Mycoscience 42: 295–299, 2001

Restriction landmark genomic scanning (RLGS) in fungi

Keisuke Tomioka[1] and Toyozo Sato[2]

[1] National Agricultural Research Center for Western Region, Zentsuji 765–8508, Japan
[2] National Institute of Agrobiological Sciences, Tsukuba 305–8602, Japan

Received 13 November 2000
Accepted for publication 27 April 2001

RLGS is a technique to detect DNA polymorphism using restriction sites as landmarks. It identifies the landmarks through direct end-labeling, two-dimensional electrophoresis and autoradiography, giving a profile with many spots to allow the scanning of numerous DNA loci. We successfully performed the technique on fungi using isolates of *Colletotrichum acutatum* and *C. gloeosporioides* in anamorphic Ascomycotina, *Rhizopus oryzae* in Zygomycotina, *Phytophthora nicotianae* in Mastigomycotina (or Oomycota) and *Rhizoctonia solani* in anamorphic Basidiomycotina. RLGS of total genomic DNA digested with three restriction enzymes, *Not* I, *Eco*R V and *Mbo* I, reproducibly gave specific profiles of ca. 400 to 1,600 spots for each isolate. A polymorphic spot appearing to reflect a genetic difference between the two *Colletotrichum* species was found in the profiles of the isolates. No other common spots were found in any combination of isolates of the two *Colletotrichum* species, and thus the other spots on the profiles were regarded as unique to each isolate. These results indicated that RLGS could be applied, as a powerful fingerprinting technique based on genetic information from the whole genomic DNA, to search for useful DNA markers for taxonomic and genomic studies on many fungal species.

Key Words——DNA fingerprinting; DNA polymorphism; genetic variation; genomic analysis; new technique.

Restriction landmark genomic scanning (RLGS) is a technique to detect DNA polymorphism (Hatada et al., 1991). It uses certain restriction sites on genomic DNA as landmarks, which are identified through direct end-labeling, two-dimensional electrophoresis and autoradiography. A single profile obtained by the technique consists of numerous spots scattered in two dimensions, allowing a large number of loci on genomic DNA to be scanned efficiently. Comparison of the profiles of closely related germplasms on the basis of differences in spot intensity can detect even minute changes of organization and/or modification of genomic DNA at a high rate, and RLGS has thus been utilized for various molecular biological studies in animals and plants (Hayashizaki et al., 1993; Hirotsune et al., 1993; Asakawa et al., 1994, 1995; Kawase, 1994; Suzuki et al., 1994; Kuick et al., 1995; Kawase et al., 1999). We tried the technique on fungi to evaluate its utility in fungal studies.

Materials and Methods

Fungi used Two isolates each of *Colletotrichum acutatum* Simmonds ex Simmonds and *C. gloeosporioides* (Penzig) Penzig et Saccardo in anamorphic Ascomycotina and one isolate each of *Rhizopus oryzae* Went et Geerligs in Zygomycotina, *Phytophthora nicotianae* van Breda de Haan in Mastigomycotina (or Oomycota) and *Rhizoctonia solani* Kühn in anamorphic Basidiomycotina were used (Table 1). The isolates were maintained on a medium consisting of 2% (w/v) agar, 0.1% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4 and 1% oatmeal at 15°C (Weitzman and Silva-Hutner, 1967; Hosoya and Otani, 1997).

DNA preparation Total DNA of each isolate for RLGS was prepared by the following procedure, which is based on the protocols reported by Murry and Thompson (1980), Garber and Yoder (1983) and Mills et al. (1994). About 5–8 g of mycelium grown in a liquid medium consisting of 0.1% NH_4NO_3, 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 1% dextrose, pH 4.8 at 25°C in the dark for 1–3 wk was rinsed with sterilized distilled water, frozen in liquid N_2, ground to powder with solid CO_2 as soon as the N_2 had evaporated, then stored with 0.5 g of sodium dodecyl sulfate (SDS) and 0.5 g of insoluble polyvinylpyrrolidone (PVP) at -80°C for more than 4 h until the CO_2 had evaporated. The powdered mycelium with SDS and PVP was suspended in 100 μl of 20 μg/μl proteinase K solution and 10 ml of heated 2% cetyltrimethylammonium bromide containing 0.2 M Tris-HCl (pH 8.0), 0.1 M EDTA and 1.4 M NaCl and incubated at 56°C for 30 min. The aqueous phase was clarified once with 15 ml of phenol/chloroform/isoamyl alcohol mixture (25 : 24 : 1, by vol.), then mixed with an equal volume of 2-propanol to precipitate the DNA. The DNA was washed with 70% and 99% ethanol, dissolved in 1 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA, then treated with RNase. The DNA solution was adjusted to 10 ng/μl for RLGS.

RLGS RLGS was carried out by the following procedure, which is based on the protocols reported by Hayashizaki et al. (1993), Asakawa et al. (1994, 1995), Kawase

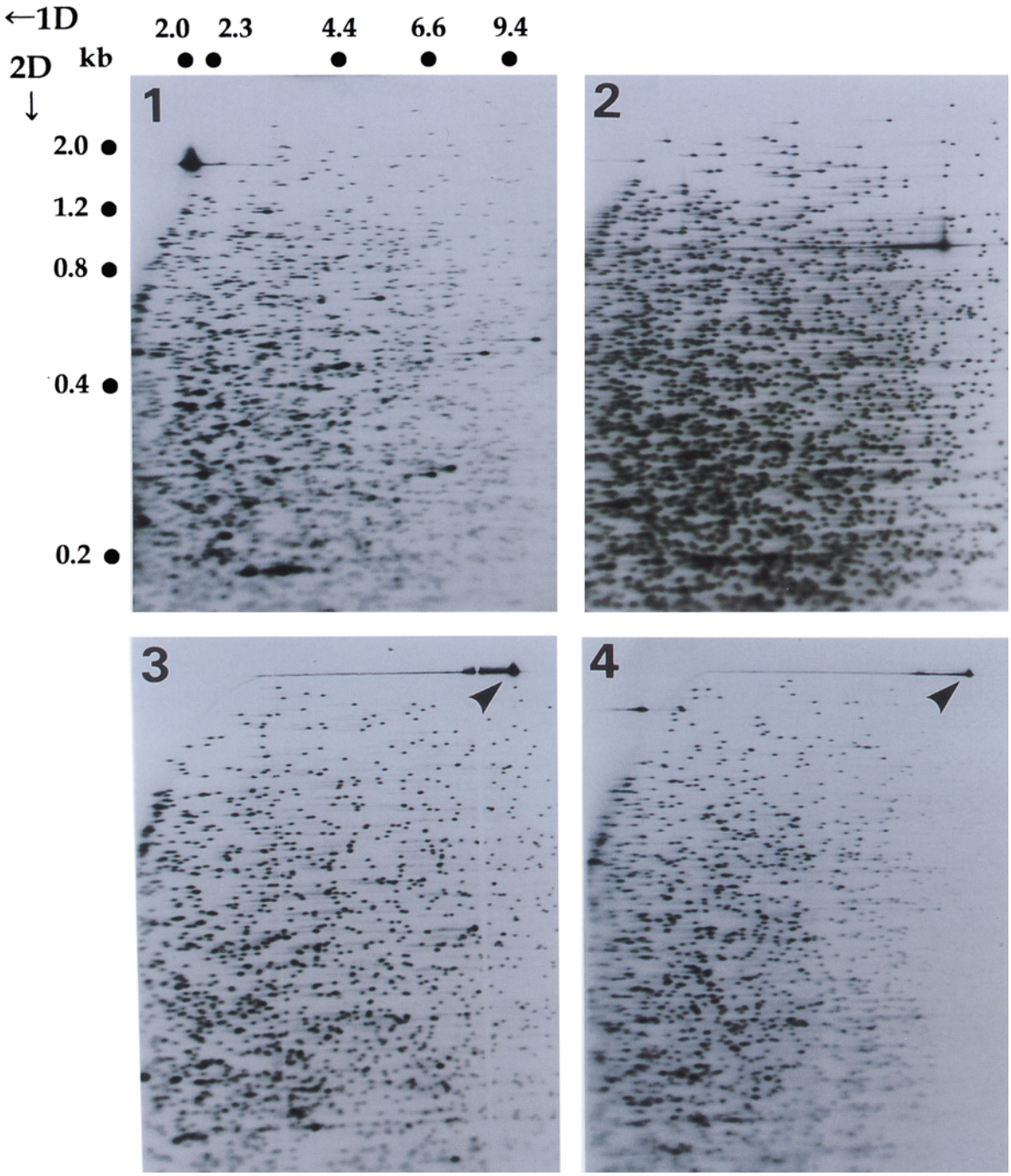

Fig. 1–4. RLGS profiles of the fungal isolates examined. The profiles obtained by 1D electrophoresis of 40 ng of DNA and autoradiography for 14 d are shown. 1. *Colletotrichum acutatum*, MAFF 306544; 2. *C. acutatum*, MAFF 306546; 3. *C. gloeosporioides*, MAFF 238043; 4. *C. gloeosporioides*, MAFF 306538. Arrowheads indicate the polymorphic spot appearing to reflect a genetic difference between *C. acutatum* and *C. gloeosporioides*.

(1994) and Kawase et al. (1999). Eighty nanograms of total DNA in 8 μl of DNA solution was incubated with 1.4 μl of 10 units/μl *Not* I, 1.4 μl of 12 units/μl *Eco*R V, 1 μl of 0.1% bovine serum albumin (BSA), 1 μl of 0.1% Triton X and 1 μl of 0.45 M Tris-HCl (pH 7.5) containing 0.09 M $MgCl_2$, 1.35 M NaCl and 9 mM dithiothreitol (DTT) at 37°C for 3 h. The *Not* I-cleaved ends of the DNA were then labeled with radioisotopes by incubating the lysate with 1 μl of 6,000 Ci/mmol [α-^{32}P] dCTP (*redi*vue; Amersham Pharmacia), 1 μl of 3,000 Ci/mmol [α-^{32}P] dGTP (*redi*vue; Amersham Pharmacia), 1 μl of 1 M DTT and 0.2 μl of 12 units/μl T7 DNA polymerase at 25°C for 15 min. The labeling reaction was stopped by adding 3 μl of 0.05 M EDTA (pH 8.0) containing 50% su-

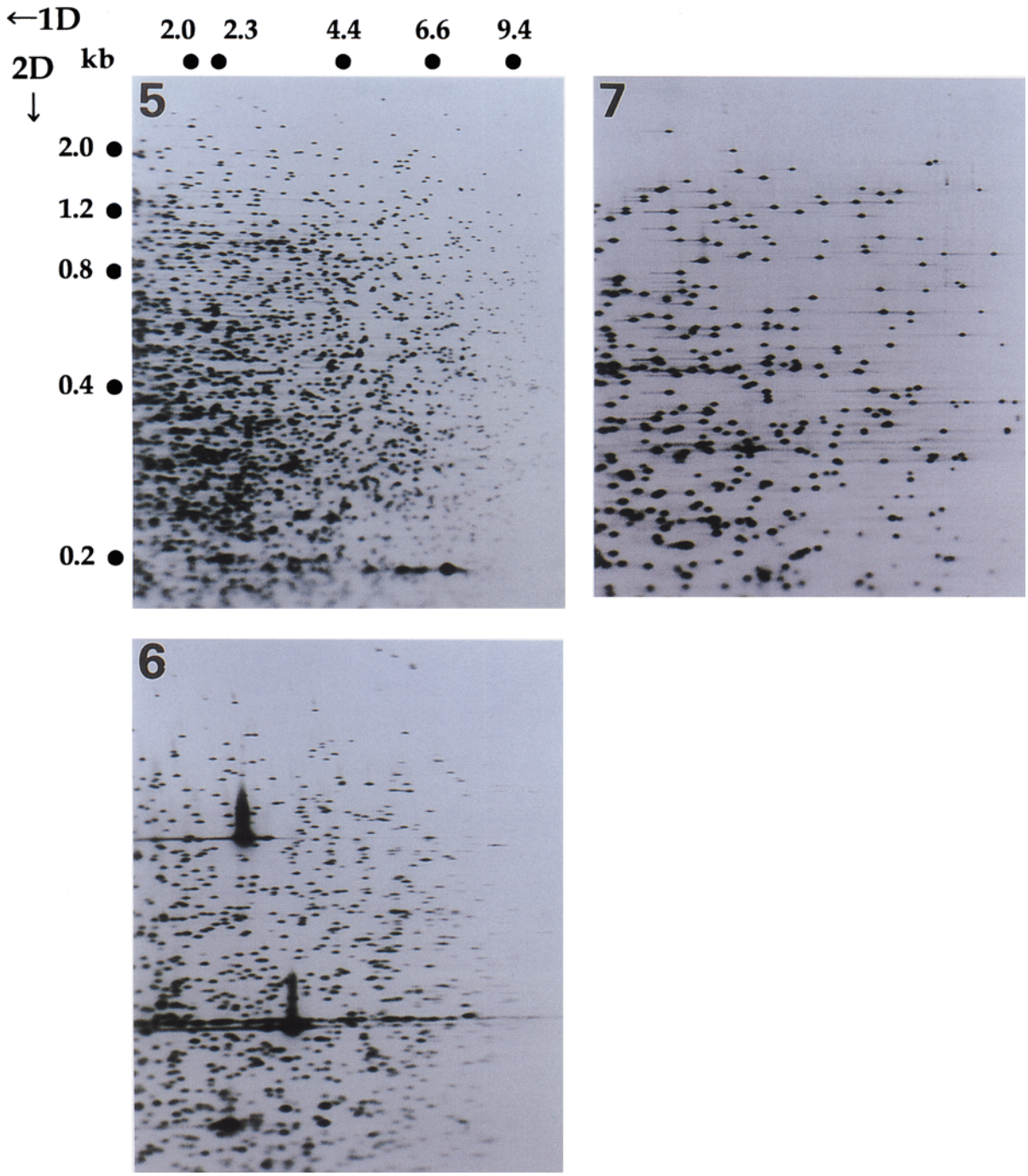

Fig. 5–7. RLGS profiles of the fungal isolates examined. 5. *Rhizopus oryzae*, MAFF 238040; 6. *Phytophthora nicotianae*, MAFF 305940; 7. *Rhizoctonia solani*, MAFF 238037.

crose, 0.5% bromophenol blue (BPB) and 0.5% xylene cyanol (XC). For the first-dimension (1D) electrophoresis, 10–40 ng of the DNA in 2.5–10 μl of the final mixture was fractionated in a vertical gel (2.4 mm in diam, 610 mm in length) in a 1D vertical electrophoresis system for RLGS (BioCraft). The gel consisted of 0.8% agarose (SeaKem Gold; FMC) and 20% sucrose. 1D electrophoresis was conducted in 0.1 M Tris (pH 8.15) containing 0.04 M sodium acetate, 3.6 mM NaCl and 4 mM EDTA at 100 V for 40 h. The gel was then equilibrated with a buffer consisting of 0.05 M Tris (pH 7.4), 0.01 M $MgCl_2$, 0.1 M NaCl and 0.01 M DTT, and the fractionated DNA was digested with *Mbo* I in situ by incubating the gel with 750 μl of the buffer containing 200 units *Mbo* I and 0.01% BSA at 37°C for 2 h. The resulting 1D gel was equilibrated with an electrode buffer consisting of 8.9 mM Tris, 8.9 mM boric acid and 2 mM EDTA (TBE buffer), and the portion (340 mm long) con-

Table 1. Fungal isolates used

Species (Subdivision)	Isolate designation[a)]	Isolation source	Collection locality	Reference
Colletotrichum acutatum (Ascomycotina: anamorphic state)	MAFF 306544	*Malus pumila*	Chiba, Japan	Sato et al. (1998)
Colletotrichum acutatum (Ascomycotina: anamorphic state)	MAFF 306546	*Malus pumila*	Ehime, Japan	Sato et al. (1998)
Colletotrichum gloeosporioides (Ascomycotina: anamorphic state)	MAFF 238043	*Malus pumila*	Ibaraki, Japan	Sato et al. (1998)
Colletotrichum gloeosporioides (Ascomycotina: anamorphic state)	MAFF 306538	*Malus pumila*	Nagano, Japan	Sato et al. (1998)
Rhizopus oryzae (Zygomycotina)	MAFF 238040	*Vigna radiata*	Tokyo, Japan	—
Phytophthora nicotianae (Mastigomycotina or Oomycota)	MAFF 305940	*Nicotiana tabacum*	Kanagawa, Japan	Yamaguchi (1976)
Rhizoctonia solani (Basidiomycotina: anamorphic state)	MAFF 238037	*Oryza sativa*	Kagawa, Japan	—

[a)] Microorganism Section, Gene Bank, Ministry of Agriculture, Forestry and Fisheries, Japan.

taining DNA fragments of approximately 1–10 kb was laid on the upper side of a slab gel (1 mm in thickness, 440 mm in width, 450 mm in height) in an apparatus for the second-dimension (2D) electrophoresis (2D slab electrophoresis system for RLGS; BioCraft). The 2D gel consisted of 5% polyacrylamide (acrylamide/methylenebisacrylamide, 29 : 1) and 1% glycerin. The 1D and 2D gels were fused together with 0.5% agarose (SeaKem Gold; FMC), then covered with 0.5% agarose (SeaKem Gold; FMC) containing 0.2% BPB and 0.2% XC. The DNA in the 1D gel was fractionated in the 2D gel by 2D electrophoresis in TBE buffer at 100 V for 40 h. The 2D electropherogram was vacuum-dried on a filter paper at 60°C for 80 min, then subjected to autoradiography by expose on an X-ray film (BioMax MS, 350 mm × 430 mm; Kodak) with an intensifying screen (BioMax MS intensifying screen; Kodak) at -80°C for 2–21 d to get a RLGS profile. Specificity of the profiles from each isolate, as well as their reproducibility and clearness, were evaluated from 2–6 profiles for each sample.

Results

Specific RLGS profiles of total DNA from each isolate were obtained reproducibly regardless of the culture time of the isolates. Satisfactory RLGS profiles were obtained by 1D electrophoresis of 20–40 ng of DNA and autoradiography for 4–14 d (Fig. 1). Satisfactory profiles were also obtained from the isolates of *Phytophthora nicotianae* and *Rhizoctonia solani* by 1D electrophoresis of 10 ng of DNA, but this amount of DNA of the other isolates produced weak signals, resulting in unclear and irreproducible profiles even when the period of autoradiography was extended to 21 d. The number of spots on the satisfactory profiles obtained under the same conditions varied from ca. 400 to 1,600, depending on the isolate (Fig. 1), and decreased in the order: *Rhizopus oryzae* (ca. 1,600 spots) > 2 spp. of *Colletotrichum* (ca. 1,100–1,400 spots) > *P. nicotianae* (ca. 800 spots) > *Rhizoctonia solani* (ca. 400 spots). Comparison of the profiles of the two *Colletotrichum* species revealed a polymorphic spot that appeared to reflect a genetic difference between the species (Fig. 1). This spot was detected on the isolates of *C. gloeosporioides* but not on the isolates of *C. acutatum*. No other common spots were found in any combination of isolates of the two *Colletotrichum* species, and thus the other spots on the profiles were regarded as unique to each isolate.

Discussion

RLGS as a new DNA fingerprinting technique in fungi In this study, fungal RLGS was successfully performed on isolates belonging to the four subdivisions of the division Eumycota. RLGS of total genomic DNA digested with three restriction enzymes, *Not* I, *Eco*R V and *Mbo* I, reproducibly gave specific profiles of ca. 400 to 1,600 spots for each isolate. Various kinds and combinations of restriction enzymes can be employed with RLGS to detect more DNA loci (Hayashizaki et al., 1993), and thus it should also provide a powerful fingerprinting technique based on genetic information from the whole genomic DNA in fungi. The present protocol from preparation of DNA materials to development of RLGS profiles should be applicable as a basal method to a large number of fungal species. Here, treated *P. nicotianae* as a fungus (Hawksworth et al., 1983; Farr et al., 1989), but if the proposal that it should be transferred from the kingdom Fungi to the kingdom Chromista in the 8-kingdom system of organisms (Cavalier-Smith, 1986; Dick, 1990, 1995; Hawksworth et al., 1995; Alexopoulos et al., 1996; Erwin and Ribeiro, 1996) is adopted, the present success of RLGS on *P. nicotianae* will represent the first demonstration of the technique in the kingdom Chromista.

Utility of RLGS in fungal studies The present study found a polymorphic spot that appears to reflect a genetic difference between *C. acutatum* and *C. gloeosporioides* in the RLGS profiles of the isolates. Other spots on the profiles were regarded as specific to each isolate. These results imply that RLGS can be uti-

lized to search for useful DNA markers indicating intra- and/or interspecific genetic variations. In this study, the number of spots on RLGS profiles also varied depending on the isolate in the following order: *Rhizopus oryzae* > 2 spp. of *Colletotrichum* > *P. nicotianae* > *Rhizoctonia solani*. The principle of RLGS shows that organisms with larger genomic sizes give RLGS profiles with the more spots (Hatada et al., 1991). This implies that organisms whose profiles have more spots also have larger genomic sizes. Although this logic cannot be directly applied to fungi such as *Rhizoctonia* spp. because of heterokaryosis, the spot number may provide one criterion for characterization of some fungal genera or species. RLGS profiles can be easily and rapidly analyzed by computerized image systems (Asakawa et al., 1995; Kuick et al., 1995; Kawase et al., 1999). Cloning of DNA fragments corresponding to spots on the profiles is possible (Hirotsune et al., 1993; Suzuki et al., 1994). RLGS, therefore, should also contribute to efficient and accurate acquisition of useful DNA markers for genomic study such as linkage mapping, base sequencing and gene isolation as well as taxonomic studies in fungi.

Acknowledgements——We thank Dr. H. Koganezawa (National Agricultural Research Center for Western Region, Japan) and Dr. M. Kawase (National Agricultural Research Center for Western Region, Japan) for their valuable advice on the present study. This work was partially funded by Science and Technology Policy.

Literature cited

Alexopoulos, C. J., Mims, C. W. and Blackwell, M. 1996. Introductory mycology, 4th ed. John Wiley & Sons, Inc., New York.

Asakawa, J., Kuick, R., Neel, J. V., Kodaira, M., Satoh, C. and Hanash, S. M. 1994. Genetic variation detected by quantitative analysis of end-labeled genomic DNA fragments. Pro. Natl. Acad. Sci. USA **91**: 9052–9056.

Asakawa, J., Kuick, R., Neel, J. V., Kodaira, M., Satoh, C. and Hanash, S. M. 1995. Quantitative and qualitative genetic variation in two-dimensional DNA gels of human lymphocytoid cell lines. Electrophoresis **16**: 241–252.

Cavalier-Smith, T. 1986. The kingdom Chromista: Origin and systematics. In: Progress in phycological research, vol. 4, (ed. by Round, I and Chapman, D. J.), pp. 309–347. Biopress, Bristol.

Dick, M. W. 1990. Phylum Oomycota. In: Handbook of Protocista, (ed. by Margulis, L., Corliss, J., Melkonian, O., and Chapman, D. J.), pp. 661–685. Jons & Bartlett Pub., Boston.

Dick, M. W. 1995. Sexual reproduction in the Peronosporomycetes (Chromistan fungi). Can. J. Bot. **73** (Suppl. 1): S712–724.

Erwin, D. C. and Ribeiro, O. K. 1996. Taxonomic position of *Phytophthora* among living organisms. In *Phytophthora* diseases worldwide, (ed. by Erwin, D. C. and Ribeiro, O. K.), pp. 1–3. APS Press, St. Paul.

Farr, D. F., Bills, G. F., Chamuris, G. P. and Rossman, A. Y. 1989. Fungi on plants and plant products in the United States, pp. 1–5. APS Press, St. Paul.

Garber, R. C. and Yoder, O. C. 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Analyt. Biochem. **135**: 416–422.

Hatada, I., Hayashizaki, Y., Hirotsune, S., Komatsubara, H. and Mukai, T. 1991. A genomic scanning method for higher organisms using restriction sites as landmarks. Pro. Natl. Acad. Sci. USA **88**: 9523–9527.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. and Pegler, D. N. 1995. Ainsworth & Bisby's dictionary of the fungi, 8th ed. CAB International, Wallingford.

Hawksworth, D. L., Sutton, B. C. and Ainsworth, G. C. 1983. Ainsworth & Bisby's dictionary of the fungi, 7th ed. Commonwealth Mycol. Inst., Kew.

Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Hatada, I., Shibata, H., Kawai, J., Hirose, K., Watanabe, S., Fushiki, S., Wada, S., Sugimoto, T., Kobayakawa, K., Kawara, T., Katsuki, M., Shibuya, T. and Mukai, T. 1993. Restriction landmark genomic scanning method and its various applications. Electrophoresis **14**: 251–258.

Hirotsune, S., Shibata, H., Okazaki, Y., Sugino, H., Imoto, H., Sasaki, N., Hirose, K., Okuizumi, H., Muramatsu, M., Plass, C., Chapman, V. M., Tamatsukuri, S., Miyamoto, C., Furuichi, Y. and Hayashizaki, Y. 1993. Molecular cloning of polymorphic markers on RLGS gel using the spot target cloning method. Biochem. Biophys. Res. Commun. **194**: 1406–1412.

Hosoya, T. and Otani, Y. 1997. Hyaloscyphaceae in Japan (1): Non-glassy-haired members of the tribe Hyaloscypheaea. Mycoscience **38**: 171–186.

Kawase, M. 1994. Application of the restriction landmark genomic scanning (RLGS) method to rice cultivars as a new fingerprinting technique. Theor. Appl. Genet. **89**: 861–864.

Kawase, M., Ishihara, J. and Tomioka, K. 1999. Detection of DNA polymorphism in Japanese rice cultivars using the restriction landmark genomic scanning (RLGS) method. Bull. Shikoku Natl. Agr. Exp. Stn. **64**: 37–46.

Kuick, R., Asakawa, J., Neel, J. V., Kodaira, M., Satoh, C. and Hanash, S. M. 1995. High yield of restriction fragment length polymorphisms in two-dimensional separations of human genomic DNA. Genomics **25**: 345–353.

Mills, P. R., Sreenivasaprasad, S. and Brown, A. E. 1994. Detection of the anthracnose pathogen *Colletotrichum*. In: Modern assays for plant pathogenic fungi, (ed. by Schots, A., Dewey, F. M. and Oliver, R. P.), pp. 183–189. CAB International, Kew.

Murry, M. G. and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8**: 4321–4325.

Sato, T., Uematsu, S., Iijima, A. and Koganezawa, H. 1998. Occurrence of apple bitter rot by grayish colony form of *Colletotrichum acutatum* in Japan and pathogenicity to apple fruits of *C. acutatum* and *Glomerella cingulata* isolated from other plants. Trans. Mycol. Soc. Japan **39**: 35–44. (In Japanese with English summary.)

Suzuki, H., Kawai, J., Taga, C., Ozawa, N. and Watanabe, S. 1994. A PCR-mediated method for cloning spot DNA on restriction landmark genomic scanning (RLGS) gel. DNA Res. **1**: 245–250.

Weitzman, I. and Silva-Hutner, M. 1967. Non-keratinous agar media as substrates for the ascigerous state in certain members of the Gymnoascaceae pathogenic for man and animals. Sabouraudia **5**: 335–340.

Yamaguchi, Y. 1976. Ecological studies of the tobacco black shank fungus under the underground condition modified by film-mulching cultivation. Bull. Kagoshima Tobacco Exp. Stn. **20**: 47–134. (In Japanese with English summary.)