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Variation in *Sclerotium rolfsii* isolates in Japan

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Sixty-seven isolates of the southern blight fungus from Japan were divided into five groups based on ITS-RFLP analysis of nuclear rDNA. Morphological characters of sclerotia varied between groups. Three groups were reidentified as *Sclerotium rolfsii*, and two resembled *S. delphinii* in RFLP patterns and/or in having large sclerotia and relatively low optimal growth temperature (28°C). Sclerotia of the latter, however, varied in size according to temperature and became indistinguishable from those of *S. rolfsii* at high temperatures. Hyphal anastomosis (imperfect fusion) was observed between different ITS-RFLP groups, as well as between different isolates belonging to the same groups. These results indicate that populations of this fungus in Japan consists of several different subgroups, although morphological differences are not always evident.

Key Words——ITS-RFLP; *Sclerotium delphinii*; *Sclerotium rolfsii*.

Sclerotium rolfsii Sacc. (teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough) is a soil-borne plant pathogenic fungus with a wide host range and worldwide distribution (Punja, 1988). Because its basidial stage (Sawada, 1912a, b; Nakata, 1926; Goto, 1930; Punja et al., 1982; Punja and Grogan, 1983) is not easily observed, this fungus is characterized by the morphology of sclerotia in most cases (Saccardo, 1911; Punja and Damiani, 1996). Consequently, little has been studied on its genetic background and/or extent of variation.

Recentry, Harlton et al. (1995) reported the variation in internal transcribed spacer regions (ITS) of ribosomal DNA of *S. rolfsii* and related fungi by RFLP (restriction fragment length polymorphisms) analysis. They also demonstrated a close affinity between *S. rolfsii* and *S. delphinii* Welch on a molecular basis, although they are morphologically distinct (Welch, 1924; Stevens, 1931; Nishihara, 1961; Punja and Damiani, 1996).

As geographical variability was considered to exist in the *S. rolfsii* population because of its life cycle strategy, we tested 67 isolates from different hosts and various parts of Japan, which country was not included in the previous paper (Harlton et al., 1995). Most isolates had been identified as *S. rolfsii* (Nonaka et al., 1990; Ishikawa et al., 1992; Tezuka et al., 1992; Uematsu et al., 1992; Shoji, 1993; Takeuchi and Horie, 1993; Uematsu et al., 1993; Ishii and Tezuka, 1994; Tezuka and Ishii, 1994; Sato et al., 1995; Shoji, 1996), but some of them differed from typical *S. rolfsii* isolates. They were isolated from tulips in the cool-temperate zone in early summer when it was too early for *S. rolfsii* to prevail.

We aimed to characterize various isolates in Japan by ITS-RFLP, colony morphology, and hyphal anastomosis, which has been used to differentiate subgroups within species (Ogoshi, 1976; Carling, 1996). Brief reports of this work were presented previously (Morikawa et al., 1997; Okabe et al., 1997a, b).

Materials and Methods

Isolates Isolates used in this study had been isolated from various hosts of diverse geographic origins (Table 1) and identified as *S. rolfsii*. Each isolate belonged to a distinct mycelial compatibility group in the preliminary pairing test.

PCR amplification and RFLP analysis PCR amplification and RFLP analysis of ITS regions of rDNA followed the methods of Harlton et al. (1995). Mycelia grown in potato-dextrose broth for 1–2 wk were used for DNA isolation according to the standard protocol of a DNA isolation kit (Easy-DNA Kit, Invitrogen, San Diego). Primers ITS1 and ITS4 (White et al., 1990) were used to amplify the ITS region, which consisted of ITS1, 5.8 S rDNA, and ITS2 regions. PCR reaction mixture (50 μl) contained 5 μl of 10 × Gene *Taq* Universal Buffer (Nippon Gene, Tokyo), 400 μM dNTP mixture (Nippon Gene, Tokyo), 20 pM concentrations of primers ITS1 and ITS4, 1 unit of *Taq* DNA polymerase (Nippon Gene, Tokyo), and 50 ng of DNA template. The thermal cycling consisted of the following steps: 5 min at 95°C for initial denaturation; 30 cycles of 1 min at 95°C for denaturation, 1.5 min at 55°C for annealing, and 2 min at 72°C for extension; and 10 min at 72°C for final extension. PCR products were digested with *Alu* I, *Hpa* II, *Rsa* I, or *Nde* II (*Mbo* I) at 37°C for 3 h, and the digests were subjected to electrophoresis in 2% agarose gel (NuSieve 3 : 1, FMC BioProducts, Rockland) with TAE buffer containing ethidium bromide (Sambrook et al., 1989).

Mycelial growth and sclerotial formation Mycelial growth rate and sclerotial formation were determined on

Table 1. Host, locality, and source of isolates used in this study.

Isolate	Host	Locality	Obtained from
S-1 (CH92S-1)	*Alstromeria* sp.	Chiba	S. Uematsu[a]
2 (CH92S-2)	*Alstromeria* sp.	Chiba	S. Uematsu[a]
3 (CH92S-3)	*Solanum mammosum*	Chiba	S. Uematsu[a]
4 (CH92S-4)	*S. mammosum*	Chiba	S. Uematsu[a]
5 (CH92S-5)	*Phalaenopsis* sp.	Chiba	S. Uematsu[a]
6 (CH87-52)	Solidaster	Chiba	S. Uematsu[b]
7 (CH87-53)	Solidaster	Chiba	S. Uematsu[b]
8 (CH91-41)	*Delphinium ajacis*	Chiba	S. Uematsu[b]
9 (CH91-42)	*D. ajacis*	Chiba	S. Uematsu[b]
10 (CH91-52)	*Chlorophytum* sp.	Chiba	S. Uematsu[b]
11 (MAFF410049)	*Cryptomeria japonica*	Fukui	MAFF Gene Bank
12 (MAFF410050)	*Coptis japonica*	Fukui	MAFF Gene Bank
13 (MAFF102020)	*Glycine max*	Gunma	MAFF Gene Bank
14 (MAFF102021)	*G. max*	Tokushima	MAFF Gene Bank
15 (MAFF102022)	*G. max*	Gifu	MAFF Gene Bank
16 (MAFF102023)	*Arachis hypogaea*	Ibaraki	MAFF Gene Bank
17 (MAFF305344)	Soil	Ishikawa	MAFF Gene Bank
18 (MAFF305545)	*Trifolium repens*	Chiba	MAFF Gene Bank
19 (CR-1)	*G. max*	Akita	T. Nakajima
20 (CR-2)	*G. max*	Akita	T. Nakajima
21	*Tricyrtis hirta*	Mie	N. Tezuka[c]
22	*Saponaria officinalis*		N. Tezuka
23	*Eupatrium fortunei*	Mie	N. Tezuka[c]
24	*Solanum tuberosum*	Mie	N. Tezuka[c]
25	*Limonium sinuatum*		N. Tezuka
26	*Aster* sp.	Mie	N. Tezuka[d]
27	*Gladiolus* sp.	Mie	N. Tezuka[e]
28	*Physostegia virginiana*		N. Tezuka
29	*Althaea rosea*		N. Tezuka
30	*Beta vulgaris* var. *cicla*	Hiroshima	N. Tezuka
31 (Co-3501-1)	*Peperomia caperata*	Tokyo	J. Takeuchi[f]
32 (CoOp-1)	*Ophiopogon japonicus*	Tokyo	J. Takeuchi[f]
33 (Co-400-1)	*Ajuga* sp.	Tokyo	J. Takeuchi[f]
34 (Co-Pa-1)	*Pachysandra terminalis*	Tokyo	J. Takeuchi
35 (Co-3-2)	*Caladium* sp.	Hachijo	J. Takeuchi
36 (Co-Ar-1)	*Aralia cordata*	Tokyo	J. Takeuchi
37 (Co-At-1)	*Atractylodes japonica*	Tokyo	J. Takeuchi
38 (Co-Ly-1)	*Licopersicon esculentum*	Tokyo	J. Takeuchi
39 (Co-Ne-1)	*Nerine* sp.	Miyake	J. Takeuchi
40 (NI91-02)	*Fragaria ananassa*	Tochigi	N. Ishikawa[g]
41	*Aralia cordata*	Tochigi	N. Ishikawa
42 (CR-8801)	*Trapa bispinosa*	Saga	T. Motomura[h]
43 (CR-8923)	*T. bispinosa*	Saga	T. Motomura
44 (CR-9001)	*T. bispinosa*	Saga	T. Motomura
45 (CR-9006)	*T. bispinosa*	Saga	T. Motomura
46 (CR-9007)	*T. bispinosa*	Saga	T. Motomura
47 (CR-9011)	*T. bispinosa*	Saga	T. Motomura
48 (CR-9014)	*T. bispinosa*	Saga	T. Motomura
49 (CR-9016)	*T. bispinosa*	Saga	T. Motomura
50 (CR-9101)	*T. bispinosa*	Saga	T. Motomura
51	*Arachis hypogaea*	Ibaraki	N. Matsumoto

Table 1. Continued.

Isolate	Host	Locality	Obtained from
S-52	*A. hypogaea*	Ibaraki	N. Matsumoto
53	*A. hypogaea*	Ibaraki	N. Matsumoto
54	*A. hypogaea*	Ibaraki	N. Matsumoto
55	*A. hypogaea*	Ibaraki	N. Matsumoto
56	*Tulipa gesneriana*	Toyama	Y. Chikuo
57	*T. gesneriana*	Toyama	Y. Chikuo
58	*Lilium* sp.	Toyama	Y. Chikuo
59	*Lilium* sp.	Toyama	Y. Chikuo
60	*Lilium* sp.	Toyama	Y. Chikuo
61	*Lilium* sp.	Toyama	Y. Chikuo
62 (W2)	*Citrullus vulgaris*		T. Sato[i]
63 (B12)	*G. max*		T. Sato[i]
64 (Y1)	*Polymnia sonchifolia*	Kagawa	T. Sato[i]
65 (F-1)	*Eupatorium fortunei*	Saitama	T. Shoji[j]
66	*Arctium lappa*	Saitama	T. Shoji
67 (I-1)	*Selaginella tamariscina*	Saitama	T. Shoji[k]

a) See Uematsu et al., 1993. b) Uematsu et al., 1992. c) Ishii and Tezuka, 1994. d) Ishii et al., 1992. e) Tezuka and Ishii, 1994. f) Takeuchi and Horie, 1993. g) Ishii et al., 1992. h) Nonaka et al.,1990. i) Sato et al., 1995. j) Shoji, 1996. k) Shoji, 1993.

Table 2. ITS-RFLP grouping of *Sclerotium rolfsii* in Japan based on four restriction enzyme digest patterns.

Group	ITS-RFLP[a]	Isolates in each group
Group 1	A2, H3, R1, M2	S-2, 3, 4, 6, 7, 10, 13, 14, 15, 21, 22, 24, 26, 27, 28, 29, 30, 31, 33, 34, 35, 39, 42, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 62, 63, 64, 66, 67
Group 2	A2, H3, R1, M3	S-1, 5, 9, 18, 23, 25, 38, 43, 44, 45, 65
Group 3	A1, H2, R2, M4	S-17
Group 4	A2, H1, R3, M2	S-32, 36, 37, 41, 56, 57, 58, 59, 60, 61
Group 5	A2, H1, R0[b], M1	S-8, 11, 12, 16, 19, 20, 40

a) Patterns were designated by Harlton et al. (1995). b) Not digested by *Rsa* I.

potato-dextrose agar (PDA) plates. Mycelial plugs (5 mm in diam) were taken from the margin of 2- to 3-d-old colonies and inoculated on the medium. Mycelial growth was determined 2 d after incubation at 23, 25, 28, 30 and 33°C,and sclerotial formation (number, size and weight) was evaluated after 40 d.

Hyphal interactions A total of 13 isolates selected from each ITS-RFLP group were paired on cellulose membranes laid over water agar in all possible combinations. Microscopic observations were made every day for 2 wk.

Results

PCR amplification and RFLP analysis Gel electrophoresis of PCR product from every isolate yielded a uniform band of approximately 730 bp. *Alu* I, *Hpa* II, *Rsa* I, and *Nde* II digestion produced 2, 3, 4, and 4 patterns, respectively (Fig. 1). Whereas most patterns corresponded to A1-2, H1-3, R1-3 and M1-4 designated by Harlton et al. (1995), one of the *Rsa* I patterns was not described before. We refer to the new pattern as "R0."

The 67 isolates used were classified into five groups by the combination of the above RFLP patterns: 1) A2, H3, R1, M2 (38 isolates), 2) A2, H3, R1, M3 (11 isolates), 3) A1, H2, R2, M4 (1 isolate), 4) A2, H1, R3, M2 (10 isolates), 5) A2, H1, R0, M1 (7 isolates) (Table 2). Groups 1, 3, and 4 corresponded to groups II (*S. rolfsii*), XI (*S. rolfsii*), and XIV (*S. delphinii*) described by Harlton et al. (1995), respectively. Groups 2 and 5 were new.

Growth and sclerotial formation The optimum temperature for mycelial growth was 28°C for most isolates of ITS-RFLP group 5 and some of group 4, whereas group 1 showed optimum growth at 30°C (Fig. 2). Group 2 showed the same pattern as group 1 (data not shown), and the single isolate of group 3 was not included in this test because it showed restricted growth.

Most isolates of group 1 produced small sclerotia (Fig. 3). Their optimum temperatures for maximum diam, number, and weight were 23–28°C (Figs. 4a, c, e). Group 4 isolates formed large sclerotia with stipes and irregular shape (Fig. 3). However, the size decreased with increasing temperature, and morphologi-

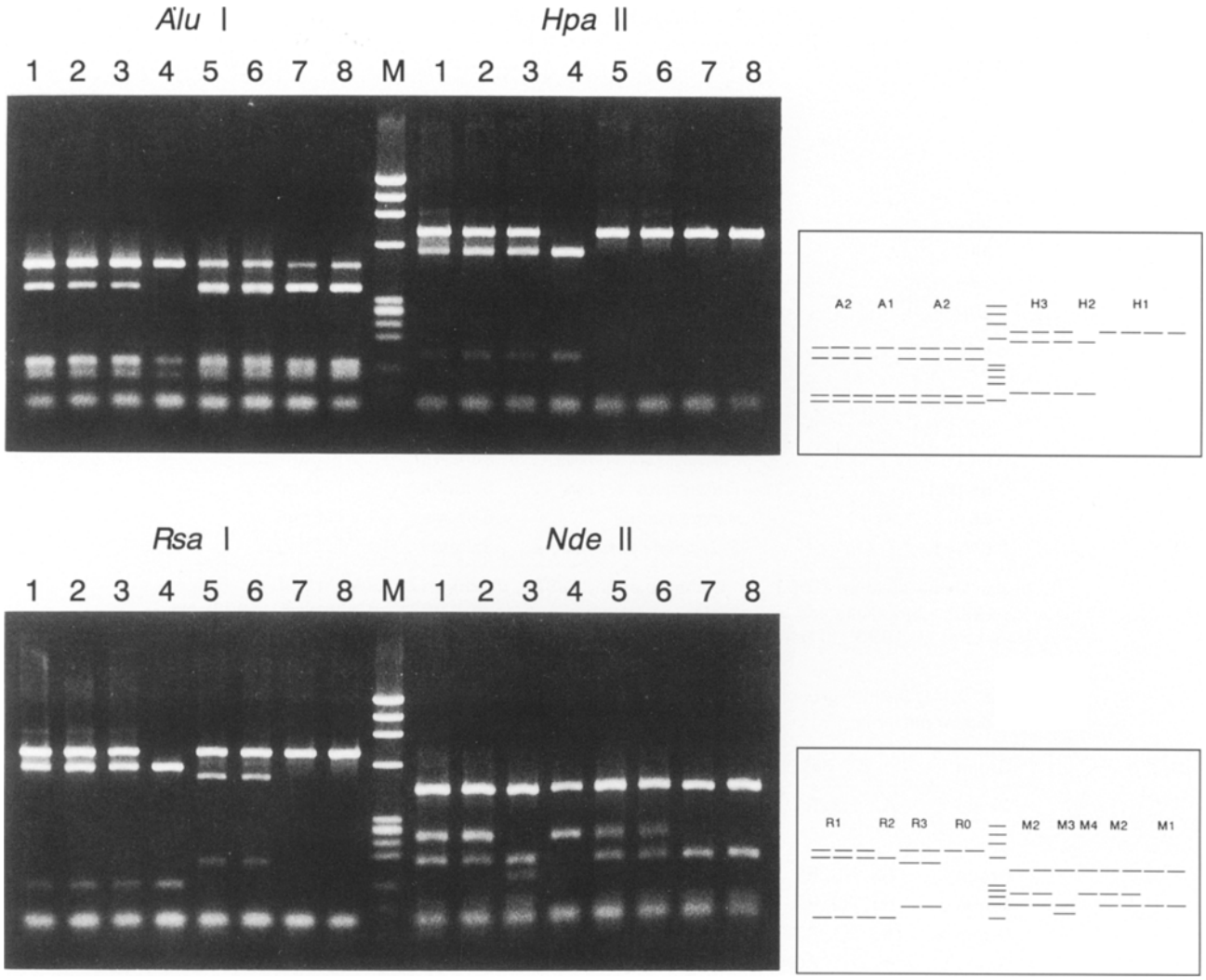

Fig. 1. ITS-RFLP patterns of Japanese isolates.
Four restriction enzymes (*Alu* I,*Hpa* II,*Rsa* I and *Nde* II) were used. Lane 1=isolate S-22 (group 1), lane 2=isolate S-42 (group 1), lane3=isolate S-43 (group 2), lane 4=isolate S-17 (group 3), lane 5=isolate S-41 (group 4), lane 6=isolate S-56 (group 4), lane 7=isolate S-8 (group 5) and lane 8=isolate S-12 (group 5), lane M=ϕX174/*Hae* III (1353, 1078, 872, 603, 310, 281, 234, 194, 118 bp).

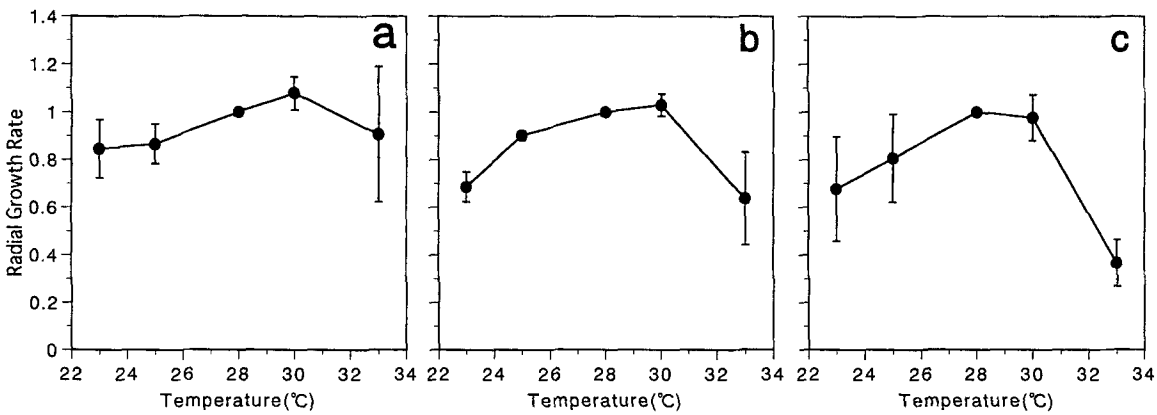

Fig. 2. Mycelial growth of groups 1 (a), 4 (b), and 5 (c).
Mycelia were incubated for 2 d at different temperatures. Data are expressed relative to the growth at 28°C. Vertical bars show the standard deviations.

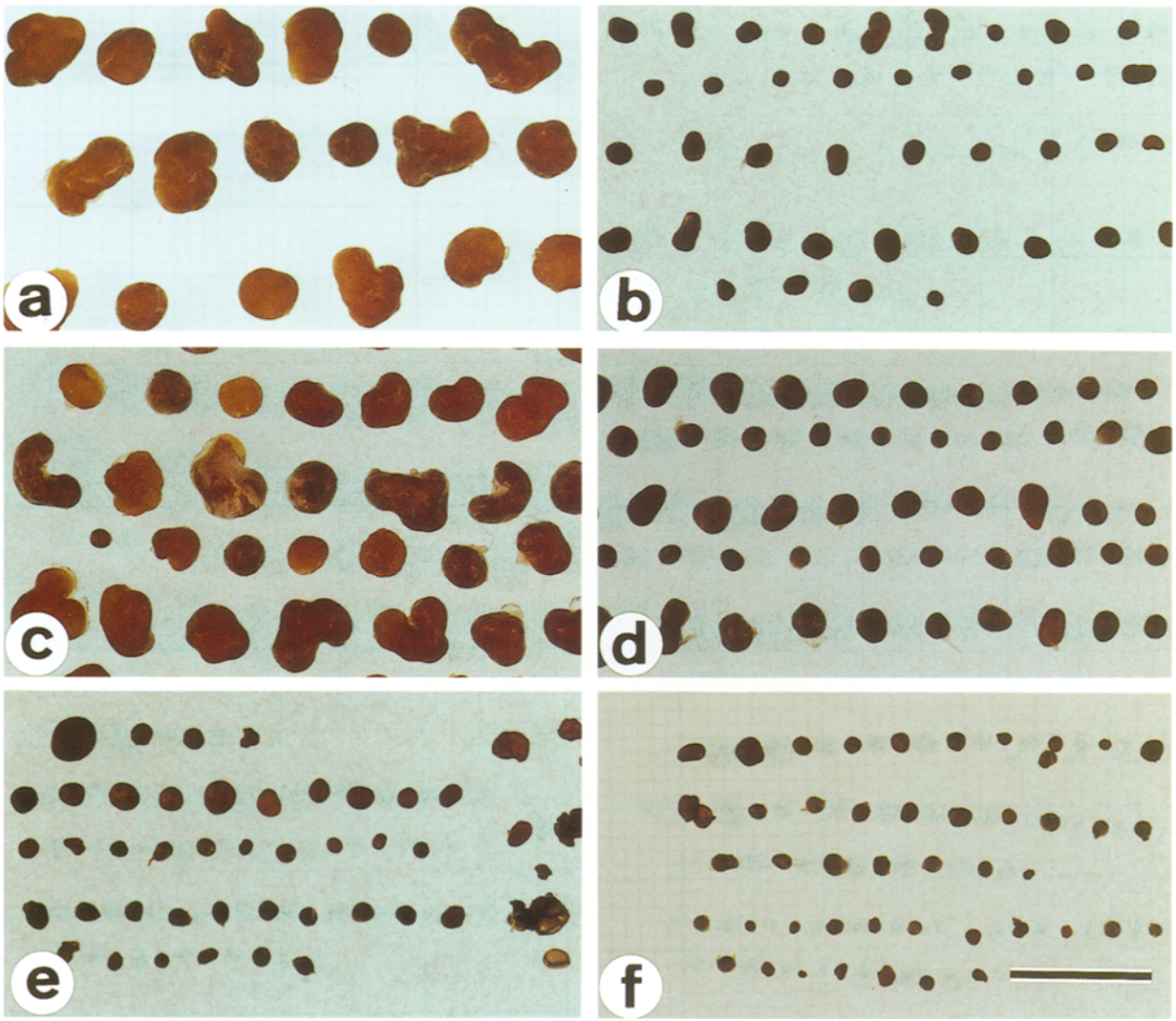

Fig. 3. Sclerotia of isolates S-58 (group 4) and 46 (group 1) produced at 23, 28, and 33°C.
a) Isolate S-58 at 23°C, b) S-46 at 23°C, c) S-58 at 28°C, d) S-46 at 28°C, e) S-58 at 33°C, f) S-46 at 33°C. Scale = 1 cm.

cal differences from groups 1 or 2 became unclear (Figs. 3, 4b). The weight was maximum at 28°C for most isolates of group 4, but the number was maximum at 30°C (Figs. 4d, f). Few sclerotia were formed by isolates of group 5, and group 3 failed to produce sclerotia. Sclerotia formation of group 2 was similar to that of group 1 (data not shown).

Hyphal interactions Mycelia of paired isolates came into contact 1–2 d after inoculation (Fig. 5a), and hyphae anastomosed (Fig. 6). Mycelia of both isolates intermingled completely in 3–4 d (Fig. 5b). Hyphal cells in the contact zone subsequently died in every combination except self-pairings, and a barrage zone was evident in 5–14 d (Fig. 5c).

Discussion

ITS-RFLP analysis revealed the existance of five groups in Japanese isolates. Each group had its own morphological characteristics and RFLP patterns, corresponding to previous results, but often differed in growth temperature reactions.

Group 1 was typical of *S. rolfsii* in sclerotial morphology and RFLP patterns and was identical to group II, one of the major groups of *S. rolfsii* in the U.S.A. and Indonesia (Harlton et al., 1995). However, group 1 differed from the description of *S. rolfsii* in the U.S.A. and South Asia, whose mycelial growth and sclerotium formation occurred at 35°C (Chowdhury et al., 1993; Punja and Damiani, 1996). Group 2, which has not been reported in other countries, was similar to group 1 in morphological characters. Groups 1 and 2 accounted for 73% of Japanese isolates, whereas groups I, III, and IV, the major groups in the U.S.A., were not found in Japan. Morphological characterization of group 3 was difficult because the only isolate available did not produce sclerotia. However, its RFLP pattern demonstrated that it corresponded to group XI (*S. rolfsii*) in Nepal (Harlton et al.,

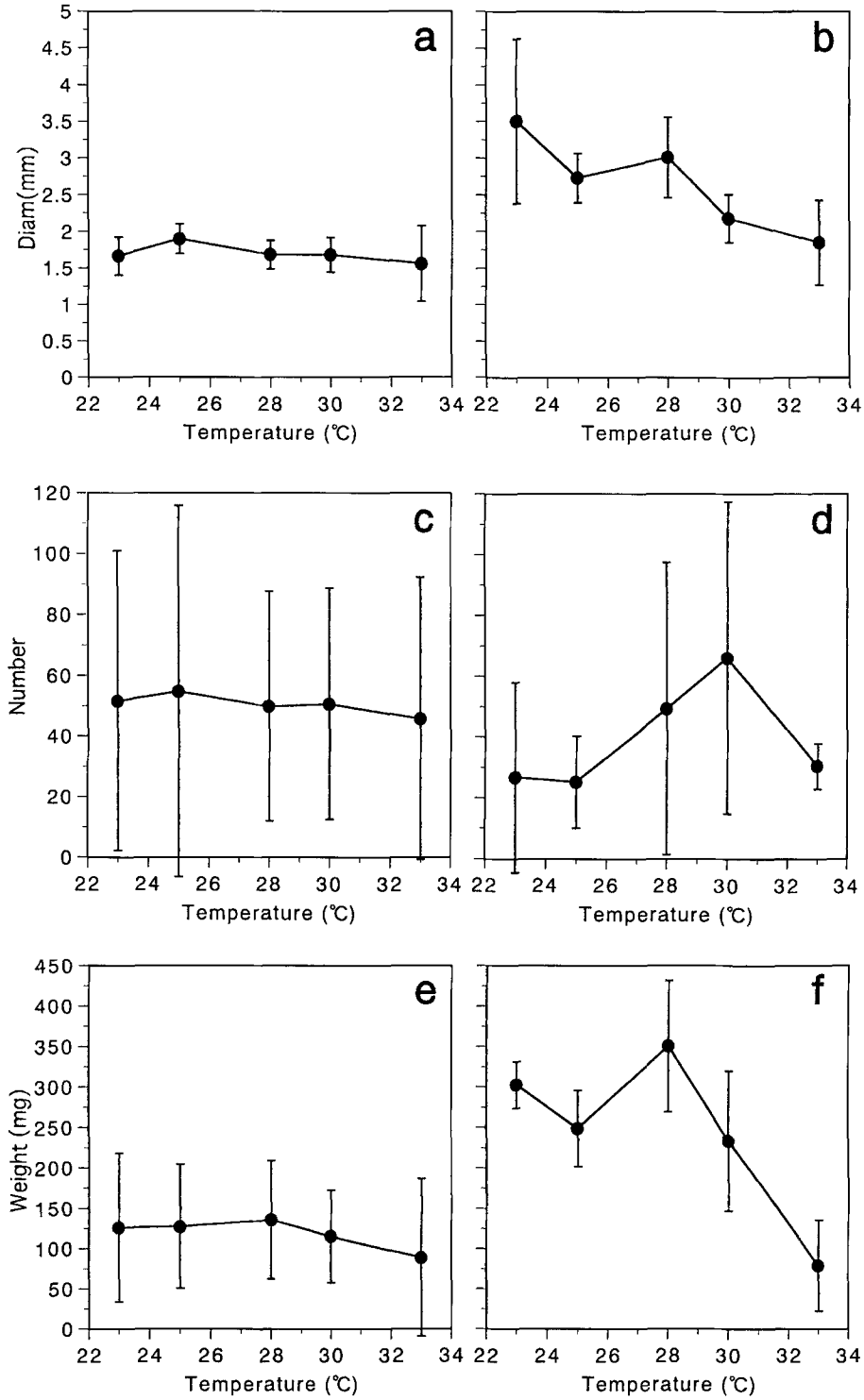

Fig. 4. Diameter, number, and weight of sclerotia produced on PDA plate (9-cm diam) by isolates of groups 1 and 2 (a, c, e) and 4 (b, d, f) at different temperatures.
Vertical bars show the standard deviations.

1995).

RFLP pattern and sclerotial morphology indicated that group 4 was similar to *S. delphinii*, as was its pathogenicity to Liliaceae and adaptation to the cool-temperate zone (Boerema and Hamers, 1988) like the Hokuriku area in Japan. Punja (personal communication) regards this group as *S. delphinii*. However, optimal temperature for mycelial growth and sclerotium formation of group 4 was

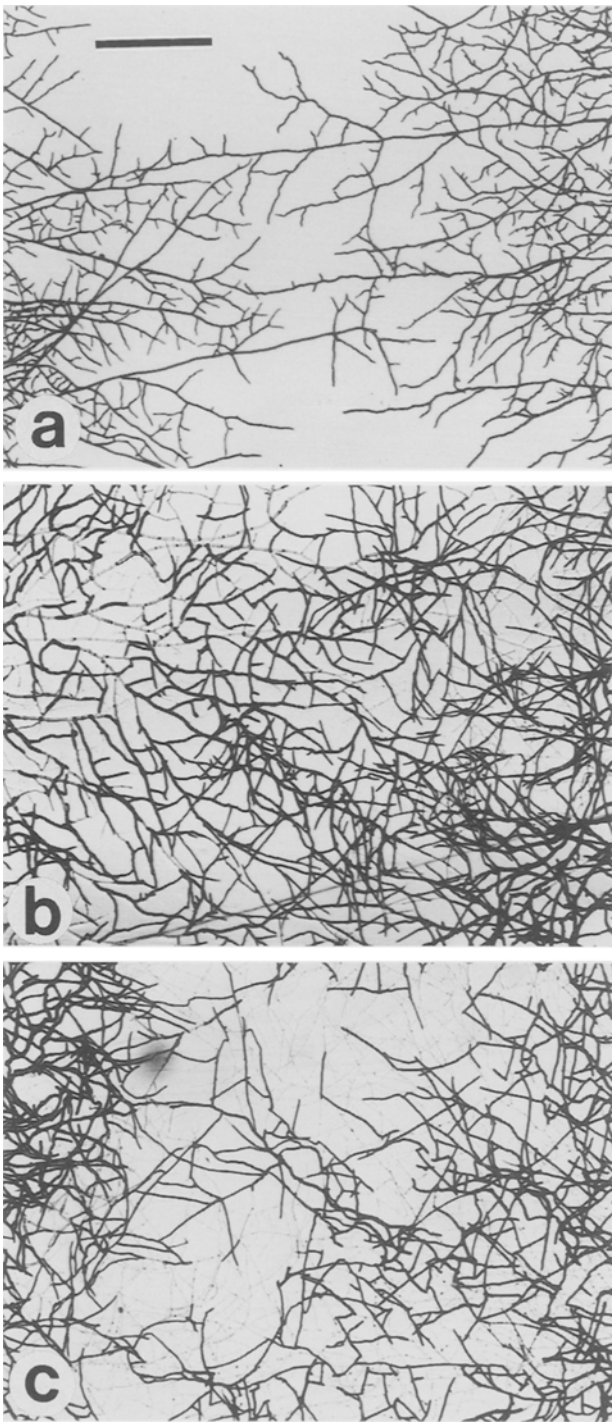

Fig. 5. Incompatible reaction between isolate S-54 (group 1, left) and 35 (group 1, right).
Mycelia of the two isolates came into contact 2 d after inoculation (a), intermingled completely after 4 d (b), and disruption of hyphal cells became apparent after 6 d (c). Scale = 400 μm.

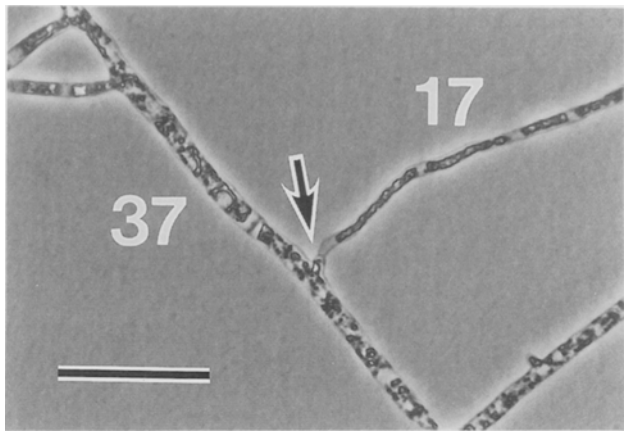

Fig. 6. Hyphal anastomosis of isolates S-17 (group 3) and 37(group 4) observed under a phase-contrast microscope. Scale = 30 μm.

higher than that of typical *S. delphinii* isolates in the U.S.A. (Punja and Damiani, 1996). Consequently, the difference in growth temperature between groups 4 and 1 was not as great as that between isolates of *S. delphinii* and *S. rolfsii* from other countries. Group 5, which had a new RFLP (*Rsa* I) pattern, was more similar to group 4 than groups 1 or 2 in the optimal temperature for mycelial growth and RFLP pattern. Although this group did not produce sclerotia easily, their morphology resembled those of *S. delphinii*.

Growth temperature reaction of each group coincided with their geographic distribution and assumed life cycle. For example, most members of group 4 were isolated from tulips and lilies in the cool-temperate zone in early summer, whereas groups 1 and 2 were mainly isolated in the warm-temperate zone in summer or early autumn (Table 1). The pattern of geographic distribution also agreed with the fact that the optimal growth temperature of Japanese isolates was lower than that of isolates from Central America and South Asia (Chowdhury et al., 1993; Punja and Damiani, 1996). It is suggested that the morphological characters of *S. rolfsii* and *S. delphinii* isolates vary continuously and are similar in the temperate zone, where the two fungi overlap.

ITS-RFLP analysis (*Hpa* II and *Rsa* I patterns) differentiated *S. rolfsii* and *S. delphinii*. However, different patterns do not always indicate different species, because the *Nde* II pattern of group 1 (*S. rolfsii*) was the same as that of group 4 (*S. delphinii*) but not those of groups 2 or 3. Some patterns seemed to be a complex of other patterns (i.e., R1 = R0 + R2, H3 = H1 + H2, and M2 = M1 + M4), suggesting that these isolates had two distinct types of nuclei (heterokaryon), genome (heterozygote), or ITS region. The complexed patterns (R1, H3 and M2) suggest the possibility of genetic exchange between groups.

We observed that hyphal anastomosis occurred between groups 1 (*S. rolfsii*) and 4 (*S. delphinii*) as well as between the isolates in the same group. This suggests genetical relatedness of the groups. However, *S. rolfsii* is considered to be homothallic (Punja and Grogan, 1983), and genetic exchange between groups through sexual recombination is less likely to occur. Further

investigations are needed to reveal genetic exchange between and within the groups. Studying the genetic background of the groups should promote the understanding of species concept of *S. rolfsii* and *S. delphinii*.

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