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ITS polymorphism within a single strain of *Sclerotium rolfsii*

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Two morphologically distinct strains, 63–76 and 63H1, were isolated from a protoplast and a hyphal tip of the parental *Sclerotium rolfsii* strain S-63, respectively. Strains 63–76 and 63H1 showed reduced mycelial growth and lacked clamp connections on hyphae. The two strains also differed from each other and from their parent in RAPD patterns generated by several primers, suggesting that 63–76 and 63H1 were homokaryons isolated from the hetereokaryon S-63. Whereas the parent S-63 belonged to ITS-RFLP group 1, RFLP patterns of internal transcribed spacer (ITS) regions of rDNA of 63–76 and 63H1 were similar to those of ITS-RFLP groups 5 and 3, respectively. The sequence similarity of ITS regions were more than 99% between 63–76 and group 5 strains, 100% between 63H1 and the group 3 strain, and 96.3% between 63–76 and 63H1. Direct sequencing failed in the parental strain S-63. S-63 was considered to contain ITS types of groups 5 and 3.

Key Words——internal transcribed spacer (ITS); *Sclerotium rolfsii*.

Sclerotium rolfsii Sacc. [teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough] is a soil-borne plant pathogenic fungus distributed from temperate to tropical regions and infects about 500 plant species (Punja, 1988; Gazaway and Hagan, 1989). Harlton et al. (1995) reported the genetic variation in internal transcribed spacer (ITS) regions of ribosomal DNA of *S. rolfsii* using restriction fragment length polymorphisms (RFLP). Okabe et al. (1998), by the same method, classified Japanese isolates into five ITS-RFLP groups which had distinct geographical distribution patterns. However, morphological differences between groups were not evident.

Harlton et al. (1995) also suggested that field isolates of *S. rolfsii* contained more than one type of ITS sequence, because single-basidiospore strains showed two distinct ITS-RFLP patterns, both of which were different from that of the parent. The sums of digested fragment sizes of field isolates often exceeded the total length of undigested ITS regions. ITS heterogeneity of *S. rolfsii* was also implied by the fact that direct sequencing of ITS regions failed, although the ITS size was determined (686 bp) (Nalim et al., 1995).

In this paper, we determined two types of ITS nucleotide sequences of homokaryons derived from a single heterokaryon strain which belonged to ITS-RFLP group 1 (Okabe et al., 1998). The ITS sequences of

Table 1. Origins and ITS-RFLP patterns of *Sclerotium rolfsii* strains.

Strain	Origin	ITS-RFLP pattern[a]
S-8	*Delphinium ajacis*, Chiba, 1991[b]	group 5 (A2, H1, R0, M1)
S-11	*Cryptomeria japonica*, Fukui, 1952[c]	group 5 (A2, H1, R0, M1)
S-17	soil, Ishikawa, 1977[d]	group 3 (A1, H2, R2, M4)
S-32	*Ophiopogon japonicus*, Tokyo, 1992[e]	group 4 (A2, H1, R3, M2)
32-32	protoplast isolate of S-32	group 4 (A2, H1, R3, M2)
S-52	peanut, Ibaraki, 1994[f]	group 1 (A2, H3, R1, M2)
S-63	soybean, Kagawa, 1994[g]	group 1 (A2, H3, R1, M2)
63-1	protoplast isolate of S-63	(A2, H3, R1, M2)
63-76	protoplast isolate of S-63	(A2, H1, R0, M1)
63H1	hyphal tip isolate of S-63	(A1, H2, R2, M4)

a) Patterns were described by Harlton et al. (1995) and Okabe et al. (1998).
b) Isolate CH91–41 of Uematsu et al. (1992).
c) MAFF410049.
d) MAFF305344
e) Isolate CoOp-1 of Takeuchi and Horie (1993).
f) Isolate 3–4 of Okabe and Matsumoto (2000).
g) Isolate B12 of Sato et al. (1995).

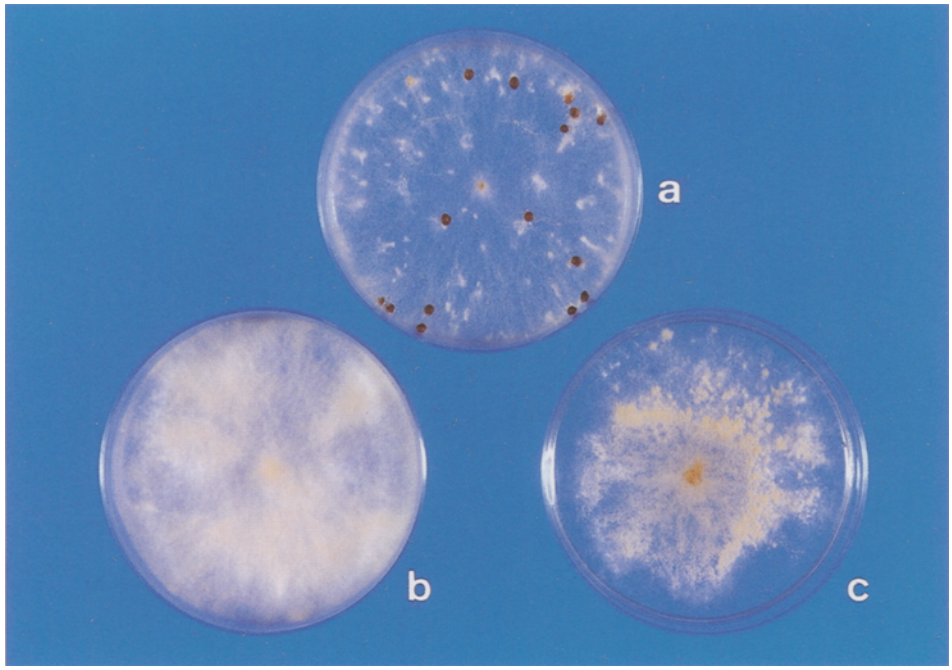

Fig. 1. Colony morphology of strains S-63 (a), 63–76 (b) and 63H1 (c) grown on PDA for 3 wk at 28°C.

component homokaryons resembled those of ITS-RFLP groups 3 and 5, suggesting a close relationship between ITS-RFLP groups 1, 3 and 5. Genetic heterogenity of *S. rolfsii* is discussed in terms of genomic composition of ITS-RFLP groups in *S. rolfsii*. A brief report of this work has been presented previously (Okabe et al., 2000).

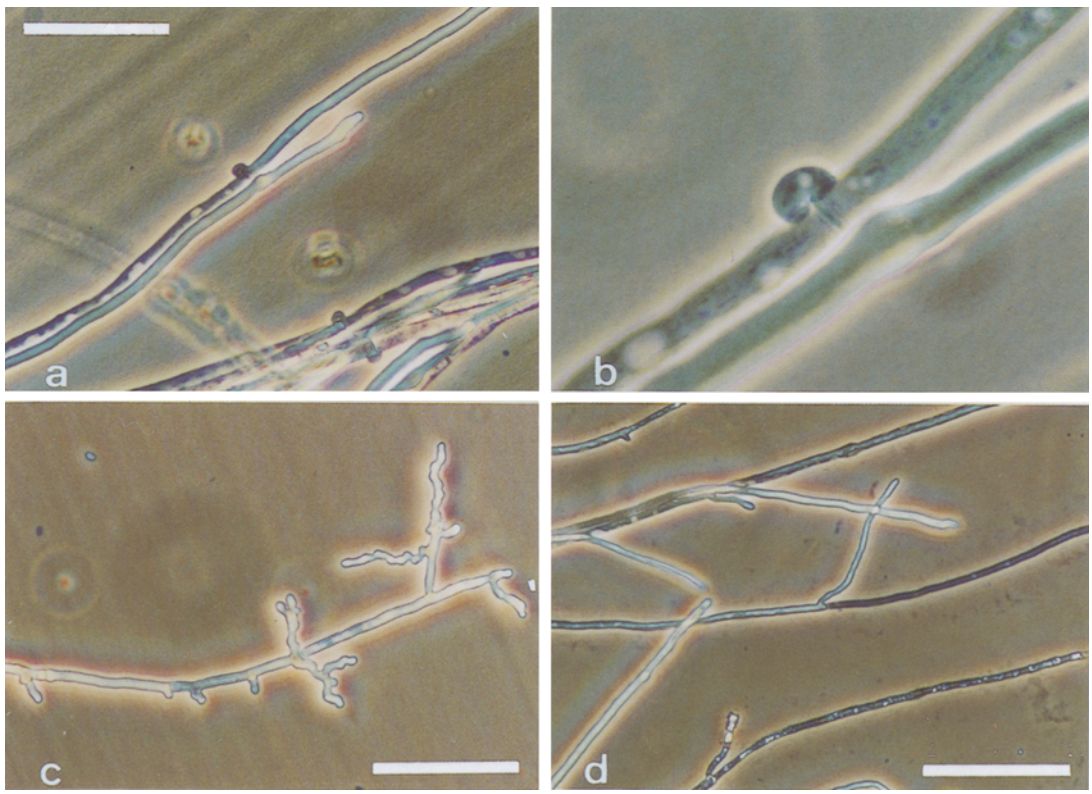

Fig. 2. Hyphal morphology of S-63 (a), 63–76 (c) and 63H1 (d). Clamps were observed on hyphae of S-63 (b: magnified hypha of a) but not of 63–76 or 63H1. Scale bars: 100 μm.

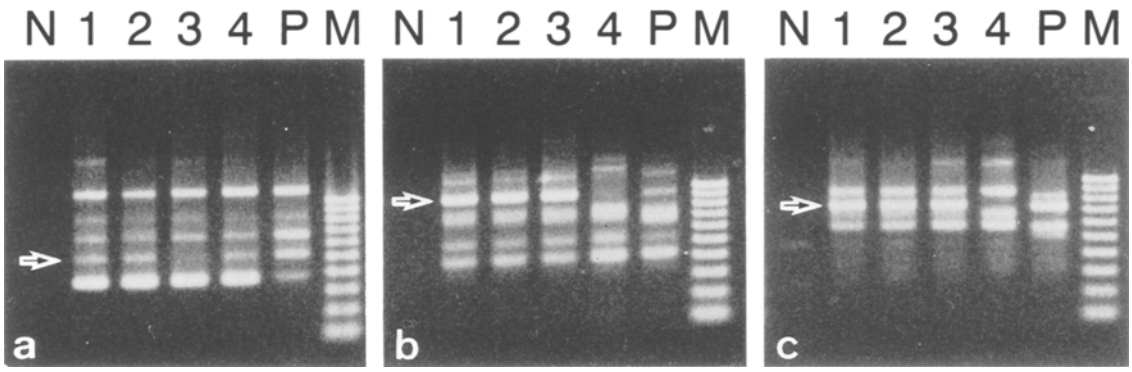

Fig. 3. RAPD patterns generated with primer OPA-15 (a), 16 (b) and 19 (c). Lane N, negative control; lane 1, S-63; lane 2, 63–1; lane3, 63–76; lane 4, 63H1; lane P, positive control (S-52); lane M, 100-bp ladder. Arrows indicate the amplification products which distinguish homokaryons from the parent heterokaryon.

Materials and Methods

Strains Strains used in this study are given in Table 1. Strain S-63, which grew normally with sclerotia on potato dextrose agar (PDA) (Fig. 1a), was used as a heterokaryotic, parental strain since this strain had clamped hyphae (Fig. 2a, b). Two homokaryotic strains were obtained, from a protoplast (63–76) and by hyphal tip isolation (63H1). Strain 63–76 had gnarled hyphae without clamp connections (Fig. 2c) and few sclerotia (Fig. 1b). Strain 63H1 formed less aerial mycelium than the original culture. Neither sclerotia nor clamp connections were observed in 63H1 (Figs. 1c, 2d). The three strains were all multinucleate. Another protoplast isolate, 63–1, which had the same cultural characteristics as S-63 and was considered to be heterokaryotic, was included in the experiments for comparison.

Random amplified polymorphic DNA (RAPD) analysis included strain S-52 with known RAPD patterns (Okabe and Matsumoto, 2000). Strains S-8, S-11, S-17, S-32 and 32–32, which had different origins and ITS-RFLP patterns, were used for comparison of ITS sequences.

Protoplast isolation Protoplasts were produced according to Nakagawa and Yamaguchi (1989) with some modification. Sclerotia collected from 1-mo-old PDA culture were incubated in 100 ml of potato sucrose broth (PSB) at 25°C for 2–3 wk with reciprocal shaking (100 strokes/min). The whole culture was disrupted in a homogenizer, mixed with an equal volume of fresh PSB, and incubated further at 25°C for 20–30 h on the shaker. The mycelia were harvested by centrifugation and washed twice in MS solution (50 mM maleic acid/0.5 M sorbitol, pH 5.8; Arima and Morinaga, 1995). About 150 mg of fresh mycelia was suspended in 1 ml of MS solution containing 1% Cellulase Y -C (Seishin Co., Tokyo) and 0.1% Novozyme 234 (Novo Nordisk, Bagsvaerd). After incubation for 30 min at 28°C, the suspension was filtered through sterile paper coffee filter to remove mycelial debris. Protoplasts were pelleted by centrifugation at 3000 rpm, washed three times in MS, and adjusted to $1–3 \times 10^5$ protoplasts per ml. Ten μl of protoplast suspension was mixed with 5 ml of regenera-

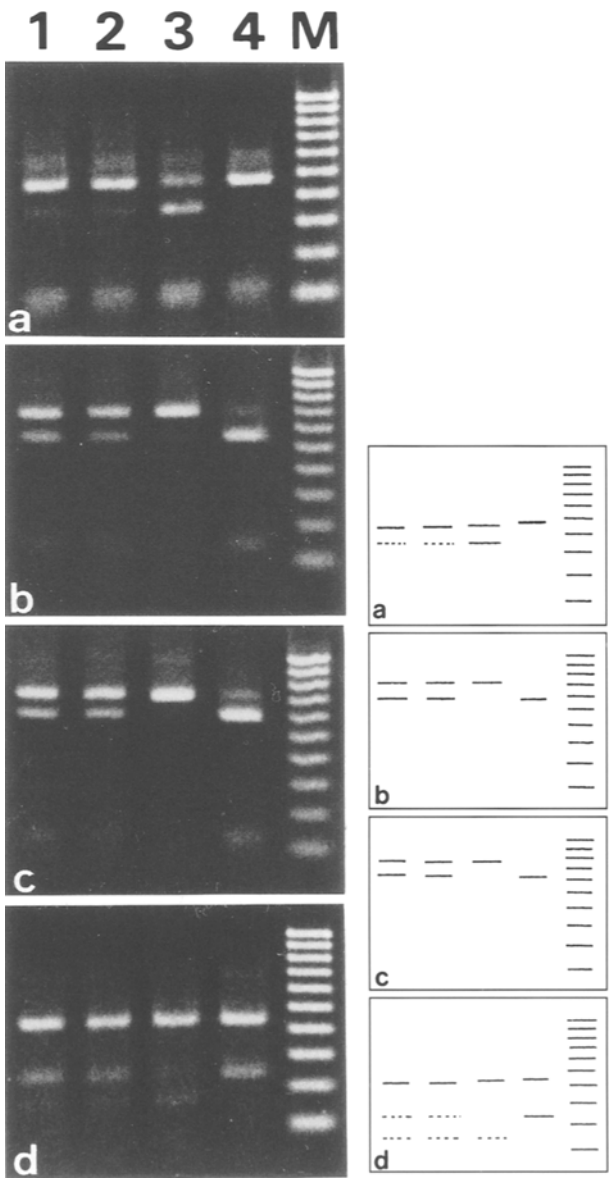

Fig. 4. ITS-RFLP patterns for *Alu* I (a), *Hpa*II (b), *Rsa*I (c) and *Sau*3AI (d). Lane 1, S-63; lane 2, 63–1; lane 3, 63–76; lane 4, 63H1; lane M, 100-bp ladder.

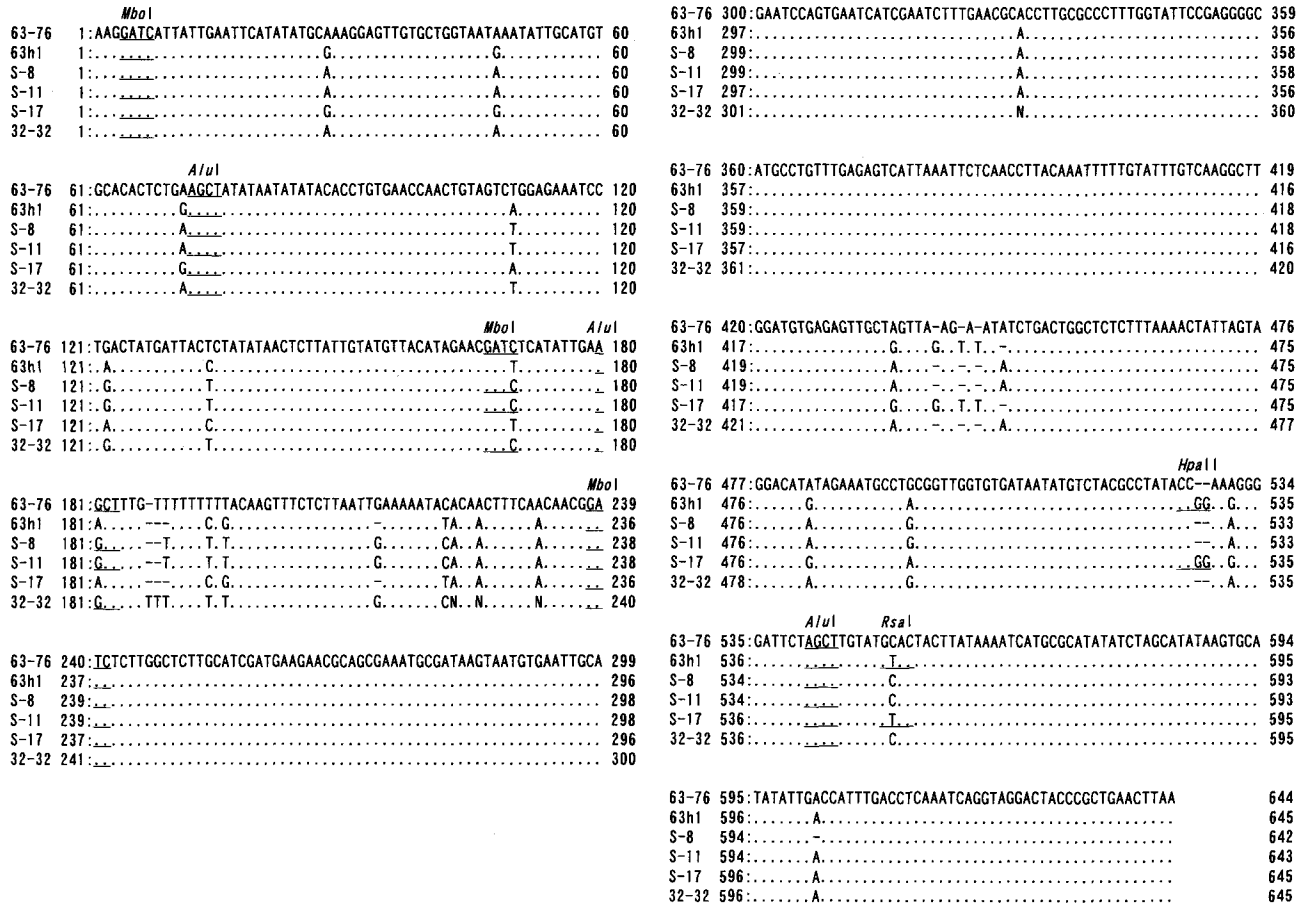

Fig. 5. Aligned DNA sequences of the internal transcribed spacers and the 5.8 S ribosomal gene. The restriction sites of *Alu* I, *Hpa*II, *Rsa*I and *Mbo*I (*Nde*II, *Sau*3AI) are underlined.
Dot (.), conserved base; dash (–), gap; N, {A, T, G, C}. The DDBJ (DNA Data Bank of Japan) accession number of the strain 63–76 is AB042626.

tion medium (Difco PDA, 39 g, and sucrose, 180 g, in 1 L of distilled water) at 42°C and dispersed in 6-cm Petri dishes. After incubation at room temperature (23–28°C) for 50 h, colonies generated from single protoplasts were transferred to PDA plates.

RAPD and ITS-RFLP analyses The procedures for DNA isolation and RAPD analysis were described previously (Okabe and Matsumoto, 2000). RAPD patterns were generated with primers OPA-09, 15, 16, 19 and OPB-13 (Operon Technologies. Inc., Alameda).

ITS-RFLP followed the method of Okabe et al. (1998). ITS regions were amplified using ITS1 and ITS4 primers (White et al., 1990) and digested with *Alu*I, *Hpa*II, *Rsa*I, or *Sau*3AI (*Nde*II). The PCR products and digests were subjected to electrophoresis for 50 min at 50 mA on 2% agarose gels in TAE buffer and stained with 0.1 μg/ml ethidium bromide solution.

Direct sequencing Direct sequencing of PCR products was conducted for both strands using Applied Biosystems sequencers 373A and 377. The sequence reaction was conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City) following the manufacturer's instructions. The primers ITS1 and ITS4 were used for the sequencing.

Sequences were initially aligned using the GENETYX-WIN 3.2 program (Software Development Co., Ltd., Tokyo) and Clustal X (Gibson et al., 1994) and later adjusted manually. Phylogenetic relationships among strains were inferred using the algorithm of the neighbor-joining method (Saitou and Nei, 1987) (NEIGHBOR) in PHYLIP 3.573c (Felsenstein, 1995), and displayed using the TreeView PPC 1.5.3 program (Page, 1998). *Laccaria bicolor* (Maire) Ort. (Martin et al., 1997), which showed the highest similarity to 63–76 in the Smith-Waterman homology search (SWN BTK 2.1.5, Paracel Inc.) and *Athelia arachnoidea* (Berk.) Julich var. *arachnoidea* (anamorph: *Rhizoctonia carotae* Rader) (Adams and Kropp, 1996) were used as outgroups.

Results

RAPD analysis Compared with the parental strain S-63, strain 63–76 lacked a 500-bp band for OPA-15, and strain 63H1 lacked a 900-bp band for OPA-16 and a 700-bp band for OPA-19 (Fig. 3). Another protoplast isolate, 63–1, had the same RAPD patterns as S-63 for all the primers.

RFLP analysis Gel electrophoresis of PCR product with

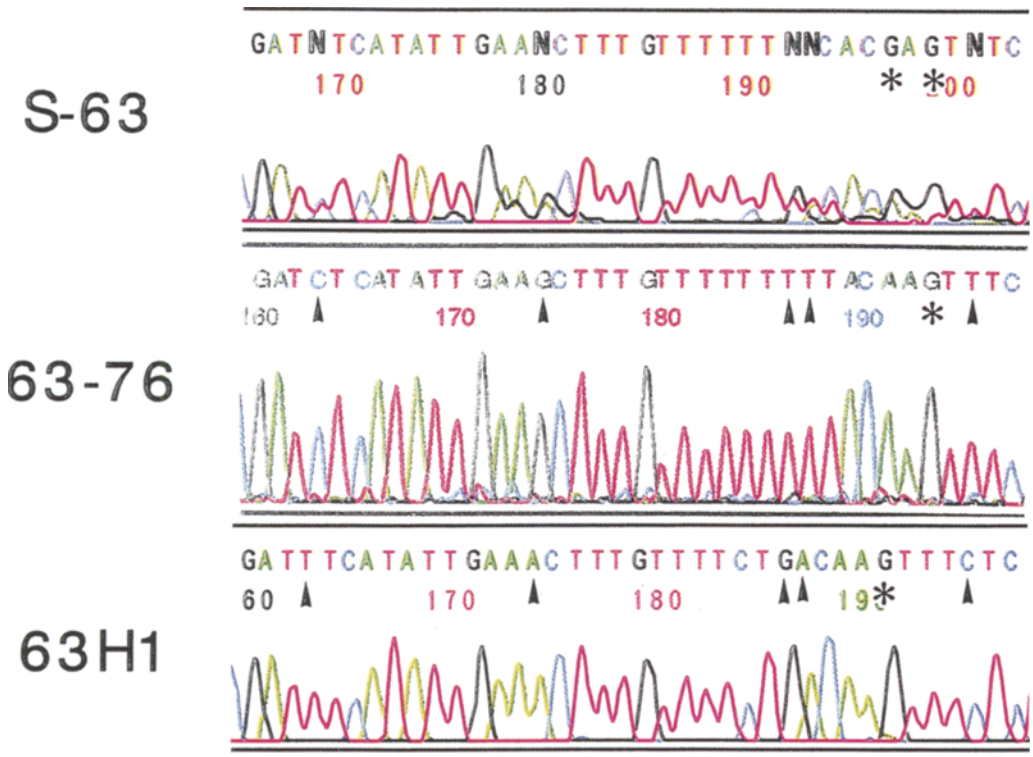

Fig. 6. Partial results of sequencing. The sequencing pattern of S-63 was the mixture of those of 63–76 and 63H1. The nucleotides were not determined (N) for S-63 where 63–76 and 63H1 mismatched (arrows). The shift in the pattern of 63–76 from that of 63H1 produced repeated peaks of G (*) in S-63.

primers ITS1 and ITS4 yielded a uniform band of approximately 700 bp from every strain. Whereas 63–1 showed the same RFLP patterns as the parental strain S-63, 63–76 and 63H1 differed from the parent and from each other (Fig. 4, Table 1). *Alu*I digestion of S-63 generated 360- and 470-bp bands, which corresponded to A2 pattern designated by Harlton et al. (1995). S-63 also showed 550- and 700-bp bands for *Hpa*II (H3 pattern), 600- and 700-bp bands for *Rsa*I (R1 pattern) and 160–, 230– and 430-bp bands for *Sau*3AI or *Nde*II (M2 pattern), and was classified as ITS-RFLP group 1 (Okabe et al., 1998). The strain 63–76 lacked the 550-bp band for *Hpa*II (H1 pattern), the 600-bp band for *Rsa*I (R0 pattern; Okabe et al., 1998) and the 230-bp band for *Sau*3AI (M1 pattern). On the other hand, 63H1 lacked the 360-bp band for *Alu*I (A1 pattern), the 700-bp band for *Hpa*II (H2 pattern), the 700-bp band for *Rsa*I (R2 pattern) and the 160-bp band for *Sau*3AI (M4 pattern).

Direct sequencing Complete sequences of ITS regions including ITSI, 5.8s ribosomal gene and ITSII were determined for strains 63–76, 63H1, S-8, S-11, S-17 and 32–32 (Fig. 5). The total ITS sizes excluding primers ITS1 (19 mer) and ITS4 (20 mer) ranged from 642 to 645 bp, and GC contents ranged from 36.7 to 37.4%. Restriction sites for *Alu*I, *Hpa*II, *Rsa*I, and *Sau*3AI (*Nde*II) were consistent with the maps of Harlton et al. (1995). Direct sequencing was incomplete for S-32, 32–32 and S-63 (Fig. 6). However, noise was relatively low for 32–32, and most nucleotides were determined.

The sequences were grouped into two types (Fig. 7). One consisted of strains S-8, S-11, 63–76 and 32–32; the other contained S-17 and 63H1.

Discussion

We used protoplast isolation to obtain homokaryons, because basidiospore strains of S-63 were not available. More than 200 protoplast isolates were screened, and 7 strains were obtained which did not have clamps on hyphae. Because homokaryons of *S. rolfsii* were binucleate or multinucleate (Punja and Grogan, 1983a) and not discriminable microscopically, RAPD and ITS-RFLP were used to examine the homokaryotic condition. Only strain 63–76 showed different patterns from the parent S-63, lacking several bands, and was confirmed to be a homokaryon (Figs. 3, 4). Another homokaryon, 63H1, was accidentally found from subcultures of S-63, suggesting that homokaryotization or monokaryotization (Masuda et al., 1995) occurred during subculture in *S. rolfsii*. RAPD and RFLP analysis suggested that 63H1 contained different types of nuclei from 63–76.

Previous DNA sequencing implied that ITS regions were heterogeneous within an isolate in *S. rolfsii* (Nalim et al., 1995; Adams and Kropp, 1996). In our experiments, direct sequencing was unsuccessful for 9 out of 12 field strains because of noise peaks. The difficulty in direct sequencing was ascribed to the mismatch between ITS types co-existing within a strain; two peaks overlapped at the positions where the nucleotide substitutions occurred in one of the heterogeneous nuclei, and

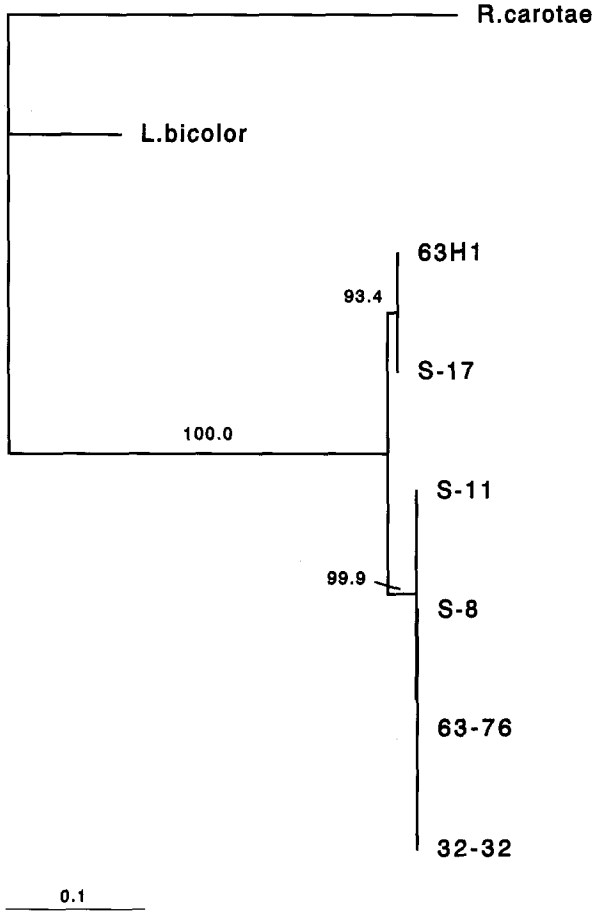

Fig. 7. A neighbor-joining tree based on distance (Kimura's two-parameter method) derived from DNA sequences of the internal transcribed spacers and the 5.8 S ribosomal gene of *Sclerotium rolfsii* strains, *Laccaria bicolor* S238N (Martin et al., 1997) and *Fiblarhizoctonia carotae* (*Rhizoctonia carotae*, teleomorph: *Athelia arachnoidea* var. *arachnoidea*) ATCC10866 (Adams and Kropp, 1996) (outgroup). Bootstrap intervals ≧50% are indicated. The bar indicates a distance of 0.1 nucleotide substitution per site.

the nucleotide insertions/deletions appeared as a shift in the sequencing pattern, which caused repeated peaks of the same nucleotides in S-63 (Fig. 6).

Noiseless sequence data were obtained from homokaryons 63–76 and 63H1, suggesting that all nuclei in these strains contained a single type of ITS. ITS sequences of 63–76 and 63H1 resembled those of ITS-RFLP group 5 and 3 isolates, implying that ITS heterogeneity in S-63 (ITS-RFLP group 1) originated from heterokaryosis between groups 5 and 3. Hyphal fusion between different ITS-RFLP groups was observed (Okabe et al., 1998) but did not result in heterokaryon formation. Heterokaryons between 63–76 and 63H1 were also not obtained.

Although most ITS copies were the same in homokaryon 63–76, minor variation was detected in ITS-RFLP patterns. The presence of the 470-bp band for *Alu*I digestion of 63–76 suggested that not all ITS copies had the *Alu*I site at position 180, as indicated by Harlton et al. (1995). These two types of ITS copies may not exist in equal proportions, and only the major type may have been sequenced. The cloning method will be needed to determine all types of ITS sequence contained in the isolate.

In most species, rDNA copies become homogenized because meiotic recombination processes including gene conversion and unequal crossing-over (Li and Graur, 1991) result in concerted evolution. ITS polymorphisms were reported for several asexual fungi (O'Donnell, 1992; Sanders, 1999), and ITS heterogeneity of S-63 also implied low significance of the sexual stage in the life cycle of *S. rolfsii*. Studies on population structure also suggested that *S. rolfsii* is asexual (Nalim et al., 1995; Okabe and Matsumoto, 2000), although basidiospores of *S. rolfsii* retain the ability to cause disease (Punja and Grogan, 1983b). Further studies on intraspecific phylogeny are needed for *S. rolfsii*, which does not conform exactly to most phylogenetic theories constructed for sexually-reproducing organisms.

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