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ORIGINAL PAPER

Ceratobasidiaceae mycorrhizal fungi isolated from nonphotosynthetic orchid *Chamaegastrodia sikokiana*

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Abstract Mycorrhizal fungi were isolated from the nonphotosynthetic orchid *Chamaegastrodia sikokiana* and identified as members of Ceratobasidiaceae by phylogenetic analysis of the internal transcribed spacer (ITS) region of ribosomal deoxyribonucleic acid. The ITS sequences were similar among geographically separated samples obtained from Mt. Kiyosumi in Chiba Prefecture and Mt. Yokokura in Kochi Prefecture. One of the isolated fungi, KI1-2, formed ectomycorrhiza on seedlings of *Abies firma* in pot culture, suggesting that tripartite symbiosis exists among *C. sikokiana*, mycorrhizal fungi, and *A. firma* in nature, and carbon compounds are supplied from *A. firma* to *C. sikokiana* through the hyphae of the mycorrhizal fungi. To our knowledge, this is the second study to suggest the involvement of Ceratobasidiaceae fungi in tripartite symbiosis with achlorophyllous orchids and photosynthetic host plants.

Keywords *Abies firma* · Ceratobasidiaceae · Ectomycorrhiza · Myco-heterotrophy · Tripartite symbiosis

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Introduction

Chamaegastrodia sikokiana Makino Makino and Maekawa is a tiny achlorophyllous orchid that is distributed from Honshu Island in Japan to western China and northern India (Seidenfaden 1994). The genus *Chamaegastrodia* consists of five achlorophyllous species, with *C. sikokiana* being the type species (Seidenfaden 1994). This orchid is usually found at the understory of broad-leaved or coniferous forests. In Japan, the shoots emerge around late June and shoot growth, flowering, fructification, and seed dispersal occur in sequence within 4 months. Clarification of mycorrhizal symbiosis would contribute to understanding the life cycle of achlorophyllous orchids. Unfortunately, however, no studies have been conducted to identify the mycorrhizal fungi of this orchid.

For many achlorophyllous orchids, symbioses with ectomycorrhizal fungi have been reported, examples of which are *Cephalanthera austinae* A. Heller with Thelephoraceae (Taylor and Bruns 1997), *Hexalectris spicata* Barnhart with Sebacinaceae (Taylor et al. 2003), and *Corallorhiza maculata* Greene and *C. mertensiana* Bong with Russulaceae spp. (Taylor and Bruns 1999). In those cases, the mycorrhizal fungi were identified from fungal deoxyribonucleic acid (DNA) sequences. Furthermore, tripartite symbiosis was confirmed in several achlorophyl-

lous orchid species. For *Rhizanthella gardneri*, the so-called underground orchid, artificial cultivation from seed germination to flowering was achieved under symbiosis with a mycorrhizal fungus isolated from *R. gardneri* rhizome and *Melaleuca uncinata* host tree (Warcup 1985). For *Corallorhiza trifida*, the development of hyphal links of mycorrhizal fungi from host trees, *Betula pendula* or *Salix repens*, was observed, and carbon transfer from the host trees to the orchid was detected (McKendrick et al. 2000).

C. sikokiana is usually found at the understory of ectomycorrhiza-forming trees, such as *Abies firma* (Pinaceae), and ectomycorrhizal roots are often found around the rhizomes of this orchid. Therefore, we supposed that this orchid might have symbiotic relationship with some ectomycorrhizal fungi. In this study, we isolated and identified mycorrhizal fungi of *C. sikokiana* and attempted to form ectomycorrhiza on *A. firma*.

Materials and methods

Orchid collection and fungal isolation

C. sikokiana rhizomes were collected from a coniferous forest of *A. firma* in Mt. Kiyosumi at 350 m above sea level, located in Kamogawa City, Chiba Prefecture, in August 2003 and 2004 and in an evergreen broad-leaved forest of *Quercus acuta* and *Cryptomeria japonica* in Mt. Yokokura at 780 m above sea level, located in Ochi-machi, Takaoka-Gun, Kochi Prefecture, in September 2005. The distance between the above two habitats is approximately 660 km. Mycorrhizal fungi were isolated from the collected rhizomes according to Warcup and Talbot (1967) with slight modifications as follows. The rhizome surface was washed with tap water and sterilized by immersing in 70% ethanol for 1 min and in sodium hypochlorite solution containing 1% available chlorine for 1 min. A piece of the sterilized rhizome, approximately 5 mm in length, was put into 1 ml of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse intracellular hyphal coils (pelotons). About 20 ml of autoclaved Modified Melin–Norkrans medium (MMN; glucose 10.0 g, $[CH(OH)COONH_4]_2$ 1.0 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $FeCl_3$ 1.2 mg, thiamine hydrochloride 0.1 mg, malt extract 3.0 g, yeast extract 2.0 g, vegetable juice (V8) 10 ml, agar 15.0 g, distilled water 1,000 ml; Marx 1969) was cooled to 45°C and poured into the Petri dish. The dish was shaken before solidification of the medium to disperse the pelotons and incubated at 25.0±0.5°C in the dark for 3 days. Fungal colonies growing from the pelotons were isolated with a sterilized scalpel.

Ectomycorrhiza formation in pot culture

In its habitat in Chiba Prefecture, *C. sikokiana* was found in a forest of *A. firma*; therefore, this woody plant species was used in the experiment to form ectomycorrhiza. *A. firma* seeds were sown in pots containing volcanic soil medium consisting of Akadama soil and Kanuma soil (1:1 v/v), which was autoclaved at 121°C for 30 min. In each pot, four *A. firma* seedlings were grown in a greenhouse for 1 year. One fungal strain, KI1-2, isolated from a *C. sikokiana* rhizome was grown in 15 ml of liquid MMN medium at 25.0±0.5°C in the dark for 1 month, and the mycelium was buried as an inoculum near the roots of *A. firma*. The inoculated seedlings were grown in a greenhouse for another 1 month and formed ectomycorrhizas were observed.

Molecular identification of isolated fungi

DNA was extracted with the CTAB method (Weising et al. 1995) from each fungus cultured on MMN medium and ectomycorrhiza formed on *A. firma*. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) was amplified from the extracted DNA by the polymerase chain reaction (PCR) with primers ITS 1F and ITS4 (Gardes and Bruns 1993) using TaKaRa Ex Taq Hot Start Version (Takara Bio, Otsu, Japan). The PCR reaction mixture contained 5 μl of template DNA sol, 0.75 U of *Taq* polymerase, 0.25 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate, and 3 μl of the supplied PCR buffer in 30 μl of the total amount. The reaction was performed on a TaKaRa PCR Thermal Cycler 480 (TaKaRa Bio) as follows: initial denaturation step at 94°C for 2 min, a subsequent step of 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min, and a final elongation step at 72°C for 5 min. The PCR products were cloned using a pT7Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI) according to the manufacturer's instructions, and plasmid DNAs were extracted from the cloned products using MagExtractor Plasmid (TOYOBO). The plasmid inserts were sequenced with the dye terminator method using sequencing primers M13-47 and RV-M. Obtained sequence data were deposited in the DNA Data Bank of Japan (DDBJ) database. All of the sequenced data were subjected to BLAST search (Altschul et al. 1997), and analogous data were downloaded from DDBJ/European Molecular Biology Laboratory/GenBank nucleotide sequence database. For the sequenced and the downloaded data, multiple sequence alignments and neighbor-joining (NJ) phylogenetic analysis (Saitou and Nei 1987) were carried out using CLUSTAL W version 1.83 (Thompson et al. 1994). For NJ analysis, evolutionary distances were estimated using Kimura's two-parameter model (Kimura

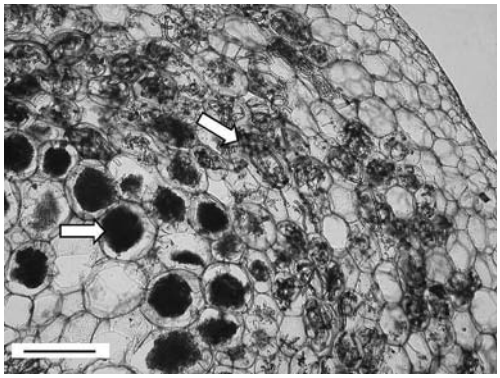

Fig. 1 Hyphal coils (*arrows*) in cortical cells of *Chamaegastrodia sikokiana* rhizome. *Bar*=100 μm

1980), and bootstrap analysis (Felsenstein 1985) was performed with 1,000 replications. The phylogenic tree was drawn using Treeview (Page 1996).

The classification of fungi corresponds to that of Kirk et al. (2001).

Results

Fungal isolation and molecular identification

Rhizomes were collected from three *C. sikokiana* individuals in a habitat in Mt. Kiyosumi in Chiba Prefecture and

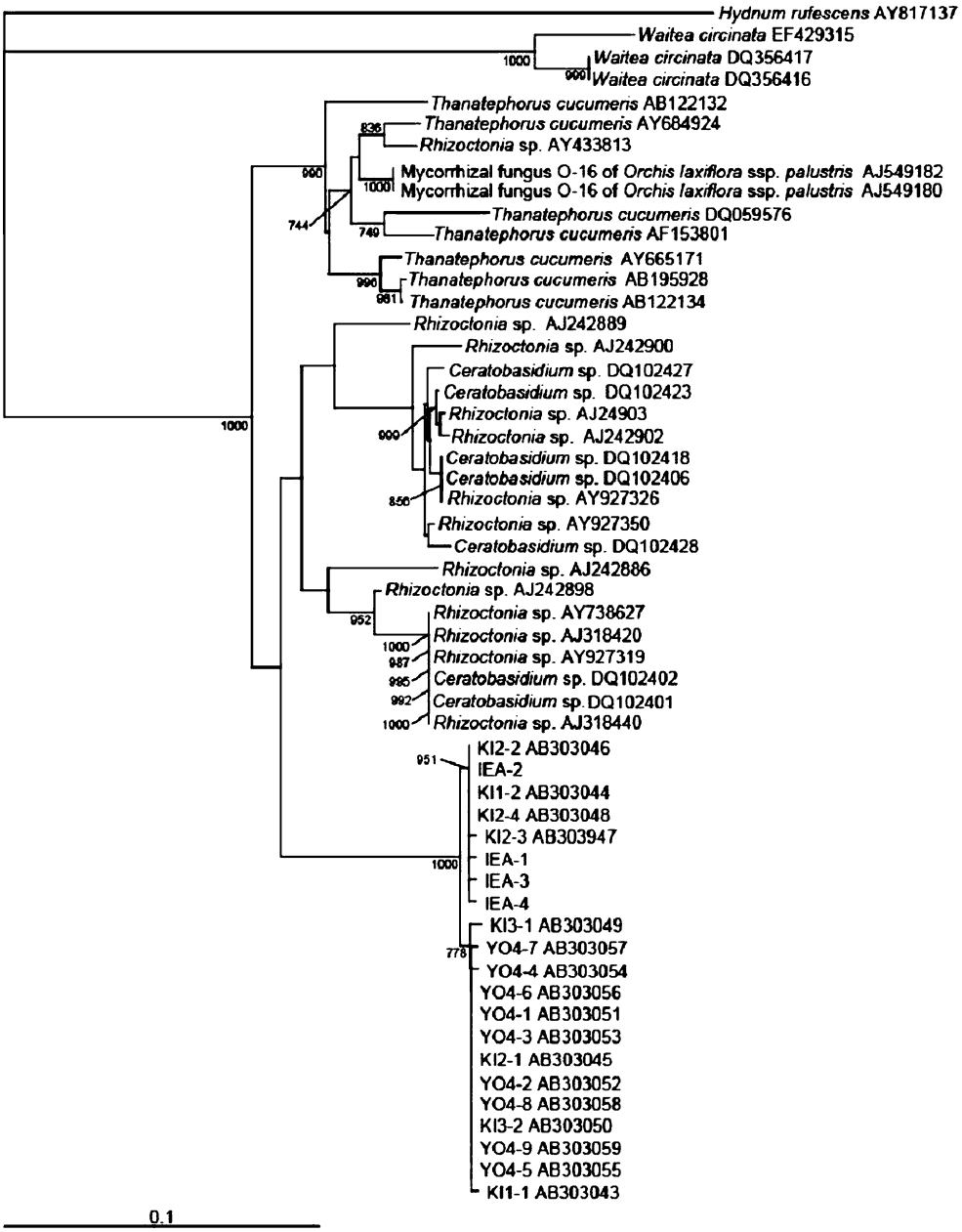

Fig. 2 Neighbor-joining phylogenetic tree showing the relationship between mycorrhizal fungi isolated from *Chamaegastrodia sikokiana* and related fungi in Ceratobasidiaceae based on sequences of the ITS region of nuclear rDNA. *Hydnum rufescens* (AY817137) in Hydnaceae, Cantharellales is the relevant outgroup species. All bootstrap values more than 70% are shown (1,000 replicates). Accession numbers of DDBJ/EMBL/GenBank nucleotide database are given for all sequences. IEA-1, IEA-2, IEA-3, and IEA-4 show isolated fungal DNA from ectomycorrhiza of *Abies firma*

one individual in a habitat in Mt. Yokokura in Kochi Prefecture. Fungal colonization was found in rhizomes that were colored brown to black. In the rhizome, most cortical cells were colonized by coiled hyphae (Fig. 1). In total, 17 strains of mycorrhizal fungi were isolated from the four rhizome samples. It was found that isolated fungi originating from the same rhizome formed apparently identical colonies. DNA sequences of the ITS regions were obtained from all 17 isolates. Phylogenetic analysis showed that the 17 isolates have very similar ITS sequence (99.6–99.9%) and are closely related to *Ceratobasidium* spp. It was also found that orchid mycorrhizal fungi isolated from *Orchis laxiflora* ssp. *palustris* were related to the isolated fungi (Fig. 2).

Ectomycorrhiza formation

KI1-2, a mycorrhizal fungus isolated from *C. sikokiana* in Mt. Kiyosumi, was used for ectomycorrhiza formation. One month after fungal inoculation, ectomycorrhizas were formed on the rootlets of *A. firma* (Fig. 3a,b). Fungal sheath and Hartig net were found in the ectomycorrhiza (Fig. 3c,d). The ITS sequence similarity between the formed ectomycorrhiza and the inoculated fungus is high at 99.8–99.9%.

Discussion

The mycorrhizal fungi of *C. sikokiana* were identified as members of Ceratobasidiaceae. One of the fungi formed ectomycorrhiza on *A. firma*, suggesting tripartite symbiosis among *C. sikokiana*, mycorrhizal fungi, and *A. firma*. No hyphal growth was observed on the Czapek•Dox medium, which has been usually used for isolation of saprobic fungi from orchid mycorrhiza, whereas active hyphal growth was observed on the MMN medium, which was usually used for ectomycorrhizal fungi. This result suggests that the isolated cultures from this orchid mycorrhiza were all ectomycorrhizal fungi. The ITS sequences amplified from the formed ectomycorrhiza have high similarity, 99.8–99.9%, to that of the inoculated fungus, KI1-2. This result strongly suggests that ectomycorrhiza was formed by the fungus isolated from *C. sikokiana*. The slight difference would be caused by the existence of different sequences among rDNA repeats or artifacts in PCR.

Because high sequence similarity was found among the mycorrhizal fungi isolated from geographically separated samples, *C. sikokiana* may have a strong relationship with this group of fungi. Further extensive study is required to examine the specificity of the relationship, in which it is

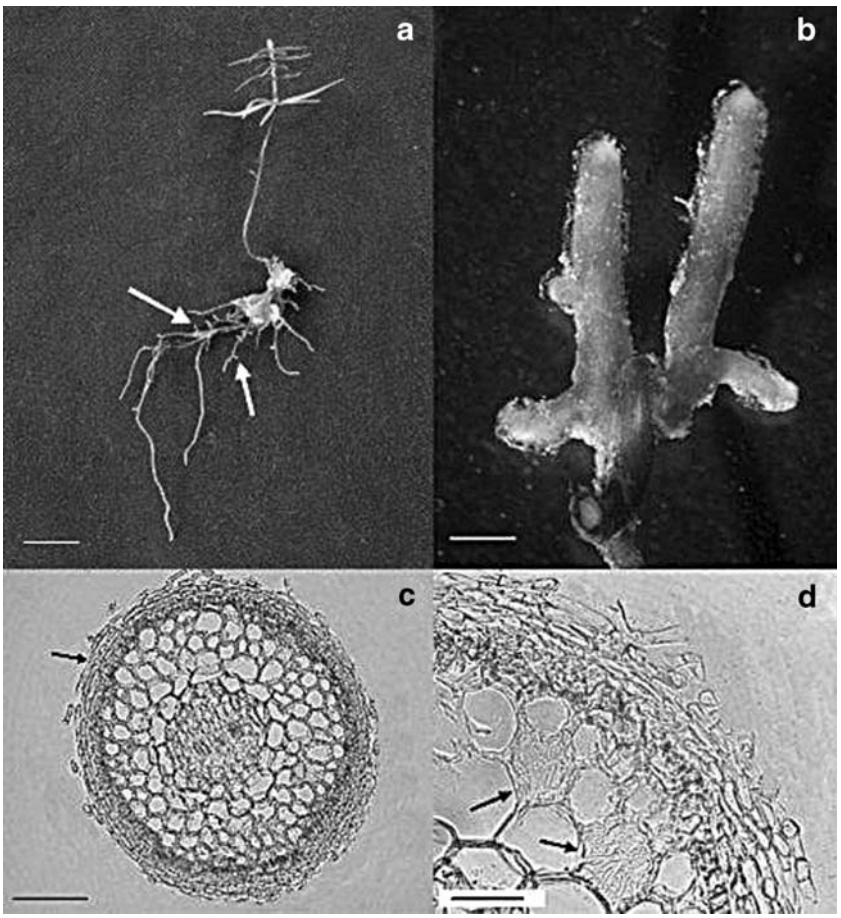

Fig. 3 Ectomycorrhiza formation on rootlets of *Abies firma* by a mycorrhizal fungus, KI2–1, isolated from *Chamaegastrodia sikokiana*. **a** *Abies firma* seedling with formation of ectomycorrhizas (*arrows*). *Bar*=1 cm. **b** Formed ectomycorrhiza. *Bar*=1 mm. **c** Cross section of the ectomycorrhiza showing a sheath (*arrow*). *Bar*=200 μm. **d** Cross-section of the ectomycorrhiza showing the Hartig net (*arrows*). *Bar*=50 μm

required to amplify fungal DNA directly from the rhizome or pelotons. A detailed investigation of ectomycorrhiza surrounding the rhizome of *C. sikokiana* is also desired to reveal the relationship.

It is well known that many orchid species have symbiotic relationships with *Rhizoctonia* spp. The anamorphic fungi, *Rhizoctonia*, are classified into three families in teleomorph, Tulasnellaceae Sebacinaceae, and Ceratobasidiaceae (Peterson et al. 1998), and orchid mycorrhizal fungi have been reported in all the three families. For Ceratobasidiaceae, orchid mycorrhizal fungi have been reported in *Ceratobasidium* and *Thanatephorus*, e.g., *Ceratobasidium cornigerum* (Bourdot) Rogers in *Thrixspermum congestum* (F. M. Bail) Dockr. (Roberts 1999), and *Thanatephorus pennatus* Currah in *Calypso bulbosa* Holzing (Currah 1987). Generally, fungi belonging to this family are regarded as saprobes. Meanwhile, a mycorrhizal fungus of an achlorophyllous orchid, *R. gardneri* R. S. Rogers, which was provisionally classified as *Thanatephorus* (*T. gardneri* Warcup, sp. nov.) based on teleomorph morphology, formed ectomycorrhiza on *M. uncinata* R. Br., *Eucalyptus* spp., and *Poranthera microphylla* Brogn (Warcup 1991). As far as we know, our present study is the second one to show the involvement of fungi in Ceratobasidiaceae in tripartite symbiosis to form orchid mycorrhiza and ectomycorrhiza. *C. sikokiana* is categorized as an endangered species in many prefectures in Japan. The results obtained in this study would be useful in considering the conservation and propagation of *C. sikokiana* in the natural habitat.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Currah RS (1987) *Thanatephorus pennatus* sp. nov. isolated from mycorrhizal roots of *Calypso bulbosa* (Orchidaceae) from Alberta. Can J Bot 65:1957–1960

Felsenstein L (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:133–118

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kirk PM, Cannon PF, David JC, Stalpers JA (eds) (2001) In: Dictionary of the fungi. 9th edn. CABI, Wallingford

Marx DH (1969) Influence of ectotrophic mycorrhizal fungi on resistance or pine roots to pathogenic infections. I. Antagonisum of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

McKendrick SL, Leake JR, Read DJ (2000) Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared phyphal connections. New Phytol 145:539–548

Page RDM (1996) An application to display phylogenetic tree personal computers. Comp Appl Biosci 12:357–358

Peterson RL, Uetake Y, Zelmer C (1998) Fungal symbiosis with orchid protocorms. Symbiosis 25:29–55

Roberts P (1999) *Rhizoctonia*-forming fungi. A taxonomic guide. The Herbarium, Royal Botanic Gardens, Kew

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Seidenfaden G (1994) The genus *Chamaegastrodia* (Orchidaceae). Nord J Bot 14:293–301

Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. Proc Nat Acad Sci USA 94:4510–4515

Taylor DL, Bruns TD (1999) Population, habitat and genetic correlates of mycorrhizal specialization in the cheating orchids *Corallorhiza maculata* and *C. mertensiana*. Mol Ecol 8:1719–1732

Taylor DL, Bruns TD, Szaro TM, Hodges SA (2003) Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. Am J Bot 90:1168–1179

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequencing weighting, position sequence gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Warcup JH (1985) *Rhizanthella gardneri* (Orchidaceae), Its *Rhizoctonia* endophyte and close association with *Melaleuca uncinata* (Myrtaceae) in western Australia. New Phytol 99: 273–280

Warcup JH (1991) The Rhizoctonia endophytes of *Rhizanthella* (Orchidaceae). Mycol Res 95:656–641

Warcup JH, Talbot PHB (1967) Perfect states of *Rhizoctonias* associated with orchids. New Phytol 66:631–641

Weising K, Nybom H, Wolff K, Meyer W (1995) DNA fingerprinting in plants and fungi. CRC, Boca Raton