APPLICATION OF THE RANDOM AMPLIFIED POLYMORPHIC DNA USING THE POLYMERASE CHAIN REACTION FOR EFFICIENT ELIMINATION OF DUPLICATE STRAINS IN MICROBIAL SCREENING

I. FUNGI

FUMIHIRO FUJIMORI and TORU OKUDA*

Department of Microbiology and Taxonomy,
Nippon Roche Research Center,
200 Kajiwara, Kamakura, Kanagawa 247, Japan

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For efficient fungal strain selection in microbial screening, we applied the random amplified polymorphic DNA (RAPD) method using the polymerase chain reaction (PCR). In order to evaluate this system, the genus *Trichoderma* was employed, because its species are difficult to distinguish from each other. We selected an appropriate oligonucleotide decamer, R28 (5'-ATGGATCCGC), determined the optimal cycles of PCR as 30 cycles, simplified the template preparation method, and determined optimal concentrations of the template and *Taq* DNA polymerase. We then examined 74 closely related strains of *Trichoderma*. The electrophoretic band patterns of the PCR products were compared. According to the statistical analysis with the phylogenetic analysis using parsimony (PAUP), the results were consistent with the morphological, physiological and ecological data on these strains. Therefore, we conclude that RAPD is a simple, efficient and reliable method for the selection of fungal strains employed in screening.

Microorganisms have traditionally been isolated and selected empirically by taxonomists in microbial screening. Such screening, however, is somewhat restricted because of repetitive encounters with possible duplicate strains. In addition, when similar strains are passed through a certain assay system based on their activity, it is difficult to say whether any one of them should be eliminated, because it takes time to examine these strains precisely. In a sense, the efficiency of a screening program depends on the skill of the taxonomist involved in eliminating such strains; the success of microbial screening does not always depend on the number of samples, but on their diversity. To improve this elimination process, we have applied the random amplified polymorphic DNA (RAPD) method using the polymerase chain reaction (PCR)[1,2]. The RAPD is now widely used for pure taxonomic works and geographical distribution studies of plant pathogens[1,3,4]. This method provides characteristically short DNA products. The polymorphisms enable one to distinguish a minor difference between closely related genotypes with high sensitivity. The method is very rapid compared to restriction fragment length polymorphisms (RFLP)[5,6]. We selected the genus *Trichoderma* in evaluating this method, because its species are difficult to distinguish from each other. In this report, we describe the establishment of a standard method of RAPD for fungi. The RAPD data are compared with the morphological, physiological, and ecological data of the strains examined. We also report briefly on the possible computerization of the data processing. Our final goal is to establish a routine method of image data analysis of the RAPD electrophoretic bands.

Materials and Methods

Strains

Trichoderma strains examined were listed in Table 1. Most strains were isolated in our laboratory

Table 1. Strains examined.

Species	No[a]/Strain/Source/Locality/Collection year
Trichoderma harzianum 1	
1[a]	NR 6891, Twig, Aichi Japan, 1988
2	NR 6964, *Xylaria polymorpha*, München Germany, 1988
3	NR 6920, Rotten wood, Gumma Japan, 1988
4	NR 6945, Leaf litter, München Germany, 1988
5	NR 5544, Soil, Kanagawa Japan, 1977
6	NR 5502, Soil, Kumamoto Japan, 1972
7	NR 6884, basidiomycete fruiting body, Nagano Japan, 1987
Trichoderma aureoviride	
8	NR 6935, Soil, Nassau Bahama, 1988
9	NR 6931, Soil, Chiba Japan, 1988
10	NR 6929, Soil, Chiba Japan, 1988
11	IMI 112086, (from IMI) Egypt
12	NR 6897, Leaf litter, Welwyn garden city United Kingdom, 1988
13	NR 6968, Soil, München Germany, 1988
14	NR 6932, Soil, Greece, 1988
15	IMI 293162, (from IMI), Gujurat India
16	IMI 304058, (from IMI), *Beta vulgaris*, Pantnagar India
17	IMI 299898, (from IMI), Ramwapur India
18	NR 5546, Soil, Kanagawa Japan, 1977
19	NR 6883, Soil, Kanagawa Japan, 1987
20	P 16560, *Pinus* sp., Okinawa Japan
21	NR 6940, Soil, Okinawa Japan, 1988
22	NR 6950, Soil, Okinawa Japan, 1988
23	NR 6938, Soil, Okinawa Japan, 1988
24	NR 6933, Soil, Geneva Switzerland, 1988
25	NR 5555, Soil, Tokyo Japan, 1977
26	NR 6934, Soil, United Kingdom, 1988
27	ATCC 18651, (from ATCC)
Trichoderma harzianum 2	
28	NR 6892, Leaf litter, Aichi Japan, 1988
29	NR 6894, Leaf litter, Aichi Japan, 1988
30	NR 6890, Wood with toadstool, Aichi Japan, 1988
31	NR 6893, Leaf litter, Aichi Japan, 1988
32	NR 6887, Aphilophorales, Aichi Japan, 1988
33	NR 6924, basidiomycete fruiting body, Gumma Japan, 1988
34	NR 6921, Rotten wood, Gumma Japan, 1988
35	NR 6923, *Polyporellus brumali*, Gumma Japan, 1988
36	NR 6925, basidiomycete fruiting body, Gumma Japan, 1988
37	NR 6954, Soil, Gumma Japan, 1988
38	NR 6944, *Pinus densiflora*, Ibaraki Japan, 1988
39	NR 6943, *Pinus densiflora*, Ibaraki Japan, 1988
40	NR 6963, basidiomycete fruiting body, Ibaraki Japan, 1988
41	NR 6926, *Amanita pantherina*, Ibaraki Japan, 1988
42	NR 6918, Soil, India, 1988
43	NR 6958, *Sasa* sp., Kanagawa Japan, 1988
44	NR 6936, Soil, Kanagawa Japan, 1988
45	NR 6967, Leaf litter, Okinawa Japan, 1988
46	NR 6895, *Pinus* sp., Shizuoka Japan, 1988
47	IMI 298371, (from IMI) Worthing United Kingdom
48	ATCC 18647, (from ATCC)
Trichoderma inhamatum	
49	NR 6930, Soil, Chiba Japan, 1988
50	NR 6953, Soil, Gumma Japan, 1988
51	NR 6915, Soil, Ibaraki Japan, 1987
52	NR 5531, Leaf litter, Kanagawa Japan, 1977
53	NR 7061, Soil, Kanagawa Japan, 1977
54	P 16561, *Pinus* sp., Okinawa
55	IMI 288110, (from IMI) Phillipines
56	IMI 300082, (from IMI), Gunnaruwa Sri Lanka
57	NR 5548, Soil, Tokyo Japan, 1977
58	IMI 292106, (from IMI), Trinidad India
Trichoderma viride	
59	NR 5510, Soil, Bratislava Czechoslovakia, 1975
60	NR 6898, Twig, Welwyn garden city United Kingdom, 1988
61	NR 6896, Leaf litter, Welwyn garden city United Kingdom, 1988
62	NR 6955, Soil, Finland, 1988
63	NR 6969, Soil, München Germany, 1988
64	IMI 293250, (from IMI), Vellayami India
65	IMI 295977, (from IMI), W. Bengal Kalyani India
66	NR 5566, Soil, Kumamoto Japan, 1977
67	IMI 304531, (from IMI), Selangor Malaysia
68	NR 5541, Leaf litter, Nagano Japan, 1978
69	NR 6937, Soil, Okinawa Japan, 1988
70	IMI 298375, (from IMI), Worthing United Kingdom
71	CBS 433.34, (from CBS)
72	FP 5566, *Pinus radiata*
73	FP 5563, *Pinus sylvestris*
74	FP 5564, *Pinus radiata*

[a] These numbers indicate the lane numbers in Fig. 6.

during the past 20 years. Some reference strains were purchased from American Type Culture Collection, Rockville, U.S.A., (ATCC); Centraalbureau voor Schimmelcultures, Baarn, the Netherlands, (CBS); and the International Mycological Institute, Egham, Surrey, UK., (IMI). Strains with an initial (P or FP) were kindly supplied by Dr. TOKUMASU of the University of Tsukuba, Tsukuba, Japan.

Morphology

The strains were examined mainly according to RIFAI[7], however, we also referred to VEERKAMPS &

GAMS[8], and BISSETT[9~12]. They were cultured on oatmeal agar or 2% malt extract agar.

Production of Metabolites

The production of isonitrile antibiotics was examined according to OKUDA *et al.*[13]. The production of brown pigments and yellow needle-like crystals was also recorded using the cultures grown on malt extract agar slants.

Total Nucleotide Preparation of *Trichoderma*

In order to simplify the template preparation, several methods of purification were compared. Liquid media, PYG and SYN were used. PYG consisted of 2% Polypepton, 1% yeast extract, and 2% glucose, while SYN consisted of 8% glucose, 0.2% NH_4NO_3, 1% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.002% $FeCl_3 \cdot 6H_2O$, and 0.0014% $MnSO_4$. A loop of conidia was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of these media and incubated on a rotary shaker at 27°C at 220 rpm for 3 days. An agar slant culture (10~20 days old) on a modified malt extract agar was also tested. The template preparation methods compared were 1) a crude supernatant of a liquid culture; 2) mycelia treated with chitinase and proteinase K; 3) a washing supernatant of agar culture; and 4) mycelia of an agar culture crushed between slide glass. For the treatment with enzymes above (No. 2), two hundred mg wet mycelia grown in PYG medium for 3 days were used as a source of template DNA. The mycelia were suspended in 350 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 0.5 mg chitinase and 300~400 mg glass beads (4~5 mm in diam.). The suspension was agitated for 1 minute and incubated at 37°C for 1 hour. A 100 μl proteinase K solution (1 mg/ml) was added to the suspension and heated at 55°C for 1 hour. As a control, a purified DNA sample was obtained using a standard method described by OMORI *et al.*[14]. These preparations were treated with phenol-chloroform followed by precipitation with ethanol. The pellet (DNA template source) was dissolved in TE buffer and the concentration of templates was determined by measuring the absorption at 260 nm.

Primers

Oligodeoxynucleotide primers were synthesized on a DNA/RNA synthesizer (Applied Biosystems, Model 392). Three primers used in this experiment were R2, 5'-AGTACAGGTC; R4, 5'-TCCTACGCAC; R28, 5'-ATGGATCCGC described by GOODWIN *et al.*[1].

RAPD Amplification

The RAPD reaction was done in a total volume of 50 μl containing 0.5 to 8 unit *Taq* DNA polymerase, 2.5 μM of a single oligonucleotide decamer, 200 μM each dNTP, 2.0 mM $MgCl_2$, 1 × buffer provided by Pro Bio or Cetus, template DNA solution. The PCR was conducted at 92°C for 1 minute 25 to 45 cycles of denaturation at 92°C for 45 seconds, annealing at 34°C for 60 seconds, extension at 72°C for 90 seconds; then the final extension at 72°C for 10 minutes in a thermoreactor (Atto, Zymoreactor II). Ten μl of the PCR product together with 500 ng of the origin and front markers were electrophoresed at 50V for 2 hours in Mupid 2 (Advance) on a 5 × 10 cm 5% polyacrylamide gel[15]. The electrophoretic bands were detected by staining with ethidium bromide. *pHY* was used as a standard marker for molecular weight. λ DNA for the origin marker and 90 bp DNA for the front marker were used to precisely determine Rf values. Various PCR cycles were also evaluated. Although GOODWIN *et al.*[1] applied PCR at 45 cycles, we tested 25 to 45 cycles in order to select optimal conditions. *Taq* DNA polymerase from Cetus was compared with that from Pro Bio and the effect of their concentration of the reaction (0.5 to 8 units per 50 μl PCR solution) was examined.

Image Analysis

The electrophoretic band patterns were photographed, and image acquisition made using an image analyzer, Bio Image (Millipore). The Rf value of each band was automatically determined by the analyzer calculating the bands of λDNA and 90 bp DNA as Rf values 0.00 and 1.00. The Rf values of individual polymorphic DNAs were treated as variable characters. When a DNA band was observed at a certain variable character, the value 1 was given; at the vacant position, the value 0 was given. The raw data of Rf values was then converted to a data matrix of 0-1 (Table 2). We compared Rf values from different

Table 2. Example of a primary data matrix for RAPD analysis.

Strain	Variable 1 ... 10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30 ... 99
Trichoderma harzianum 1																					
NR 6891	1	1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0
NR 6964	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0
NR 6920	1	0	0	1	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0
NR 6945	0	1	0	0	1	0	1	0	0	1	1	1	0	0	1	1	0	0	0	0	1
NR 5544	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0
NR 5502	1	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0
NR 6884	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0

Variables correspond to 100 × Rf value.

Table 3. Example of a new data matrix for RAPD analysis.

Strain	New variable 1 ... 10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30 ... 99
Trichoderma harzianum 1																					
NR 6891	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
NR 6964	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
NR 6920	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
NR 6945	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
NR 5544	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
NR 5502	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1
NR 6884	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0	1

Variables correspond to 100 × Rf value.

gels, taking their deviations into consideration. A set of three adjacent variable characters was thus grouped in a new variable character. For example, the variable character from 1 to 3 was renamed 1, character 2 to 4 became 2, and character 3 to 5 became 3. When the value 1 existed within this new column, a new value 1 was given. If not, 0 was given. A new data matrix with fuzziness was finally completed as shown in Table 3.

Analysis Using PAUP

The 0-1 matrix data obtained as above was entered in a computer (Apple, Macintosh IIci or LC II) for statistical analysis. The phylogeny analysis using parsimony, the PAUP vers. 3.0s was used to construct dendrograms[16]. The PAUP bootstrap with a heuristic search (1,000 times replication) was used in finding the most parsimonious trees.

Results

Morphological Analysis of *Trichoderma* Strains

Among the 74 strains of *Trichoderma* listed in Table 1, 10 strains were identified as *T. inhamatum* Veerkamp & Gams, 28 as *T. harzianum* Rifai, 20 as *T. aureoviride* Rifai and 16 as *T. viride* Pers.: Gray. On the average, the conidia of *T. viride* were the largest, the second were those of *T. aureoviride*, and *T. inhamatum* has the smallest conidia. Some strains of *T. harzianum* formed larger conidia that were similar to *T. viride*. These strains had a similar branching pattern of conidiophores to that of *T. viride*, as well. However, since they formed smooth conidia, they were distinct from *T. viride*. Therefore, as we reported previously[17], the *T. harzianum* forming larger conidia were tentatively renamed *T. harzianum* 1, whereas the other strains of *T. harzianum* were tentatively assigned as *T. harzianum* 2 (Table 4).

Detection of Secondary Metabolites

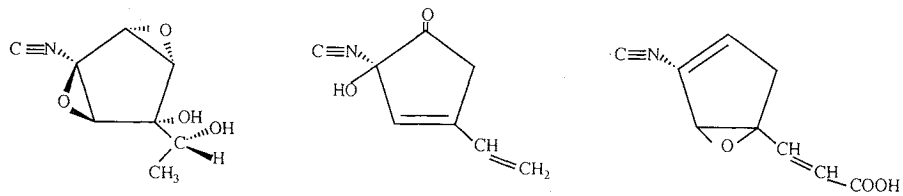
Among the 74 strains examined, 16 strains produced at least one component of the isonitrile antibiotics

Table 4. Average size of conidia and phialides of *Trichoderma* spp.

Species	Conidia		Phialides	
	Size (μm)	Length/width ratio	Size (μm)	Length/width ratio
T. inhamatum	2.5~3.0×2.0~2.5	1.10~1.19	4.5~6.0×2.5~3.5	1.37~2.01
T. harzianum 1	3.5×3.0~3.5	1.07~1.20	6.5~8.0×2.5~3.5	2.03~2.79
T. harzianum 2	2.5~3.5×2.0~3.0	1.10~1.17	5.0~7.5×3.0~3.5	1.70~2.33
T. aureoviride	3.0~3.5×2.5~3.0	1.13~1.29	6.0~10.5×2.5~4.0	1.53~3.93
T. viride	3.5~4.5×3.0~4.0	1.05~1.27	7.0~9.5×2.5~4.0	1.84~3.16

Fig. 1. Isonitrile antibiotics.

A, isonitrin C (trichoviridin); B, isonitrin D; C, isonitrinic acid E (dermadine).



A B C

Fig. 2. Flow chart of the template DNA preparation for RAPD.

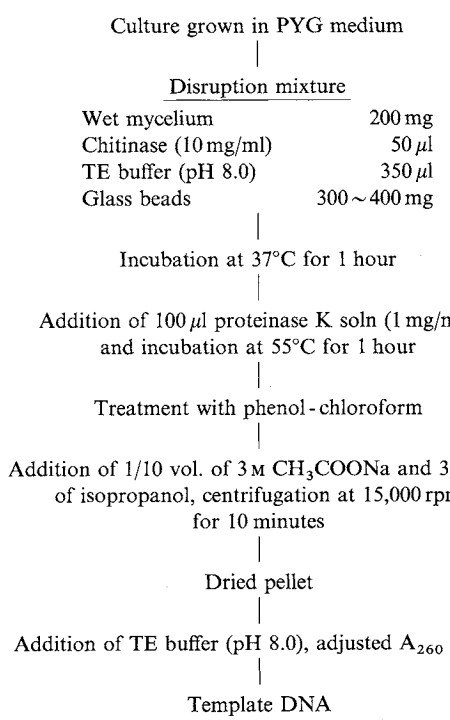
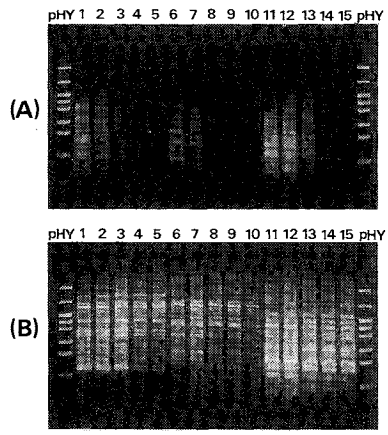


Fig. 3. Effect of PCR cycles to electrophoretic bands.



(A) Reaction without template DNA. (B) Reaction with template DNA. Lanes 1 to 5, primer R2; lanes 6 to 10, primer R4; lanes 11 to 15, primer R28. Lanes 1, 6 and 11, 45 cycles; lanes 2, 7 and 12, 40 cycles; lanes 3, 8 and 13, 35 cycles; lanes 4, 9 and 14, 30 cycles; lanes 5, 10 and 15, 25 cycles. DNA used was purified from *Trichoderma* sp.

(Fig. 1). The producers were restricted to *T. harzianum* 1 or *T. viride*[13,17]. On the other hand, the production of yellow crystals or dark red brown pigments was observed in cultures of *T. inhamatum*, *T. harzianum* 2, and *T. aureoviride*. There was no strain that produced both isonitrile antibiotics and these pigments.

Establishment of a Standard Method for RAPD

Although several methods have already been reported for RAPD analysis,[1,2] we had to determine a standard method in order to compare a large amount of data at one time. It was also essential to minimize the deviation between gels for computerization. Most methods of DNA template preparation could be used; however, treatment of wet mycelia grown in PYG medium plus glass beads with chitinase and proteinase K was found to be comparable to the purified DNA (Fig. 2). In addition, this method was less laborious permitting routine operation for a large number of samples. The DNA template solution was to be more than 0.1 at A_{260} for *T. aureoviride*, *T. harzianum* 1, and *T. inhamatum*, while it was to be more than 1.0 for *T. harzianum* 2 and *T. viride*, because a lower concentration of DNA from the latter two groups created a fewer bands in electrophoresis (data not shown). Therefore, we determined the optimal

Fig. 4. Dendrograms for *T. viride* using primers R2 (A), R4 (B) and R28 (C).

The number in front of the strain number indicates the number in the Table 1 and Fig. 6.

concentration of the template as 1.0 at A_{260} nm so that almost uniform number of electrophoretic bands can be obtained. According to the evaluation of cycles for PCR, even without a template, PCR produced non specific products after more than 35 cycles of reactions (Fig. 3), thus we conducted reactions in less than 30 cycles. With any of the three primers, around 10 to 30 clear DNA bands appeared between one hundred base pairs and 2 Kbp. Around 10 to 30 bands were sufficient for the statistical analysis to obtain reliable and reproducible results within a short time. Fig. 4 shows dendrograms of 16 *T. viride* strains using 3 different primers, which demonstrated that all three trees were essentially identical. Primer R28 was the most suitable because it was able to amplify a larger number of DNA segments distributed in a certain wider range of DNA size. This made analysis easier and more accurate. The *Taq* DNA polymerase from either Cetus or Pro Bio could be used, but the latter produced a larger number of electrophoretic bands, probably due to its greater stability at high temperature. Finally, the standard procedure for RAPD was established as summarized in Fig. 5. By this

Fig. 5. Flow chart of the PCR for RAPD.

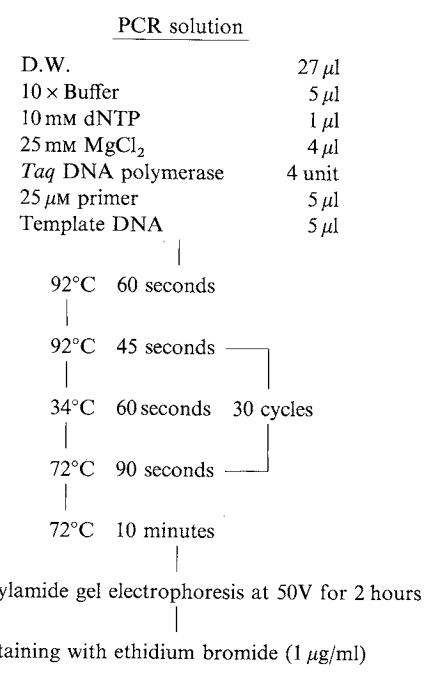
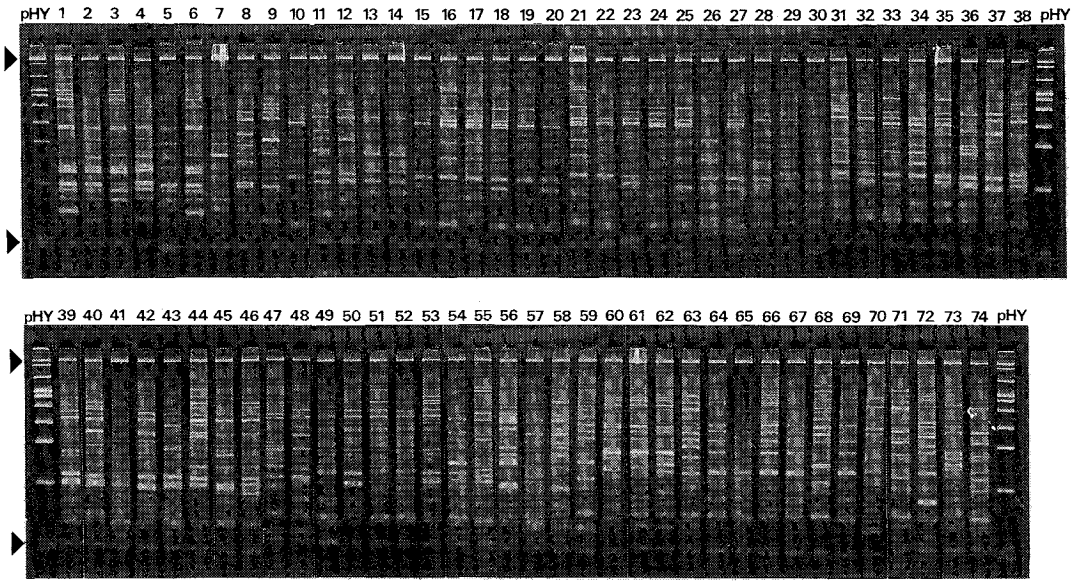


Fig. 6. RAPD electrophoretic results of the 74 strains of *Trichoderma* spp. by using primer R28.



The lane number indicates the number in Table 1. pHY, pHY marker. Each lane contained λDNA and 90 bp DNA (arrowheads).

standard method, we minimized the deviation of Rf values in the electrophoresis within 0.03 even between different gels. The RAPD of 74 *Trichoderma* strains using primer R28 is shown in Fig. 6.

Discussion

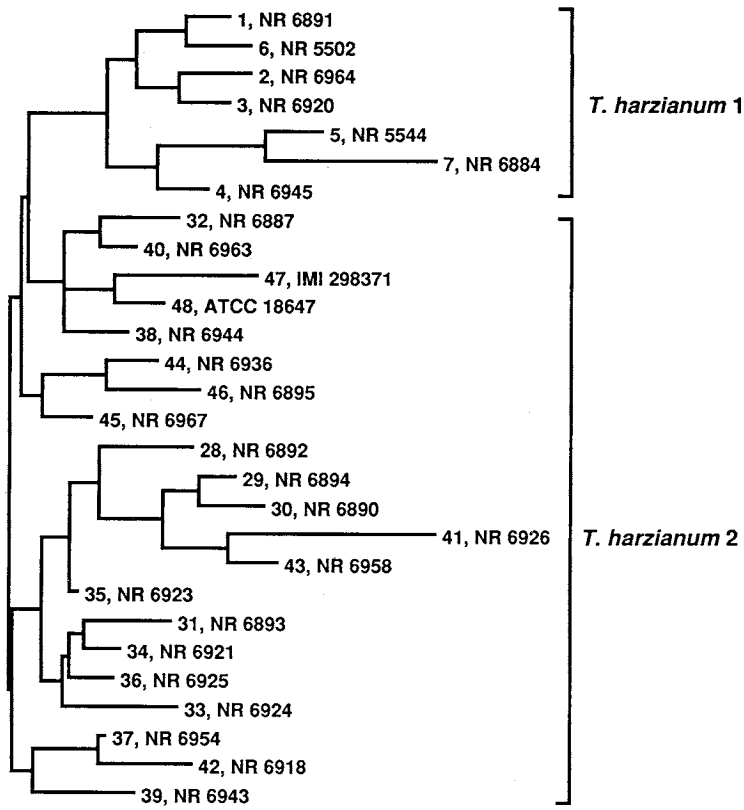
By minimizing the Rf value deviation between gels and converting the matrix described previously, we were able to process a large amount of data at one time.

Fig. 7 shows the dendrogram constructed by the PAUP based on the RAPD results in Fig. 6. All strains of *T. harzianum* 1 were clearly separated from *T. harzianum* 2. The conidia of *T. harzianum* 1 were on average $3.5 \times 3.0 \sim 3.5\,\mu m$. The size of their conidia was larger than those of *T. harzianum* 2, which were $2.5 \sim 3.5 \times 2.0 \sim 3.0\,\mu m$. The phialides of *T. harzianum* 1 were more slender (length/width ratio, 2.03~2.79) than those of *T. harzianum* 2 (length/width ratio, 1.70~2.33). Furthermore, *T. harzianum* 1 characteristically produced some of isonitrile antibiotics (Fig. 1). No strains included in *T. harzianum* 2 produced these antibiotics, however some strains produced yellow needle-like crystals in malt extract agar slants or dark red brown soluble pigments in agar.

T. inhamatum is characterized by the formation of densely packed, short and almost globose phialides[8]. However, there were several strains that had characteristics which were somewhere in between *T. harzianum* 2, *T. inhamatum*, and *T. aureoviride*. It was quite difficult to clearly distinguish these three species from each other[11,12,17] as well as clealy delineate the three by electrophoretic band analysis.

Interestingly, several strains isolated from locations in the same vicinity formed one cluster.

Fig. 7. Dendrogram for *T. harzianum* 1 and *T. harzianum* 2 by the PAUP using bootstrap algorithm (Primer, R28).



The number in front of the strain number indicates the number in the Table 1 and Fig. 6. The branch length represents the distance between strains.

T. harzianum 2 NR 6890, NR 6892, and NR 6894 were combined in a cluster, and they were isolated from different substrates collected in Aichi Prefecture. *T. harzianum* 2 NR 6921, NR 6924 and NR 6925 were also clustered together, isolated from samples collected in Gumma Prefecture. In fact, *T. harzianum* 2 NR 6921, NR 6924 and NR 6925 showed almost the same cultural and physiological characteristics. They produced yellow needle-like crystals and dark red brown pigments in agar. They did not grow at 37°C and very slowly at 34°C, attaining a diameter of less than 15 mm after 4 days. While *T. harzianum* 2 NR 6890, NR 6892, and NR 6894 did not produce yellow crystals but secreted red brown pigments in agar. They showed luxuriant growth at 34°C, attaining a diameter of more than 50 mm after 4 days.

Similarly interesting results were obtained for *T. viride*.: *T. viride* IMI 293250, NR 5566, NR 6937, IMI 295977, IMI 304531 and FP 5566. They grew well at 34°C, attaining a diameter of more than 50 mm after 4 days, whereas the other *T. viride* strains did not grow at 34°C. The fast growers all produced isonitrile antibiotics; IMI 293250, NR 5566, NR 6937, IMI 295977, and IMI 304531 (parsimoniously connected according to Fig. 4) came from areas with warmer climates; India, Malaysia, and sourthern Japan such as Kumamoto and Okinawa; whereas, all the other *T. viride* strains were derived from temperate or northern countries. Among these temperate *T. viride* strains, three antibiotic producers, NR 5510, NR 5541 and CBS 433.34 were closely associated (Fig. 4). A similar result was also obtained for *T. aureoviride* (data not shown). The dendrogram constructed by the PAUP therefore demonstrated that RAPD results were consistent with morphological, physiological and ecological characteristics, and expressed differences between fungal strains.

Conclusion

Recently, techniques based on the PCR have been used to detect polymorphisms in various plants[18], animals[19], and fungal species[20]. Decameric primers are used to reproducibly amplify segments of genomic DNA from a wide variety of species[21~23]. The arbitrarily-primed polymerase chain reaction (AP-PCR) and random amplified polymorphic DNA (RAPD) generate fingerprints of DNA products. Major advantages of both techniques over traditional RFLP are that rapid analysis is possible even with a small amount of partially purified DNA samples. RAPD has therefore been applied to taxonomic works and ecological studies to distinguish small genetic differences among strains, races, varieties, and species[1,3,4]. The method is also useful for genetic mapping studies[22].

RAPD analysis with PCR proved to be a powerful tool in the elimination of duplicate strains in the microbial screening program. The electrophoretic band patterns of 74 strains of *Trichoderma* correlated well with their morphological and cultural properties, metabolite production profiles and ecological data. Based on RAPD data, it was possible to select several strains by eliminating the other similar strains. Although the data is not shown, RAPD analysis with several other fungal genera gave similar results. Therefore, we conclude that the RAPD method is a simple, efficient and reliable method for the selection of fungal strains employed for screening.

In addition, we succeeded in the establishment of a standard method for RAPD to minimize the deviation of Rf values between gels less than 0.03. Through image acquisition of electrophoretic band patterns by an image analyzer, the image data can be converted to Rf value data. The Rf value data is then converted to 0-1 data matrices. The matrix data is analyzed either by PC or main frame, so that the whole process can be automated.

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References

1) GOODWIN, P. H. & S. L. ANNIS: Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. 57: 2482~2486, 1991
2) WILLIAMS, J. G. K.; A. R. KUBELIK, K. J. LIVAK, J. A. RAFALSKI & S. V. TINGEY: DNA polymorphisms amplified

by arbitrary primer are useful as genetic markers. Nucleic Acids Res. 18: 6531~6535, 1990
3) STRONGMAN, D. B.; Discrimination between *Hirsutella longicolla* var. *longicolla* and *Hirsutella longicolla* var. *cornuta* using random amplified polymorphic DNA fingerprinting. Mycologia 85: 65~70, 1993
4) YOON, C.-S. & D. A. GLAWE: Association of random amplified polymorphic DNA markers with stromatal type in *Hypoxylon truncatum sensu* Miller. Mycologia 85: 369~380, 1993
5) MAYER, W.; R. MORAWETZ, T. BÖRNER & C. KUBICEK: The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. Curr. Genet. 21: 27~30, 1992
6) CARDER, J. C. & D. J. BARBARA: Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycol. Res. 95: 935~942, 1991
7) RIFAI, M. A.: A revision of the genus *Trichoderma*. Mycological Papers No. 116, 1~56. IMI, Surrey, United Kingdom, 1969
8) VEERKAMP, J. & W. GAMS: Los hongos de Colombia-VIII. Some new species of soil fungi from Colombia. Caldasia 8: 709~717, 1983
9) BISSETT, J.: A revision of the genus *Trichoderma*. I. Section *Longibrachiatum* sect. nov. Can. J. Bot. 62: 924~931, 1984
10) BISSETT, J.: A revision of the genus *Trichoderma*. II. Infrageneric classification. Can. J. Bot. 69: 2357~2372, 1991
11) BISSETT, J.: A revision of the genus *Trichoderma*. III. Section *Pachybasium*. Can. J. Bot. 69: 2373~2417, 1991
12) BISSETT, J.: A revision of the genus *Trichoderma*. IV. Additional notes on section *Longibrachiatum*. Can. J. Bot. 69: 2418~2420, 1991
13) OKUDA, T.; A. FUJIWARA & M. FUJIWARA: Correlation between species of *Trichoderma* and production patterns of isonitrile antibiotics. Agric. Biol. Chem. 46: 1811~1822, 1982
14) OMORI, Y.; M. TSUMURA, T. ICHIDA & K. YAMASATO: Disrupting of fungus and actinomycetes cell with Cryo Clean Blaster (CCB). Japanese J. Freez. Dry. 36: 36~40, 1990
15) PANG, J.-P.; L.-C. CHEN, L.-F. O. CHEN & S.-C. G. CHEN: DNA polymorphisms generated by arbitrarily primed PCR in rice. Biosci. Biotech. Biochem. 56: 1357~1358, 1992
16) SWOFFORD, D. L.: PAUP, phylogenetic analysis using parsimony, version 3.0s. Illinois Natural History Survey, Champaign, Illinois, USA. 1991
17) OKUDA, T.; M. YANAGISAWA, S. MATSUKUMA & A. YAMAGUCHI: *Trichoderma* forming green globose conidia. Abstracts of the Fouth International Mycological Congress, p 43, Regensburg, Germany, 1990
18) KLEIN-LANKHORST, R. M.; A. VERMUNT, R. WEIDE, T. LIHARSKA & P. ZABEL: Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). Theor. Appl. Genet. 83: 108~114, 1991
19) LEHMANN, P. F.; D. LIN & B. A. LASKER: Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbial. 30: 3249~3254, 1992
20) BROUSSEAU, R.; A. SAINT-ONGE, G. PRÉFONTAINE, L. MASSON & J. CABANA: Arbitrary primed polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. J. Appl. Entomol. 59: 114~119, 1993
21) WILDE, J.; R. WAUGH & W. POWELL: Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA marker. Theor. Appl. Genet. 83: 871~877, 1992
22) WELSH, J.; C. PETERSEN & M. MCCLELLAND: Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucleic Acids Res. 19: 303~306, 1991
23) KHUSH, R. S.; E. BECKER & M. WACH: DNA amplification polymorphisms of the cultivated mushroom *Agaricus bisporus*. Appl. Environ. Microbiol. 58: 2971~2977, 1992