DNA-fingerprinting : one of the molecular methods used for the reclassification of some species of the Trichoderma aggregate

Autor(en): **Meyer, W. / Lieckfeldt, E. / Morawetz, R.**

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DNA-Fingerprinting, one of the molecular methods used for the reclassification of some species of the *Trichoderma* aggregate.

W. Meyer[1,2]; **E. Lieckfeldt**[1]; **R. Morawetz**[3]; **Th. Börner1** and **C. P. Kubicek**[3]

[1] Institut für Genetik, Biochemie und Mikrobiologie
Fachbereich Biologie, Humboldt Universität zu Berlin
Invalidenstrasse 43, 0-1040 Berlin, Germany
[2] IFZ Biotechnologische Forschungs- und Entwicklungs GmbH
Alt-Stralau 52/54, 0-1017 Berlin, Germany
[3] Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU-Wien, Getreidemarkt 9
A-1060 Wien, Austria

Summary: We have analysed 9 different species of the filamentous fungus *Trichoderma* and 3 mutants of *T. reesei* (QM6a; QM9123; QM9414) for the presence of hypervariable loci in their genomes by hybridization with simple repeat oligonucleotides [(CT)8; (GTG)5; (GATA)4] and the DNA of the phage M13. On the base of the DNA-fingerprints obtained, we suggest a reclassification of the *Trichoderma* aggregate into five groups:

I. *T. reesei; T. todica*
II. *T. polysporu, T. longibrachiatum, T. koningii, T. pseudokoningii*
III. *T. virgatum*
IV. *T. saturnisporum*
V. *T. harzianum*

These results contradict the claim that *T. reesei* is a subspecies of *T. longibrachiatum*. Furthermore, hybridization with (CA)8 allowed a subdivision of group II, wherein *T. pseudokoningii* formed subgroup IIb, which is highly homolgous, yet distinct to subgroup IIa. The results show that DNA-fingerprinting analysis may be used to reclassify the *Trichoderma* aggregate.

DNA-fingerprinting offers a simple and reliable alternative method to resolve taxonomic problems and to "label" strains of filamentous fungi.

Introduction

Besides being an instrument of medical and forensic diagnostics, DNA-fingerprinting is rapidly becoming a tool for the interpretation of evolutionary or familary relationships, and is also applicable to questions of systematics and taxonomy. Humans, mammals, birds, fishes, plants, bacteria and protozoa belong to the investigated organisms. Until now there is only little information in the literature about DNA-fingerprinting of fungi. DNA-fingerprinting has been used to reassess species identification of commercially important or phytopathogenic strains (Braithwaite and Manners 1989; Förster et al. 1990; Meyer et al. 1991).

The fungus *Trichoderma* is an ubiquitous soil organism which has attracted strong interest because of the ability of some species to produce large quantities of cellulases and other hydrolytic enzymes.

The *Trichoderma* group is particularly difficult to classify. Most workers follow the Rifai (1969) scheme based on grouping of species into an aggregate. A very important species is *T. reesei*. The strain QM6a from the South Pacific has become the progenor of hundreds of mutants which have served as the almost exclusive source of cellulase production (El-Gregory et al. 1990). Its taxonomic position is however still unclear: based on Rifai (1969), it is considered as *T. longibrachiatum*, being a morphologically distinct subspecies. Here we report on the successful use of DNA-fingerprinting for the reclassification of 9 species of the Trichoderma aggregate.

Methods

DNA-fingerprints (specific hybridization patterns) were obtained by hybridization of DNA probes or oligonucleotides to restriction fragments of genomic DNA (Jeffreys et al. 1985; Epplen 1988) (Fig. 1). The nucleotide sequences used as probes are homologous to hypervariable repetitive sequences which are referred to as "minisatellite DNAs" and "simple repetitive sequences". Minisatellite DNA consists of about 15-30 bp long sequence motifs repeated in tandem at various loci. They show a high degree of variability. They were first described from human DNA and found to be individual-specific (Jeffreys et al. 1985). The simple repetitve sequences used in DNA-fingerprinting are made of tandemly arrange motifs of only 2-10 bp. They also show an extreme degree of variability (Epplen 1988).

An important precondition for DNA-fingerprint analysis is the preparation of high molecular and clean genomic DNA from cell material. It is splitted into fragments of definite size by the use of restriction endnucleases.

Individual sequence differences in the genomic DNA lead to a RFLP (restriction fragment length polymorphism) which increased in the course of evolution:

1) through the mutation of the recognition sequences of the restriction enzyme;

2) through differentiation in the length of DNA sequences between two recognition sequences (a variable copy number of the repetitive sequence motif).

One can not recognize these differences with the eye because ot the large number and the variety of restriction fragments (10'000 - 100'000 per

restriction enzyme). Therefore the specific hypervariable DNA-sequences are marked by hybridization with a suitable DNA probe and made visible by autoradiography on X-ray film.

DNA probes used for the DNA-fingerprinting of *Trichoderma*.

The choice of the DNA probe is important for the number of detectable restriction fragments and for the resulting polymorphism.

We used the following DNA probes:

1) M13 wildtype phage DNA; protein-III-gen consensus sequence: GAGGTGGNGGNTCT (Reference: Vassart et al. 1987).

2) oligonucleotides corresponding to repetitive sequences originally found in snake: (CT)8; (GTG)5; (GACA)4; (CA)8 (Reference: Ali et al. 1986).

Table I: The following species were analysed:

Number	species/strain	origin
1	*Trichoderma todica*	ATCC 36936
2	*Trichoderma reesei* QM 6a	ATCC 13631
3	*Trichoderma reesei* QM 9123	ATCC 24449
4	*Trichoderma reesei* QM 9414	ATCC 26921
5	*Trichoderma longibrachiatum*	CBS 816.68
6	*Trichoderma polysporum*	ATCC 18650
7	*Trichoderma koningii*	P. J. Stadler, TU Wien
8	*Trichoderma pseudokoningii*	ATCC 60641
9	*Trichoderma saturnisporum*	ATCC 18903
10	*Trichoderma virgatum*	ATCC24961
11	*Trichoderma harzianum*	ATCC 36042

Results

- We studied different combinations of DNA probes and restriction enzymes to obtain DNA-fingerprints from the investigated *Trichoderma* species.
- The M13 wildtype phage DNA and the oligonucleotides (GACA)4, (CT)8, (CA), and (GTG)5 were found to be well suited probes for DNA-fingerprinting in all species and strains tested.
- Concerning the restriction enzymes used, those recongizing six nucleotides (EcoRI, BamHI, PstI) were found to be superior to those recognizing only four (AluI, HinfI).
- The results obtained suggest a reclassification of the *Trichoderma* aggregate into five groups:

group I:	*Trichoderma reesei*
	Trichoderma todica
group II:	*Trichoderma longibrachiatum*
	Trichoderma polypsorum
	Trichoderma koningii
	Trichoderma pseudokoningii
group III:	*Trichoderma virgatum*
group IV:	*Trichoderma saturnisporum*
group V:	*Trichoderma harzianum*

- Using other restriction enzymes and DNA probes we could carry out a further sub-classification of group II:

group IIa:	*Trichoderma longibrachiatum*
	Trichoderma polypsorum
	Trichoderma koningii
group IIb:	*Trichoderma pseudokoningii*

- *T. reesei* could be clearly distinguished from *T. longibrachiatum,* to whom it was for a long time considered to be identical (Rifai 1969). Homology between *T. reesei* and *T. longibrachiatum* was only 25% which justifies claiming that these two species are different.
- Groups I-V share homologies of the hybridizing restriction fragments to each other of less than 20%, whereas IIa and IIb more than 50%.
- The three strains of the groups III-V do not appear to be related neither to group I or II nor to each other, and are thus considered to represent separate groups.
- *T. todica* and *T. reesei* produced identical DNA-fingerprints in all experiments. Therefore, *T. todica* and *T. reesei* should be defined as a single species within the *Trichoderma* aggregate.

Conclusions

Taxonomic evaluation and identification of fungi imperfecti is a tedious trait, since in most cases only morphological and nutritional criteria could be used as tools. Here we have used DNA-fingerprinting for the determination of phylogenetic relationship within the *Trichoderma* aggregate (Rifai 1969). We could show that it is a powerful tool for the differentiation and reassessement of species classification. We have analysed 9 different species of the filamentous fungus *Trichoderma* and three mutant strains of *T. reesei* (QM 6a, QM 9123, QM 9414) for the presence of hypervariable loci in their genomes by

hybridization with M13 wildtype phage DNA and simple repeat oligonucleotiedes (GTG)5, (CT)8, (CA)8, (GACA)4. We suggest a reclassification of the *Trichoderma* aggregate into five groups. It is especially noteworthy that by means of this technique, *T. reesei* strains were clearly distinguished from *T. longibrachiatum*, to whom they were for a long time considered to be identical. In contrast, *T. todica* and *T. reesei* produced indentical fingerprints. *T. todica* is a rather rarely studied and late discovered species. Our results favour the proposal that these two species should be defined as a single species within the *Trichoderma* aggregate.

It is interesting to note that the new group-classification suggested in this paper correlates very well with the one recently proposed from the relative position of two cellulase genes (cbhI and cbhII) on restriction fragments (Morawetz et al. 1991). We could show that the high cellulase procuding *Trichoderma* species are found in groups I and II only.

The present results offer a promising perspective for a reappraisal of the classification of commercially important *Trichoderma* strains.

DNA-fingerprinting offers a simple and reliable alternative method to resolve taxonomic problems and to "lablel" strains of filamentous fungi.

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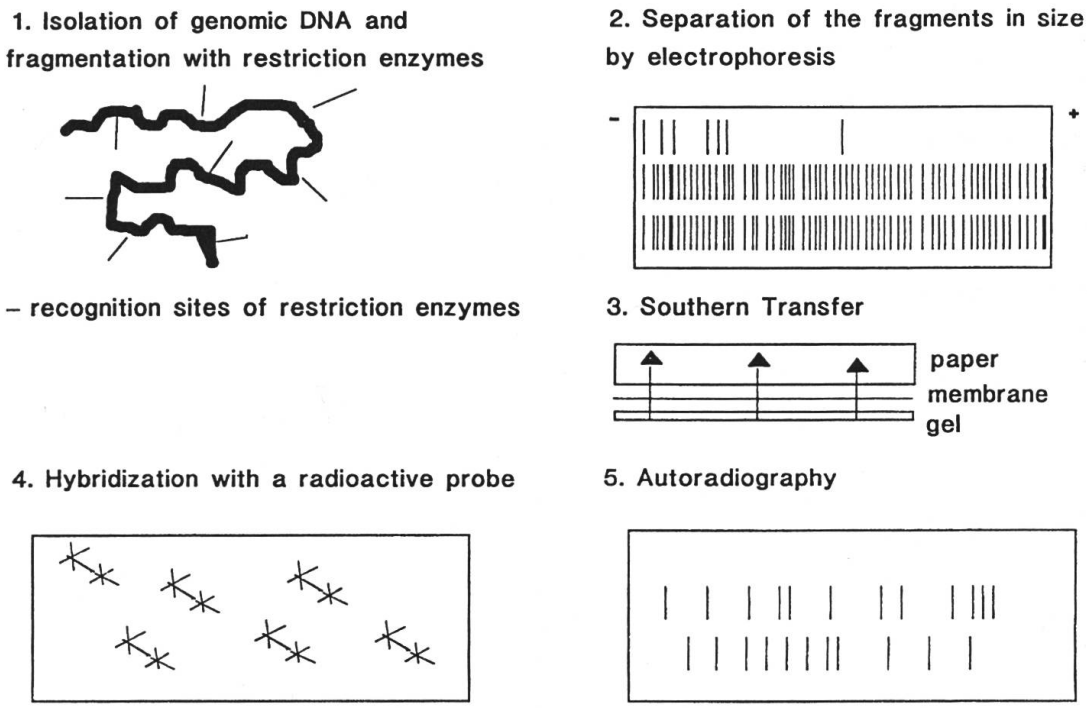

Figure 1: Method of the DNA-Fingerprinting

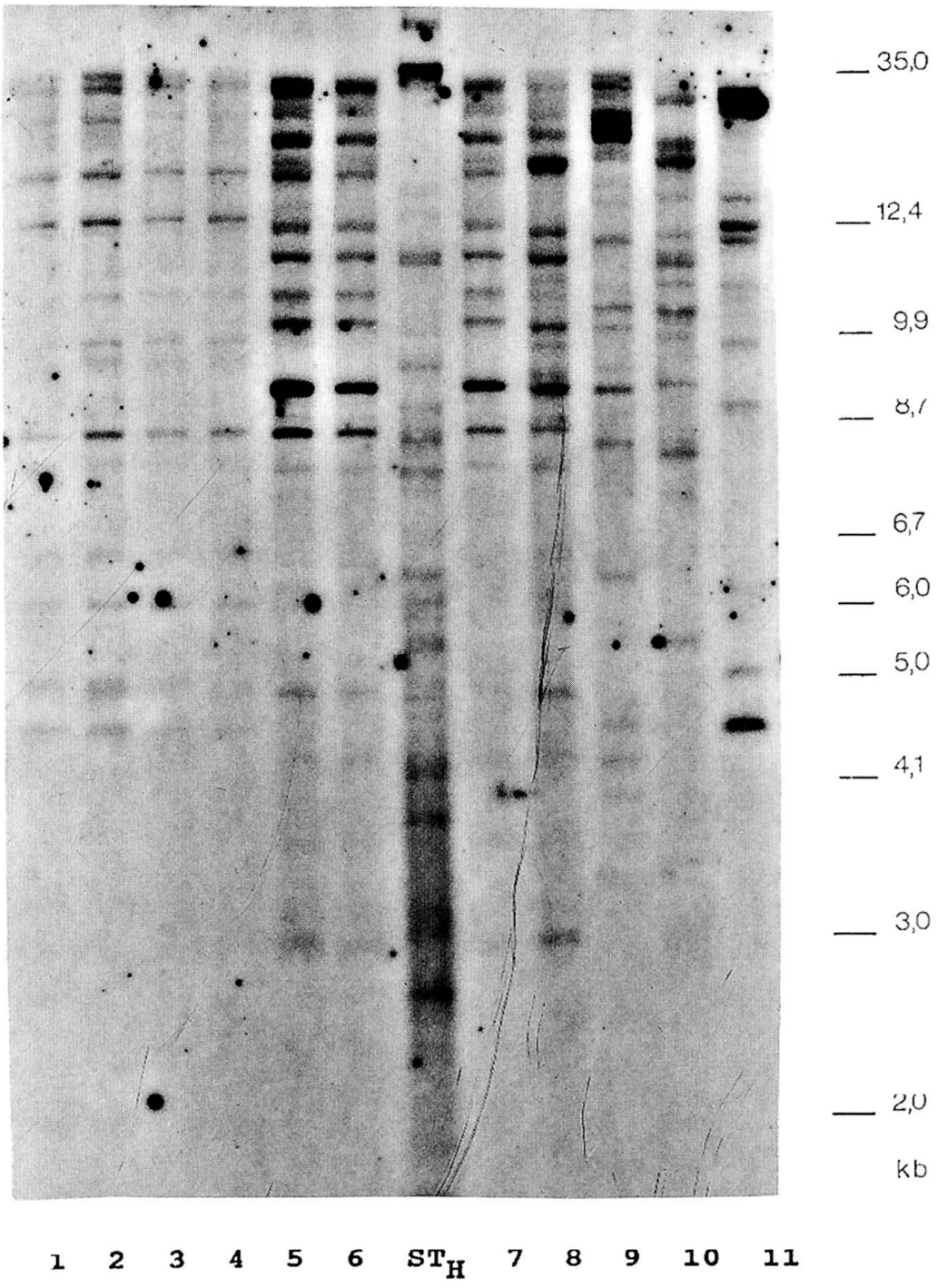

Figure 2: Hybridization of BamHI digested genomic DNA with M13 wildtype phage DNA
1-11 = number of strains (see table 1); ST_H = Human marker digested with HinfI.

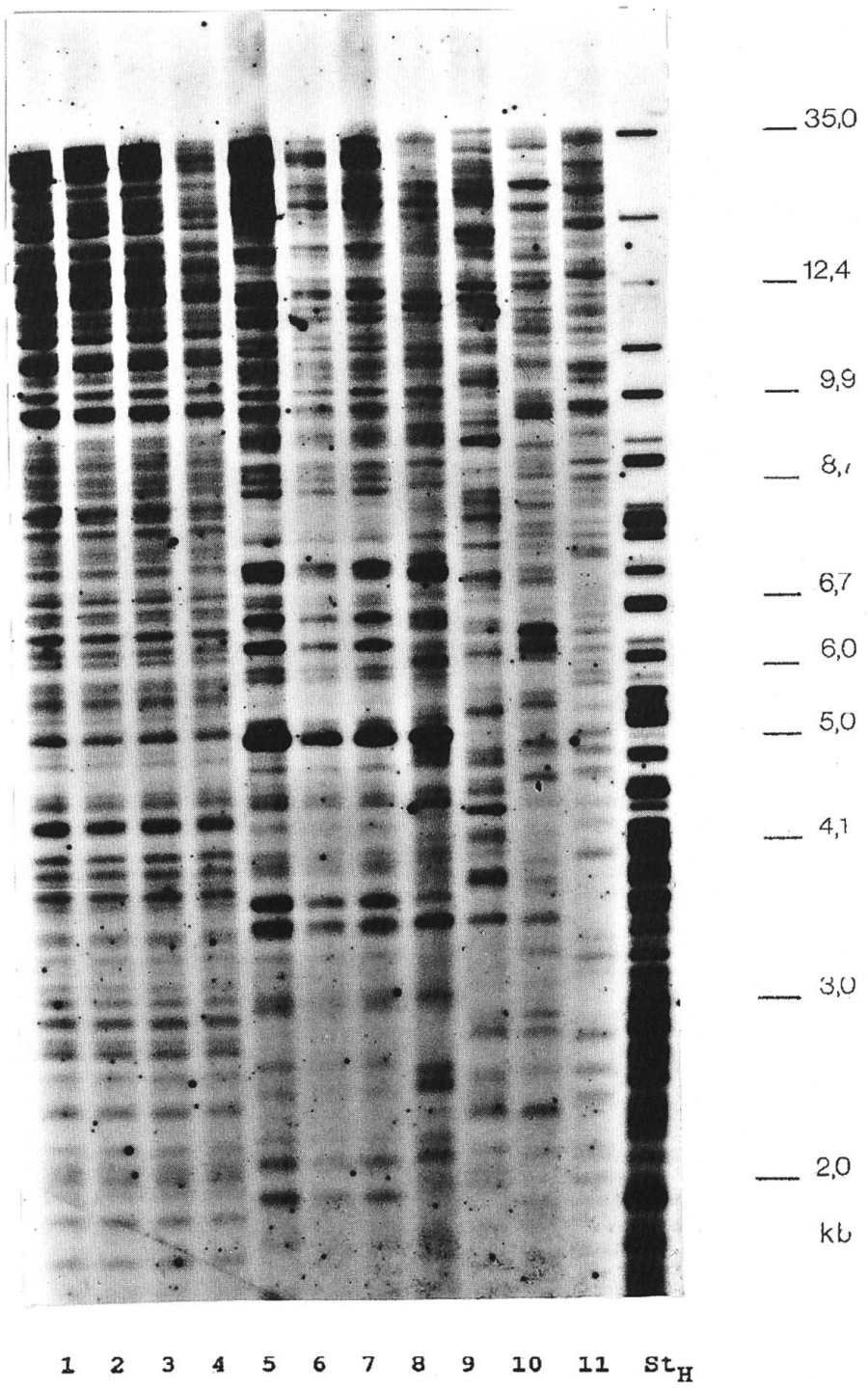

Figure 3: Hybridization of BamHI digested genomic DNA with $(GTG)_5$
1-11 = number of strains (see table 1); ST_H = Human marker digested with HinfI.

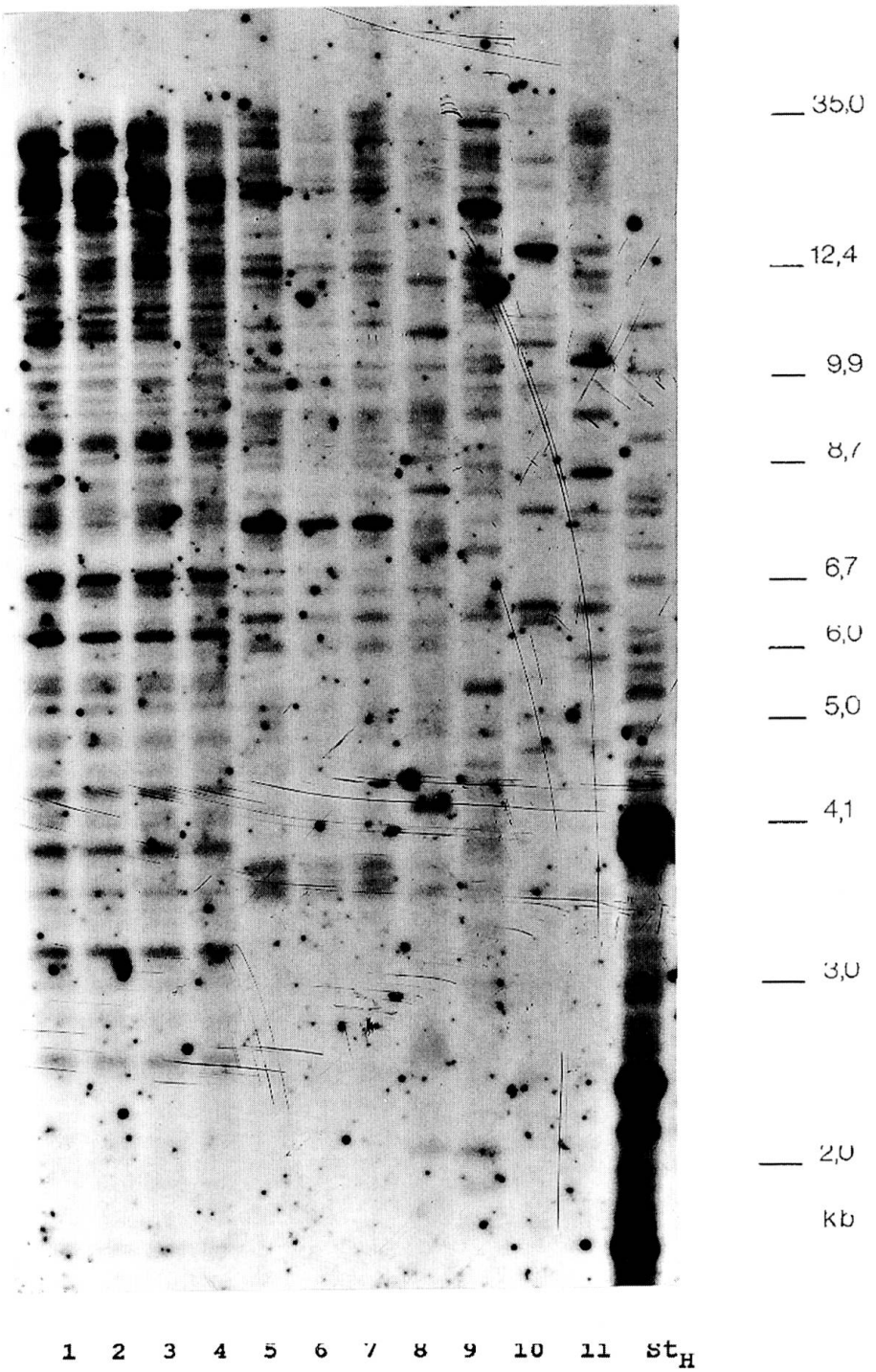

Figure 4: Hybridization of BamHI digested genomic DNA with $(CT)_8$
1-11 = number of strains (see table 1); ST_H = Human marker digested with HinfI.

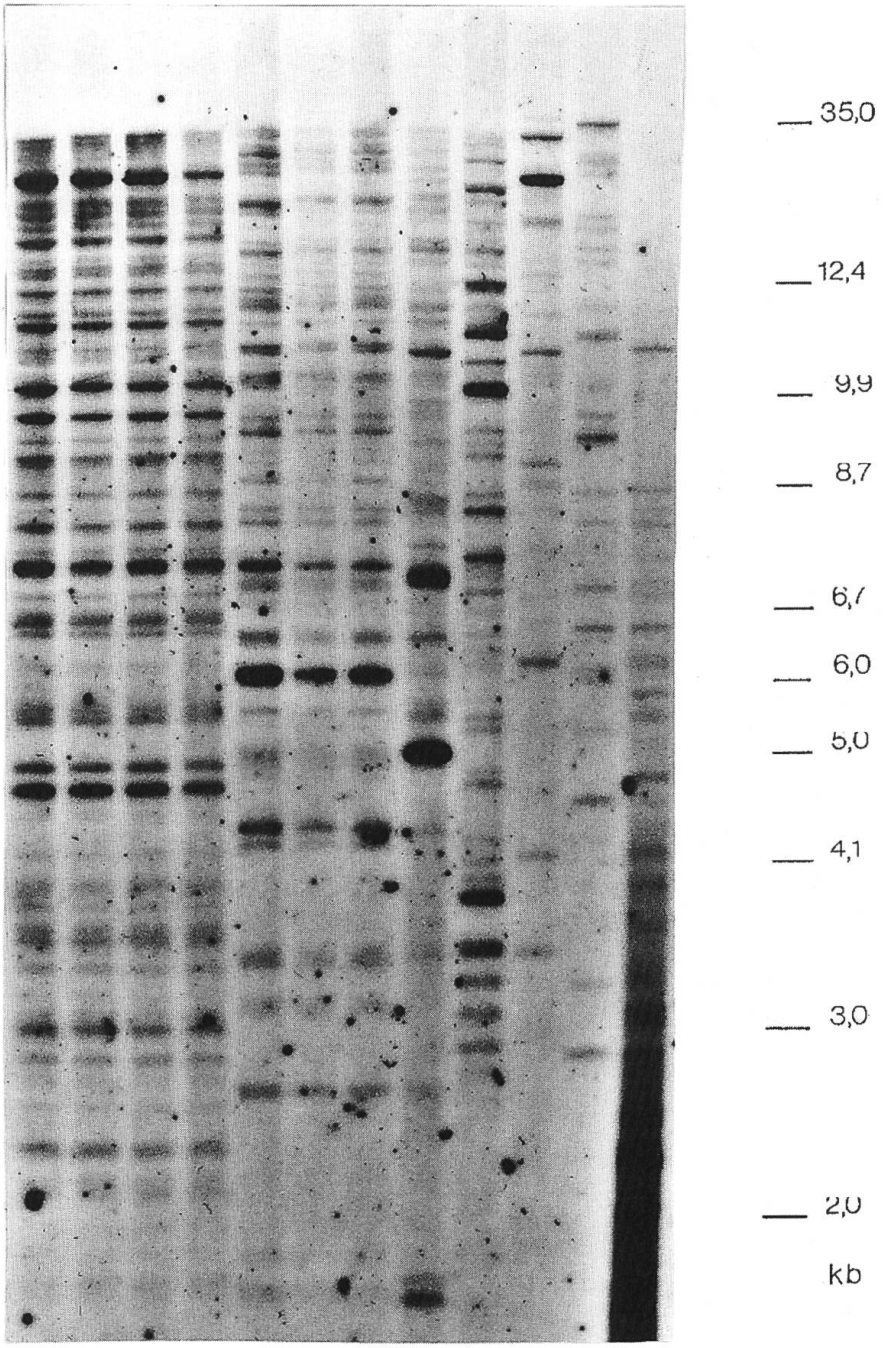

Figure 5: Hybridization of BamHI digested genomic DNA with $(CA)_8$
1-11 = number of strains (see table 1); ST_H = Human marker digested with HinfI.

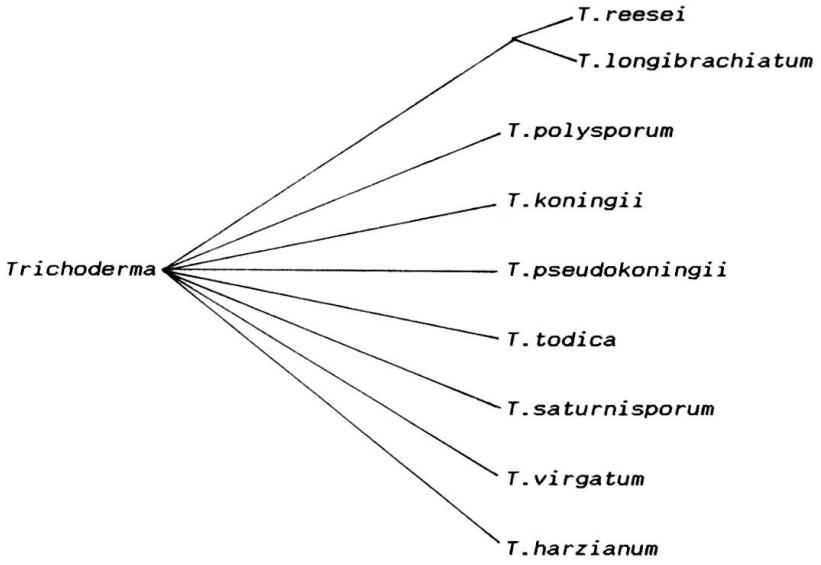

Figure 6: Old classification of the *Trichoderma* aggregate according to Rifai (1969)

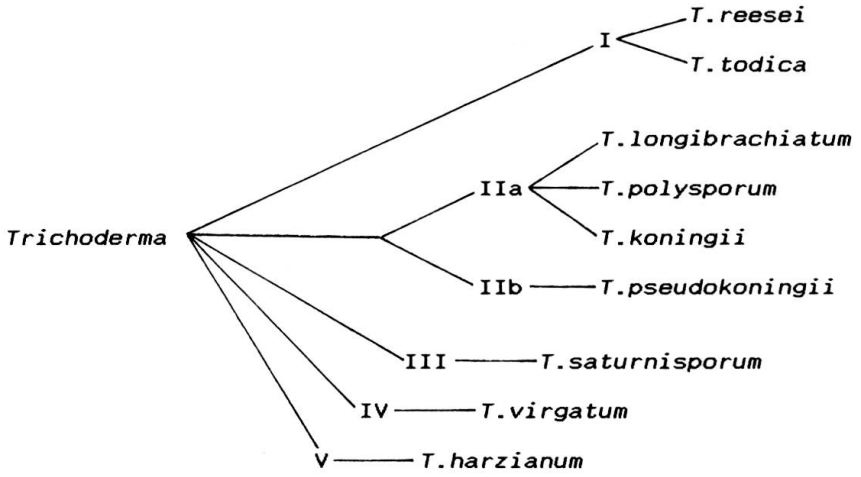

Figure 7: New classification of the *Trichoderma* aggregate suggested according to our results

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