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AFLP Fingerprinting of *Tricoderma reesei* Strains

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Amplified Fragment Length Polymorphism (AFLP), a novel molecular fingerprinting technique, modified by using one PstI restriction enzyme, has been applied to assess the genomic diversity of *Trichoderma reesei* strains and mutants deriving from different world collections. The genetic relationship among all AFLP pattern based on the Jaccard's similarity coefficient were transformed to build an unweighted pair group method with arithmetic mean dendrogram. The statistical analysis of obtained results enabled the classification of *T. reesei* strains into two main clusters. Nine of them were classified as group I and next four strains into group Ⅱ. The analysis revealed the existence of one subgroup composed of 291 and 292 strains within group Ⅱ. Six subgroups were present within group I, the first formed by 289; the second composed by 290 and 298; the third formed by 300; the fourth including 293 and 294; the fifth formed by 296; and the sixth composed by 295 and 299. In general, the AFLP analysis has differentiated all strains tested in this study. AFLPs revealed the lower level of diversity among group Ⅱ, whereas for strains in group I, the level of diversity was higher.

Key words: *Trichoroma reesi*, AFPL, fingerprinting

INTRODUCTION

The fungus *Trichoderma reesei* (Reese *et al*., 1950) of the subphylum *Pezizomycotina* is a mesophilic fungus which is one of the most commonly used industrial protein production organisms (Arvas, 2007). It is an asexual form of the *Hypocrea jecorina* (Kuhls *et al*., 1996), atropical soft rot fungus, and was discovered as the jungle rot that degraded soldier's tents and uniforms in the Pacific during World War Ⅱ (Reese, 1976). *Trichoderma reesei* is very often used as one of the best protein expression systems. The efficient secretory ability and the cheap and easy cultivation of *T. reesei* make it a useful organism for the large–scale production of enzymes for a variety of industrial applications. Industrial strains of *T. reesei* can achieve protein production levels of up to 100 g/l (Cherry and Fidantsef, 2003). Originally *T. reesei* produce mainly cellulases and hemicellulases. The differences between cellulases induced and non–induced conditions and a mutant strain producing abundant cellulases compared to a parent strain have been also very intensive studied (Foreman *et al*., 2003). The modern production strains are genetically engineered to increase the enzyme production and often to remove the expression of unwanted enzymes, like cellulases during the production of xylanases (Paloheimo *et al*., 2003). For these reasons the fast and precisely method for strains identification are very important in industry level of biotechnology. The Amplified Fragment Length Polymorphism (AFLP), is a technique for fingerprinting DNA of any origin and complexity i.e. human, plant, and microbial (Valsangiacomo *et al*., 1995; Mueller *et al*., 1996; Blears *et al*., 1998; Suazo and Hall, 1999; Terfework *et al*., 2001). The standard AFLP method consists of (i) a restriction–digestion of genomic DNA using two restriction enzymes (a rare and a frequent cutters), (ii) ligation of synthetic adaptors with DNA sequences corresponding to the sticky ends of restricted DNA products, (iii) preselective PCR amplification of restriction DNA fragments with two primers complementary to the adaptor–ligated ends, (iv) selective amplification with two primers complementary to the adaptors and restriction site sequences with additional one to three selective nucleotides at the 3' ends (Vos *et al*., 1995). The use of selective primers reduces the complexity of amplified products. Only those fragments with complementary nucleotides, extending beyond the restriction site, are amplified by selective primers under stringent annealing conditions. The amplified products are then resolved by polyacrylamide electrophoresis and molecular genomic polymorphisms are identified by the presence or absence of DNA fragments and by comparison of the generated DNA profiles for each sample.

For the rapid genomic characterization of organisms a modified AFLP protocol was developed. The major modifications to the standard AFLP procedure included: (i) using only one restriction enzyme, one adaptor and one primer, (ii) agarose gel electrophoresis and ethidium bromide staining to analyse amplicons (Suazo and Hall, 1999; Tyrka *et al*., 2002). Such modifications maintain high reproducibility of AFLP method, which serve as reliable molecular tool to generate genetic markers in different organisms. Modified AFLP procedure is being used extensively for genetic mapping in plants and animals

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and for DNA fingerprinting in microorganisms including fungi (Mueller *et al.*, 1996).

In this paper we describe usefulness of AFLP technique for study the genomic diversity and for identification of *T. reesei* strains.

MATERIALS AND METHODS

Fungal strains and cultivation

Trichoderma reesei strains (Table 1) were obtained from Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin and deposited at the Fungal Culture Collection (FCL), The Department of Biochemistry, Maria Curie–Sklodowska University, Lublin, Poland. The strains were maintained in 2% malt–extract agar and cultivated in a Mandels – Andreotti (MA) minimal medium (1978) with modifications as described by Labudova and Farkas (1983). The culture medium was prepared combining 500 ml mineral salt solution (2.8 g/l $(NH_4)_2SO_4$, 4.00 g/l KH_2PO_4, 0.60 g/l $MgSO_4$), 480 ml 0,1 M phosphate buffer (35.6 g/l $Na_2HPO_4 \cdot 2H_2O$ (0.2 M) and 0.2 M citric acid added to adjust a pH of 5.6), 20 ml mineral trace element solution (0.250 g/l $FeSO_4 \cdot 7H_2O$, 0.080 g/l $MnSO_4 \cdot H_2O$, 0.070 g/l $ZnSO_4$ and 0.100 g/l $CaCl_2 \cdot 2H_2O$), 0.3 g/l urea, 2.0 g/l peptone, 0.5 g/l Tween 80 and 7.00 g/l lactose (all chemicals by POCh, Poland). The fungi cultures were incubated 10 days at 26˚C on above medium containing additionally 1.5% agar. Conidia obtained after above cultivation in the concentration 10^8/l were used for inoculation the liquid media.

Trichoderma strains were cultured stationary in 250 ml Erlenmeyer flask containing 100 ml liquid MA medium for 5 days at 26˚C. Broth cultures were then harvested by centrifugation at 10 000xg for 10 minutes and used for DNA extraction.

Isolation of total DNA

The mycelia from 100 ml liquid cultures were DNA extracted according to the method of Borges *et al.* (1990). To extract DNA, 0.5 to 2.0 g of fresh mycelium was ground in liquid nitrogen. The mycelial powder was transferred to a sterile test tube containing 15 ml of cold spermidine–SDS buffer (4 mM spermidine, 10 mM EDTA, 0.1 M NaCl, 0.5% SDS, 10 mM β–mercaptoethanol, 40 mM Tris–HCl pH 8.0) and thoroughly shaken for 20 minutes. The mixture was immediately extracted with 1 volume of double distilled phenol. After a second phenol extraction, the aqueous phase was extracted with 1 volume of chloroform – isoamyl alcohol (24:1) and centrifuged (10 000xg, 10 min, 4˚C). To the resulting aqueous phase 10% of that volume of 3 M sodium acetate pH 5.5 was added. DNA was then precipita]ted by addition of 2 volumes of cold 96% ethanol (–20˚C), and recovered by centrifugation at 10 000xg, 4˚C for 10 minutes. DNA was dried in a vacuum exsiccator (Sigma, USA), redissolved in 1 ml of sterile MilliQ water and stored at −20˚C. The quality and quantity of genomic DNA was accurately measured with spectrophotometric absorbency at 260 nm and 280 nm respectively.

Ribonuclease treatment

Extracted nucleic acids were digested with the RNase A (Sigma, USA) according to manufacturer protocol. The final RNase concentration was 10 μg/ml. The reaction mixture was incubated for 30 min at room temperature.

AFLP analysis

The AFLP reactions were referenced as described by Vos *et al.* (1995) with some modifictions. Adapters and primers were synthesized by Genset Oligos, France and IBB PAN, Poland.

The genomic DNA (1 μg) was digested in 30 μl final volume with 20 U of PstI (Fermentas, Lithuania) using as enzyme buffer the O+ Buffer supplied by the manufacturer, for 18 hours at 37˚C. The quality and quantity of the amplification product was examined by means of agarose (0.8%) gel electrophoresis stained with ethid-

Table 1. List of fungal strains used in this study

Original strain name	Strain number in FCL	Strain description
18/10	289	Metabolic mutant of *T. reesei* QM9414
18/12	290	*T. reesei* RUT C-30
18/13	291	Heterocaryons between *T. reesei* QM9414 and *T. reesei* RUT C-30
18/16	292	*T. reesei* D-862H (D2H)
M7	293	Mp7 strain mutant
Mp1	294	*T. reesei* mutant
Mp3	295	*T. reesei* mutant
Mp4	296	*T. reesei* mutant
Mp5	299	*T. reesei* mutant
Mp7	297	*T. reesei* mutant
Mp8	298	*T. reesei* mutant
MCG-77	300	*T. reesei* mutant
–	262	Wild strain of *T. reesei* QM9414

ium bromide and visualised under UV fluorescence as a smear across bromophenol blue. The ligation solution containing the double–stranded oligonucleotides adapters, DNA digested with PstI, 5 U T4 DNA polymerase (Fermentas, Lithuania) and 10X T4 ligase buffer (400 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8) was incubated for 4 hours at 37°C (25 μl final volume). Ligated DNA was then precipitated with a mixture of 3 M sodium acetate, pH 5.5 and cold 96% ethanol (−20°C in the ratio 1:25) to remove unbound adapters. DNA was harvested by centrifugation (14 000xg, 4°C, 15 minutes) and dried in a vaccum centrifuge. The debris of DNA was dissolved in 50 μl of sterile water and used as a template in amplification reaction. PCRs were performed in a 50 μl total volume which consisted of: 1X reaction buffer (Fermentas, Lithuania), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq DNA Polymerase (Fermentas, Lithuania), 10 pmol of each primer, 0.5 μl of targeted digested and ligated genomic DNA. All amplification reactions were performed in a T–personal thermal cycler (Biometra, Germany) with the conditions as follows: 94°C for 2 min 30 s followed by 35 cycles of 40 s at 94°C, 40 s at 48–58°C (annealing termperature depend on primers Tm) and 50 s at 72°C. The final cycle was followed by an additional 10 min at 72°C. The PCR products were stored at 4°C until further analysis. The adapters and primers employed for AFLP are shown in Table 2.

Electrophoresis and imaging

A 5 μl aliquot of the reaction mixture was combined with 1 μl of loading buffer and the preparation was electrophoresed on a 2% agarose gel in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8,0). The gel was run at 120 V in TBE on a horizontal gel electrophoresis system (Agagel Mini, Biometra) for about 2 hours. The gels were stained with ethidium bromide and photographed on a UV transilluminator (Vilber Lourmat, France).

Data analysis

Electropherograms were analysed using GeneTools software (Syngene, USA). Polymorphic AFLP markers were manually scored as binary data for the presence or absence of fragments between 150 and 3000 bp. A table

Table 2. List of oligonucleotide primers and adapters

	Adaptor name	Adaptor sequence 5'-3'	Melting temperature [°C]
1.	Pst I AF	C T C g T A g A C T g C g T A C A T g C A	51°C
2.	Pst I AR	T g T A C g C A g T C T A C	42°C
	Primer name	**Primer sequence 5'-3'**	**Annealing temperature [°C]**
1.	Pst 01	g A C T g C g T A C A T g C A g A A g	57.3°C
2.	Pst 02	G A C T G C G T A C A T G C A G A A T	48.2°C
3.	Pst 03	G A C T G C G T A C A T G C A G A C A	50.4°C
4.	Pst 04	G A C T G C G T A C A T G C A G A G T	48.2°C
5.	Pst05	G A C T G C G T A C A T G C A G A T A	48.2°C
6.	**Pst 06**	**G A C T G C G T A C A T G C A G A T C**	50.4°C
7.	Pst 06a	G A C T G C G T A C A T G C A G A T C G A T C	56.4°C
8.	Pst 07	G A C T G C G T A C A T G C A G A T G	50.4°C
9.	Pst 08	G A C T G C G T A C A T G C A G C A C	52.6°C
10.	Pst 09	G A C T G C G T A C A T G C A G G A A	50.4°C
11.	Pst 10	G A C T G C G T A C A T G C A G G A G	52.6°C
12.	Pst 11	G A C T G C G T A C A T G C A G A A A	48.2°C
13.	Pst 12	G A C T G C G T A C A T G C A G A A C	50.4°C
14.	Pst 13	G A C T G C G T A C A T G C A G A C C	52.6°C
15.	**Pst 14**	**G A C T G C G T A C A T G C A G A C G**	51.8°C
16.	Pst 14a	G A C T G C G T A C A T G C A G A C G A C G T	55.1°C
17.	Pst 15	G A C T G C G T A C A T G C A G A C T	50.4°C
18.	Pst 16	G A C T G C G T A C A T G C A G A G A	50.49°C
19.	Pst 17	G A C T G C G T A C A T G C A G A G C	52.6°C
20.	Pst 18	G A C T G C G T A C A T G C A G A G G	52.6°C
21.	**Pst 19**	**G A C T G C G T A C A T G C A G A T T**	48.2°C
22.	**Pst 19a**	**G A C T G C G T A C A T G C A G A T T C A T G**	53.9°C
23.	Pst 20	g A C T g C g T A C A T g C A g C A A	57.3°C

The primers used for the analysis are in bold. Selective nucleotides are underlined.

containing these binary information were used to calculate Jaccard's pairwise coefficients of similarity as implemented in the program FreeTree version 0.9.1.50 (Hampl *et al*., 2001). Cluster analysis by UPGMA method (unweighted pair–group method with arithmetic averages) was performed. The phylogenetic trees were visualized and edited using TreeView software version 1.6.6 (Page, 1996).

RESULTS AND DISCUSSION

The different high–cellulase producing *T. reesei* mutants isolated worldwide presented in Table 1 represent the fungi line from Natick (USA) – the QM strains (Mandels *et al*., 1971), from Rutgers University (USA) – the Rut strains (Montenecourt and Eveleight, 1979), Indian Institute of Technology (India) the D strains (Mishra *et al*., 1982), Technical Research Centre of Finland (VTT) – the 18/ strains (Nevalainen *et al*., 1994) and Department of Biotechnology, Human Nutrition and Science of Food Commodities – the M strains.

AFLP is capable of simultaneously screening many different DNA regions distributed randomly throughout the genome for restriction site variation(s) using a limited set of generic primers (Mueller and Wolfenbarger, 1999) and is also known to be extremely useful for genetic variability studies of closely related organisms, as has been shown at the population, species and superspecific level (Brown, 1996; Majer *et al*., 1996). AFLP markers are well suited for distinguishing closely related organisms at the species to strain level (Leissner *et al*., 1997; Hynes *et al*., 2006). In this study, we used PCR–based fingerprinting method to assess genetic differences among *Trichoderma reesei* strains. In the analyses, extracted DNA of thirteen *T. reesei* strains was digested with PstI rare cutting restriction endonuclease. A simplified AFLP protocol, using only PstI, was also used by Mueller *et al*. (1996) to detect genetic differences among 14 symbiotic fungi of the fungus growing ant, *Cyphomyrmex minutus*.

In preliminary analysis, all 23 primers successfully amplified AFLP bands from all 13 isolates of the fungus. Although large numbers of restriction fragments were obtained for each primer, only 4 primers (Pst06, Pst14, Pst19 and Pst19a) were used for the data analysis because they were the only ones that generated polymorphic fragments (Table 2). The obtainment of monomorphic banding pattern when using other primers demonstrates most likely close genetic similarity among strains of interest (metabolic mutants of *T. reesei*). Each of the fourth primer generated fingerprint pattern markedly distinct from the other primers, even when primers differed by only one nucleotide in the extension. A total of three to six selective bases were found to provide a sufficient complex pattern for fragment analysis (Table 3). According to Vos *et al*. (1995) primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants, although selectivity is still acceptable with primers having more selective nucleotides. Janssen *et al*. (1996) have showed that the choice of the restriction enzymes and the length and composition of selective nucleotide will determine the complexity of the final AFLP fingerprint. In turn, Welsh and McCleland (1990) showed that the final PCR banding pattern depends on the number of determinats e.g. annealing temperature, Mg2+ concentration and the length of primers.

AFLP is a powerful tool in molecular fingerprinting and for studying relationships among microbial isolates. Since its first application for distinction and identification of fungi (Majer *et al*., 1996), many different fungal isolates have been studied using the AFLP technique (Mueller *et al*., 1996; Schmidt *et al*., 2004; Urbanelli *et al*., 2007; Brooker *et al*., 2008). The characterization of *Trichoderma* species has traditionally been based on morphology or, more recently, on molecular data (Druzhinina *et al*., 2006). The applied AFLP process provided characteristic markers to differentiate among the analysed *T. reesei* strains. The number of legible bands produced high variation and ranged from 8–23 fragments for one lane. A total of 822 well defined bands were observed in the four AFLP gels. The high sensitivity and the large number of bands observed demonstrates that AFLP analysis is a robust and efficient method for detecting differences between *Trichoderma* strains. Primer Pst06 amplified the highest number of fragments (244), while the fewest bands (154) were observed with the primer Pst19. On average, 205,5 AFLP markers were

Table 3. AFLP primers used in the analysis of *Trichoderma reesei* strains, number and range of length of all amplified DNA fragments, number of polymorphic fragments and percentage of polymorphism

Primer name	Primer's selective bases	No. of all fragments	Fragment range length [bp]	No. of polymorphic fragments	Percentage of polymorphism
Pst06	ATC	244	193–2172	47	19.3
Pst14	ACG	239	167–2202	63	26.3
Pst19	ATT	154	437–2987	12	7.8
Pst19a	ATTCATG	185	228–2469	81	43.8
Sum		822		203	
Average		205.5		50.7	24.6

amplified per primer, in the size range from 167 to 2987 bp. A total of 203 reliable polymorphic bands (24.6% polymorphism) were observed across all thirteen isolates, which corresponded to an average of 50,7 polymorphic bands per primer combination. In the four primer systems, the primer Pst19a produced the most polymorphic bands and 81 reliable bands were observed. Primer Pst19 amplified the fewest (12) number of polymorphic bands. Our results showed, as earlier observed by Majer *et al*. (1996) and Gril *et al*. (2010), that there is a connection between the level of detected polymorphism and the chosen primers. The main AFLP characteristics for 4 primers are presented in Table 3.

The genetic relationship among all AFLP patterns of *T. reesei* based on the Jaccard's similarity coefficient (1912) were transformed to build an unweighted pair group method with arithmetic mean (UPGMA; phenotype–based) dendrograms shown in Fig. 1. Statistical analysis of AFLP data enabled the classification of *T. reesei* strains into two main clusters (groups). Out of 13 strains analysed, 9 were classified as group I and 4 next strains as group II. These two major groups were further divided into subgroups. The separation into group I and II was strongly supported by a bootstrap value of 100%. The analysis revealed the existence of one subgroup composed of 291 and 292 strains within group II. Six subgroups were present within group I, the first formed by 289; the second composed by 290 and 298; the third formed by 300; the fourth including 293 and 294; the fifth formed by 296; and the sixth composed by 295 and 299 (Fig. 1). In general, the AFLP analysis has differentiated all strains tested in this study. AFLPs revealed the lower level of diversity among group II, whereas for strains in group I, the level of diversity was higher.

In conclusion, PCR–based approach allowed us to group all strains of *Trichoderma reesei* in a clear manner, according to AFLP profiles. The exact characterization and identification of *Trichoderma* strains is an important first step in systematically utilizing the full potential of fungi in specific applications (Lieckfeldt *et al*., 2001). Genetic characterization is fundamental to correctly identify the individuals used in the industry – to rapidly detect strain of interest. In view of the results of the present study, complex AFLP patterns were obtained using four different primers and genomic similarity analyses derived from qualitative data enabled us to differentiate *T. reesei* strains which was impossible based on morphological criteria. Moreover, a large numbers of AFLP markers was obtained without any genetic information on the species, suggesting that AFLPs may be an excellent alternative to other molecular markers, *e.g*., microsatellites.

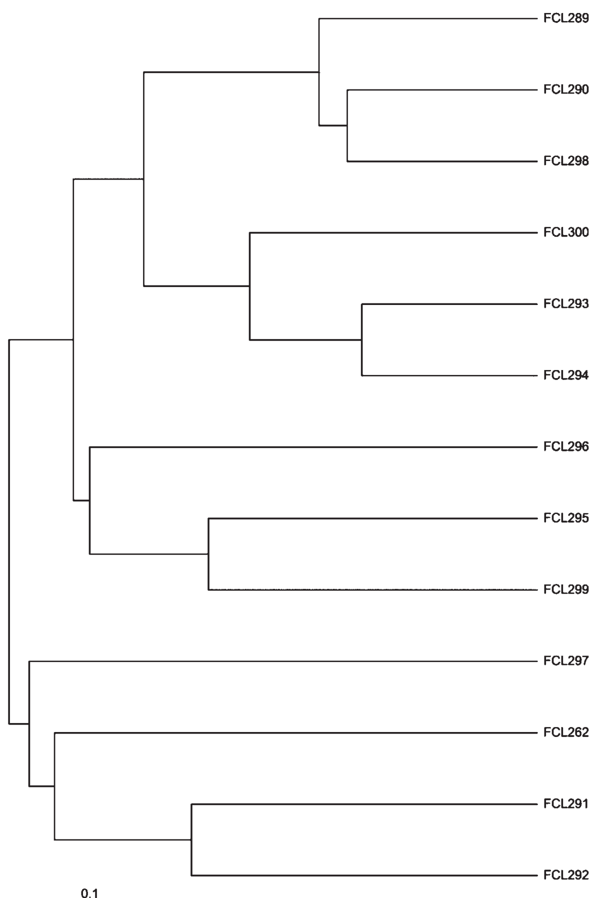

Fig. 1. UPGMA tree based on polymorphic AFLP markers for 13 investigated *Trichoderma reesei* strains.

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