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Comparison of *Cenangium* Dieback Fungus Isolated from Three Different Species of Pine

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Dieback of pine branches or twigs with brown needles occurs most commonly on *Pinus* species after severe winter in Korea. In this study, *Cenangium ferruginosum* was isolated from infected stems, branches, and twigs of *Pinus koraiensis* (C1), *P. densiflora* (C2), and *P. thunbergii* (C3). Morphological and cultural characteristics of the isolates were then compared. There were no significant differences in the morphological characteristics of conidia and ascospores produced by the three isolates. However, cultural differences were observed among the isolates. Optimum temperatures for mycelial growth of C1, C2, and C3 were 15, 20, and 20°C, respectively. C1 produced a few conidia and no ascospores, while C2 and C3 produced abundant ascospores and conidia. While optimum temperatures for mycelial growth ranged from 15 to 20°C, mycelial growth was also relatively good at lower temperatures of 5-10°C. Conidiomata and conidia were produced on MSA (malt extract soya peptone agar) after 25-30 days of incubation in the dark at 15°C. Apothecia were produced by altering culture condition from 15 to 20°C, and incubating for 35-60 more days. Optimum temperature for ascospore and conidium germination was 20°C. RAPD analysis revealed that there was high similarity of 0.78 between C2 and C3, and low similarity of 0.31 between C2 or C3 and C1.

Keywords : apothecia, ascospore, *Cenangium ferruginosum*, dieback fungus, *Pinus* species.

Cenangium species known as the causal agent of *Cenangium* dieback of pines are ascomycetous fungi which usually damage several coniferous trees. Japanese red pines planted as a landscape tree in urban forests and recreation areas are often infected with this fungus, but group-dyings in pine forests are most common these days after severe environmental conditions (Sinclair and Hudler, 1980). Since the first report of the disease on *Pinus koraiensis* at Gapyung and Cheongpyung area in Korea in 1989, group-dyings have been observed to occur in Kangwon and Kyunggi provinces every year. Shirai (1911) and Kobayashi and Mamiya (1961) reported the disease on *P. thunbergii* and *P. densiflora*, respectively, in Japan. Kowalski (1998) reported the outbreak on *P. sylvestris* over one million hectares of land in Poland. The disease occurs widely on *P. thunbergii* in Tailen, China. The Korea Research Association of Tree Protection has also surveyed the disease in 1999.

Cash and Davison (1940) classified the genus *Cenangium* into *C. ferruginosum, C. abietis,* and *C. atropurpureum* by the size of apothecium and the color of excipulum. Ferchau and Johnson (1956) reported that *C. ferruginosum* was a markedly variable species, with the variations in ascocarps most likely being expressed as a partial function of the host, maturity of the fructification, and as a result of environmental conditions at the time of fruiting. They concluded that *C. atropurpureum* was merely a variant of *C. ferruginosum,* and that *C. atropurpureum* was synomymous with *C. ferruginosum.* Funk (1981) described the morphological characteristics of anamorph and teleomorph of *C. ferruginosum.* Vloten and Gremmen (1953) described the morphological and cultural characteristics of *C. ferruginosum.* Kobayashi and Mamiya (1963) reported that optimum pH and temperature for mycelial growth of the isolate from *P. densiflora* were pH 4-5 and 10-25°C, respectively. They also reported that optimum temperature and humidity required for ascospore germination were 25°C and 100% RH, respectively. However, comparisons of isolates from different species of pine were not carried out because of the slow growing characteristic of this fungus. Thus, more researches are needed to fully understand the biology and ecology of this fungus for the development of effective management strategy. The objective of present study was to investigate and compare the characteristics of anamorph and teleomorph of *C. ferruginosum* isolates from three different species of pines namely, *P. koraiensis, P. densiflora,* and *P. thunbergii.* These characteristics include spore bearing structures, sporulation, morphological characteristics of spores, spore germination, and cultural characteristics.

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Materials and Methods

Fungal isolation. *Cenangium* dieback fungal isolates were obtained from infected branches of *P. koraiensis* in the experimenal forest of Kangwon National University in Hongchon; *P. densiflora* in Seoul; and *P. thunbergii* in Tailen, China. Single ascospores were collected from mature apothecia on the bark fixed with vaseline on the inner surface of the lid of a petri dish containing 1.5% water agar. Germinating ascospores on 1.5% water agar were transcultured on PDA (potato dextrose agar) (Table 1). Fungal identifications were already described in a previous paper (Lee et al., 1998).

Cultural characteristics. To investigate the proper composition of culture media and optimum culture conditions, mycelial growth was compared by inoculating agar discs (ϕ 5 mm) with mycelium on various culture media (Table 2) at different pH and temperatures. Radial mycelial growth was compared after 15 days of incubation in the dark at 15°C. Mycelial growth at various ranges of temperature (5-30°C) or pH (4-10) on MSA medium (3% malt extract, 0.3% soya peptone, 1.5% agar) supplemented with hot-water extract from *P. thunbergii* was also measured after culturing in the dark at 15°C for 15 days. All experiments were repeated three times.

Sporulation and microscopic observations. Fungal isolates were grown on MSA in the dark at 15°C for 1 month. Morphological characteristics of 100 conidia from each isolate were observed. After producing ascospores in apothecium by culturing at the altered incubation temperature (20°C) for 2 more months, morphological characteristics of apothecia and ascospores were also compared with the structures produced on naturally-infected trees by sectioning with freezing microtome (MICROM HM505E) and observing under light microscope (NIKON Eclipse E800).

Spore germination. Fungal isolate (C2) from *P. densiflora*, which produced the most abundant conidia and ascospores among isolates, was used to investigate the effect of temperature on spore germination. After harvesting both condia and ascospores from culture media, concentrations of spore suspension were adjusted at 1.72×10^8 spores/ml and 4.5×10^6 spores/ml, respectively. Fifty (50) microliters of each spore suspension was spread on 1.5% water agar and incubated for 15 days at various temperatures (15, 20, 25, and 30°C). Spore germination was observed under a light microscope. All treatments were repeated three times.

RAPD analysis. Genomic total DNA was extracted from the cultured mycelium using the CsCl method described by Yoon et al. (1991). RAPD procedure used in this experiment was described by Schots et al. (1994). The primers used were Random primer Kit B Amplitaq DNA Nos. 1,3,5,7,11,12,14,15,16,17 (Operon Tech.). DNA amplification was performed in a thermal cycler (Mini Cycler™, MJ Research) with an initial denaturation step of 3 min at 94°C (1 cycle), followed by 43 cycles of 1 min denaturation at 94°C, 1 min annealing at 35°C, and 2 min for extension at 72°C with a final extension of 5 min at 72°C. PCR products were visualized using ethidium bromide staining after electrophoresis (Mupid 21, Cosmo Bio Co.) in 1.5% agarose gel (1x TBE buffer) at 100V for 30 min. The UPGAMA program of Gel Documentation System (Bio-Rad) was used to estimate the genetic relationships among *Cenangium* isolates.

Table 1. Host and origin of *Cenangium ferruginosum* isolates used in this experiment

Isolates	Host	Origin
C1	*Pinus koraiensis*	Chunchon, Korea
C2	*P. densiflora*	Seoul, Korea
C3	*P. thunbergii*	Tailen, China

Table 2. Composition of various culture media used in comparing mycelial growth of *Cenangium ferruginosum* isolates

Medium	Composition
PDA	Potato Dextrose Agar (Difco)
PDA(Pk)[a]	Potato Dextrose Agar + HWE[e] (*P. koraiensis*)
PDA(Pd)[b]	Potato Dextrose Agar + HWE (*P. densiflora*)
PDA(Pt)[c]	Potato Dextrose Agar + HWE (*P. thunbergii*)
MSA[d]	Malt Extract Agar (Difco)
MSA(Pk)	Malt Extract Soya Peptone Agar + HWE (*P. koraiensis*)
MSA(Pd)	Malt Extract Soya Peptone Agar + HWE (*P. densiflora*)
MSA(Pt)	Malt Extract Soya Peptone Agar + HWE (*P. thunbergii*)

[a]PDA (Pk): PDA containing hot-water extract from *P. koraiensis*.
[b]PDA (Pd): PDA containing hot-water extract from *P. densiflora*.
[c]PDA (Pt): PDA containing hot-water extract from *P. thunbergii*.
[d]MSA: 3% Malt extract, 0.3% Soya peptone, 1.5% Agar.
[e]HWE: hot-water extracts from branches or twigs of *Pinus* species.

Results

Cultural characteristics. Mycelial growth on the culture media supplemented with extracts from pine trees was greatly increased as compared with those on non-supplemented media. All three isolates showed good growth on MSA containing hot-water extract from *P. densiflora*, and

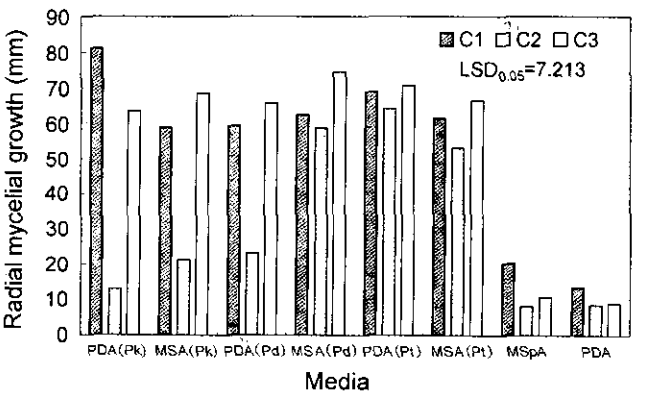

Fig. 1. Comparison of *Cenangium ferruginosum* isolates from the three species of pine in radial mycelial growth on various culture media. C1: isolates from *Pinus koraiensis*, C2: isolates from *P. densiflora*, C3: isolates from *P. thunbergii*. Radial mycelial growth was measured at 15 days after incubation in the dark at 15°C.

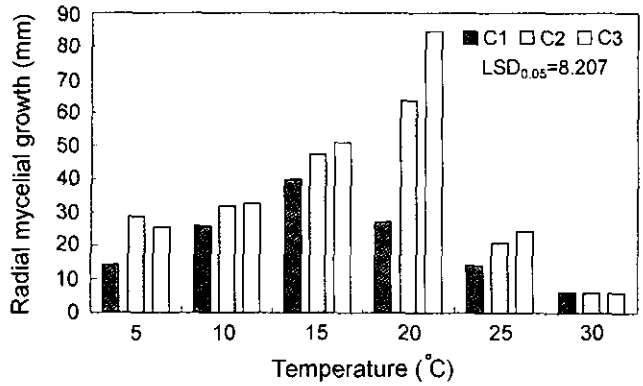

Fig. 2. Effect of temperature on radial mycelial growth of *Cenangium ferruginosum* isolates from the three species of pine. C1: isolates from *Pinus koraiensis*, C2: isolates from *P. densiflora*, C3: isolates from *P. thunbergii*. Radial mycelial growth was measured at 15 days after incubation on MSA(Pt) in the dark at 15°C.

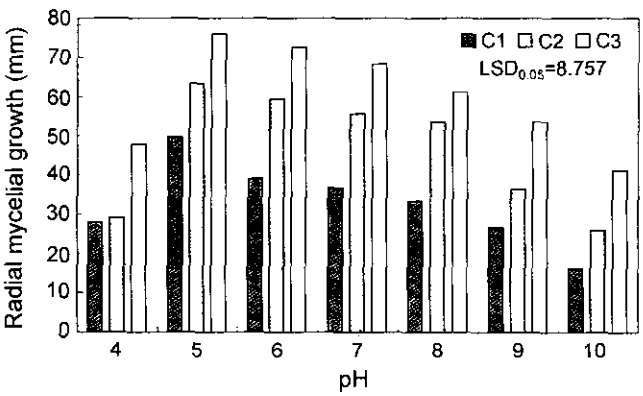

Fig. 3. Effect of pH on radial mycelial growth of *Cenangium ferruginosum* isolates from the three species of pine. C1: isolates from *Pinus koraiensis*, C2: isolates from *P. densiflora*, C3: isolates from *P. thunbergii*. Radial mycelial growth was measured at 15 days after incubation on MSA(Pt) in the dark at 15°C.

on MSA and PDA containing hot-water extract from *P. thunbergii*. Radial mycelial growth on these media ranged from 59 to 74 mm for 15 days (Fig. 1). Optimum temperature for C2 and C3 was 20°C, but that for C1 was 15°C (Fig. 2). Mycelial growth was the best at pH 5, and gradually decreased with the increase in pH (Fig. 3).

Sporulation and morphological characteristics of spores. Conidiomata and conidia were produced on MSA after 25-30 days of incubation in the dark at 15°C (Fig. 4B). However, the production of apothecia and ascospores required more incubation periods at the altered incubation temperature of 20°C. Incubation periods required for apothecia and ascospore formation varied from 35 to 60 days, depending on the fungal isolates (Table 3).

Conidiomata produced *in vitro* were dark brown to black, and globoid. The mass of conidia was produced in the form of sticky droplets, milky white to yellowish white in the early stage, and changed into brown to dark brown through time (Fig. 4B). Conidia were bacilliform (Fig. 4C).

Table 3. Incubation period and temperature required for the production of asexual and sexual structures of *Cenangium ferruginosum* isolates

Isolate	Host	Structures	Incubation period/temperature		
			30 days/ 15°C	60 days/ 20°C	90 days/ 20°C
C1	*Pinus koraiensis*	Asexual	−	−	+
		Sexual	−	−	−
C2	*P. densiflora*	Asexual	+++		
		Sexual	−	++	
C3	*P. thunbergii*	Asexual	+++		
		Sexual	−	−	+

−: no; +: sparse; ++: fair; +++: abundant production of asexual or sexual structures.

Apothecia produced on MSA (Fig. 4H, 4J) did not show significant differences from apothecia on naturally-infected host (Fig. 4F, 4G) in size and morphology. However, they were darker in color than that of natural structures. Hyphae density in medullary excipulum of apothecium produced on artificial culture media was lower than those of naturally-developed apothecium. Ascospores were hyaline, broadly ellipsoid, and non-septate (Fig. 4I). Comparisons in size of conidia and ascospores of three isolates are shown in Table 4.

Effect of temperature on spore germination. Conidial germination was observed 10-20 days after culturing. Optimum temperature for germination was 20°C, but germination rate was lower than 0.04% with poor mycelial growth after germination (Fig. 4D). On the other hand, optimum temperature for ascospore germination was 20°C (Fig. 5). After germination of single conidium and ascospore, they again produced conidia after 30 days of incubation in the dark at 15°C. Additional incubation at 20°C for 60 days resulted in the formation of apothecia and ascospores from 70% of single ascospore and 30% of single conidium.

Genetic relationships among three isolates from different species of pine. Primer No. 14 amplified the same PCR products for the three *Cenangium* isolates. There was no PCR product in C1 isolate amplified by primer Nos. 3 and 4, but C2 and C3 isolates were not amplified by primer No. 16. The other primers produced very similar PCR products between C2 and C3 isolates, but not with C1 isolate (Fig. 6). Phylogenetic tree of the RAPD profiles by UPGAMA program revealed that C2 and C3 belonged to one group with high similarity of 0.78, while C1 was another group with low similarity of 0.31 (data not shown).

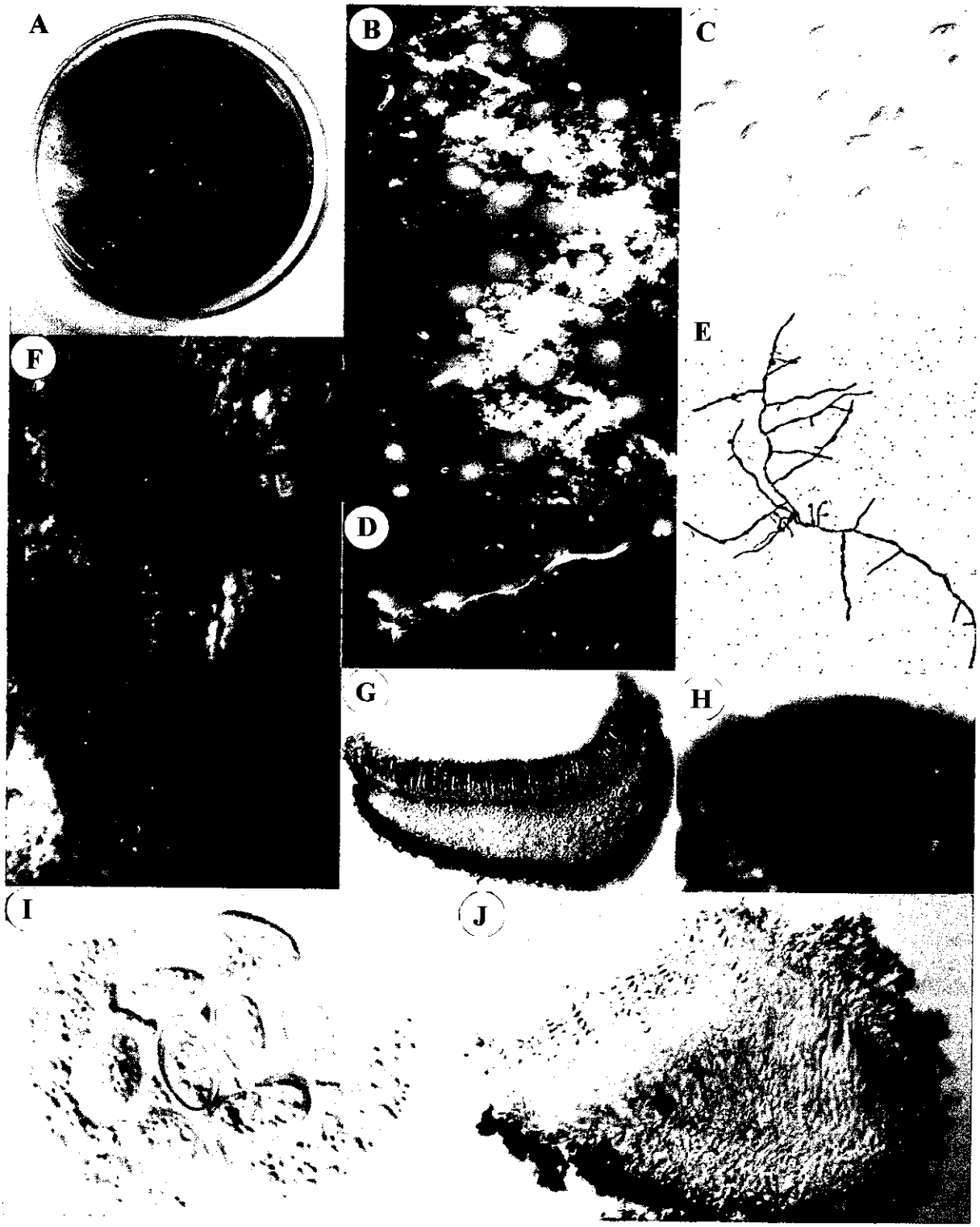

Fig. 4. Mycological characteristics of *Cenangium ferruginosum* isolate. (**A**) Typical colony morphology of *C. ferruginosum* isolate on MSA (malt extract soya peptone agar), (**B**) Conidiomata and mass of conidia shown in the form of sticky and shiny droplets on MSA after 25-30 days of incubation in the dark at 15°C, (**C**) Bacilliform conidia, (**D**) Germinating conidia, (**E**) Growing branched hyphae on 1.5% water agar, (**F**) Top view of mature and open apothecia naturally developed on infected branches of a pine tree, (**G**) Longitudinal section of the apothecium produced on natural host showing lined-up asci, internally formed ascospores, and dense hyphae in medullary excipulum, (**H**) Mature and partially open apothecia produced on artificial culture media after 60-90 days of incubation, (**I**) Eight-spored ascus, (**J**) Longitudinal section of an apothecium produced on artificial culture media.

Table 4. Comparison of conidium and ascospore size of *Cenangium ferruginosum* isolated from three species of pine

Isolate	Host	Size (μm)	
		Conidium	Ascospore
C1	*Pinus koraiensis*	2.9-4.5 × 1.0-2.0 (3.7 × 1.5)[a]	4.9-7.3 × 7.3-11.0 (5.8 × 9.3)
C2	*P. densiflora*	1.9-5.0 × 1.0-2.0 (3.3 × 1.2)	4.9-6.1 × 8.5-11.0 (5.8 × 10.6)
C3	*P. thunbergii*	1.9-4.5 × 1.0-2.0 (3.3 × 1.3)	5.4-6.1 × 9.3-11.0 (5.9 × 10.1)

[a] Values in parenthesis indicate the mean of 100 observations.

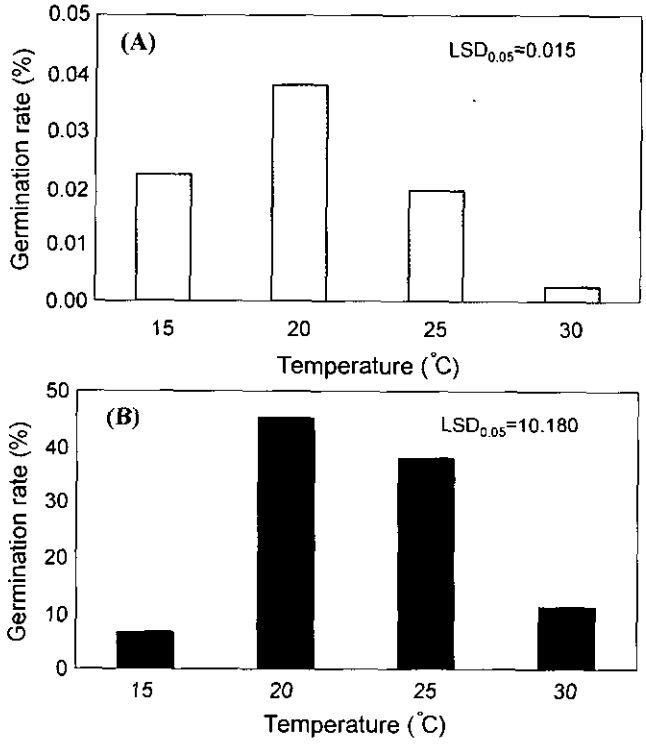

Fig. 5. Effect of temperature on conidia (**A**) and ascospores (**B**) germination of *Cenangium ferruginosum* isolate (C2) at various temperatures. Spore suspension was spread and incubated on 1.5% water agar for 10-20 days. Each datum indicates the mean of 9 replications.

Discussion

It was difficult to find significant differences in morphological characteristics, i.e., shape and size of conidia and ascospores from all isolates tested. However, there were cultural differences among isolates in terms of optimum temperature for mycelial growth and sporulation. C2 and C3 isolates grew well at 20°C and produced abundant spores, but C1 grew well at 15°C and showed poor sporulation. C1 isolate showed relatively lower mycelial growth than those of C2 or C3 at the same temperature and pH conditions. Generally, optimum temperature for mycelial growth was 15-20°C, and mycelial growth was better at 5-10°C than at 25-30°C. In addition, mycelial growth almost stopped at 30°C or above. Thus, these isolates showed typical characteristics of psychrophylic fungi. These characteristics are also associated with the frequent occurrence of *Cenangium* dieback of pines after severe winter and drought (Sinclair et al., 1980). Apothecia were produced by altering incubation temperature from 15 to 20°C, and optimum temperature for ascospore germination was 20-25°C. These ranges of temperature are associated with the biology and ecology of *Cenangium*, i.e., apothecial formation, maturity, and dissemination of ascospores in nature (Sinclair et al., 1987; Forrest Research Institute, 1991). In the comparison of culture media, the best medium for mycelial growth was composed of 3% malt extract, 0.3% soya pep-

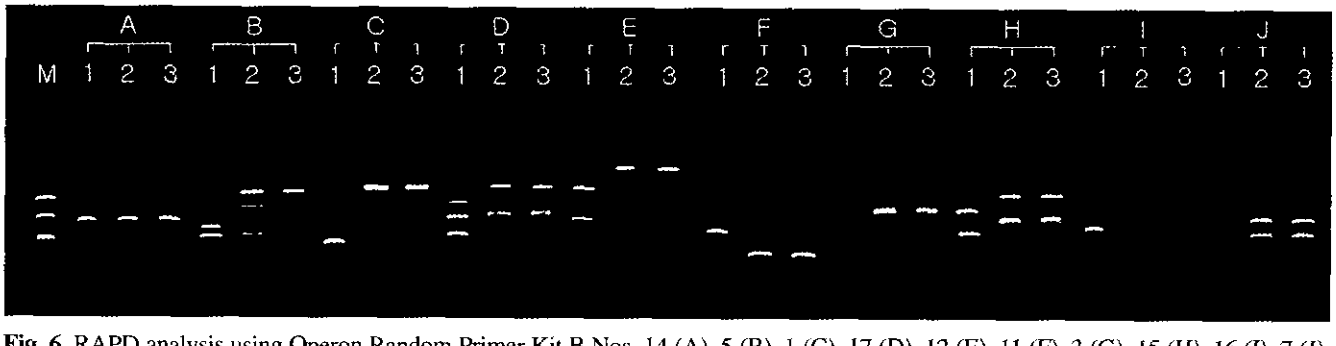

Fig. 6. RAPD analysis using Operon Random Primer Kit B Nos. 14 (A), 5 (B), 1 (C), 17 (D), 12 (E), 11 (F), 3 (G), 15 (H), 16 (I), 7 (J), respectively. Lane 1 to 3 represent the PCR products from *Cenangium ferruginosum* C1, C2, and C3 isolates, respectively. M: PCR marker (Promega).

tone, 10% hot-water extract from pines, and 1.5% agar. This medium gave relatively good mycelial growth, even though Kobayashi and Mamiya (1963) reported that this fungus was very slow growing.

Optimum pH for mycelial growth was 5, but mycelial growth was relatively good at a wide range of pH 4-10. This is not common because most of the plant pathogenic fungi do not grow well at basic conditions. Furthermore, this fungus did not show rapid decline in mycelial growth even below pH 4 or above 10. Optimum temperature for ascospore germination was 20-25°C, and this range of temperature might be related with the ecological characteristics of the pathogen, which are usually disseminated at humid and cool weather by rain. The mean temperature from July to August, that is the period of ascospore dissemination, was reported at 24-26°C. The presence or absence of pathogenicity for this fungus is still uncertain (Boyce, 1948; Fink, 1911; Kujala, 1950; Sinclair and Hudler, 1980; Smerlis, 1973; Weir, 1921). However, Lee et al. (1998) proved the pathogenicity of this fungus by inoculating C1 isolate on 3-year-old pot-grown *P. koraiensis* seedlings, and killing them within 2 months after inoculation. Vloten and Gremmen (1953) reported that conidia were not involved in the infection process. Conidia germinated at very low rate, but they could grow normally if germinated (Fig. 4E). Therefore, it must be assured whether the conidia can induce the disease or not. *C. ferruginosum* seems to be a homothallic fungus because apothecia and ascospores were produced from 70% of single ascospores or 30% of single conidium. RAPD results for genetic relatedness analysis showed the differences among the three isolates. Genetic similarity between C2 and C3 was relatively high at 0.78, while those between C1 and C2 or C3 was low at 0.31. Furthermore, based on the cultural characteristics, C2 isolate showed higher similarity with C3 isolate in mycelial growth at various ranges of temperature and sporulation than C1. *Cenangium* isolates tested differed in cultural and genetic characteristics even if there were no differences in morphological characteristics. More isolates from different pine trees in various locations may be required for the analysis of genetic relatedness among them.

Acknowledgments

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