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FULL PAPER

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Heterothallic life cycle in the white root rot fungus *Rosellinia necatrix*

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Abstract To prepare homologous DNA fragments as restriction fragment length polymorphism (RFLP) markers, the genes encoding phenol oxidase, chitinase, and xylanase were amplified from genomic DNA of *Rosellinia necatrix* strains. RFLP analysis using the amplified DNA fragments as probe was carried out, with segregation of the markers among two sets of F_1 progenies isolated from an independent perithecium. RFLP was frequently found using *rpo*1 as the RFLP marker among strains of *R. necatrix*, which was isolated from single ascospores and the circumference of the perithecium. In each set, RFLPs of some F_1 progenies were different from that of the parent strain. Random amplified polymorphic DNA (RAPD) also revealed that several strains, which were of different genotypes from the parent strain, were contained in the single ascospore culture isolated from the same perithecium. From these results, it is suggested that another strain, which was genetically different, was required for mating and development of the ascus in *R. necatrix*. Therefore, the life cycle in *R. necatrix* was presumed to be heterothallism. This is the first report about a heterothallic life cycle in *R. necatrix*.

Key words Ascospore · Heterothallism · Life cycle · RFLP · *Rosellinia necatrix*

Introduction

The filamentous ascomycete *Rosellinia necatrix* (Hartig) Berlese is a commercially important, soilborne, root pathogen affecting a wide range of plant species. It is the causal agent of white root rot disease, and host plants infected by the fungus quickly wither and die. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines and apple and pear trees, among other crops. Unfortunately, despite its importance, little is known about its nuclear phase, including karyotype and heterokaryosis, and the genetic differentiation of this fungus. Moreover, its mating type is also unknown as well as whether this fungus is heterothallic or homothallic in its life cycle. To exterminate white root rot disease, we must first understand its genetic features, including heterokaryosis and mating systems.

Electrophoretic karyotyping using pulsed-field electrophoresis is a good tool for estimating heterokaryosis. This technique detects differentiation of chromosomes among strains, especially with respect to molecular size (Zolan 1995), but it is difficult to compare the number of chromosomes and their polymorphisms because achieving protoplast formation in this fungus is problematic. Moreover, classical genetic methods are not applicable because the development of the ascus of this fungus on artificial medium has not yet been reported. Therefore, to reveal the life cycle of *R. necatrix*, other methods for detecting karyotypic differences must be developed. Recently, we developed a method for estimating chromosome number in *R. necatrix* using telomeric fingerprinting methods (Aimi et al. 2002b). In that report, both field-isolated strains and monospore-isolated strains had the same chromosome number. Therefore, it was suggested that the karyotypes in all *R. necatrix* strains were the same. However, the genetic relationship between the putative parent strain and its progeny have not yet been clarified.

In this study, we report genetic difference among single ascospore cultures isolated from the same perithecium, using some restriction fragment length polymorphism (RFLP) markers, that were newly amplified and cloned as a single copy gene. The present study is a part of our attempt to better understand the genetics and life cycle of *R. necatrix*, including mating type and vegetative incompatibility.

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Table 1. *Rosellinia necatrix* strains used in this study

Strain	Nuclear phase	*rpo*1	RAPD pattern[a]			Characteristics
			OP16	OP19	OP13	
Location: Chiba, Japan						
W153	Dikaryon	*rpo*1-2	A	D	F	Mycelium of W153 strain (parent strain) was isolated from circumference of a perithecium in the field
W462	Dikaryon	*rpo*1-2	A	E	G	These four F_1 progenies were isolated from the same perithecium from which the W153 strain was isolated
W463	Dikaryon	*rpo*1-1	B	E	G	
W465	Dikaryon	*rpo*1-1	A	D	G	
W466	Dikaryon	*rpo*1-1	A	E	G	
Location: Hyogo, Japan						
W427	Dikaryon	*rpo*1-1	B′[b]	E	H	Mycelium of W427 strain was isolated from circumference of a different perithecium in the field
W442	Dikaryon	*rpo*1-2	C	D	I	These five F_1 progenies were isolated from the same perithecium from which the W427 strain was isolated
W443	Dikaryon	*rpo*1-1	B	D′[c]	I	
W444	Dikaryon	*rpo*1-1	B	E	I	
W445	Dikaryon	*rpo*1-2	C	E	H	
W446	Dikaryon	*rpo*1-1	B	E′[d]	H	

[a] Random amplified polymorphic DNA (RAPD) patterns correspond with Fig. 3
[b] B′; main band is similar to B
[c] D′; main band is similar to D
[d] E′; main band is similar to E

Materials and methods

Strains and culture conditions

The field-isolated (W8 strain) and single ascospore isolates of *R. necatrix* used in this study (Table 1) were originally obtained from Dr. Naoyuki Matsumoto (National Institute of Agro-Environmental Science, Japan). The mycelium of the W8 strain was isolated from a field in Okayama, Japan. Two sets of single ascospore isolates (F_1 progenies) were used in this study; the first set consists of four F_1 progenies isolated from the same perithecium (W462, W463 W465, and W466 strains) and their parent strain (W153 strain), and the second set consists of five F_1 progenies isolated from the same perithecium (W442, W443, W444, W445, and W446 strains) and their parent strain (W427 strain) (Table 1). The mycelium of the W153 strain (parent strain) was isolated from the circumference of a perithecium in the field, and four F_1 progenies (W462, W463, W465, and W466 strains) were isolated from the same perithecium. The mycelium of the W427 strain was isolated from the circumference of a different perithecium in the field, and five F_1 progenies (W442, W443, W444, W445, and W446 strains) were isolated from the same perithecium. PDA (potato extract, 2% glucose, 2% agar) medium was used for maintaining the fungus. The strains were grown in PD (potato extract, 2% glucose) medium for later DNA extraction.

DNA extraction

DNA was extracted from lyophilized tissue of the *R. necatrix* strains using the CTAB (hexadecyltrimethylammonium bromide) procedure, essentially as described previously (Zolan and Pukkila 1986). The extracted DNAs were suspended in 20 µl TE [50 mM Tris-HCl, 10 mM ethylenediammotetraacetic acid (EDTA) (pH 8.0)], and then electrophoresed in 1% agarose gels along with control lambda DNA (0.1 µg). The gels were stained with ethidium bromide (0.5 µg/ml), and photographed under UV light. The amount of each DNA present was estimated by comparing the ethidium bromide fluorescence intensity of the control lambda DNA with those of the *R. necatrix* DNAs.

Polymerase chain reaction and cloning

Polymerase chain reaction (PCR) primers that were used for amplification of DNA fragments containing the partial phenol oxidase, chitinase, and xylanase genes are shown in Table 2. These primers were designed based on complementarity to a conserved portion of fungal protein sequences, which were phenol oxidase (Saloheimo et al. 1991; Giardina et al. 1995; Smith et al. 1998), chitinase (Blaiseau and Lafay 1992; Pishko et al. 1995; Giczey et al. 1998), and xylanase (Apel-Birkhold and Walton 1996; Perez-Gonzalez et al. 1996; Wu et al. 1995). PCRs were conducted in 100-µl reaction volumes. Each reaction tube contained approximately 100 ng DNA template, 10 µl 10 × PCR buffer II, 1.5 mM $MgCl_2$, 200 µM each dNTP, 1 pM each primer pair, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA). The thermal cycling parameters were initial denaturation at 95°C for 12 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 2 min, and extension at 72°C for 1 min; a final extension at 72°C for 10 min was done at the end of the amplification. All PCR products were subcloned into pT7-Blue T-vector (Novagen, Madison, WI, USA), and the resultant recombinant plasmid DNAs were sequenced. DNA sequencing was carried out in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) using the chain termination procedure with a BigDye Terminator cycle sequencing kit

Table 2. Polymerase chain reaction (PCR) primers used for amplification of genes in this study

Primer	Nucleotide sequence	Amino acid sequence	Target gene	Amplified gene (fragment size, bp)
BaLccF	5′-GGNACNTTYTGGTAYCAYWSNCA-3′	GTFWYHSH	Laccase	*rpo*1 (1181)
BaLccR	5′-TGNCCRTGNARRTGRAANGGRTG-3′	HPFHLHGH	Laccase	*rpo*2 (1089)
ChiF2	5′-YTNGGNTTYGAYGGNATRPO-3′	WLGFDGID	Chitinase	*rch*1 (360)
ChiR	5′-WARTCRTANGCCATNARRTT-3′	NLMAYDY	Chitinase	*rch*2 (315)
XylMBF	5′-GTNGGNGGNAARGGNTGG-3′	VGGKGW	Xylanase	*rxy*1 (483)

(Applied Biosystems) with M13 universal primers. The deduced amino acid sequences of amplified DNA fragments were analyzed for homology with a protein database using BLASTX (Altschul et al. 1997) on the World Wide Web at http://www.ddbj.nig.ac.jp/E-mail/homology-j.html).

Southern hybridization

Genomic DNAs from *R. necatrix* strains were isolated and digested with appropriate restriction endonucleases, separated by agarose gel electrophoresis and blotted onto nylon membrane Hybond-N+ (Amersham Biosciences, Piscataway, NJ, USA). The blots were probed with DNA fragments using the Gene Image random primed nucleic acid labeling and detection system (Amersham Biosciences). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

Random amplified polymorphic DNA-PCR

The nucleotide primers set used for random amplified polymorphic-PCR (RAPD-PCR) (Williams et al. 1990) were purchased from Qiagen (Tokyo, Japan). OP-26-13 (5′-GTTTTCGCAG-3′), OP-26-16 (5′-GATCACGTAC-3′), and OP-26-19 (5′-GATCATAGCC-3′) were used in this study. PCRs were conducted in 20-μl reaction volumes. Each reaction tube contained approximately 10 ng DNA template, 2.0 μl 10× PCR buffer, 200 μM each dNTP, 1 pM each primer, and 0.5 U of AmpliTaq polymerase (Applied Biosystems). The thermal cycling parameters were initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 40°C for 2 min, and extension at 72°C for 2 min; a final extension at 72°C for 10 min was done at the end of the amplification. PCR products were subjected to separation by agarose gel electrophoresis and photographed after staining with ethidium bromide.

Results and discussion

Amplification and cloning of DNA fragments used for RFLP markers

To prepare homologous DNA fragments as RFLP markers, which were encoding phenol oxidase, chitinase, and xylanase, PCR was carried out using genomic DNA from the W8 strain as template. The selected genes were expected to be present as a single copy gene in the haploid genome, and their nucleotide sequences were expected to be diverse among *R. necatrix* strains. Primers BaLccF and BaLccR amplified two products, *rpo*1 and *rpo*2, of 1180 and 1090 bp, respectively. The homology of deduced amino acid sequences of partial *rpo*1 and *rpo*2 was less than 30%. Deduced amino acid sequences of partial *rpo*1 and *rpo*2 have 52% identity with *lac*2 of *Gaeumannomyces graminis* var. *tritici* Walker (Litvintseva and Henson 2002) and 60% identity with laccase of *Fusarium proliferatum* (Matsushima) Nirenberg (Kwon et al. 2001), respectively. Therefore, the two genes were not alleles. Primers ChiF2 and ChiR amplified two products, *rch*1 and *rch*2, of 360 and 315 bp, respectively. The homology of deduced amino acid sequences of partial *rch*1 and *rch*2 was less than 35%. Deduced amino acid sequences of partial *rch*1 and *rch*2 have 55% identity with *chi*B of *Aspergillus nidulans* (Eidam) Winter (DNA database accession number, AF314225) and 49% identity with *chi*C of *A. nidulans* (DNA database accession number, D87063), respectively. Therefore, the two genes were not alleles, and the two chitinase genes may have different functions. Primers XylMBF and XylMBF amplified a product, *rxl*1, of 483 bp. The deduced amino acid sequence of partial *rxy*1 has 79% identity with *cgx*B of *Chaetomium gracile* Udagawa (Yoshino et al. 1995). The five PCR products were labeled with fluorescein and used as probes for later RFLP analysis (Fig. 1; Table 2).

RFLP analysis

Genomic DNAs from putative parent strains and monospore isolates (see Table 1) were isolated and digested with several restriction endonucleases and then blotted onto nylon membranes. The blots were probed with five amplified fragments such as *rpo*1, *rpo*2, *rch*1 *rch*2, and *rxy*1. When *rpo*2, *rch*1, *rch*2, and *rxy*1 were used as probes, no RFLP was observed (Fig. 2B–E) among putative parent strain and monospore isolates, in spite of examination with seven restriction endonucleases. Therefore, the nucleotide sequence of *rpo*2, *rch*1, *rch*2, and *rxy*1 were conserved and not variable in *R. necatrix* strains. However, when *rpo*1 was used as a probe, RFLP were detected in spite of the absence of a *Bam*HI site in the probe (Fig. 2A).

Two groups of RFLP were observed in each of the digests among single ascospore isolates. One group had *rpo*1-1 type RFLP of 2.5 kbp, which was same as the pattern for

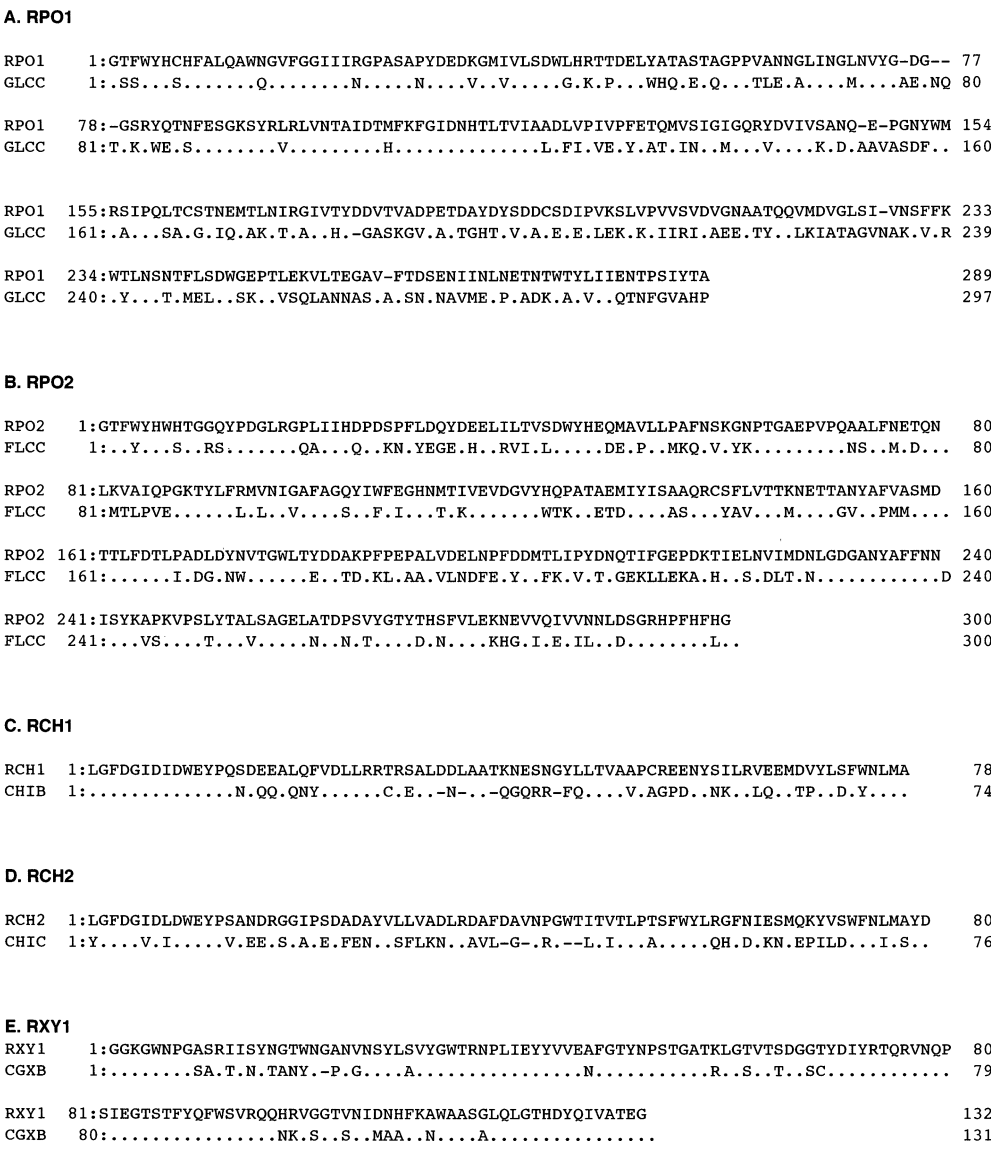

Fig. 1. Comparison of the deduced amino acid sequences. Amino acid residues identical to those in the proteins of *Rosellinia necatrix* are indicated by *dots*. *RPO1*, *R. necatrix* phenol oxidase 1; *GLCC1*, *Gaeumannomyces graminis* var. *tritici* laccase (Litvintseva and Henson 2002); *RPO2*, *R. necatrix* phenol oxidase 2; *FLCC*, *Fusarium proliferatum* laccase (Kwon et al. 2001); *RCH1*, *R. necatrix* chitinase 1; *RCH2*, *R. necatrix* chitinase 2; *CHIB* and *CHIC*, *Aspergillus nidulans* chitinase B and C, respectively (accession numbers in DDBJ are D87063 and AF314225, respectively); *RXY1*, *R. necatrix* xylanase 1; *CGXB*, *Chaetomium gracile* xylanase B (Yoshino et al. 1995)

the W427 strain. The other group had *rpo*1-2 type RFLP of 2.0 kbp, which was same as the pattern observed for the W153 strain. This polymorphism was the result of the presence of a *Bam*HI site in *rpo*1-2 and its absence in *rpo*1-1 (Fig. 2A). All ascospore isolates had either a *rpo*1-1 or a *rpo*1-2 pattern; hence, the detected fragments are alleles of *rpo*1. Because of an unknown reason, the background was very high; however, only single major fragments about 2.5 or 2.0 kbp in size, respectively, were observed in all digests. Thus, only a single copy of *rpo*1 may be present in the genome of single ascospore isolates and their parent strain that were isolated from the perithecium, suggesting that it is indeed a dikaryon, but that the single ascospore isolates and their parent strains which were isolated from the perithecium may actually be homokaryons (see Table 1) (Aimi et al. 2002b).

The segregation of *rpo*1 was also examined among two sets of F_1 progenies that were isolated from two independent perithecia in the field (see Table 1). The first set consists of four F_1 progenies isolated from the same perithecium (W462, W463, W465, and W466 strains) and their parent strain (W153 strain); the second set consists of five F_1 progenies isolated from the same perithecium (W442, W443, W444, W445, and W446 strains) and their parent strain (W427 strain) (see Table 1). Surprisingly, the RFLP of some F_1 progenies was different from their parent strain. For example, parent strain W427 and F_1 progenies such as W443, W444, and W446 had *rpo*1-1; however, W442 and W445 strains, which were isolated from the same perithecium, had *rpo*1-2. In another set, the same phenomenon was also observed. F_1 progenies such as W463, W465, and W466 had *rpo*1-1, although the parent strain W153 and F_1 progeny such as W462 had *rpo*1-2. Although the number of F_1 progenies was not sufficient, frequently an RFLP different from that of the parent strain was observed in each set of F_1 progeny.

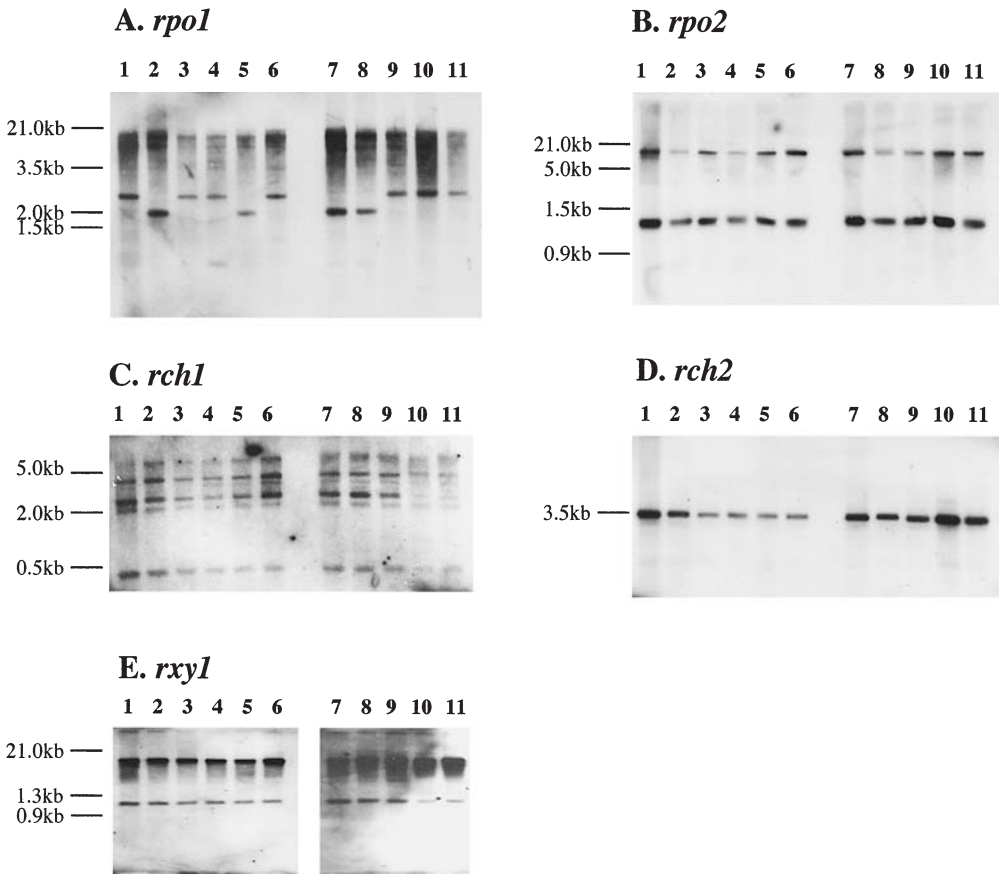

Fig. 2. Southern blot analysis of *Bam*HI-digested DNA from *R. necatrix* isolated from single ascospores and their parent strain. The position and size in kilobases are indicated on the *left*. *Lanes 1–11* correspond to W427, W442, W443, W444, W445, W446 W153, W462, W463, W465, and W466 strains. The W427 strain is the putative parent strain of W442, W443, W444, W445, and W446, which were single ascospore isolates; W153 is the putative parent strain of W462, W463, W465, and W466, which were single ascospore isolates

RAPD analysis

To confirm that the RFLP of *rpo*1 between parent strain and F_1 progenies was due to mating with the different parent strain used in this study, RAPD analysis was also performed. By amplification with OP-26-13, OP-26-16, and OP-26-19 as primers, similar polymorphisms were observed (Fig. 3). In the case of amplification with OP-26-13, parent strain W153 showed pattern F; however, monospore isolates W462, W463, W465, and W466 showed pattern G. Parent strain W427 and monospore isolates W442, W443, and W444 showed pattern H; however, monospore isolates W446 and W445 showed pattern G. In the case of amplification with OP-26-16, parent strain W153 and monospore isolates W462, W465, and W466 showed pattern A; however, the W463 strain showed pattern B. Parent strain W427 and monospore isolates W443, W444, and W446 showed pattern C; however, monospore isolates W442 and W445 showed pattern B. In the case of amplification with OP-26-19, parent strain W153 and monospore isolates W465 showed pattern D; however, W462, W463, and W466 showed pattern E. Parent strain W427 and monospore isolates W444 and W445 showed pattern E; however, monospore isolates W442, W443, and W446 showed pattern D. These phenomena supported the idea that some F_1 progeny in each set were carrying genetically different genes from parent strains used in this study; it is suggested that another strain mated with the W427 or W153 strain, and then heterokaryons were produced before development of ascus and ascospore. Moreover, these results suggested that the RFLP of *rpo*1 was not the result of recombination during meiosis.

We have reported that single ascospore isolates belong to a different mycelial compatibility group (MCG) and imperfectly fuse among the strains isolated from the same perithecium (Aimi et al. 2002a). This phenomenon suggested that the life cycle of this fungus might be the homothallic system. However, our finding suggests that the other strain, which was genetically different from the strain isolated from a perithecium, was required to develop ascus and ascospores. This phenomenon indicates that mating and ascus development occurred between two genetically different strains. Therefore, this fungus might have a heterothallic life cycle (Fig. 4). Recently, we developed a method for estimating chromosome number in *R. necatrix* using telomeric fingerprinting methods (Aimi et al. 2002b). That report suggests that both field-isolated strains and monospore-isolated strains may be a dikaryotic homokaryon. Moreover, other RFLPs except for *rpo*1 were not observed. Thus, observation of RFLP of *rpo*1 and RAPD patterns was not due to reconstruction of the genome and mobile genetic elements during the homothallic life cycle. Therefore, these results strongly suggested that observation of RFLP of *rpo*1 and RAPD patterns were due to mating

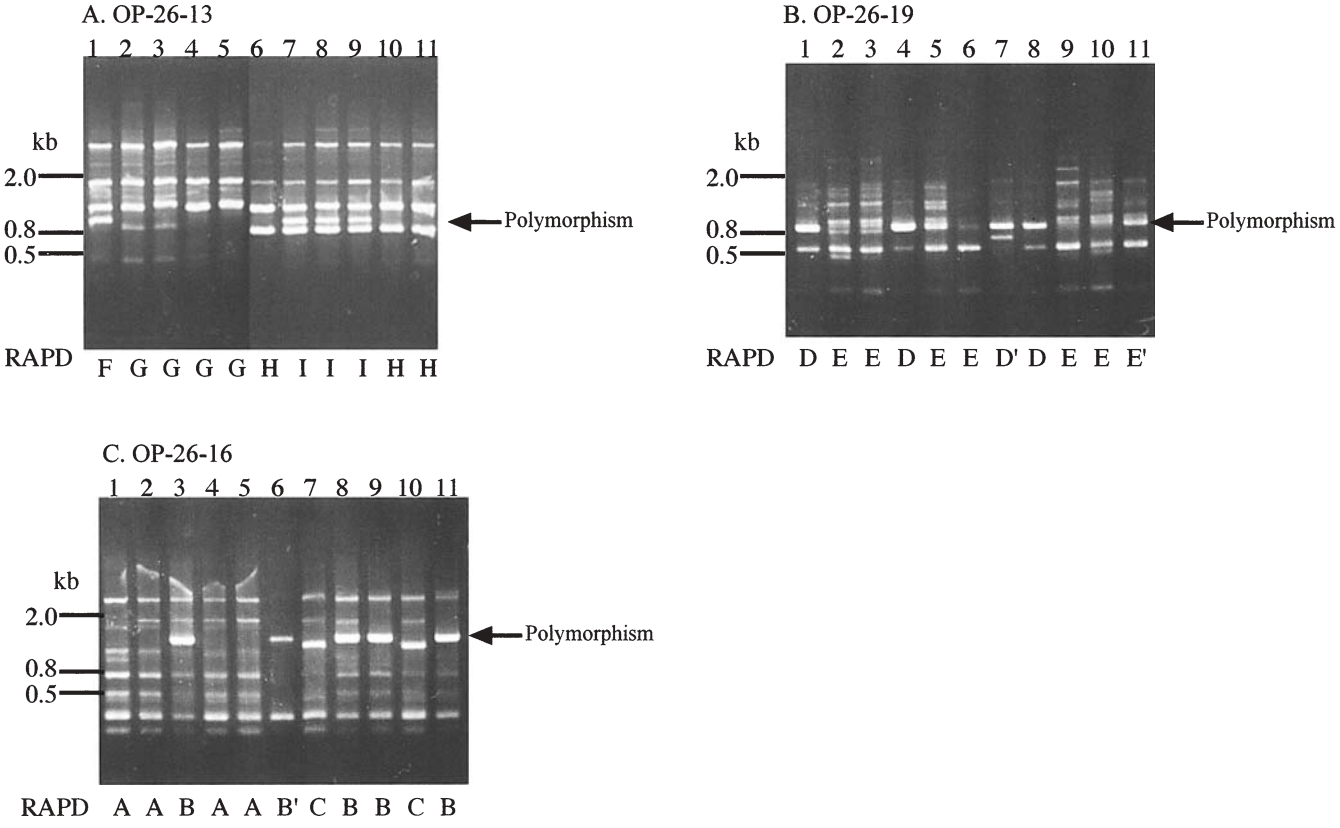

Fig. 3. Random amplified polymorphic DNA (RAPD) analysis of DNA from *R. necatrix* isolated from single ascospores and their parent strain: **A** OP-26-13; **B** OP-26-19; **C** OP-26-16. In each panel, *lanes 1–11* correspond to W157, W462, W463, W465, W466, W427, W442, W443, W444, W445, and W446 strain. W427 strain is the putative parent strain of W442, W443, W444, W445, and W446, which were single ascospore isolates; W153 is the putative parent strain of W462, W463, W465, and W466, which were single ascospore isolates. The RAPD pattern corresponds with Table 1. *Dashes*: B′, main band is similar to B; D′, main band is similar to D; E′, main band is similar to E

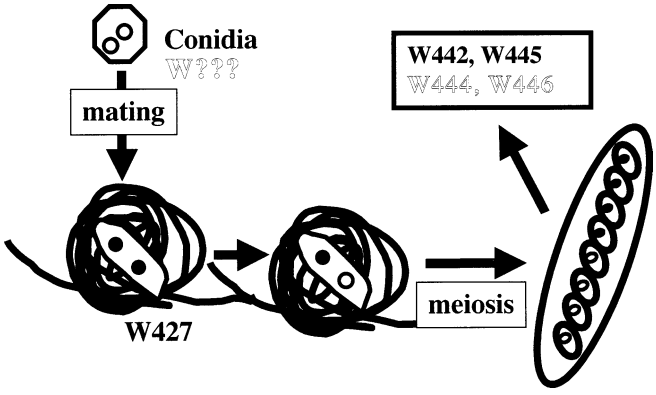

Fig. 4. Supposed life cycle of *Rosellinia necatrix*

between genetically different dikaryotic homokaryons and formation of a heterokaryon before meiosis.

Mycelial compatibility including cell fusion with cell death in *R. necatrix* was very complex because of imperfect fusion among the strains isolated from the same perithecium (Aimi et al. 2002a). Synnemata development has been reported by Nakamura et al. (2000), and conidia were observed on the synnemata. Thus, these results indicate that mycelium fusion was very rare and difficult in this fungus, and that interaction and attachment between mycelium and conidia may be required for mating and heterokaryon formation. These findings are well known in heterothallic ascomycetes *Neurospora crassa* Shear et Dodge (Davis 2000). Further study, such as identification of mating type is required, however. This is the first report about an evidence of heterothallic life cycle in *R. necatrix*.

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