cystidia (A) and surface view (B) of the fungal sheath of type 7. 8. Cystidia of type 8. 9. Cystidia of type 9. 10. Cystidia (A) of type 11 and their arrangement (B). 11. Cystidia of type 12. Bar=10 μm.

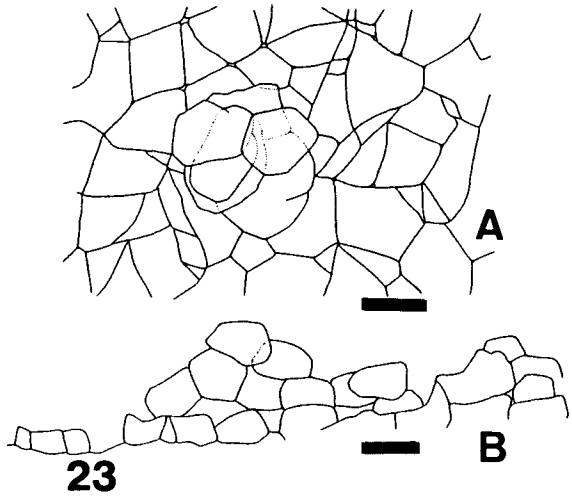


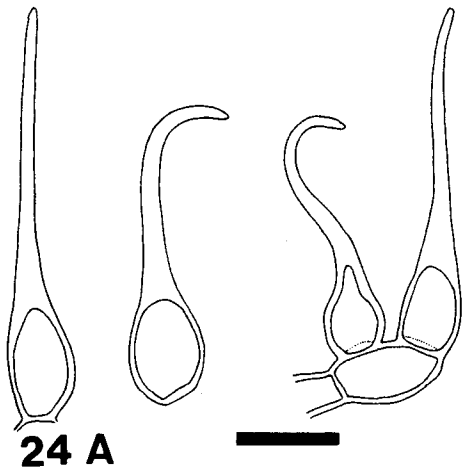
Figs. 12–17. Morphological characteristics of the ectomycorrhizas III.
12. Cystidia of type 13. 13. Cystidia (left) and emanating hyphae (light) of type 14. 14. Cystidia and connected emanating hyphae of type 15. 15. Cystidia of type 16. 16. Cystidia of type 17. 17. Cystidia of type 18. Bar = 10 μm.

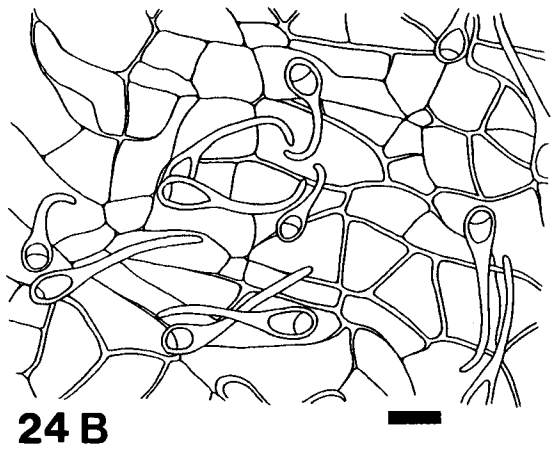


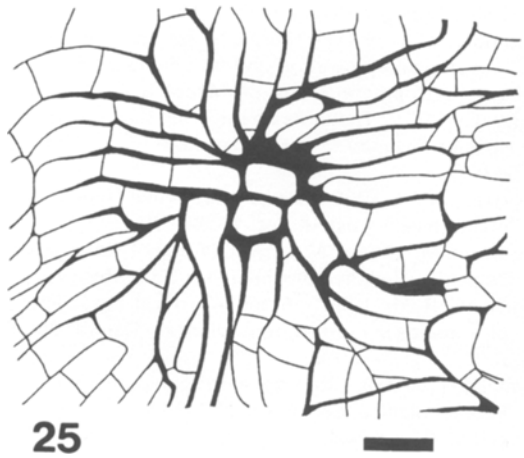
Figs. 18–21. Morphological characteristics of the ectomycorrhizas IV.
18. Cystidia of type 19. 19. Micrograph of laticiferous hyphae in the surface layer of the fungal sheath of type 38. 20. Fluorescent micrographs of type 40. Emanating hyphae (A) and a mycorrhizal tip (B). 21. Cystidia (A) and surface view (B) of the fungal sheath of type 45. Bar=10 μm.

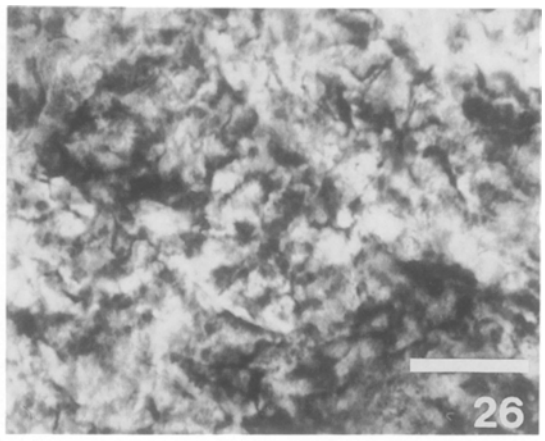












Figs. 22–26. Morphological characteristics of the ectomycorrhizas V.
22. Cystidia of type 46. 23. Surface view of the fungal sheath of type 47 bearing mounds of flattened cells. A. Dosal. B. Vertical aspect. 24. Cystidia (A) and surface view (B) of the fungal sheath of type 52. 25. Surface view of the fungal sheath of type 54. 26. Micrograph of surface view of the fungal sheath of type 56. Bar=10 μm.

pine trees, and 16 on roots of pine seedlings (Table 3). Thus 40 types occurred only on mature trees, 4 types only on seedlings, and 12 types on both. Ten of the 15 mycorrhizal types observed in plot B were also found in plot A (Table 3). No particular dominant (frequent occurrence) type was observed, except for type 12, which was prominent on seedlings.

Discussion

Fewer characters were used for the classification of ectomycorrhizal types in the present study (Fig. 1) than those reported by Agerer (1987–1994), which may have resulted in inadequate description of the types. However, our previous study on mycorrhizal synthesis (Yamada and Katsuya, 1995) suggested that each of the 56 mycorrhizal types was related to a fungal species. Of these, only 4 types were produced by a known fungal species, *R. delica*, *R. mariae*, *R. nigricans* and *C. geophilum*, respectively. It was also inferred that mycorrhizal types 1, 11, 12, 16, 18, 20, 28, 40 and 43 were related to *Tuber*, *Russula*, *Lactarius* and *Hebeloma*, but the causal fungi could not be identified at the species level. Many of the other types, in which the mycobiont could not be assigned to a specific taxonomic state, also showed characteristic cystidia or other specific morphological characteristics (Table 2). These observations indicate that the "plan view" (Chilvers, 1968; Ingleby et al., 1990; Agerer, 1991) is informative for the characterization of ectomycorrhizas on *P. densiflora*. Mycorrhizal type 40 has verrucose emanating hyphae, which show a positive reaction to UV and violet irradiation. Furthermore, fluorescent staining (Duckett and Read, 1989) revealed the hyphal arrangement of the fungal sheath (data not shown). This method may be useful for further characterization of certain mycorrhizal types, particularly those lacking cystidia or showing a poorly organized arrangement of the tissues of the fungal sheath. Every mycorrhizal type needs further proof or reconfirmation of the precise relation with a fungus (or fungi) experimentally by means of mycorrhizal synthesis or DNA analysis.

No particular mycorrhizal type was observed at a high frequency (Table 3). Although quantitative data (e.g., length of mycorrhiza or number of mycorrhizal tips) are not available for these mycorrhizal types, no conspicuously dominant mycorrhizal types could be identified in the present plots, as has been reported previously for pine stands (Zak and Bryans, 1963; Lamb and Richards, 1970; Danielson, 1984; Bruns, 1995). The number of mycorrhizal types per unit area in the present study was higher than that hitherto reported in coniferous stands (Zak and Bryans, 1963; Lamb and Richards, 1970; Chu-Chou, 1979; Thomas et al., 1983; Danielson, 1984; Bruns, 1995). This observation does not contradict the absence of conspicuously dominant mycorrhizal types as suggested above. The difference in mycorrhizal types between the two plots was obvious (Table 3), although the number of samplings in plot B was relatively limited. These facts suggest that the association of *P. densiflora* with ectomycorrhizal fungi is diversified and varies with the stand.

Ectomycorrhizal succession in relation to forest age was critically reviewed by Newton (1992) and Molina et al. (1992) in terms of mycorrhizal fungal strategy. The successional mechanism was mainly ascribed to the change in the soil environment including either biotic or abiotic factors, understory vegetation, and corresponding mycorrhizal fungal strategy, rather than the tree age. On the other hand, sharing of the same mycorrhizal fungal hyphal networks between mature trees and understory seedlings in the forest was stressed (Newton and Pigott, 1991). Eleven mycorrhizal types among the 15 observed on seedlings were also observed on mature trees in the present study. This fact reflects the importance of hyphal networks as inocula for mycorrhizal infection on *P. densiflora* seedlings in the forest, whereas the age of tree itself is presumably less important for mycorrhizal association of the pine. However, 4 of the 15 mycorrhizal types observed on seedlings in the present study were seedling-specific. In particular, type 11 was distinct (putatively *Russula* sp.) and frequently observed (Table 3). This observation indicates the presence of specific associations of ectomycorrhizal fungi in relation to the age of the host plant as reported by Tonkin et al. (1989) on synthesized ectomycorrhiza of *Eucalyptus marginata* Donn ex Sm. inoculated with *Pisolithus tinctorius* (Pers.) Cok et Couch.

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