Trichoderma populations from alkaline agricultural soil in the Nile valley, Egypt, consist of only two species

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The biodiversity of *Trichoderma* was studied in the Northern half of the Nile valley in Egypt. 20 strains were isolated from 9 different geographic locations, representing 19 different habitats, all with a pH between 7.3 and 8.4. Only *T. harzianum* (three ITS1/2 haplotypes and three RAPD-genotypes) and the anamorph of *Hypocrea orientalis* were found. One of the *T. harzianum* haplotypes (4 strains) is new. The occurrence of *T. harzianum* haplotypes and of *H. orientalis* appeared to be essentially independent of the habitat (pH, plant, soil type), and also did not correlate with biochemical properties (cellulase and chitinase activity) of the individual strains. These two taxa seem to be indigenous to the Nile valley, their presence not being influenced by the agricultural history of the soils.

Some *Trichoderma* spp. are economically important because of their production of industrial enzymes (cellulases and hemicellulases), antibiotics, and their action as biocontrol agents (KUBICEK & PENTTILÄ 1998, SIVASITHAMPARAM & GHISALBERTI 1998, HJELJORD & TRONSMO 1998). As the latter application implies the introduction of *Trichoderma* into the rhizosphere of a given ecosystem, a knowledge of the *Trichoderma* taxa indigenous in different soils and climates will contribute criteria influencing the choice of strains to be applied.

The occurrence of *Trichoderma* spp. in soil has been the subject of several investigations. Most of them were performed on ecosystems of the Northern, temperate hemisphere (DANIELSON & DAVEY 1973, WIDDEN & ABITBOL 1980, NELSON 1982), but no reports on cultivated soils have been published. While some of these studies contain very detailed information about the biochemical properties of the taxa detected, and their physico-chemical preferences, they suffer from the fact that species identification had been performed exclusively by morphological analysis and according to the key of RIFAI (1969), both of which are now known to lead to ambiguous results.

The valley of the river Nile in Egypt is one of the most ancient geographic areas that were cultivated by mankind (HAMDAN 1961). The Nile Valley and Nile Delta make up a total of about three percent of Egypt's area, and almost all Egyptian farms are located in this densely populated region. Water from the Nile enables farmers in the valley and delta to raise various crops around the year. The chief winter crops – in order of importance – include clover, wheat, and beans and other vegetables. The main summer crops are cotton, corn, rice, and millet. Cotton is Egypt's most important economic crop. The soil of the Nile valley essentially consists of silt and loamy sand, which is carried down by the river from its springs in Southern Etiopia (SAID 1993), and which provides a pH > 7.0 that is not very suitable for *Trichoderma*. The occurence of *Trichoderma* in Egyptian soils has been the subject of several earlier studies (MOUBASHER & ABDEL-HAFEZ 1987, MAZEN & SHABAN 1983, SHABAN 1986). However, similar to the above-cited studies, also these used morphological analysis for taxon identification only, and the species identity of the detected isolates is thus unclear.

Because of the very ancient nature of Egyptian cultivated soils, we felt that an enumeration of the *Trichoderma* taxa would yield interesting information. The purpose of the present work was therefore to assess the biodiversity of *Trichoderma* in the Northern part of the Nile valley including the Nile delta. We found that, independent of the crop and the physicochemical properties of the soil, they contained only two species of *Trichoderma*, viz. *T. harzianum* and the anamorph of *Hypocrea orientalis*.

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Tab. 1: Strains, locations and habitat properties*

Strain	Taxon	Location	Geographic Location	Habitat	Soil and soil properties				Gene Accession No.
					Type	pH	T.S.S.	O.M	Sand-clay-silt (% weight)	
Y1	*T. harzianum* 3	Beni-Suief	29.05N/31.05E	Corn	Clay	7.8	0.9	2.6	20.46/35.22/44.32	AJ606382
Y2	*T. harzianum* 3	Beni-Suief	29.05N/31.05E	Maize	Clay- Loamy	7.3	0.8	2.85	43.65/27.17/29.18	AJ606383
Y3	*T. harzianum* 3	El-Minia	28.5N/30.40E	Watermelon	Clay- Loamy	7.5	1.2	2.3	43.26/25.93/30.81	AJ606384
Y4	*T. harzianum* 3	El-Minia	28.5N/30.40E	Broad-bean	Clay- Loamy	8.1	0.9	2.8	27.84/34.53/37.63	–**
Y5	*T. harzianum* 2c	El-Faiyum	29.20N/30.50E	Cotton	Clay	8.02	1.9	2.9	20.0/29.71/50.29	AJ606385
Y6	*T. harzianum* 2c	El-Faiyum	29.20N/30.50E	Maize	Clay	7.6	1.04	2.3	24.16/34.87/40.97	AJ606386
Y7	*T. harzianum* 2c	El-Faiyum	29.20N/30.50E	Wheat	Clay	8.1	0.99	2.8	21.16/36.31/42.53	AJ606387
Y8	*T. harzianum* 2c	El-Faiyum	29.20N/30.50E	Wheat	Clay	8.4	1.5	3.1	18.78/10.52/70.70	AJ606388
Y9	*T. harzianum* 3	El-Faiyum	29.20N/30.50E	Corn	Clay	8.3	1.6	2.7	21/33.38/45.62	AJ606389
Y10	*T. harzianum* 3	El-Minia	28.5N/30.40E	Maize	Clay- Loamy	8.1	0.8	2.8	31.75/39.81/28.44	AJ606390
Y11	*H. orientalis*	El-Minia	28.5N/30.40E	Clover	Sandy-Loamy	7.7	0.7	1.8	81.79/1.90/16.33	AJ606391
Y12	*T. harzianum* 3	Beni-Suief	29.05N/31.05E	Citrus	Sandy-Loamy	8.0	0.9	2.1	69.99/7.58/22.43	AJ606392
Y13	*T. harzianum* 3	Beni-Suief	29.05N/31.05E	Clover	Sandy-Loamy	8.1	0.9	1.2	52.42/30.02/17.06	AJ606393
Y14	*T. harzianum* 4	Banha	30.29N/31.11E	Wheat	Clay	7.5	0.26	2.75	10/70 /20	AJ606394
Y15	*T. harzianum* 3	Zefta	30.42N/31.14E	Onion	Clay	8.1	0.45	3.20	10/72/18	AJ606395
Y16	*H. orientalis*	Tanta	30.47N/31.00E	Clover	Clay	7.3	0.45	3.38	10/70/20	AJ606396
Y17	*H. orientalis*	El-Mansura	31.3N/31.52E	Orange	Clay	8.0	0.25	2.15	10/60/30	AJ606397
Y18	*H. orientalis*	El-Mansura	31.3N/31.52E	Maize	Clay	7.4	0.43	3.17	10/70/20	– **
Y19	*H. orientalis*	El-Kharga	25.27N/30.32E	Wheat	Loamy	7.7	0.12	2.05	28/27/45	AJ606398
Y20	*H. orientalis*	Alexandria	31.14N/29.57E	Guava	Sandy-Loamy	7.9	2.1	2.05	73/7/20	AJ606399

* Numbers after the *T. harzianum* strains indicate the ITS1 and 2 haplotype;

** These two strains were identified by identity (similarity index > 0.95) of their RAPD patterns to isolates Y3 and Y17, respectively.

Material and methods

Collection of soil samples and isolation

Twentythree samples from cultivated soils were collected in March, April and May 1999 from different Egyptian Governorates. 3 cm of the top soil were removed and 5 subsamples then taken at random to a depth of 15 cm for each site. The soil samples were then transfered into sterile polyethylene bags and transported to the laboratory. There, all subsamples from one site were combined to yield one composite sample representing the location, exposed to ambient temperature to reach a humidity degree of 50 % and sieved through a mesh of 2 mm.

The isolation plate technique was used for the isolation of *Trichoderma* (JOHNSON et al. 1959), using TME medium (*Trichoderma* medium E; PAPAVIZAS & LUMSDEN 1982) which contains (per l): 200 ml V8 juice, 1 g glucose, and 100 mg PCNB (pentachloronitrobenzene; added after autoclaving). Isolates were identified by sequence analysis of their ITS1 and 2 region (KUBICEK et al. 2003) as explained in more detail in the results section. The strains so isolated are listed in Tab. 1 are stored at Botany Depatment, Faculty of Science, El-Minia University. Representative species from this work have also been deposited at CBS.

Chemical analysis of the soil

The type of the soil was determined by the hydrometer method (PIPER 1955). Chemical analysis included total soluble salts (T.S.S.) and organic matter (O.M.) and was done according to JACKSON (1958). pH was measured in water.

DNA extraction, PCR methods, and DNA sequencing

DNA was isolated from fresh mycelium by the CTAB method (MESSNER et al. 1996). A region of nuclear rDNA, containing the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA gene, was amplified by PCR using the primer combinations SR6R and LR1 as described by WHITE et al. (1990).

Five µl from each PCR reaction were electrophoresed on 1.5 % agarose minigels (containing 0.5 mg/l ethidium bromide) for 1 h in 1x Tris-acetate buffer (SAMBROOK, FRITSCH & MANIATIS 1989). The PCR products were revealed under UV-light. Template DNA (100 µl) was directly prepared from PCR products by purifying it with a commercial kit (Cleanmix; Fa. Talent), and sequenced with the aid of a LI-COR 4000L automatic sequencing system (MIDDENDORF et al. 1992), using cycle-sequencing (Robocycler 40; Stratagene) with the Thermo Sequenase-kit (Amersham LIFE SCIENCE) as described previously (KINDERMANN et al. 1998).

RAPD-PCR was performed as described by MESSNER et al. (1996), using the following primers: M13 (5'-GAGGGT GGCGGTTCT-3'; MEYER et al. 1991), V5 (5´ dTGCCGA GCTG; CAETANO-ANOLLES, BASSAM & GRESSHOFF 1992) and V6 (5´ dTGC AGCGTGG; LOPANDIC et al. 1996). RAPD analysis performed by the Phoretix 1D Advanced v5.20 software (Non Linear Dynamics).

Sequence analysis

DNA sequences were aligned first with Clustal X 1.81 (THOMPSON et al. 1997) and than visually adjusted, based on the algorithm of WATERMAN (1986) using Genedoc 2.6 (NICHOLAS & NICHOLAS, 1997).

Cellulase and Chitinase production and assay

To measure the capability of individual isolates to form and excrete cellulases in liquid culture, 250-ml Erlenmeyer flasks containing 50 ml of cellulose Czapek's liquid medium (containing (in g/l): native cellulose (N-Prolabo, El Naser Pharmaceutical Chemical Co., Egypt) 20; $MgSO_4 \cdot 7H_2O$, 0.29; KCl, 0.29; NH_4NO_3, 1.09; K_2HPO_4, 0.99; Fe^{2+}, 0.0029; Mn^{2+}, 0.0029; Zn^{2+}, 0.0029; distilled water one litre; pH 6.3) were autoclaved, and used to cultivate the various *Trichoderma* isolates for 10 days at 28 °C. Culture filtrates were obtained by centrifugation at 5000 rpm (15 min).

The cellulolytic activity of the filterates was measured by incubating 0.25 ml culture filtrate with 0.1 ml 1 % carboxymethylcellulose (N-Prolabo, El Naser Pharmaceutical Chemical Co., Egypt) and 0.15 ml 0.1 M sodium acetate buffer, pH 4.5 for 60 min at 40 °C, and subsequent determination of the released sugars by the method of SOMOGI (1953).

Incubations without substrate served as controls. Activities are expressed as units (1 U = 1 µmole of sugar released per min under the conditions of the assay) per ml culture filtrate.

The ability to form chitinases was investigated by growing the *Trichoderma* isolates in the same medium, yet using chitin (shrimp shells ; N-Prolabo, El Naser Pharmaceutical Chemical Co., Egypt) as a carbon source. To measure chitinase activity, 10 ml aliquots of the culture filtrates were mixed with 1 ml of a 1 % (w/v) suspension of chitin (shrimp shells) and 3 ml of 1M sodium-phosphate buffer, pH 7.0, and incubated for 60 min at 40 °C. Incubations without substrate served as controls. The incubation was terminated by boiling the reaction mixture. The debris was separated by filtration, and the released N-acetyl-glucosamine released subsequently determined by the method of REISSING, STROMINGER & LELOIR (1955). Activities are expressed as units (1 U = 1 µmole of sugar released per min under the conditions of the assay) per ml culture filtrate.

Cluster analysis of enzyme activity data was performed by hierarchical cluster analysis (SPSS Software, SPSS Inc. USA; NORUSIS, 1993).

Results

Only two taxa are present in soils from the Nile valley

Trichoderma was isolated from the combined fractions of twenty-three soils, and their ITS1 and 2 sequences analyzed (Tab. 1). Seventeen of them yielded sequences which were characteristic of members of the *T. harzianum* lineages (KULLNIG et al. 2000, CHAVERRI et al. 2003). Microscopical examination was

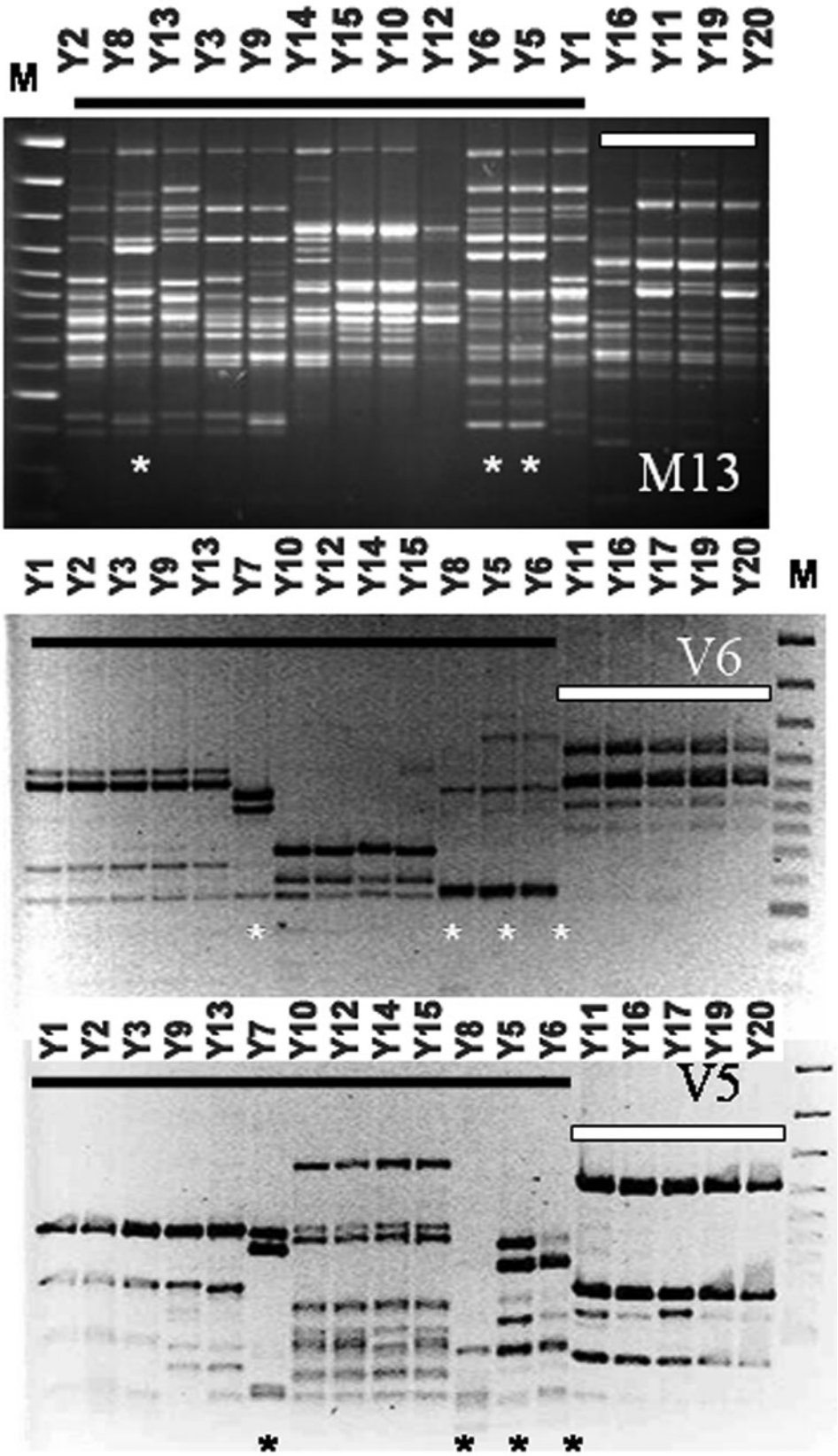

Fig. 1a: RAPD analysis of the isolates from this study; fragment pattern observed with primers M13, V6 and V5. The black horizontal bar indicates isolates of *T. harzianum*, the white bar isolates of *H. orientalis*. *T. harzianum* haplotype 2 c is indicated by an asterisc. M indicates Mr markers. RAPD patterns of isolates Y4 and Y18 are not shown, but were identical (> 95 %) to Y1 and Y20, respectively, and thus considered cospecific with them.

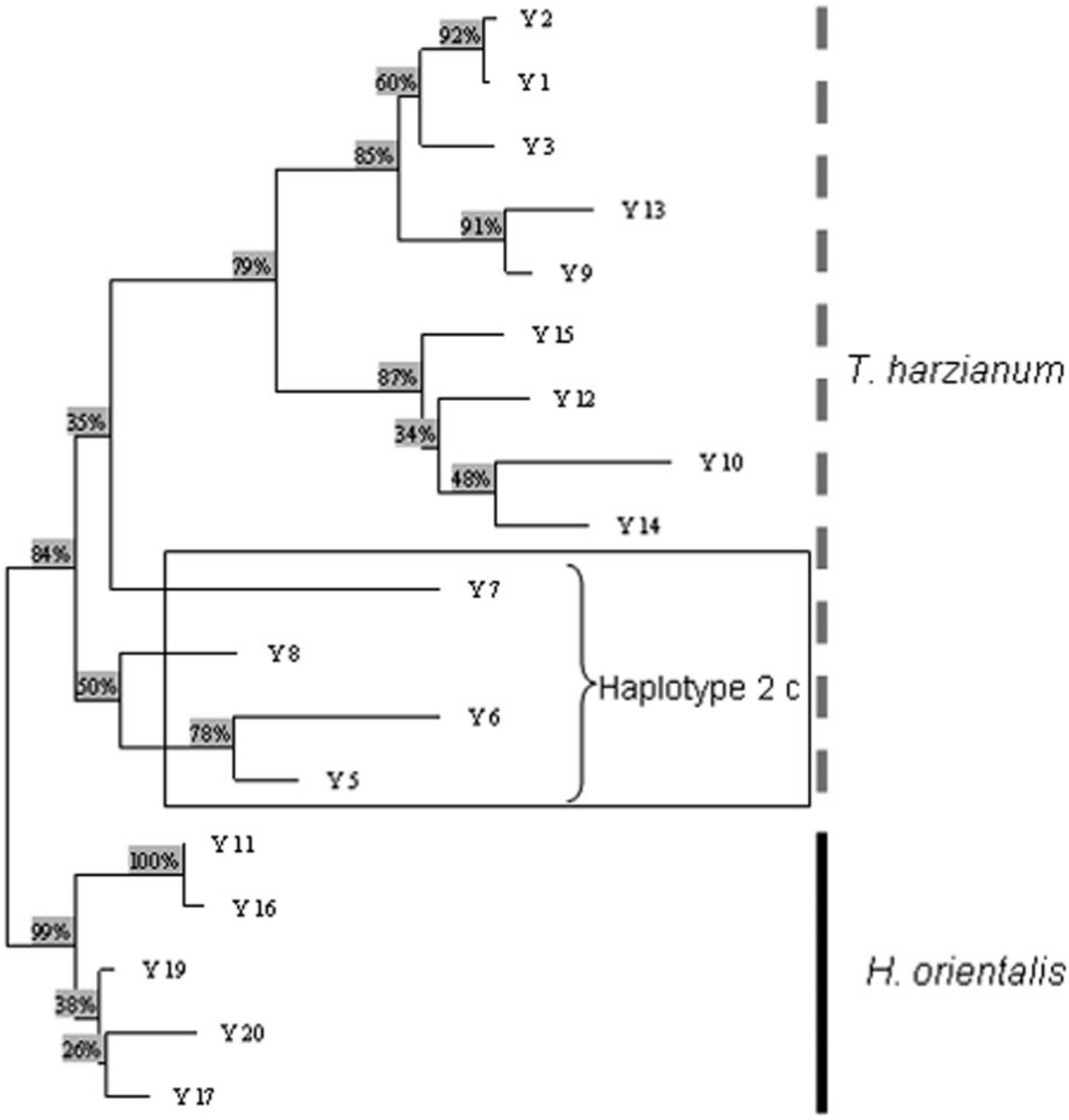

Fig. 1b: RAPD analysis of the isolates from this study; phylogenetic tree based on combined analysis of RAPD analysis with all three primers. The clade of haplotype 2c is boxed and emphasized by a bracket. Numbers over the branches indicate bootstrap coefficients (500 replicas).

consistent with this identification (data not shown). Among these seventeen strains, three of the five haplotypes defined by KULLNIG et al. (2000) were found. Haplotype 3 accounted for the majority of these isolates and was found independent of the soil type and the geographic location. Haplotype 4 was represented by a single isolate only (Y14), from a wheat field at Banha. Four strains (Y5, Y6, Y7 and Y8) represented a new haplotype which is closely related to haplotype 2a and 2b and thus termed 2c. Interestingly, this haplotype was detected in soils from El-Faiyum only, albeit from different habitats (cotton, wheat, maize). Phylogenetic analysis, including sequences of ex-type strains of closely related taxa (cf. KULLNIG-GRADINGER et al. 2002) clearly placed the new haplotype 2c into the clade containing the other haplotypes of *T. harzianum*, and its identity as *T. harzianum* is thus approved (data not shown).

Six strains formed two closely related haplotypes (differing in a single nt in ITS2), whose sequence was identical to that of *Hypocrea orientalis*. Differentiation of this taxon from *T. longibrachiatum* is not trivial, as their ITS1 sequence are absolutely identical, yet they differ in a diagnostic hallmark in ITS2. The single nucleotide polymorphism in ITS2 occurs in a region which is otherwise conserved in section *Longibrachiatum* and therefore does not jeopardize this identification. In addition, a morphological examination was consistent with the identity of these isolates as *T. longibrachiatum/H. orientalis* (data not shown).

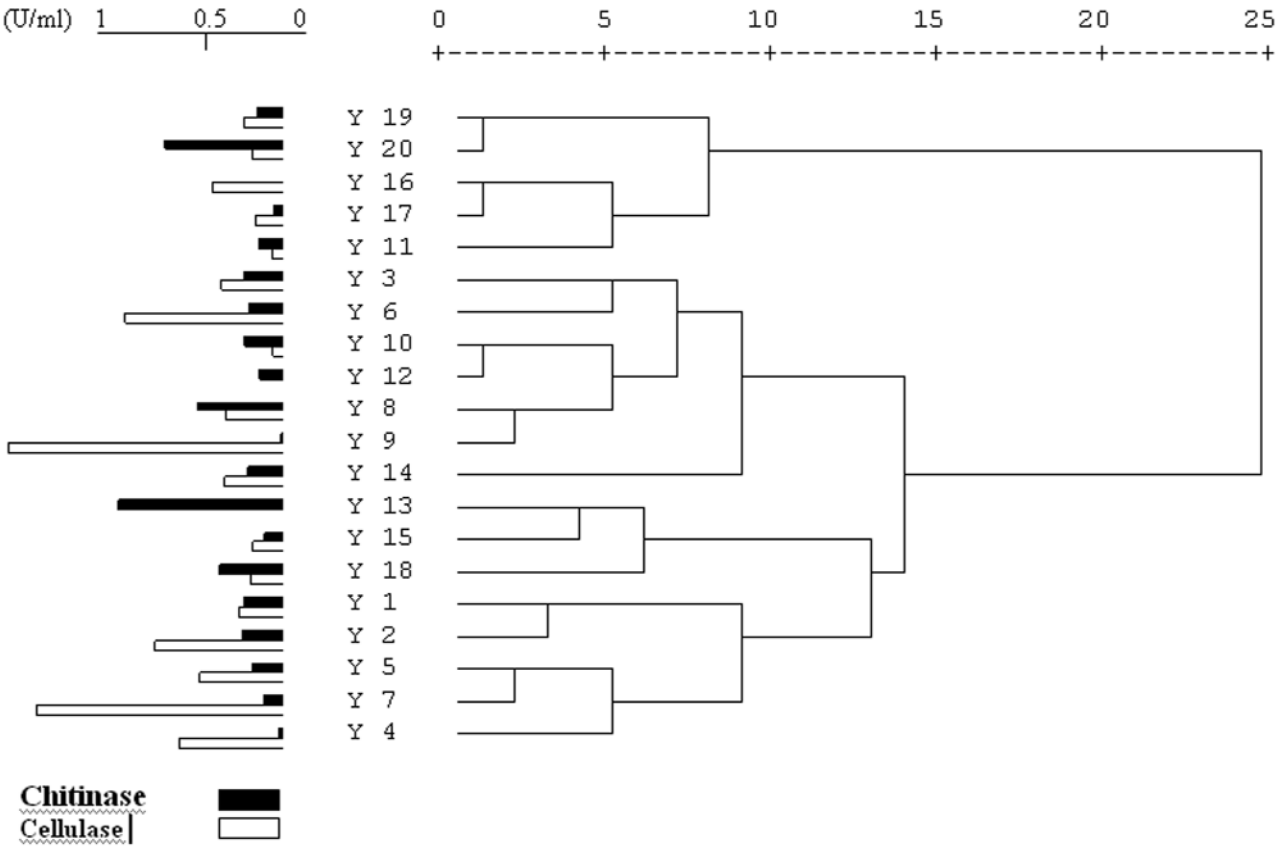

Fig. 2: Cluster analysis of cellulase and chitinase formation by the isolates from this study, and their respective enzyme activities.

Genetic and biochemical diversity of *T. harzianum* and *H. orientalis* from the Nile valley

In order to learn about the genetic and biochemical diversity of the two taxa identified in this study, we first performed RAPD analysis with three different primers [M13, (GTG)5, (GATA)4] which were previously shown to work well with isolates of various *Trichoderma* species (TURNER et al. 1996; LIECKFELDT et al. 1998; 2001; KULLNIG, SZAKACS & KUBICEK 2000). The results are shown in Fig. 1 a. *H. orientalis* isolates revealed only little genetic diversity, whereas the *T. harzianum* clade split into three major subgroups: haplotype 2c was clearly differentiated, although isolate Y7 exhibited a unique RAPD fingerprint with all three primers and clustered basal to haplotypes 3 and 4 (Fig. 1 b). Haplotype 3 exhibited two RAPD patterns, indicated by two clades in cluster analysis (Fig. 1 b), of which one also include the single isolate of haplotype 4. An overlapping of RAPD pattern within haplotypes 3 and 4 is also consistent with previous data by KULLNIG, SZAKACS & KUBICEK (2000). Genetic similarity within these clades was generally > 80 %, using Nei's similarity index (NEI & LI 1979). Almost identical RAPD patterns were sometimes observed in strains from strongly diverging geographic origin, indicating that they are present as a single clonal population within the investigated area. No correlation between RAPD pattern and the habitat could be detected.

To learn about the biochemical diversity of the two *Trichoderma* taxa from the Nile valley, we examined their ability to grow on cellulose and chitin, respectively, and to form the corresponding extracellular hydrolytic enzymes. These two substrates and enzyme systems were chosen becuase of the importance of cellulose and chitin degradation in rhizosphere competence and antagonism against fungal root pathogens (PETERBAUER et al. 1992; KUBICEK et al. 2001). The results were subjected to hierarchical cluster analysis (Fig. 2). Although *H. orientalis* exhibited a tendency to form higher cellulase activities than *T. harzianum*, and the latter to form higher chitinase activities than *H. orientalis*, some strains showed significant exceptions to this. Neither cellulase nor chitinase activities correlated with the habitat from which the strains were isolated.

Discussion

Many of the earlier studies on the occurence of *Trichoderma* in woodland or agricultural soil are ambiguous. When molecular methods for species identification are used, the biodiversity of *Trichoderma* in the investigated areas was found to be even higher, sometimes even in the same soil sample (KULLNIG et al. 2000, KUBICEK et al. 2003, WUSZKOVSKY et al. 2003). In

contrast, the results from the present study showed the occurrence of two *Trichoderma* taxa only in a total of twentythree soils, which is the lowest number of taxa which has been reported from a comparable number of soils so far. One of the two taxa was *T. harzianum*, which was also the most abundant species in all earlier investigations (KULLNIG et al. 2000; KUBICEK et al. 2003; WUSZKOVSKY et al. 2003). The other one, isolated from six soil samples, was identified as the anamorph of *H. orientalis*, a member of section *Longibrachiatum* (SAMUELS et al. 1998). Although *H. orientalis* has been described from Yunnan (P.R China) and New Zealand, the anamorph of *H. orientalis* has so far been isolated from Sierra Leone, South Africa, and India only; its occurrence in soil of Egypt would therefore be consistent with an Afro-Indian distribution of this taxon. It is noteworthy that it accounted for 24 % of the isolates obtained in this study: members of section *Longibrachiatum* were so far very underrepresented in the other geographic areas investigated (1.2 % in Central Asia and the Himalaya, KULLNIG et al. 2000; 7 % in South East Asia, KUBICEK et al. 2003; 2 % in Central Europe, WUSZKOVSKY et al. 2003). We have recently initiated an investigation on the biodiversity of *Trichoderma* in Africa (I. Druzhinina, A. Koptchinski, G. Szakacs, J. Bissett, C.P. Kubicek, unpublished data), and our preliminary results indeed confirm that members of section *Longibrachiatum* are present in higher frequencies in soils from this continent.

T. harzianum is probably the genetically most diverse taxon of the genus. KULLNIG et al. (2000) described several ITS1 and 2 haplotypes, of which the majority also correlated well with RAPD-based isolate typing. In this context, it is interesting that the *T. harzianum* haplotypes found in this study are rare: one (2c) has not been found before. Isolates of haplotype 4 have so far been recovered from soils of Nepal (KULLNIG et al. 2000), Costa Rica and South Africa (unpublished data), the Phillippines (C.P. Kubicek, M. Lübeck, unpublished data; see also CUMAGUN et al. 2000), and Austria (WUSZKOVSKY et al. 2003). CHAVERRI et al. (2003), studying the evolution and biodiversity of *T. harzianum*, included only haplotype 3, using isolates from Japan, Cameroon and Austria, which clustered in their lineages V and VI. Attempting to trace back the evolutionary origin of *T. harzianum*, they reported that the SHIMODAIRA-HASEGAWA (1999) test did not refute the hypothesis of an origin from South America or Central Asia. The *T. harzianum* haplotypes found in this study would be compatible with an origin of haplotype 4 from South America via South and Central Asia. Work on the reassessment of a biogeographic phylogeny of *T. harzianum*, based on a complete set of haplotypes from all geographic regions, is currently in progress in our laboratory (I. Druzhinina, J. Bissett, C.P. Kubicek, unpublished data).

The reason for this low degree of biodiversity of *Trichoderma* in the Nile valley soil may be related to its alkaline pH value. DANIELSON & DAVEY (1973) stressed that the pH of the soil is one of the most critical parameters for *Trichoderma* propagation. KREDICS et al. (2003) reported that species of *Trichoderma* grow optimally around pH 4.0 – 5.0, and exhibit little or no growth below 2.0 and above 6.0. The pH of the soils used for isolation of *Trichoderma* in this study varied between 7.4 and 8.4. Our results therefore show that *Trichoderma* can also be isolated from such adverse habitats, but with a lower abundance than in other soils. While it is possible that the two species found have a general tolerance to high pH, we rather speculate that their long term presence in the soil has led to corresponding adaptation to it. This assumption is also consistent with our findings that we could not detect any correlation between the plant species in the rhizosphere of which the respective samples were taken, the physical or chemical properties and composition of the soil, or its pH and the taxa or haplotypes of *Trichoderma* recovered. Correlations between any of these characters and the two taxa were essentially random, and we thus believe that the populations of *T. harzianum* and *H. orientalis* detected in this study are generally indigenuous components of the soil in the Nile valley.

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