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Variation in Southern Blight Fungus in Japan Detected by ITS-RFLP Analysis

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Abstract
PCR-RELP analysis of the ITS region in the ribosomal RNA gene cluster revealed that Japanese isolates of the southern blight pathogen consisted of 5 groups. Groups 1, 2 and 3 corresponded to *Sclerolium rolfsii*, whereas groups 4 and 5 were similar to *S. delphinii*, a related species of *S. rolfsii*. Most of the isolates belonging to groups and 2 were detected in the central to southwestern regions of Japan, while groups 3, 4 and 5 were distributed in the central to northern regions, Although the ITS-RFLP groups also differed in the size of the sclerotial, the sclerotial size varied with the incubation temperature and became indistinguishable at high temperatures. These groups showed hyphal anastomosis with one another, indicating their conspecificity.

Discipline: Plant disease
Additional key words: Sclerotium rolfsii, Selerotium delphinii

Introduction

Sclerotium rolfsii Saccardo (teleomorph; *Atheria rolfsii* (Curzi) Tu & Kimbrough) is a soil-borne plant pathogenic fungus, which infects approximately 500 species belonging to 100 plant families[6] including soybean, peanut, and other leguminous crops. Infection occurs at the level of or just below the soil surface, causing basal stem rot and blighting of the leaves, referred to as southern blight. Southern blight is characterized by the presence of white mycelium and brown sclerotia, about the size of mustard seeds on diseased plants (Fig. 1a, b). Sclerotia act as the primary inoculum, since they are the sole organ capable of withstanding adverse conditions. Asexual spores are not produced, and the sexual stage is seldom observed[14,15]. Hot and humid weather is conducive to sclerotial germination and mycelial growth, and consequently the disease is more serious in subtropical and tropical regions than in temperate regions[19].

Although *S. rolfsii* does not usually prevail in the cool temperate zone, southern blight has also been reported in the central to northern regions of Japan[9,11,16], suggesting the existence of subgroups of *S. rolfsii*, which may be adapted to relatively low temperatures. Another possibility is that the isolates reported from cool regions belong to *S. delphinii* Welch, a related species to *S. rolfsii*. *S. delphinii* is known to infect ornamental plants grown in temperate regions in North America, e.g. *Delphinium* sp.[18], scilla[10], lilies and iris[17]. Differentiation of these 2 species based on morphological criteria is difficult although they may be distinguished by the size of the sclerotia[13,17].

Recent advances in molecular techniques have enabled inter- and intraspecific grouping of fungi[3] with few morphological differences. The analysis of restriction fragment length polymorphisms (RFLPs) of the internal transcribed spacer (ITS) region of ribosomal RNA genes (rDNA) indicated the presence of genetic variation in *S. rolfsii*[8,12]. In this paper, we reviewed the genetic variation of the Japanese isolates of the southern blight fungus and their geographic distribution. We also evaluated the species concept in *S. rolfsii*.

ITS-RFLP analysis

ITS-RFLP analysis was first applied to *S. rolfsii* and related species by Harlton et al.[8]. They tested 119 *S. rolfsii* isolates and 12 *S. delphinii* isolates from the U.S.A. and other countries, using restriction enzymes *Alu* I, *Hpa* II, *Rsa* I and *Mbo* I. *S. delphinii* was distinguished from *S. rolfsii* by the *Rsa* I pattern, and *S. rolfsii* isolates were

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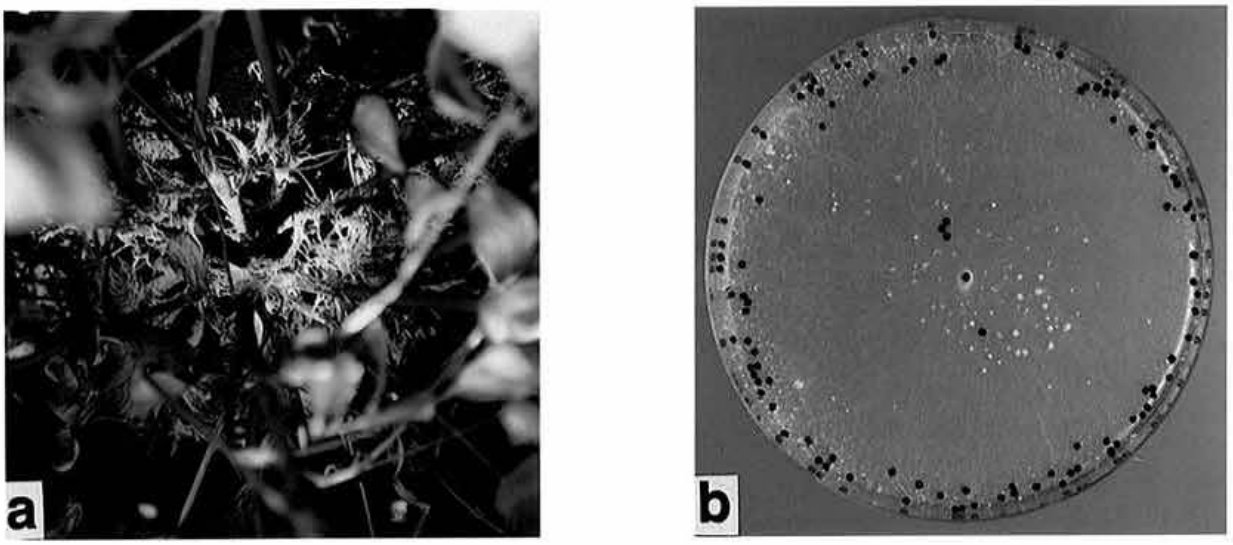

Fig. 1. Mycelia and sclerotia formed on a peanut plant (a) and potato-dextrose agar (b)

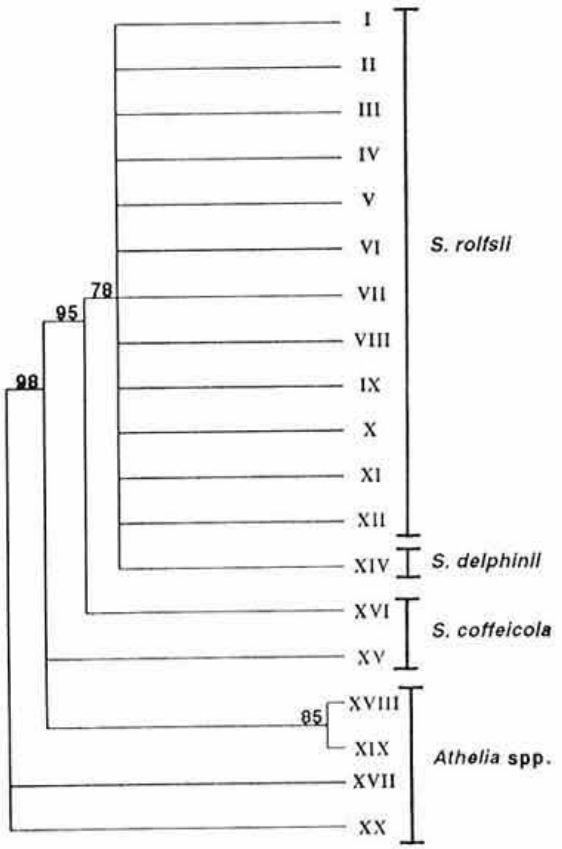

Fig. 2. Putative phylogenetic relationships between three *Sclerotium* and three *Athelia* species based on *Alu* I, *Hpa* II, *Rsa* I, and *Mbo* I restriction sites of internal transcribed spacer (ITS) region[8]

classified into 12 groups (I to XII). They also suggested the existence of phylogenetic relationships (Fig. 2).

To determine the geographical variability in the *S. rolfsii* population, we examined 67 isolates from different hosts and various regions of Japan, and classified them into 5 ITS-RFLP groups (Figs. 3 and 4)[12]. Groups 1 and 2 were distributed in the southwestern region of Japan, which is located in the warm temperate zone, and groups 3, 4 and 5 were found in the central to northeastern regions of Japan, which are cool temperate regions. Groups 1 and 3 corresponded to groups II and XI, respectively, and group 4 showed the same pattern as that of *S. delphinii* from the U.S.A. Groups 2 and 5 were newly found, and group 2 was similar to group 1, whereas group 5 was close to group 4.

ITS-RFLP analysis indicated that isolates resembling *S. delphinii* were distributed in the central to northern regions in Japan, whereas *S. rolfsii* occurred in the central to southwestern regions. The boundary was not clear, and they overlapped in the Kanto and Hokuriku areas. All the groups except for group 3 were found in Tokyo, suggesting that trade of seedlings and bulbs had introduced various *Sclerotium* strains.

Growth temperature reaction of ITS-RFLP groups

The most evident morphological difference between *S. rolfsii* and *S. delphinii* lies in the sclerotial size: *S. delphinii* produces larger sclerotia than *S. rolfsii*[17]. We observed sclerotial formation in several isolates of groups 1, 2 and 4 grown on potato dextrose agar (PDA) plates at various temperatures. Group 4 produced larger sclerotia than group 1 (Fig. 5). However, the size decreased with

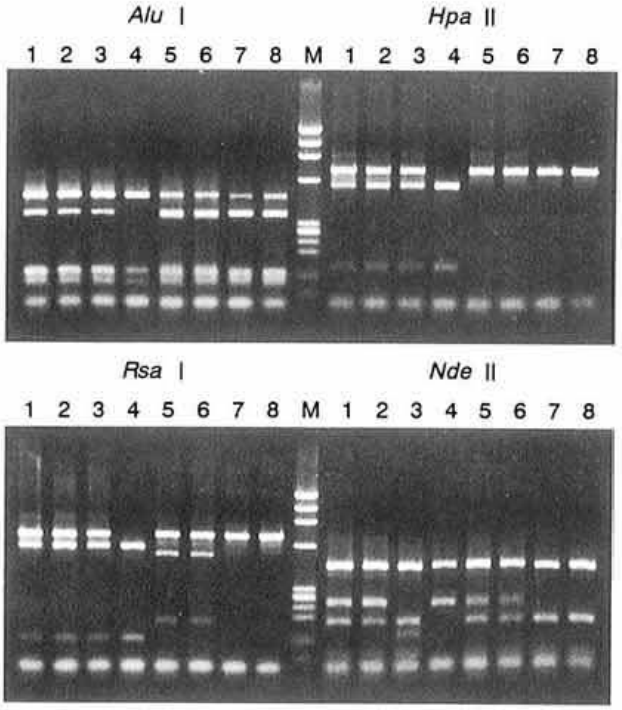

Fig. 3. ITS-RFLP patterns of Japanese isolates[12]
Four restriction enzymes (*Alu* I, *Hpa* II, *Rsa* I and *Nde* II) were used.
Lane 1: isolate S-22 (group 1).
2: isolate S-42 (group 1).
3: isolate S-43 (group 2).
4: isolate S-17 (group 3).
5: isolate S-41 (group 4).
6: isolate S-56 (group 4).
7: isolate S-8 (group 5).
8: isolate S-12 (group 5).
M: $\phi\chi$ 174 / *Hae* III(1353, 1078, 872, 603, 310, 281, 234, 194, 118bp).

Fig. 4. Distribution of ITS-RFLP groups in Japan
Numbers of isolates of groups 1,2,3,4 and 5 from each location are shown in parentheses, respectively.

increasing temperature, and the difference between groups 1 and 4 became less distinct. Sclerotial size of group 2 was similar to that of group 1 (data not shown). Few sclerotia were formed by isolates of group 5, and group 3 failed to produce sclerotia.

S. delphinii and *S. rolfsii* also differed in the optimal temperature for mycelial growth. *S. rolfsii* grew better at high temperatures[13] than *S. delphinii*, which is in agreement with the fact that *S. rolfsii* is prevalent in tropical and warm temperate regions whereas *S. delphinii* occurs in cool temperate regions. In our observations of the Japanese isolates, the growth temperature response was different among the ITS-RFLP groups but did not vary as much as that between *S. delphinii* and *S. rolfsii* in foreign countries. Group 1 showed optimum growth on PDA at 30°C (Fig. 6), but the growth rate decreased at 33°C whereas *S. rolfsii* isolates from the U.S.A. and South Asia grew well even at 35°C [5,13]. The optimal growth temperature was 28°C for most of the isolates of group 5 and some of group 4, but other isolates of group 4 grew most rapidly at 30°C. Group 2 showed the same pattern as group 1 (data not shown), and one isolate of group 3 was not included in this test because it showed restricted growth.

Hyphal anastomosis between different RFLP groups

Conspecificity can be determined by hyphal interactions in basidiomycetous fungi. Hyphae of the same isolate often fuse (or anastomose) with one another, forming a hyphal network. Anastomosis may also occur between hyphae of different strains of the same species but usually results in the death of fused cells and in the subsequent formation of a boundary line between the mycelia. Hyphal anastomosis does not occur between different species[7] nor between genetically isolated populations within a species complex[3].

Hyphal anastomosis was observed between different RFLP groups, when they were paired on water agar plates (Fig. 7). Fused hyphal cells in the contact zone subsequently died in every combination except for the self-pairings, suggesting that all the groups should be considered as the same genetic entity. Some researchers proposed the term *S. rolfsii* var. *delphinii* to accommodate *S. delphinii* in the *S. rolfsii* complex[2].

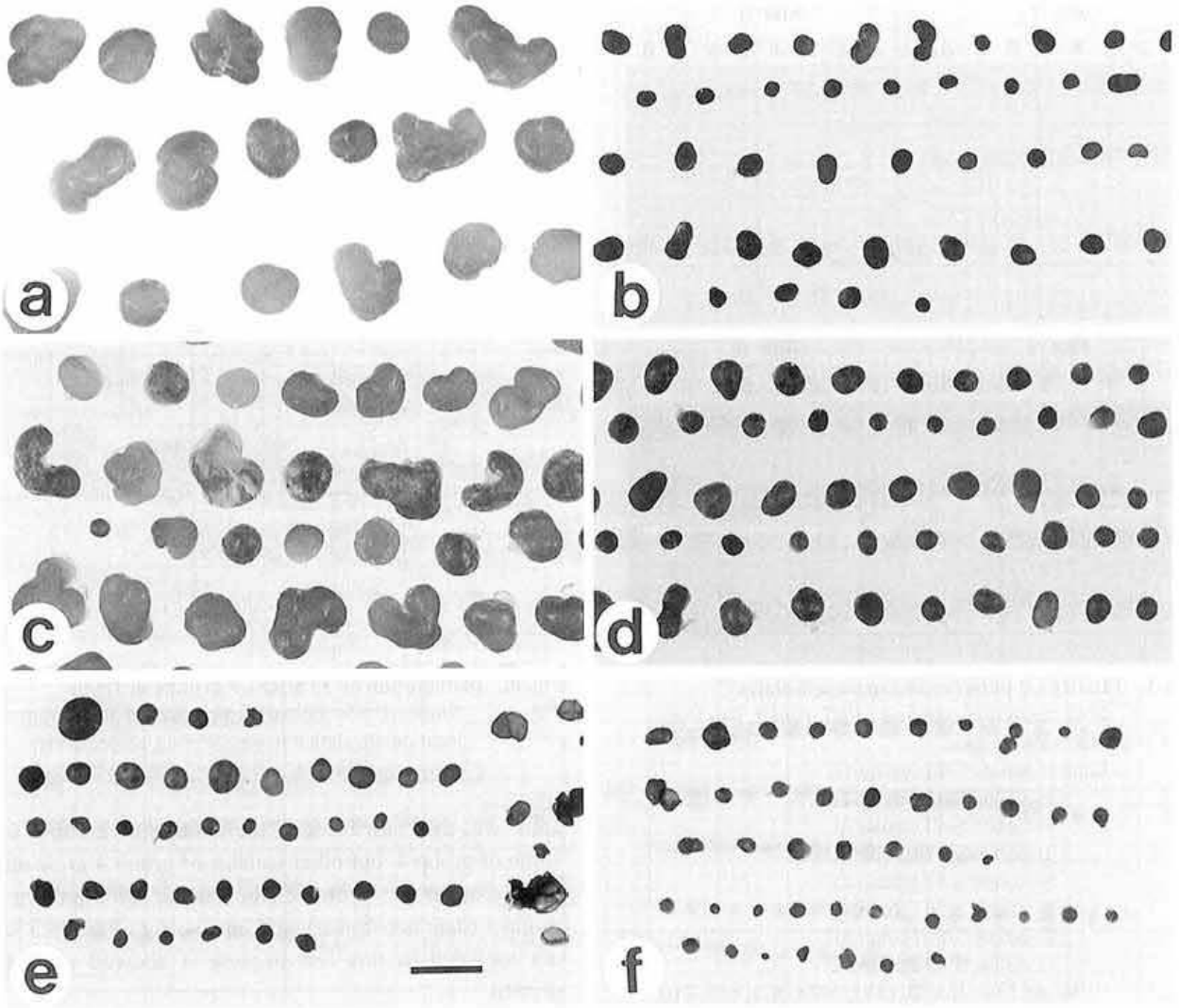

Fig. 5. Sclerotia of isolates S-58 (group 4) and S-46 (group 1) produced at 23, 28, and 33°C[12]
a: Isolate S-58 at 23°C. b: S-46 at 23°C. c: S-58 at 28°C. d: S-46 at 28°C.
e: S-58 at 33°C. f: S-46 at 33°C. Scale bar = 5 mm.

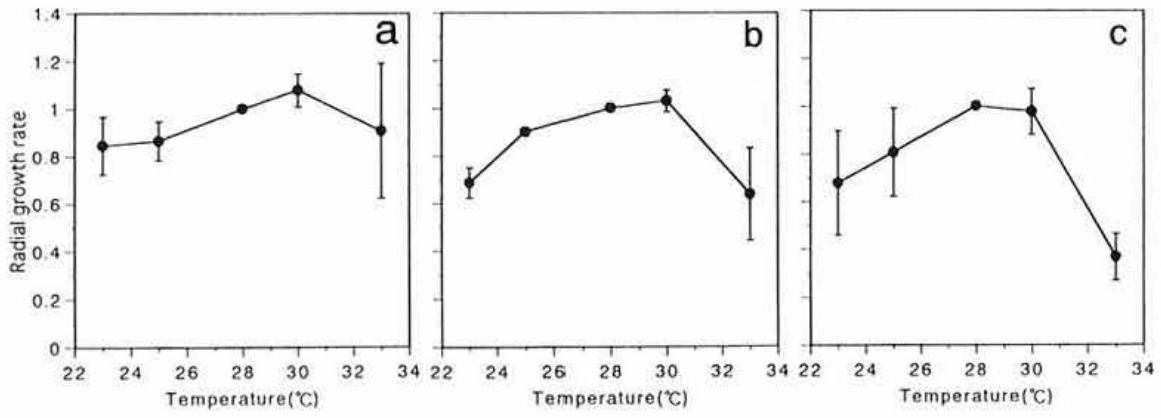

Fig. 6. Mycelial growth of groups 1 (a), 4 (b), and 5 (c) [12]
Mycelia were incubated for 2 days at different temperatures. Data are expressed relative to the growth at 28°C. Vertical bars show the standard deviations.

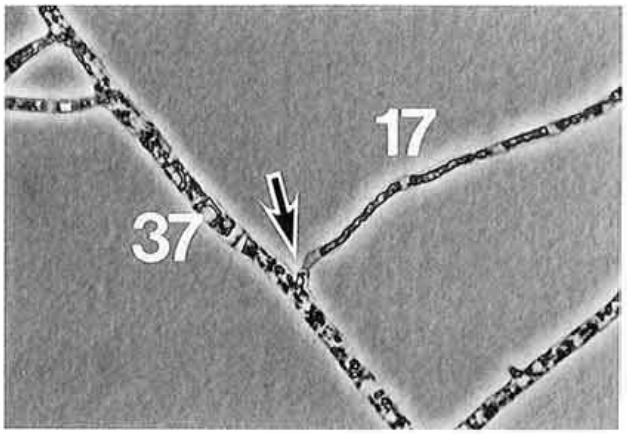

Fig. 7. Hyphal anastomosis of isolates S-17 (group 3) and S-37(group 5) observed under a phase-contrast microscope[12]

Conclusion

ITS-RFLP analysis suggested the existence of population differentiation in the southern blight fungus in Japan. The groups from southern regions included *S. rolfsii*, and those distributed in the northern regions corresponded to *S. delphinii* in a broad sense. Before ITS-RFLP analysis was applied, *S. rolfsii* and *S. delphinii* had been distinguished by the sclerotial size and optimal growth temperature. However, sclerotial morphology was found to vary with the incubation temperature, and optimal growth temperature did not enable to distinguish ITS-RFLP groups in Japan. Lack of significant differences within the southern blight fungus may be due to the less distinct climatic differences between localities in Japan, and indicate the existence of intergrades between the 2 species. *S. rolfsii* and *S. delphinii* are considered to represent 2 extremes of continuous variation.

Information about the mating behavior of *S. rolfsii* and *S. delphinii* is limited, and the biological species concept[1] is not easily applicable to these fungi. The phylogenetic tree based on ITS-RFLP analysis indicated a close relationship between these species (Fig. 2). Moreover, hyphal anastomosis also suggested their incomplete segregation as separate species. Further investigations are required to reveal the genetic background of the groups and to reassess the species concept of *S. rolfsii* and *S. delphinii*.

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