

***Entoloma aprile* (Agaricales, Entolomataceae) new to Japan, with notes on its mycorrhiza associated with *Populus maximowiczii* in cool-temperate deciduous forests of Hokkaido**

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An entolomatoid fungus, *Entoloma aprile* is newly recorded from Japan. Basidiome morphology of the species is described and illustrated based on the Japanese specimens. ITS sequences were obtained from Japanese *E. aprile* and three related species, viz. *E. clypeatum* f. *clypeatum*, *E. clypeatum* f. *hybridum* and *E. sepium*. Basidiomata of *E. aprile* were highly homologous (99.4 % – 100.0 %) among our collected specimens and were separated from other known *Entoloma* spp. in the dendrogram based on ITS sequences. This results supported our morphological observation that *E. aprile* differed from those of allied species. Mycorrhizae of *E. aprile* on *Populus maximowiczii* in the cool-temperate deciduous forests of Hokkaido, Japan were observed by stereo, light and scanning electron microscopy for the first time. In studied mycorrhizae, the root cap, meristem, and apical region of the cortex disappeared. Fungal hyphae of the mycorrhizae invaded root tissues. Hyphal sheath outside of the cortex was composed of plectenchymatous and pseudoparenchymatous hyphae divided into three layers according to hyphal thickness and arrangement. Those morphological characteristics suggest that mycorrhizae of *E. aprile* are of the same type as those of allied *Entoloma* species associated with rosaceous and ulmaceous plants.

Keywords: entolomatoid fungi, mycorrhizal morphology, phylogenetic analysis, salicaceous plants, taxonomy.

The genus *Entoloma* (Fr.) P. Kumm. belongs to Agaricales (Basidiomycota) and consists of saprotrophic and mycorrhizal fungi, of which some of the latter are known to be associated with rosaceous and ulmaceous plants (Noordeloos 1980, 1981a, b; 2004, Agerer & Waller 1993, Szűcs & Végelyi 1998, Kobayashi & Hatano 2001, Kobayashi 2004, 2007; Gryndler *et al.* 2009). While five species, one variety and three forms of *Entoloma* which are associated with those plants have hitherto been described (Noordeloos 1980, 1981b, 2004), only two species and one form viz. *Entoloma clypeatum* (L.) P. Kumm. f. *clypeatum*, *E. sepium* (Noulet & Dass.) Richon & Roze and *E. clypeatum* f. *hybridum* (Romag.) Noordel. have been recorded from Japan (Imazeki & Hongo 1987, Kobayashi *et al.* 2003). Although several unidentified species of *Entoloma* that can be morphologically distinguished from each other have been collected under various rosaceous and ulmaceous plants (Kobayashi 2004, 2007), comprehensive studies on taxonomy and phylogeny of those fungi have not been conducted yet. Recently, during our investigations of mycobiota in cool-temperate deciduous forests of Hokkaido, Northern Japan, an unidentified, entolomatoid fungus that was associated with *Populus maximowiczii* A. Henry has been collected in early spring. According to our morphological observations of Japanese collections, we identified this agaric as *E. aprile* (Britzelm.) Sacc. Phylogenetic analysis of Japanese specimens of *E. aprile* and allied members of this genus were also supported by our morphological observations. Therefore, we describe and illustrate *E. aprile* based on our Japanese collections as the first record of this species from Japan, with notes on its phylogenetic relationships to allied *Entoloma* species.

Entoloma aprile has been recorded under various rosaceous, ulmaceous, and betulaceous plants in Europe and North America (Largent 1994, Breitenbach and Kränzlin 1995, Noordeloos 1980, 1981b, 2004). So far, however, no salicaceous plants have been recorded as the mycorrhizal host of *E. aprile*. Furthermore, mycorrhizae formed by *E. sepium* on *Rosa* sp. and *Prunus domestica* L. revealed that the fungus “invades and almost completely destroys root meristem and young root cells” (Agerer & Waller 1993), indicating that their mycorrhizal relationship is not ectomycorrhizal. In addition, mycorrhizae of *E. clypeatum* f. *hybridum* present the same morphological characteristics as *E. sepium* (Kobayashi & Hatano 2001, Kobayashi 2004, 2007). The mycorrhizal morphology of *E. aprile*, however, has not been studied yet. Therefore, in this paper, we examine the morphology of mycorrhizae formed by *E. aprile* on *P. maximowiczii*. Additionally, we discuss the genetic relationships between basidiomata of *E. aprile* and mycorrhizae on *P. maximowiczii* based on the analysis of DNA sequences.

Materials and Methods

Basidiome morphology

The specimens examined in this study are deposited in the mycological herbaria of the National Museum of Nature and Science, Tsukuba, Japan (TNS) and the Hokkaido University Museum, Sapporo, Japan (SAPA). Macroscopic characters were described by observations on fresh material. Guaiac reaction of fresh basidiomata was tested following Largent *et al.* (1977). For light microscopic observations, free-hand sections of the fresh specimens were mounted in water, 3 % or 5 % KOH (wt/vol), and phloxine B solution on glass slides. Randomly selected basidiospores were measured under a light microscope at 1000× magnification with oil immersion objective. In length measurements, the apiculus for basidiospores are excluded. The abbreviations 'Q' and 'Qave.' indicate the ratio of length to width of basidiospores and its average, respectively. The surface features of basidiospores were also observed by scanning electron microscopy (SEM). For SEM, air-dried pileus samples were attached with double-sided adhesive tape on specimen holders, then gold coated and examined with a JFC-1100 (JEOL, Tokyo, Japan). They were examined with a JSM-T330A SEM (JEOL, Tokyo, Japan) operating at 20.0 kV. The morphological terms applied were referred to Noordeloos (1992, 2004).

Mycorrhizal morphology

Mycorrhizal samples of *E. aprile* (SAPA 1160) were collected from the cool-temperate deciduous forest of the Miyagaoka Park, Nishi-ku, Sapporo-shi, Hokkaido (43° 4' N, 141° 15' E) on May 5, 2008 (Fig. 1). The forest is dominated by *P. maximowiczii*, *Quercus crispula* Blume, *Acer mono* Maxim., *Maackia amurensis* subsp. *buengeri* (Maxim.) Kitam., and *Ulmus davidiana* var. *japonica* Nakai. Based on the methods of Kobayashi & Hatano (2001), mycorrhizae were collected as follows. Three cubic cores of 10 cm edge were excavated from soil under basidiomata of *E. aprile*. Mycorrhizal fungi in the soil cores were identified by rhizomorphal connections between basidiomata and mycorrhizal rootlets. The water-soaked mycorrhizal samples were cleaned from soil particles using fine forceps and brushes.

Macroscopic characters of mycorrhizae were described by observations on fresh material with a stereomicroscope. For light microscopic observations, free-hand longitudinal sections of mycorrhizae were cut with a razor blade under a stereomicroscope, and were then mounted in water or 3 % (wt/vol) lactophenol on glass slides. Samples were examined with a Leica DM LB microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) with Nomarski interference contrast. Longitudinal sections of mycorrhizae were observed also by scanning electron microscopy (SEM). For SEM, small pieces (ca. 2.5 mm) of my-

corrhzal sections were fixed with 2 % glutaraldehyde solution over 24 h, and then rinsed 2 or 3 times with distilled water, followed by dehydration in a graded series of ethanol, passed through 100 % isoamylacetate, and dried with a HCT-2 Critical Point Dryer (Hitachi, Tokyo, Japan). Then, samples were attached with double-sided adhesive tape on specimen holders, and coated with platinum-palladium using an E-1030 Ion Sputter Coater (Hitachi, Tokyo, Japan). Samples were examined with a S-4200 SEM (Hitachi, Tokyo, Japan) operating at 15.0 kV. Some descriptive terms were based on Agerer (1987–1993) and Kobayashi & Hatano (2001).

Phylogenetic analysis

DNA was extracted from basidiomata and mycorrhizae which were already examined by us for their morphological features (Tab. 1). Additional samples of mycorrhizae of *Entoloma* sp. on *P. maximowiczii* (SAPA 1161) were collected from the cool-temperate deciduous forest of the Ishikari shelterbelt forest, Shinko-nishi, Ishikari-shi, Hokkaido (43° 1' N, 141° 28' E) on May 12, 2007, which is dominated by *P. maximowiczii*, *Salix* sp., and *U. davidiana* var. *japonica* (Fig. 1, Tab. 1). Collecting methods followed Kobayashi & Hatano (2001). Moreover, dried specimens of basidiomata of three allied *Entoloma* species from the mycological herbarium of the Osaka Museum of Natural History, Osaka, Japan (OSA) were also used for DNA extraction (Tab. 1). Methods of DNA extraction followed the protocol of ISOPALNT II (Nippon Gene Co., Ltd., Tokyo, Japan). The ITS region of genomic

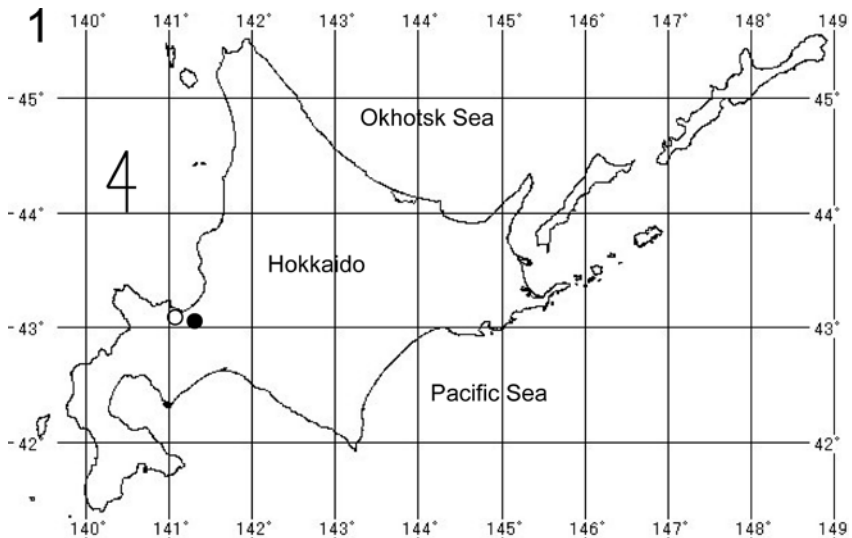


Fig. 1. – Collection sites for samples of mycorrhizae. White and black circles indicate Miyagaoka Park, Sapporo, and Ishikari shelterbelt forest, Ishikari, respectively.

Tab. 1. – Taxa/specimens of *Entoloma* used for ITS sequencing. (BA = basidiomata; MC = mycorrhizae)

Species	Specimen no.	Material	Locality	Collecting date	DDBJ accession no.
<i>Entoloma aprile</i>	TNS-F-24626	BA	Ishikari, Hokkaido, Japan	24 May 2006	AB520845
<i>E. aprile</i>	TNS-F-24627	BA	Ishikari, Hokkaido, Japan	12 May 2007	AB520846
<i>E. aprile</i>	TNS-F-24628	BA	Sapporo, Hokkaido, Japan	20 May 2007	AB520847
<i>E. aprile</i>	SAPA 1160	MC	Sapporo, Hokkaido, Japan	5 May 2008	AB520848
<i>Entoloma</i> sp.	SAPA 1161	MC	Ishikari, Hokkaido, Japan	12 May 2007	AB520849
<i>E. clypeatum</i> f. <i>clypeatum</i>	OSA-MY-4081	BA	Nagaokakyo, Kyoto, Japan	7 May 1994	AB520850
<i>E. clypeatum</i> f. <i>clypeatum</i>	OSA-MY-4082	BA	Kyoto, Kyoto, Japan	19 May 1994	AB520851
<i>E. clypeatum</i> f. <i>clypeatum</i>	OSA-MY-4085	BA	Kyoto, Kyoto, Japan	19 May 1994	AB520852
<i>E. clypeatum</i> f. <i>clypeatum</i>	OSA-MY-4086	BA	Kyoto, Kyoto, Japan	19 May 1994	AB520853
<i>E. clypeatum</i> f. <i>hybridum</i>	OSA-MY-5000	BA	Kumamoto, Japan	7 Apr 1996	AB520854
<i>E. clypeatum</i> f. <i>hybridum</i>	OSA-MY-5004	BA	Kyoto, Kyoto, Japan	11 Apr 1995	AB520858
<i>E. sepium</i>	OSA-MY-5001	BA	Naka, Ibaraki, Japan	29 Apr 1999	AB520855
<i>E. sepium</i>	OSA-MY-5002	BA	Kyoto, Kyoto, Japan	29 Apr 1995	AB520856
<i>E. sepium</i>	OSA-MY-5003	BA	Nagaokakyo, Kyoto, Japan	7 Apr 1996	AB520857

rDNA was amplified with the primer pairs ITS1-F (5'-CTTGGTCATT-TAGAGGAAGTAA-3') and ITS4-B (5'-CAGGAGACTTGTACACG-GTCCAG-3'), as described by Gardens and Bruns (1993) to use KOD plus polymerase (Toyobo Co., Ltd., Tokyo, Japan) instead of Taq polymerase. The PCR product was purified using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA) using the primer ITS1-F. Multiple alignments of the ITS sequences were performed, and the nucleotide substitution rate was calculated. A phylogenetic tree was constructed by the neighbor-joining method (Kimura 1980, Saitou & Nei 1987) using the program CLUSTAL W (Thompson *et al.* 1994) with bootstrap values based on 1000 replications (Felsenstein 1985).

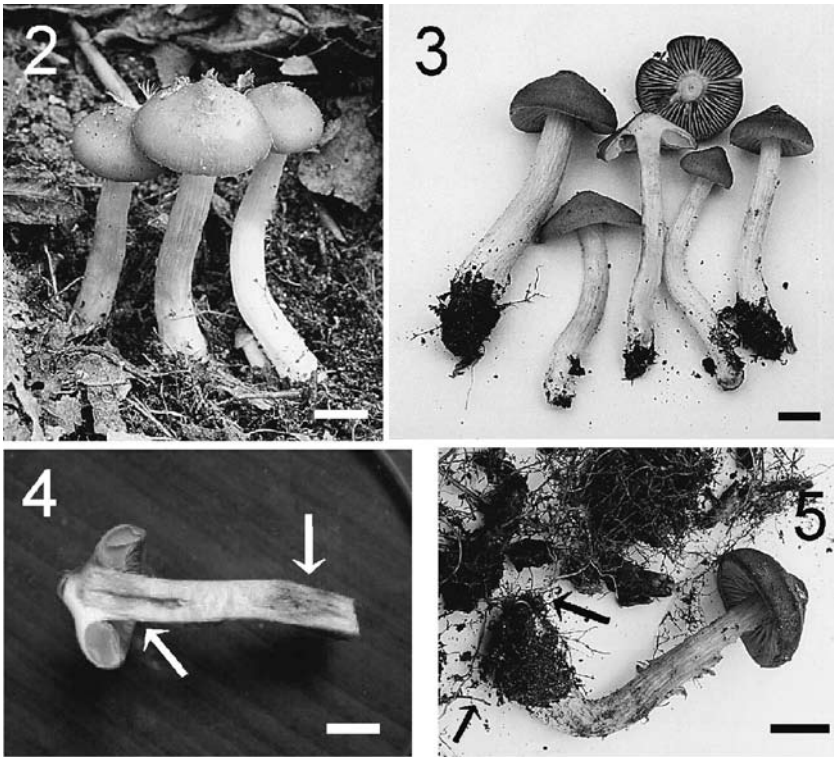
Taxonomy

Entoloma aprile (Britzelm.) Sacc., Syll. Fung. 5: 696. 1887. – Figs. 2–8.

Basidiomata (Figs. 2–3) entolomatoid. – Pileus 20–27 mm broad, conical at first, then campanulate to convex-applanate, usually with acute to conical umbo, or convex with an umbo, brown to dark brown when dried, smooth, shiny, hygrophanous, viscid to subviscid when moist, covered with radially fibrillose striae, striae sometimes split at marginal zone, marginal edge incurved for a long time, context white, subcutis pale brown, not changing in color by guaiac. – Lamellae subdistant, sinuate-adnate, pinkish brown, thickened, ventricose, 5–5.5 mm broad, edge smooth, not changing in color by guaiac. – Stipe 34.5 × 6.7–7.1 mm, cylindrical, pale grayish brown, paler at apex, equal, sometimes compressed, tapering or thickened toward the base, often L-like bended, covered with longitudinal striate fibrils, with tomentose fibrils or sometimes erect squamules, base covered with white mycelial strands associated with rhizomorphs (Fig. 5); context white, hollow, subcutis brown, slowly changing into bluish green by guaiac (Fig. 4). – Odor farinaceous to rancid. Spore print pinkish-brown.

Pileipellis a ixocutis, uppermost layer consisting of 2–5 µm broad, filamentous, periclinal to erect hyphae, tip of the hyphae lanceolate to narrowly-fusiform, colorless or with yellowish brown intracellular pigments, sometimes with amorphous, granulose to reflective elements, smooth-walled or encrusted; subpileipellis of 10–15 µm broad, fusiform to cylindrical hyphae with blackish-brown intracellular pigments, without clamp-connections. – Stipitipellis cylindrical, regular, terminal cells with clavate to cylindrical caulocystioid elements, 37–70 × 6–13 µm, thin to slightly thick-walled, encrusted with intracellular pigments, clamp-connections absent or rarely present. – Cystidia absent. – Lamella trama consisting of cylin-

drical to fusiform hyphae, up to 100 μm long, 5–16 μm broad, containing distinct intracellular pigments, clamp-connections absent or rare. – Stipe trama of cylindrical to fusiform hyphae, up to 80 μm long, 9–19 μm broad, containing distinct intracellular pigments, clamp-connections absent or rare. – Basidia (Fig. 7) 50–70 \times 12–16 μm , slender clavate, (1-)2-4-spored, sterigmata up to 11 μm long, with basal clamp-connections. – Basidiospores (Figs 6, 8) 9–11(–11.5) \times 8–10 (–10.5) μm , $Q = 1.0\text{--}1.2\text{--}(1.4)$, $Q_{\text{ave.}} = 1.1$, isodiametric to heterodiametric, 5-7-angled, slightly thick-walled, straw-yellow to pinkish brown, with a short appendage, containing one or a few oil drops.



Figs. 2-5. – *Entoloma aprile* (TNS-F-24627): **2.**, **3.** Mature basidiomata. **4.** Sectioned basidioma with positive guaiac reaction in the flesh of stem (arrows). **5.** Basidioma with rhizomorphs (arrows) connected to mycorrhizae. Bars 1.5 cm.

Habitat. – Basidiomata usually gregarious to fasciculate in cool-temperate broad-leaved deciduous forests, appearing in early spring, especially in late April to early May.

Distribution. – Japan (Hokkaido, new record), Europe (Noordeloos 1980, 1981b, 2004, Breitenbach & Kränzlin 1995), and North America (Largent 1994).

Toxicity. – Weakly poisonous fungus causing gastrointestinal disorder.

Japanese name. – Haruno-togari-ippunshimeji ('April Pinkgill', newly named here).

Material examined. – JAPAN, Hokkaido, Ishikari Prov., Ishikari-shi, Shinko-nishi, Ishikari shelterbelt forest, 20 May 2000, *leg.* C. Takehashi & S. Takehashi, *det.* S. Takehashi (TNS-F-24622 = SAPA 1158); same locality, 29 May 2005, *leg.* C. Takehashi, *det.* S. Takehashi (TNS-F-24625); same locality, 24 May 2006, *leg.* C. Takehashi and S. Takehashi, *det.* S. Takehashi (TNS-F-24626); same locality, 12

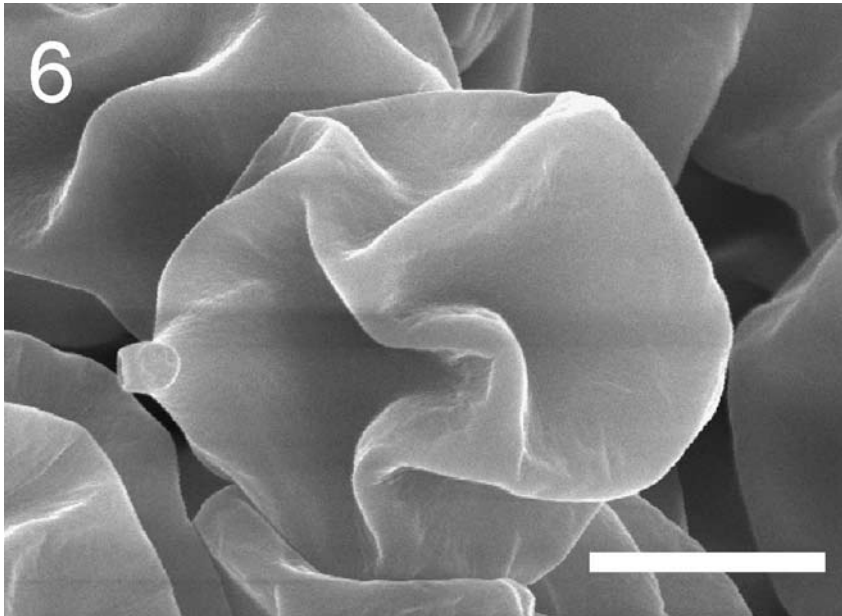
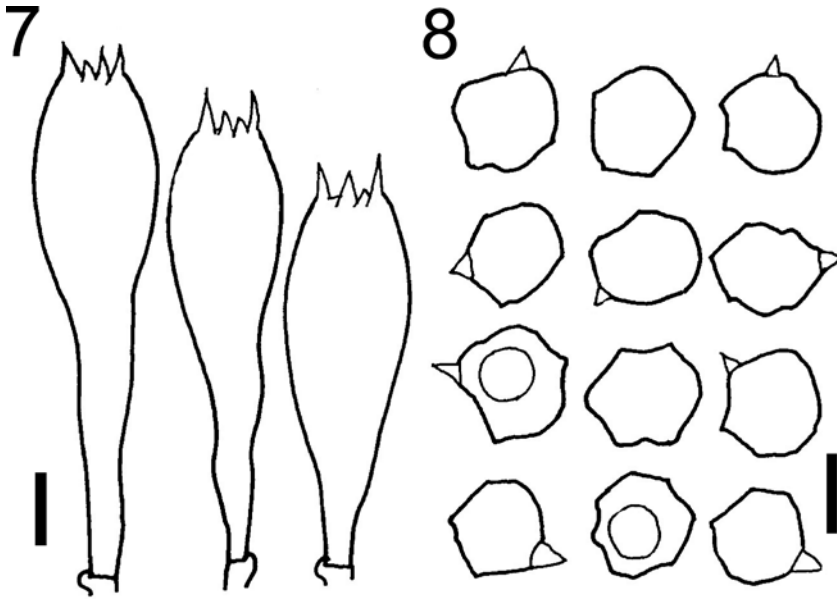


Fig. 6. – SEM micrograph of a basidiospore of *E. aprile* (TNS-F-24627). Bar 3 μm .

May 2007, *leg.* C. Takehashi, T. Kasuya, T. Hosino, S. Koshimizu and S. Takehashi, *det.* S. Takehashi (TNS-F-24627); JAPAN, Hokkaido, Ishikari Prov., Sapporo-shi, Nishi-ku, Miyagaoka Park, 20 May 2007, coll. T. Takazawa, S. Koshimizu and S. Takehashi, *det.* S. Takehashi (TNS-F-24628 = SAPA 1159); same locality, 5 May 2008, coll. S. Takehashi and T. Kasuya, *det.* S. Takehashi (TNS-F-18596).

Remarks. – Studied Japanese specimens are well characterized by: slender basidiomata, pileus acute or with conical umbo, stipe relatively long, odor farinaceous, positive guaiac reaction, and microscopically by 5-7-angled basidiospores and relatively short hyphae (up to 100 μm) in the pileus and lamella trama. These morphological characters suggest its placement in the genus *Entoloma*, subgenus *Entoloma*, section *Nolanidea* (Fr.) Quél., but also because the studied specimens have been collected from broad-leaved forests in early spring.

Hitherto, nine taxa belonging to section *Nolanidea*, viz. *E. aprile*, *E. bloxamii* (Berk. & Broome) Sacc., *E. clypeatum* f. *clypeatum*, *E. clypeatum* var. *defibulatum* Noordel., *E. clypeatum* f. *hybridum*, *E. clypeatum* f. *pallidogriseum* Noordel., *E. clypeatum* f. *xanthophyllum* Noordel., *E. pseudolividium* Largent, and *E. sepium* are known worldwide (Largent 1994, Breitenbach & Kränzlin 1995, Noordeloos 1980, 1981b, 1992, 1998, 2004). Morphological characteristics of the specimens examined are in good agreement with previous descriptions of *E. aprile* (Largent 1994, Breitenbach & Kränzlin 1995, Noordeloos 1992,



Figs. 7–8. – Microscopic features of *E. aprile* (TNS-F-24627): 7. Basidia (bar = 10 µm). 8. Basidiospores (bar = 3.5 µm).

1998, 2004). In the section *Nolanidea*, the studied specimens are clearly distinguished from *E. clypeatum* f. *clypeatum*, *E. clypeatum* var. *defibulatum*, *E. clypeatum* f. *xanthophyllum*, and *E. clypeatum* f. *pallidogriseum* by their positive guaiac reaction. Moreover, it differs from *E. clypeatum* f. *hybridum*, *E. sepium*, *E. bloxamii* and *E. pseudolividium* in the following morphological features: *E. clypeatum* f. *hybridum* with a stipe staining brownish-orange; the stipe of *E. sepium* stains reddish when bruised; *E. bloxamii* with a greenish stipe; *E. pseudolividium* has a yellowish pileus and pale yellowish lamellae.

On the other hand, the studied Japanese specimens are ecologically very similar to *E. vernum* S. Lundell, *E. hirtipes* (Schumach.) M.M. Moser, *E. plebejum* (Kalchbr.) Noordel., and *E. sericeoides* (J.E. Lange) Noordel. by its fruiting season., The latter four species, how-

ever, clearly differ from the Japanese fungus by the following features: *E. vernum* grows in coniferous forests and does not have a farinaceous odor; the pileipellis of *E. hirtipes* is not an ixocutis and it has cheilocystidia; *E. plebejum* has polygonal to nodulose basidiospores; *E. sericeoides* has centrally depressed pileus and basidia without basal clamp-connections.

Mycorrhizal morphology

Rhizomorphs of *E. aprile* were connected to lateral roots of *Populus maximowiczii* growing near the basidiomata. Therefore, *P. maximowiczii* could be identified as probable mycorrhizal host of *E. aprile* in the study site.

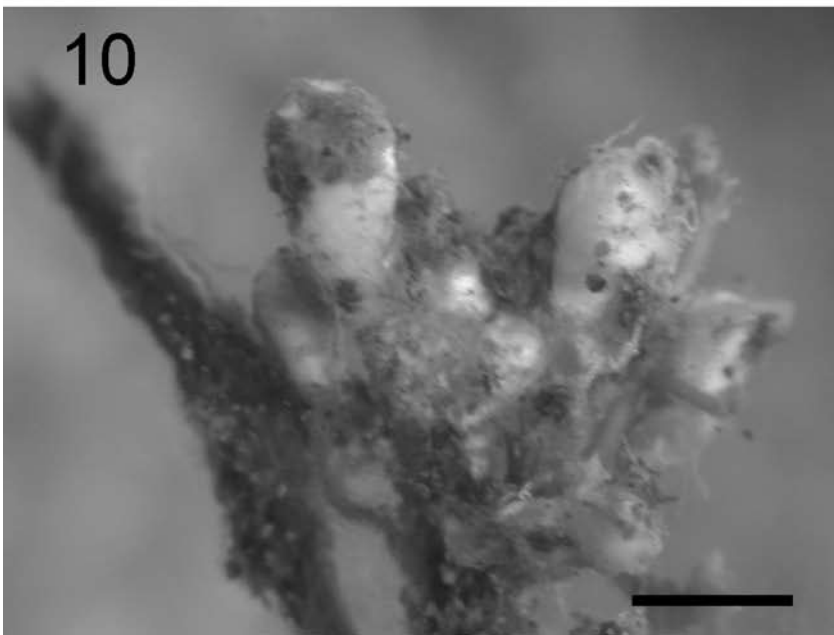
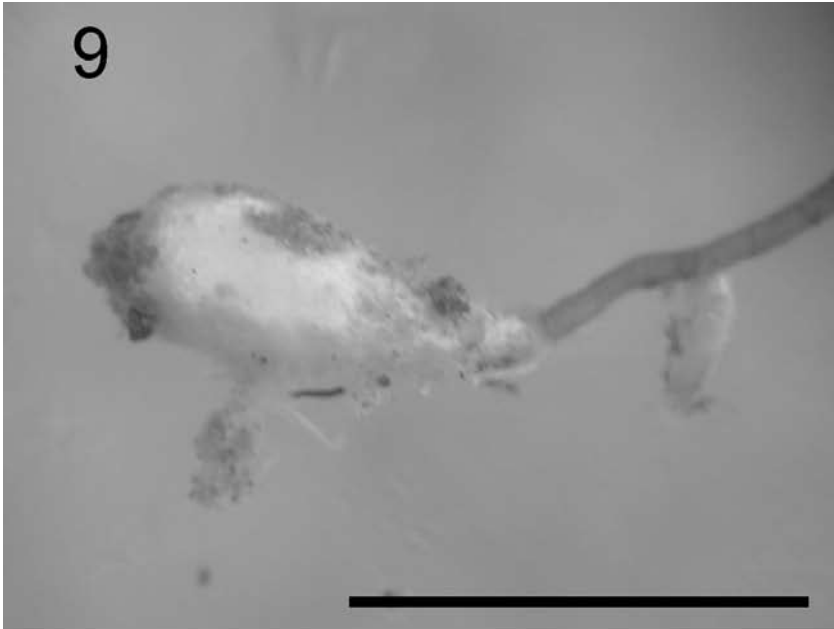
Macroscopic characters

The mycorrhizae of *E. aprile* on *P. maximowiczii* (Figs. 9–10) were unramified, drum-stick shaped, 1–5.5 mm long, 1–3.5 mm in diameter, silvery white to ochraceous, and covered with woolly to felty texture and adhering soil debris. Tips of the mycorrhizae were cylindrical to clavate. In longitudinal sections of mycorrhizae, a thick gelatinous and whitish hyphal sheath was observed around the root cortex (Fig. 11). Rhizomorphs were cream colored to somewhat pale ochraceous, embedded in a thin mycelium; several mycorrhized root tips were connected. No colored mycelia were observed around rhizomorphs and mycorrhizae.

Remarks. – The characters are morphologically similar to those of *E. clypeatum* f. *hybridum* (on *R. multiflora*) mycorrhizae, but the surface color of the latter is almost white (Kobayashi & Hatano 2001) and they have pinkish mycelia around rhizomorphs and mycorrhizae (Kobayashi & Yamada 2003). The surface structures of *E. sepium* mycorrhizae on *Rosa* sp. (Agerer & Waller 1993) also resemble those of *E. aprile*, but the latter can be distinguished by its cream to somewhat pale ochraceous rhizomorphs.

Microscopic characters

In longitudinal sections of most mycorrhized root tips, a distinct hyphal sheath was observed outside the root tissue (Figs. 12–13, HS). The cortex of the mycorrhizae remained in the basal part (Figs. 12–13, C), whereas root cells had disappeared and were replaced by the fungal hyphae in the apical part. In the apical parts of mycorrhizae, the root cap and meristem had disappeared and were replaced by fungal hyphae (Fig. 12, arrow and Fig. 14). Invading hyphae in the apical part were somewhat ramified, undulated, elongated, 1.5–3 μm in diameter. The stele of the roots had remained.



Figs. 9-10. – Mycorrhizae of *E. aprile* on *P. maximowiczii* (SAPA 1160): **9.** Plan view of a mycorrhiza on a lateral root (bar = 4 mm). **10.** Several mycorrhizae on a lateral root (bar = 2 mm).

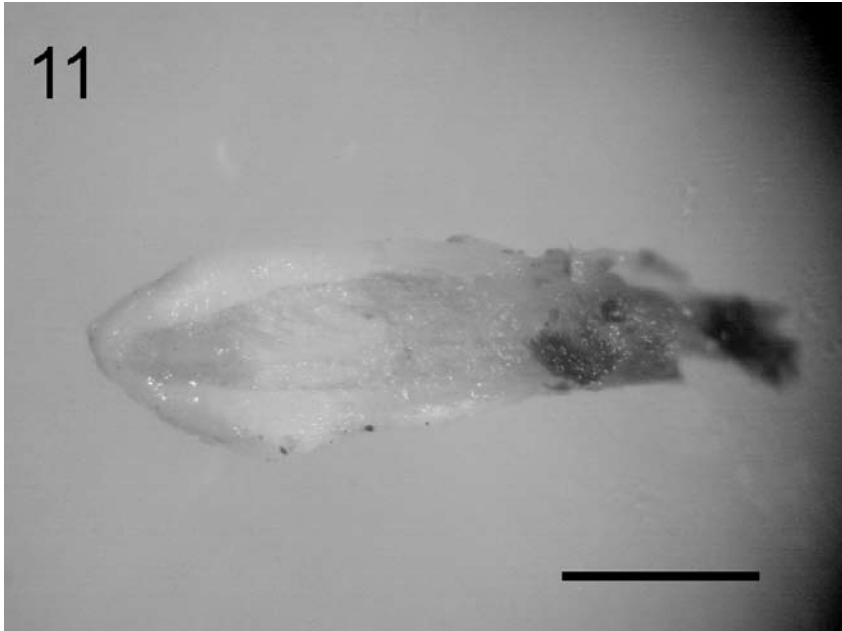
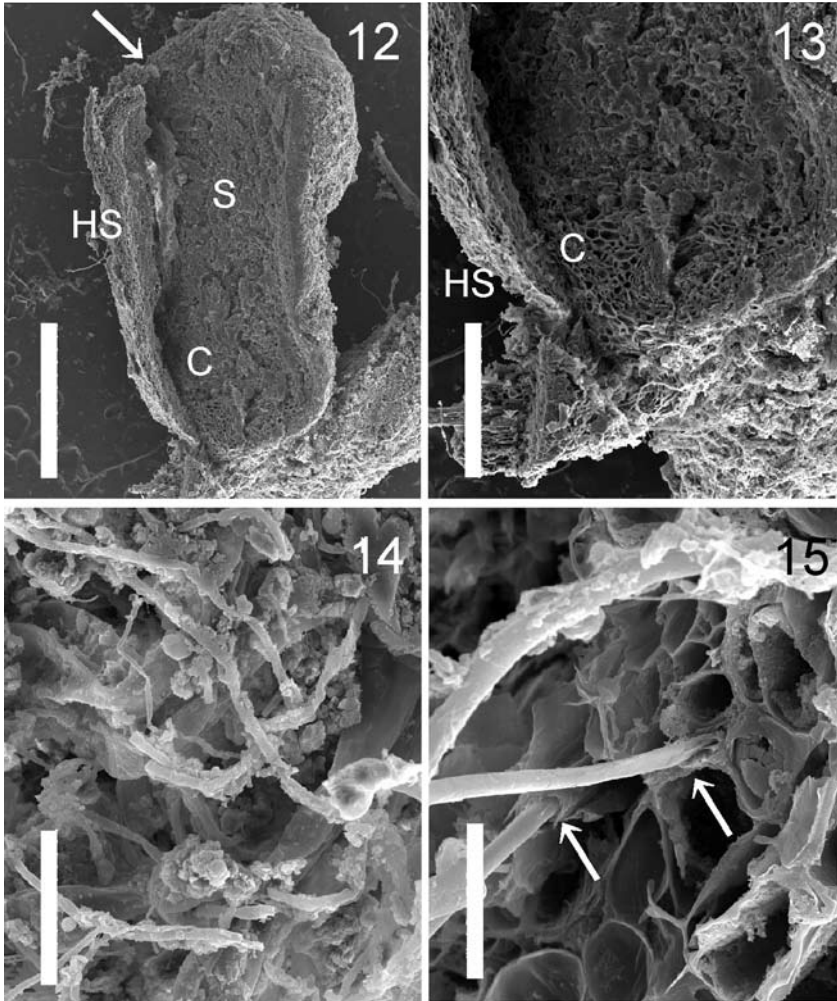


Fig. 11. – A longitudinal section of a mycorrhizized root tip with a thick, gelatinous hyphal sheath (SAPA 1160). Bar 3 mm.

The fungal hyphae in the basal part somewhat invaded outer cortical cells (Fig. 15, arrows). Epidermal cells were not observed under the hyphal sheath. Outer cortical cells in this part sometimes contained dark granules (Figs. 16–17). Root cells with intact nuclei were present close to the cells invaded by hyphae (Fig. 18). Microsclerotia of unidentified dark septate endophytes (DSEs) were observed in some outer cortical cells in the basal part of mycorrhizae (Fig. 19, arrows).

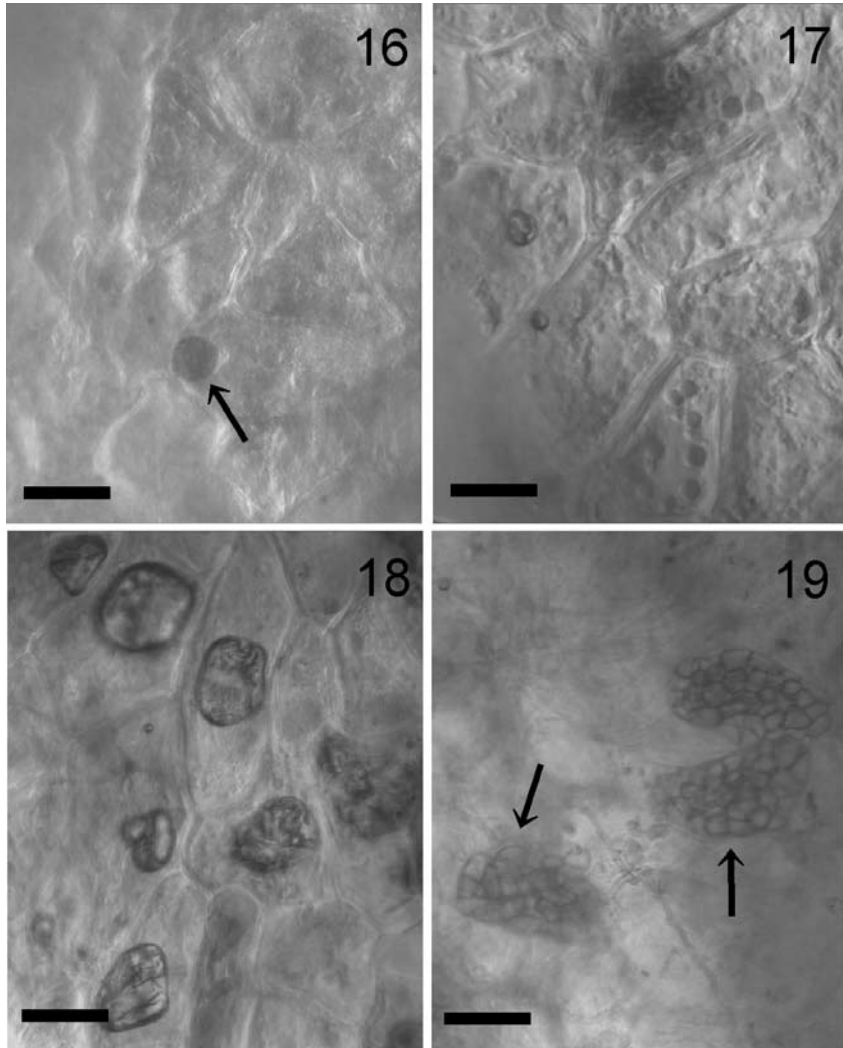
The hyphal sheath was composed of three layers according to hyphal thickness and arrangement. The outermost layer (Fig. 20) was a loose plectenchymatous tissue composed of subparallel, colorless, clampless and gelatinous-walled hyphae, 2–6.5 μm in diameter. The middle layer (Fig. 21) was composed of loose interwoven hyphae, 2–4.5 μm in diameter, colorless, clampless, and with gelatinous walls (Fig. 21, black arrows). This layer was intermixed with pseudoparenchymatous cells, 1.5–30 μm in diameter, colorless to ochraceous, and extremely gelatinous (Fig. 21, white arrows). The inner layer (Fig. 22–23) was a pseudoparenchymatous tissue composed of globose to subglobose or somewhat ovoid, colorless to ochraceous, extremely gelatinous cells of 1.5–35 μm in diameter.

Remarks. – In our morphological observations of the mycorrhizae of *E. aprile* on *P. maximowiczii*, hyphal sheaths were formed on root tips, but apical root cells had disappeared in mycorrhizal speci-



Figs. 12–15. – SEM micrographs of longitudinal sections of the mycorrhizae (SAPA 1160): **12.** A mycorrhiza: hyphal sheath (HS) covered with root tissue; stele (S) and cortex of the basal part (C) remained, but the root cap, meristem, and apical region of the cortex disappeared (arrow) (bar 0.5 mm). **13.** Basal part of the mycorrhiza (bar = 100 μ m). **14.** Apical part of the mycorrhiza: root cells replaced by fungal hyphae (bar = 10 μ m). **15.** Fungal hyphae in outer cortical cells (arrows) (bar = 10 μ m).

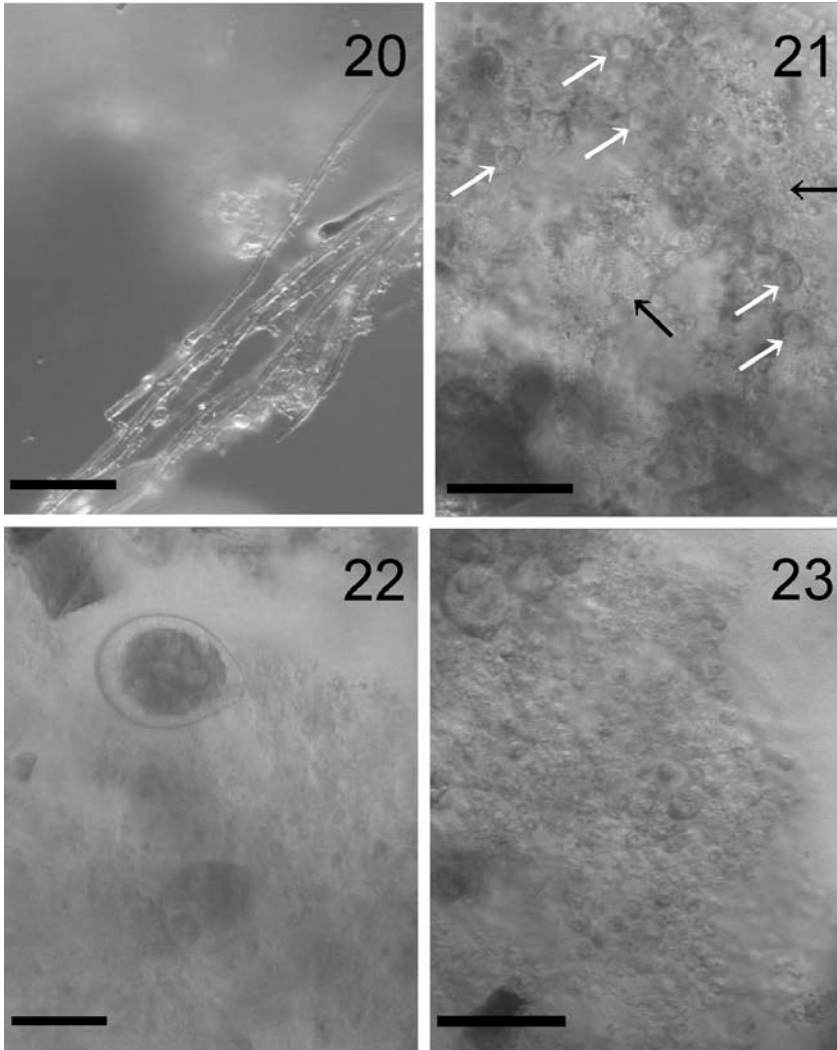
mens examined. Moreover, invading hyphae were observed in the basal part of the outermost cortical cell layer, but a typical Hartig net was not formed. These morphological features suggest that the mycorrhizae of *E. aprile* on *P. maximowiczii* are not ectomycorrhizal: they are morphologically similar to mycorrhizae of *E. sepium* on *Rosa* sp. and *P. domestica* (Agerer & Waller 1993), and of *E. clypeatum* f. *hybridum* on *R. multiflora* (Kobayashi & Hatano 2001). Therefore, we think that



Figs. 16–19. – Nomarski interference contrast micrographs of longitudinal sections of mycorrhizae (SAPA 1160): **16.** A large dark granule of an outer cortical cell in the basal part (arrow). **17.** Several small dark granules of outer cortical cells in the basal part. **18.** Root cells with intact nuclei near cells with invaded hyphae. **19.** Microsclerotia of unidentified DSE fungi (arrows) within the outer cortical cells in the basal part of mycorrhized root tips. Bars 10 μm .

entolomatoid fungi associated with rosaceous and ulmaceous plants have the ability to form their structurally unique mycorrhizae on various plants, including members of salicaceous species.

However, ectomycorrhizal entolomatoid fungi associated with salicaceous plants have been reported in previous studies (Antibus *et al.* 1981, Linkins & Antibus 1982, Graf & Brunner 1996). The ectomy-



Figs. 20–23. – Nomarski interference contrast micrographs of a hyphal sheath (SAPA 1160): **20.** The outer layer, composed of plectenchymatous tissue (bar = 20 μm). **21.** The middle layer, intermixed with plectenchymatous (black arrows) and pseudoparenchymatous (white arrows) tissues (bar = 10 μm). **22.** Two large, spherical to oval, pseudoparenchymatous cells in the inner layer (bar = 10 μm). **23.** Numerous small, spherical, pseudoparenchymatous cells in the inner layer (bar = 10 μm).

corrhizae of *Entoloma sericeum* (Bull.) Quél. on *Salix rotundifolia* Trautv. form a thick mantle of clamped hyphae running parallel to the root axis and an epidermal Hartig net out of which no invasion of root cells was observed (Antibus *et al.* 1981, Linkins & Antibus 1982). Besides, the ectomycorrhizae of *E. alpicola* (J. Favre) Noordel. on *S. herbacea* L. have a thick, two-layered mantle of clamped hyphae and form

a typical Hartig net, also without any intracellular hyphae (Graf & Brunner 1996). From these facts, we think that entolomatoid fungi associated with salicaceous plants form two types of mycorrhizae depending on the fungal species, viz. the unique morphological type described above and typical ectomycorrhizae. For a better understanding of mycorrhizal symbiosis between entolomatoid fungi and diverse plants, more comprehensive studies on their species-specific mycorrhizal morphology are required.

Microsclerotia of unidentified DSE fungi (Fig. 19, arrows) were observed in some outer cortical cells in the basal part of *E. aprile* mycorrhizae. Our mycorrhizal specimens have been collected from cool-temperate deciduous forests of Hokkaido, northern Japan (Fig. 1). Perhaps, DSEs frequently colonize roots of *P. maximowiczii* in those forests because plants growing in cool, nutritionally poor, alpine or sub-alpine ecosystems have a high incidence of DSEs (Haselwandter & Read 1980, Stoyke *et al.* 1992, Hambelton & Currah 1997). Not infrequently, plant roots form typical mycorrhizal associations and, in addition, become colonized by DSE fungi (Peterson *et al.* 2004). However, no reports on additional colonization of DSEs in non-ectomycorrhizal, unique types of mycorrhizae formed by entolomatoid fungi including *E. aprile* have hitherto been published before the present study.

Phylogenetic analysis

ITS sequences were obtained from our collected specimens of basidiomata and mycorrhizae from Hokkaido, and allied three *Entoloma* species collected from other parts of Japan (Table 1). Phylogenetic comparisons of our specimens with allied specimens on ITS sequences of *Entoloma* spp. and related genera are shown in Fig. 24. Basidiomata of *E. aprile* were highly homologous (99.4 % – 100.0 %) among our collected specimen and were separated from other known *Entoloma* spp. in the dendrogram based on ITS sequences. These results support our morphological observation that *E. aprile* differs from *E. clypeatum* f. *clypeatum*, *E. clypeatum* f. *hybridum*, and *E. sepium* in Japan, and is therefore a new record for the Japanese mycobiota.

The sequences of *P. maximowiczii* mycorrhizae from Miyagaoka Park, Sapporo, were also highly homologous (96.7 % – 97.3 %) with that of *E. aprile* basidiomata. Thus, we could confirm that *P. maximowiczii* is the host plant of *E. aprile*. On the other hand, the sequences from mycorrhizae on *P. maximowiczii* from Ishikari shelterbelt forest, Ishikari, were highly homologous with *E. clypeatum* f. *clypeatum* (99.5 % – 100.0 %), *E. clypeatum* f. *hybridum* (92.9 % and 98.9 %) and *E. sepium* (99.7 % and 99.8 %), and clearly separated from *E. aprile* samples. Consequently, we have to assume that at least two species of *Entoloma* share a very similar niche in these forests.

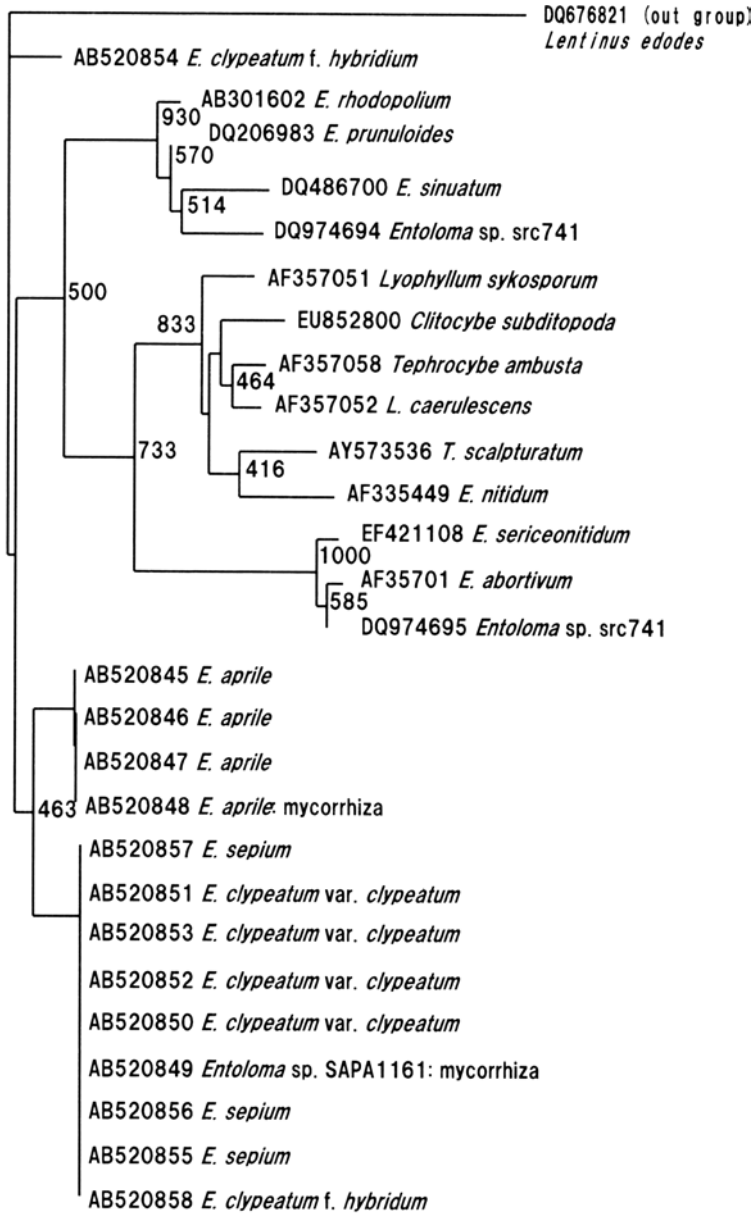


Fig. 24. – Neighbor-joining (NJ) tree based on sequences of the internal transcribed spacer ITS1-5.8S-ITS2 region for phylogenetically related species in the genus *Entoloma*. Bootstrap percentages (from 1000 replications) greater than 40 % are shown at branch points.

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