Mycol Progress
DOI 10.1007/s11557-009-0621-5

ORIGINAL ARTICLE

MALDI-TOF MS of *Trichoderma*: a model system for the identification of microfungi

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Received: 26 March 2009 / Revised: 25 August 2009 / Accepted: 26 August 2009

Abstract This investigation aimed to assess whether MALDI-TOF MS analysis of the proteome could be applied to the study of *Trichoderma*, a fungal genus selected because it includes many species and is phylogenetically well defined. We also investigated whether MALDI-TOF MS analysis of peptide mass fingerprints would reveal apomorphies that could be useful in diagnosing species in this genus. One hundred and twenty nine morphologically and genetically well-characterized strains of *Hypocrea* and *Trichoderma*, belonging to 25 species in 8 phylogenetic clades, were analyzed by MALDI-TOF MS mass spectrometry. The resulting peak lists of individual samples were submitted to single-linkage cluster analysis to produce a taxonomic tree and were compared to ITS and *tef1* sequences from GenBank. SuperSpectra™ for the 13 most relevant species of *Trichoderma* were computed. The results confirmed roughly previously defined clades and sections. With the exceptions of *T. saturnisporum* (Longibrachiatum Clade) and *T. harzianum* (Harzianum Clade), strains of individual species clustered very closely. *T. polysporum* clustered distantly from all other groups. The MALDI-TOF MS analysis accurately reflected the phylogenetic classification reported in recent publications, and, in most cases, strains identified by DNA sequence analysis clustered together by MALDI-TOF MS. The resolution of MALDI-TOF MS, as performed here, was roughly equivalent to ITS rDNA. The MALDI-TOF MS technique analyzes peptides and represents a rough equivalent to sequencing, making this method a useful adjunct for determination of species limits. It also allows simple, reliable, and quick species identification, thus representing a valid alternative to gene sequencing for species diagnosis of *Trichoderma* and other fungal taxa.

Keywords Taxonomy · MALDI-TOF MS · Mass spectrometry · Hypocreales

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Introduction

Trichoderma (*Ascomycota*; teleomorph: *Hypocrea*) is a species-rich genus of microfungi. The approximately 130 described species of *Trichoderma* are dispersed to all latitudes, habitats, and climatic zones (Klein and Eveleigh 1998). They are usually encountered in soils (Domsch et al. 2007; Klein and Eveleigh 1998), but may also colonize diverse habitats such as water-damaged building materials or indoor dust (Thrane et al. 2001), mushroom production facilities (Komon-Zelazowska et al. 2007; Samuels et al. 2002), animal feed (Caballero et al. 2007), marine sponges (Sperry et al. 1998), and sapwood of trees (Evans et al. 2003). *Trichoderma* species are used in the biological

Published online: 17 September 2009

control of plant disease and as stimulators of plant growth (Harman et al. 2004), while some species are able to degrade polysaccharides and related macromolecules (Bénitez et al. 1998; Bigly and Tenkanen 1998; Koivula et al. 1998; Kubicek and Penttilä 1998; Lorito 1998). Still others cause opportunistic infections in immunosuppressed subjects (Kuhls et al. 1999), or produce mycotoxins (Degenkolb et al. 2008a) and polypeptide antibiotics (peptaibiotics) (Degenkolb and Brückner 2008; Degenkolb et al. 2008b; Toniolo and Brückner 2009). Thus, species of *Trichoderma* have an impact on human activities, and it is critical to accurately circumscribe and identify species in order to communicate information about them.

Until the mid-1990s, the taxonomy of *Trichoderma* was based entirely on microscopic phenotype with the result that many, or most, reports of *Trichoderma* in the literature prior to that time may be based on misidentified strains (Degenkolb et al. 2008a; Kullnig et al. 2001). In the mid-1990s, DNA sequence analysis assumed the major role in species recognition (Samuels 2006; Druzhinina et al. 2006), and today all described species of *Trichoderma* can be identified using DNA sequences that can be found either through a dedicated web site (Druzhinina et al. 2006, http://www.isth.info/) or in GenBank.

The currently accepted taxonomic paradigm is phylogenetic reconstruction based on sequencing of multiple genes (Taylor et al. 2000). Clades are examined for phenotypic apomorphies or biogeographic signals. Evolution has progressed at a much faster rate at the molecular level than at the level of phenotype. Increasingly, as more strains from wider geographic regions are included in phylogenetic analysis, it has become difficult or even impossible to recognize morphologically defined taxa (e.g., Crouch et al. 2006; Hughes et al. 2007). In *Trichoderma*, the number of phenotypic characters, including microscopic and colony characters and growth rates, is too small to account for the number of species indicated by molecular phylogenetic analysis. If evolution has progressed more rapidly at the genomic level than at the level of physical phenotype, then secondary metabolites should provide useful taxonomic characters. Some authors (Degenkolb et al. 2006a, b, 2008a; Kubicek et al. 2008a) found that the patterns of peptaibiotics and hydrophobins tend to be species-specific. The analysis of polypeptides has the advantage over, for example, substrate utilization, because they directly reflect the genome.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is one way of analyzing these polypeptides. The technique allows rapid and reliable identification of bacterial taxa (Bright et al. 2002; Dickinson et al. 2004; Fenselau and Demirev 2001; Friedrichs et al. 2007; Hathout et al. 1999; Lasch et al. 2008; Pignone et al. 2006; Ryzhov et al. 2000; Smole et al. 2002) and has already been successfully applied to the identification of specimens belonging to the fungal genera *Serpula, Conidiophora, Antrodia, Penicillium*, and *Candida* (Chen and Chen 2005; Qian et al. 2008; Schmidt and Kallow 2005). Recently, MALDI-TOF MS has also been used to study the peptaibiomics (Degenkolb and Brückner 2008; Degenkolb et al. 2008a, b; Neuhof et al. 2007a; Toniolo and Brückner 2009) and for direct identification of hydrophobins of *Trichoderma* (Neuhof et al. 2007b).

The results so far obtained with bacterial and fungal organisms suggest that analysis of polypeptides using MALDI-TOF MS may be a relatively cheap, rapid, and reliable method for species identification in microfungi. With this in mind, we started this project to assess whether MALDI-TOF MS analysis of proteomics could be applied to the study of a genus of microfungi. *Trichoderma* was selected because of its economic importance, because it includes many species and there exists a robust phylogeny for it, which will enable validation of the MALDI-TOF MS results. We also investigated whether MALDI-TOF MS analysis would reveal apomorphies that could be useful in separating species.

Materials and methods

Fungal strains

One hundred and twenty nine morphologically and genetically well-characterized strains of *Hypocrea* and *Trichoderma*, belonging to 25 species in 8 phylogenetic clades (Brevicompactum, Hamatum, Harzianum, Longibrachiatum, Polysporum, Stromaticum, Virens, and Viride), were analyzed (Table 1). Strains were grown on PDA (potato dextrose agar, BD Diagnostics, Sparks, MD) agar plates at 25°C for 48 h. The species were selected so as to represent a great diversity in the genus while also including closely related species.

MALDI-TOF MS

Results of preliminary MALDI-TOF MS analyses of *Trichoderma* strains grown on SNA (defined low nutrient medium; Nirenberg 1976) and PDA at two different temperatures (25 and 30°C), at varying incubation times (from 2 to 10 days), showed that the best profile spectra (quality and number of peaks) were obtained when using young mycelium grown on PDA agar plates, before visible onset of sporulation (data not shown). The main analysis was then carried out using 2-day-old material.

Growing mycelium was scraped from the culture plates and spotted onto wells of a 48-position stainless steel FLEXImass™ target plate (Shimadzu Biotech, Kyoto,

Table 1 *Trichoderma/Hypocrea* strains analyzed by MALDI-TOF MS

Taxon[a]	Strain[b]	Geographic origin	Clade	*tef*1	ITS
T. ghanense	ATCC 208858=IAM 13109= G.J.S. 95-137	Ghana	Longibrachiatum	AY937423	
	G.J.S. 04-313	Peru			
	G.J.S. 04-323	Peru			
	G.J.S. 04-335	Peru			
	G.J.S. 07-28	Ghana			
	G.J.S. 07-29	Ghana			
T. longibrachiatum	G.J.S. 91-10	Brazil	Longibrachiatum		
	G.J.S. 95-73	USA (TX)			
	G.J.S. 96-149	Kenya			
	G.J.S. 00-07	Mexico			
	CBS 118640=G.J.S. 04-31	Mexico		DQ297069	
	G.J.S. 07-21	Ghana			
T. citrinoviride	CBS 258.85	USA (NC)	Longibrachiatum		
	CBS 817.91=CTR 79-225	USA (NJ)			
	CBS 818.91=CTR 79-290	USA (NC)			Z31013
	CBS 636.92=IMI 352472= G.J.S. 92-8	Canada			X93963
	G.J.S. 93-1	USA (NC)			
	G.J.S. 94-14	USA:(PA)			
T. reesei	CBS 102271=G.J.S. 97-177	Brazil	Longibrachiatum		
	CBS 102270=G.J.S. 97-178	Brazil			
T. pseudokoningii	ATCC 18646	Australia	Longibrachiatum		X93985
	CBS 354.97=G.J.S. 81-300	New Zealand		AY937429	DQ083025, X93971
T. saturnisporum	ATCC 18903	USA	Longibrachiatum		Z48726
	CBS 335.92	Unknown			X93973
	CBS 886.72	South Africa		AY937414	X93974
	ATCC 20898=G.J.S. 99-3	USA (NY)			
	G.J.S. 06-66	Vietnam			
T. effusum	DAOM 230007	India	Longibrachiatum	AY937419	DQ083008
T. sinense	DAOM 230004	Taiwan	Longibrachiatum	AY750889	
	G.J.S. 00-72	Reunion			
H. novaezelandiae	CBS 639.92=ATCC 208856= G.J.S. 81-265	New Zealand	Longibrachiatum	AY937448	DQ083019, X93969
H. orientalis	CBS 243.63	New Zealand	Longibrachiatum	Y376052, AY937421	X93965
T. gamsii	G.J.S. 04-09	USA (TX)	Viride	DQ307541	DQ315459
	CBS 120072=G.J.S. 05-111	Italy			
	G.J.S. 06-07	Sardinia			
	G.J.S. 06-09	Sardinia			
	G.J.S. 06-10	Sardinia			
	G.J.S. 06-11	Sardina			
	G.J.S. 06-12	Sardinia			
	G.J.S. 06-13	Sardinia			
	G.J.S. 06-14	Sardinia			
T. viridescens	G.J.S. 89-142	USA (NC)	Viride	AY376049	DQ109532, DQ315426, X93987
	G.J.S. 99-175	Australia		DQ307521	DQ315437
	G.J.S. 04-81	Italy			

Table 1 (continued)

Taxon[a]	Strain[b]	Geographic origin	Clade	*tef*1	ITS
	G.J.S. 04-232	Mexico			
	TR 5	USA (OR)		DQ307525	DQ315443
T. viride	CBS 101526	Netherlands	Viride	AY376053	
	NRRL 6955=G.J.S. 99-13	Finland			
	G.J.S. 05-104	Italy			
T. atroviride	CBS 142.95	Slovenia	Viride	AF456891, AY376051	AF456917
	DAOM 196932	Canada (QE)		AF456884	X93948
	G.J.S. 95-36	New Zealand			
	G.J.S. 00-59	Japan			
	G.J.S. 01-209	Cameroon			
	G.J.S. 04-73	Italy			
	G.J.S. 06-305	Ecuador			
T. koningii	ATCC 64262	Hungary	Viride	AY376046	AY380903
	CBS 989.97=IMI 378801= G.J.S. 89-122	USA (MD)		AY376045	AY380902
	CBS 987.97=G.J.S. 92-18	Canada		DQ289005	DQ323435
	CBS 460.96=IMI 374797= G.J.S. 96-119	Netherlands		DQ289003	DQ323424
	CBS 119061=G.J.S. 00-156	USA (PA)		DQ288991	DQ323433
T. hamatum	DIS 216d	Ecuador	Hamatum	EU856313	EU856288
	DIS 358e	Ecuador		DQ109544	EU856283
	DIS 358 h	Ecuador		FJ463286	FJ442658
	ICMP 16296=G.J.S. 98-170	New Zealand		DQ109544	DQ109530
	G.J.S. 04-325	Peru		EU856318	EU856293
	G.J.S. 04-368=Hoitink TH382	USA (OH)		DQ151582	DQ151583
	CBS 123309=G.J.S. 05-18	Cameroon		EU856315	EU856290
	CBS 123055=G.J.S. 07-63	Peru		EU856314	EU856289
T. pubescens	DAOM 166162	USA (NC)	Hamatum	AY750887	DQ083016
	DIS 391b	Cameroon		EU856303	EU856279
	G.J.S. 01-207	Cameroon		EU856304	EU856280
T. asperellum	CBS 123775=G.J.S. 91-1	South Africa	Hamatum		
	G.J.S. 02-62	Cameroon		EF186001	
	G.J.S. 02-63	Cameroon		EF186000	
	G.J.S. 02-65	Cameroon		EF185998	
	G.J.S. 02-66	Cameroon		EF185999	
	G.J.S. 02-144	Costa Rica			
	G.J.S. 03-35	Russia			
	G.J.S. 04-18	USA			
	G.J.S. 04-72	Italy			
	G.J.S. 04-103	Vietnam			
	G.J.S. 05-94	Israel			
	G.J.S. 05-226	Cameroon		EU248629	
	G.J.S. 05-359	Cameroon			
	G.J.S. 06-24	Vietnam			
	G.J.S. 06-159	Nigeria			
	G.J.S. 06-283	Cameroon			
	G.J.S. 06-292	Cameroon			
	G.J.S. 06-314	Cameroon			
	G.J.S. 07-66	Brazil			

Table 1 (continued)

Taxon[a]	Strain[b]	Geographic origin	Clade	*tef*1	ITS
T. polysporum	TR 46=BBA 70309	USA (WI)	Polysporum	AY750885	DQ112552
	CBS 820.68=ATCC 18650=TR 100	Germany		AY750886	Z48814
T. harzianum	DIS 217a	Ecuador	Harzianum	FJ463319	FJ442243
	DIS 314f	Cameroon		FJ463400	FJ442259
	DIS 325ai	Ecuador		FJ463359	FJ442271
	G.J.S. 97-106	Thailand		AF443939	AF443921
	G.J.S. 00-12	Mexico			
	G.J.S. 01-105	Russia	Type group of harzianum		
	G.J.S. 05-107	Italy		FJ463329	FJ442679
	G.J.S. 06-111	Cameroon		FJ463312	FJ442631
T. undescribed	DIS 252e	Ecuador	Harzianum		FJ442618
T. aggressivum f. *aggressivum*	DAOM 222156	Canada (ON)		AF348098	AF456924
	IMI 393970=G.J.S. 99-29	USA (PA)		AF348094	AF345950
T. aggressivum f. *europaeum*	CBS 689.94	United Kingdom		FJ467645	FJ442606
T. stromaticum	DAOM 231097=ATCC 204425=G.J.S. 97-180	Brazil	Stromaticum		
	CBS 101875=DAOM 231100= ATCC 204426=G.J.S. 97-183	Brazil		AY937418	
	G.J.S. 00-110	Brazil			
	G.J.S. 03-50	Brazil			
	G.J.S. 04-190	Peru			
	G.J.S. 04-331	Peru			
	G.J.S. 06-320	Ecuador			
	G.J.S. 07-78	Brazil			
	G.J.S. 07-88	Brazil			
T. crassum	G.J.S. 05-114	Italy	Virens		
T. virens	DAOM 167651	USA (GA)	Virens		
	DIS 162	Costa Rica		FJ463367	FJ442669
	CBS 123790=G.J.S. 01-287	Ivory Coast		AY750894	DQ083023
	G.J.S. 04-220	Peru			
	G.J.S. 05-109	Italy			
	CBS 249.59	USA (GA)		AF328559	AF099005
T. brevicompactum	CBS 112443=IBT 40867	Papua New Guinea	Brevicompactum	EU338281	EU330943
	CBS 112444	Caribbean		EU338296	EU330938
	CBS 112447	Mexico		EU338300	EU330942
	G.J.S. 04-380	USA (NY)		EU338292	EU330935
	CBS 109720=DAOM 231232= G.J.S. 04-381	USA (NZ)		AY937453, EU338299	DQ000635, EU330941
	CBS 119572=IBT 40865= G.J.S. 05-174	Iran		EU338295	EU330937
	CBS 119571=IBT 40838= G.J.S. 05-178	Iran		EU338293	EU330934
	CBS 121154=G.J.S. 05-355	Cameroon		EU338283	EU338330

[a] *T Trichoderma, H Hypocrea*

[b] ATCC American Type Culture Collection (Manassas), CBS Centraalbureau voor Schimmelcultures (Utrecht), CTR C. T. Rogerson Collection (New York Botanical Garden Bronx), DAOM Department of Agriculture-Mycology (Ottawa), DIS Harry Evans and Keith Holmes collection CABI, G.J.S. Gary J. Samuels collection (Beltsville), ICMP International Collection of Micro-Organisms from Plants (Lincoln), IMI International Mycologial Institute (New Zealand), NRRL Agricultural Research Service Culture Collection (Peoria), TR Earl Nelson collection (maintained at the USDA-ARS Beltsville Collection)

Japan). Then, 0.5 µl of matrix solution containing 75 mg/ml 2,5-dihydroxybenzoic acid (DHB) in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid was immediately added to the sample. The solvent was allowed to evaporate at room temperature and formation of DHB crystals was controlled visually. MS analyses were performed in positive linear mode in the range of 2,000–20,000 mass-to-charge ratio (*m/z)* with delayed, positive ion extraction (delay time: 104 ns with a scale factor of 800) and an acceleration voltage of 20 kV on an AXIMA Confidence (Shimadzu Biotech) mass spectrometer, equipped with a 50-Hz nitrogen laser (pulse width: 3 ns).

Averaged profile spectra fulfilling the quality criteria were collected from 20 laser shot cycles. For every sample, 2×50 averaged profile spectra were stored and used for analysis. All spectra were processed by the MALDI-TOF MS Launchpad 2.8 software (Shimadzu Biotech) with baseline correction, peak filtering and smoothing.

Analysis of the spectra

The resulting peak lists of individual samples were exported to the SARAMIS™ software package (AnagnosTec, Potsdam, Germany) and submitted to single-linkage cluster analysis to produce taxonomic trees (0.08% error, range from *m/z* 3,000 to 20,000). SuperSpectra™ for the 13 most relevant species of *Trichoderma* were computed and integrated in the SARAMIS™ database for future rapid identification of new samples (Table 2). SuperSpectra™ are used for automatic microorganism identification and contain characteristic genus, species, and strain biomarkers that are representative for the respective group of microorganisms. Each *Trichoderma* SuperSpectrum™ contains 22–31 biomarkers.

Sequence analyses

The ITS and *tef1* sequences of some of the strains (Table 1) were used for UPGMA cluster analysis (bootstrap, 1,000 replicates; model, number of nucleotide differences) and the resulting phylogenetic trees were then compared to the dendrograms obtained with the MALDI-TOF MS results.

Results

A unique mass spectral fingerprint, in the range *m/z* 2,000–20,000 was identified for 2-day-old mycelium of the 129 strains studied. Five *T. viride* strains could not be analyzed because they produced mass spectra of low quality.

The number of ions composing the spectra ranged from 31 to 291 (mean=102.71, median=97 for the Longibrachiatum Clade; 99.05 and 100 for the Viride and Hamatum Clades; and 102.80 and 103 for the Brevicompactum, Harzianum, Stromaticum, and Virens Clades). This indicated a statistically homogeneous sample situation among the different Clades.

MALDI-TOF MS spectra of some relevant Clades were selected (Fig. 1). All of them presented dominating peaks (according to their intensity) between *m/z* 6,000 and 8,000. A second group of dominant peptides was observed in the mass range between *m/z* 2,000 and 5,000. Few proteins could be detected after *m/z* 9,000, and the last peaks were present at about *m/z* 12,200.

For 13 species, the number of strains was sufficient for the creation of SuperSpectra™ (Table 2). Mass peaks between *m/z* 3,000 and 12,200 were selected. The two dominant mass ranges described above were reflected in the SuperSpectra™ by the presence of many significant peaks inside these two regions.

Mass profiles of each strain were imported into the SARAMIS™ database for further statistical analysis. Single-linkage dendrograms obtained using the SARAMIS™ software package were derived from percentages of similarity (0.08% error) between strains. For *Trichoderma*, this similarity was relatively low when dealing with species and ranged from 53% for *T. polysporum* to 79% for *T. aggressivum*.

Different dendrograms were constructed and the results confirmed roughly previously defined clades and sections (Fig. 2c). With the exceptions of *T. saturnisporum* (Longibrachiatum Clade) and *T. harzianum* (Harzianum Clade), strains of individual species clustered very closely (Figs. 3, 4 and 5). *T. polysporum* clustered distantly from all other groups (38% similarity; Fig. 2).

Phylogenetic trees from ITS and *tef1* sequences showed in general the same major clustering as the MALDI-TOF MS dendrogram, with Clades Hamatum and Viride clearly separated from the other Clades (Fig. 2). According to the ITS tree, Clade Longibrachiatum clustered close to Clades Hamatum and Viride. In the *tef1* and MALDI-TOF MS dendrograms, and it was more related to the other Clades (e.g., Harzianum, Stromaticum, Brevicompactum). Sequence analyses of the *tef1* gene showed that Clade Brevicompactum was very distant from all the others, which was not observed by ITS sequencing or MALDI-TOF MS analysis. For both trees, *T. polysporum* clustered separately from the other groups, but the MALDI-TOF MS dendrogram provided more evidence for this difference (Fig. 2c).

Longibrachiatum Clade

Within the Longibrachiatum Clade, *T. ghanense*, *T. reesei*, and *T. citrinoviride* showed a relatively high degree of similarity (63%) and formed a well-defined group in the dendrogram (Fig. 3). Morphologically indistinguishable *T. saturnisporum* strains were separated into two distinct

Table 2 Characteristic masses retained for the creation of SuperSpectra™ of the most relevant *Trichoderma* species

Mass (*m/z*)[a]												
aspe[b]	*gams*	*atro*	*koni*	*hama*	*virs*	*ghan*	*citr*	*satu*	*long*	*brev*	*stro*	*vire*
3026.4	3026.4	3026.5	3026.3	3026.6		3025.3	3027.1		3032.9	3026.1		
												3034.0
								3047.5				
3058.9					3059.8							
											3291.6	3291.0
										3303.3		
					3312.0							
					3359.5							
	3365.7	3365.9						3366.6	3366.5			3366.5
							3372.5					
			3381.6									
											3435.0	
							3507.9					
									3536.9			
			3557.6									
								3602.0				
				3622.5								
	3671.2	3671.4	3671.3									
					3678.6							
		3684.5		3685.8							3685.3	
	3690.9											
							3698.4					
			3708.1						3707.3			
						3721.8						
											3798.3	
			3868.5									
												3875.8
							3881.6					
				3898.7								
		3913.6										
3932.9	3933.1	3932.7	3933.3	3933.8	3934.0	3932.7	3933.5		3934.4	3932.3	3934.6	3933.7
							4001.5		4001.6			
			4015.0						4014.8			4017.4
								4030.3				
4036.7												
							4043.7					
			4054.1									
		4060.4										
												4064.5
				4110.5						4107.4		
											4151.1	
										4168.3		
			4215.3									
	4229.9	4230.0										
					4295.4						4295.8	
												4300.4
4306.5	4306.4	4306.2										

Table 2 (continued)

Mass (*m/z*)[a]												
aspe[b]	*gams*	*atro*	*koni*	*hama*	*virs*	*ghan*	*citr*	*satu*	*long*	*brev*	*stro*	*vire*
								4314.8				
		4321.4			4323.2							
										4336.7		
												4367.2
4435.0												
		4505.7							4505.2	4505.3		
										4506.4		
								4513.8				
			4663.2									
4720.1												
4819.9					4819.4							
				4842.0								
								4850.8				
					4855.6							
		4877.1	4877.0									
	4906.9											
4911.3												
												5030.0
										5035.2		
							5042.5					
				5152.9						5149.7		
												5159.7
								5168.0				
		5213.4										
			5218.7									
	5226.7			5227.3								
							5247.3					
							5260.6					
				5443.0								
	5457.3											
		5481.0	5480.9									
								5510.4		5505.9		5508.2
						5517.9						
5526.5												
						5536.9						
												5586.4
										5615.5		
						5630.3						
			5641.8				5637.4					
	5670.8											
								5681.1	5678.0			
						5691.9						
				5715.1							5711.1	
												5722.0
							5765.5			5761.5		
				5770.1								
5783.0			5783.9		5783.7							

Table 2 (continued)

Mass (m/z)[a]												
aspe[b]	*gams*	*atro*	*koni*	*hama*	*virs*	*ghan*	*citr*	*satu*	*long*	*brev*	*stro*	*vire*
									5807.2			
											5842.0	
								5855.1				
			5888.6									
												5909.3
										6006.7		
						6055.7						
	6058.2			6058.9	6059.3		6058.5	6060.3		6057.3		
									6069.5			
							6097.4	6099.1		6096.1	6098.8	6097.7
	6123.1	6123.3	6123.2	6124.1								
												6135.7
		6145.2										
						6154.1						
			6221.4									
							6233.3					
						6290.9						
										6329.9		
	6465.0		6465.2									
				6528.6					6526.7			
										6547.7		
											6587.0	6586.0
										6611.6		
	6627.6			6628.5	6628.1		6627.8	6629.8	6628.6			
					6656.3							
	6710.0	6710.1	6710.0	6710.9						6709.1	6711.3	6710.3
					6722.8							
							6748.3		6748.7			
											6810.5	
							6834.0		6834.0			
									6846.1			
											6874.8	
				6902.4		6899.1						
				6954.3	6953.9							
												6974.5
6983.7	6980.1											
		7002.7										
					7017.5	7019.1	7019.2					
								7052.7				
											7060.0	
	7072.4								7076.6			
7129.9		7132.6										
							7139.3					
	7147.1											
									7152.9	7156.5		
			7230.7									
				7248.8								

Table 2 (continued)

Mass (*m/z*)[a]												
aspe[b]	*gams*	*atro*	*koni*	*hama*	*virs*	*ghan*	*citr*	*satu*	*long*	*brev*	*stro*	*vire*
					7258.3							
		7270.0										
				7277.2								
7346.0	7346.6	7347.0	7346.7									
					7361.0							
		7370.7		7376.0							7373.0	
	7386.1											
							7399.0					7400.6
									7415.9		7416.5	
							7429.0					
		7445.2	7442.1			7447.8						
		7459.2			7459.9							7460.6
7475.0						7479.5		7477.1				
									7489.9			
	7580.1											
					7601.0						7598.5	7596.1
			7739.6									
												7755.2
							7765.7			7766.0	7769.7	
						7778.2						
	7793.3			7798.0								
7808.6												
								7828.6				
7869.4	7871.2	7870.1	7872.3	7871.1	7869.8	7868.9	7869.5	7872.5	7870.5	7868.5	7870.9	7870.6
		7897.6										
											7943.2	
						8003.7						
								8062.4	8061.3			
8076.0												
							8090.2					
				8109.8	8105.9							
			8112.1	8110.3								
8138.6												
	8152.5											
											8191.1	
										8218.2		
				8224.8								
							8276.6					
						8310.1			8314.5			
					8329.6							
									8338.5		8333.1	
8355.6						8358.6						
								8374.6				
			8592.0		8592.0						8592.9	
												8602.1
8614.5	8614.8	8614.9										
			8621.8		8619.4				8621.2			

Table 2 (continued)

Mass (*m/z*)[a]												
aspe[b]	*gams*	*atro*	*koni*	*hama*	*virs*	*ghan*	*citr*	*satu*	*long*	*brev*	*stro*	*vire*
								8631.1				
		8647.3										
										8695.5		
					8768.3							
8783.0												
											8803.2	8799.4
		9013.9				9009.8			9010.7			
								9028.1				
9641.8	9640.7											
										9657.2		
									9673.0			
												10056.6
				10306.2						10300.2		
												10318.0
								10335.7				
	10452.6			10453.6								
						10477.3						
	10914.3											
										11225.4		
						11258.1	10255.1					
										11295.8		
	11341.1											
							11385.2					
	11701.8											
											11997.1	
12127.9			12120.2									
												12156.7

[a] 0.08% error

[b] aspe *T. asperellum*, atro *T. atroviride*, brev *T. brevicompactum*, citr *T. citrinoviride*, gams *T. gamsii*, ghan *T. ghanense*, hama *T. hamatum*, koni *T. koningii*, long *T. longibrachiatum*, satu *T. saturnisporum*, stro *T. stromaticum*, vire *T. virens*, virs *T. viridescens*

groups. This separation in the dendrogram was not so clear at the level of MALDI-TOF MS mass spectra. In fact, profiles of two representative strains of this species showed similar mass profiles and much the same peak intensities (Fig. 6) The single *Hypocrea orientalis* strain was close to, but distinct from, strains of *T. longibrachiatum* (68% similarity), as also shown in the *tef1* taxonomic tree (Fig. 2b).

Hamatum and Viride Clades

At the species level, *tef1* and MALDI-TOF MS taxonomic trees were clearly more discriminative than the ITS dendrogram, showing a clear separation of *T. gamsii*, *T. koningii*, *T. viridescens*, and *T. atroviride*, as well as *T. hamatum* and *T. pubescens* (Fig. 2). Cluster analysis of the MALDI-TOF MS results showed that strains of *T. viride* did not cluster with other members of the Viride Clade (Fig. 4). MALDI-TOF MS mass spectra belonging to *T. viride* were the most difficult to obtain because of the generally low number of masses showing up in the analysis.

Within the Hamatum Clade *T. hamatum* and *T. pubescens* clustered in the same branch (65.5% similarity), while *T. asperellum* was more similar to *T. gamsii* in the Viride Clade (69% similarity). The same close clustering between *T. hamatum* and *T. pubescens* was seen in the ITS and *tef1* trees (Fig. 2a,b). As for the MALDI-TOF MS dendrograms, *T. asperellum* was more similar to members of the Viride Clade in the *tef1* tree (Fig. 2b).

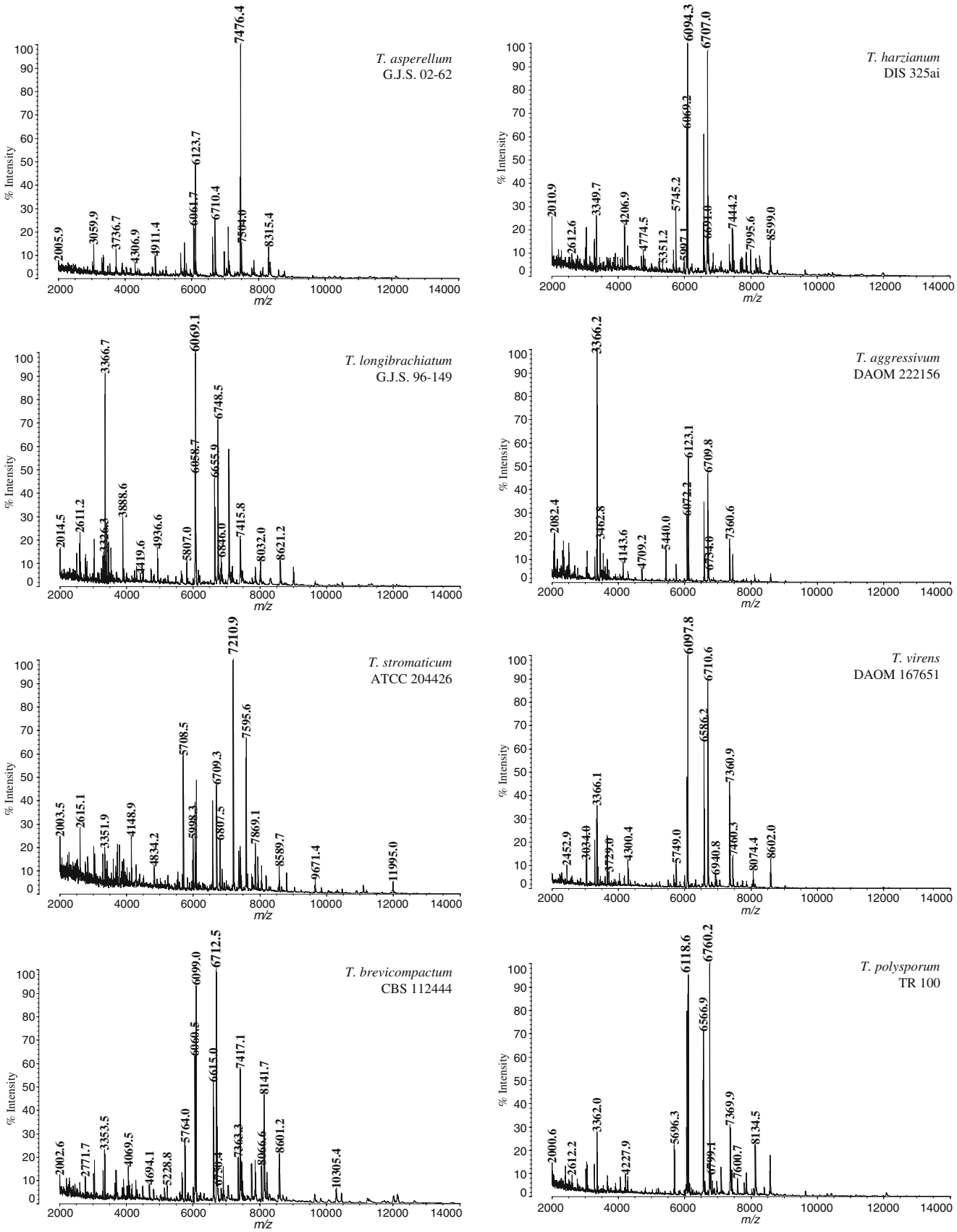


Fig. 1 MALDI-TOF mass spectra of relevant Clades

The 19 *T. asperellum* strains analyzed by MALDI-TOF MS were divided among three clades (I, II, and III; Fig. 4). Strains in clade I were the most homogeneous of the whole set of isolates studied, with at least 83% similarity. Inspection of the mass spectra of representatives of clades I, II, and III, respectively (Fig. 7), showed the typical dominating peaks between *m/z* 6,000 and 8,000. Within this mass range, however, the profiles of prevalent proteins differed according to the clades (*m/z* 7,472 in clade I and about 7,426, 7,465, and 7643 in both clades II and III). Within the *m/z* 2,000 and 5,000 mass range, the dominating peaks were less numerous, in particular for *T. asperellum* G.J.S 05-94 (Fig. 7), compared to other species of *Trichoderma* (Fig. 1).

Brevicompactum, Harzianum, Stromaticum and Virens Clades

The MALDI-TOF MS analysis included the Brevicompactum, Harzianum, and Stromaticum Clades in a single group (Fig. 5). Clade Virens was separated, with 52% similarity to the others. The Brevicompactum, Stromaticum, and Virens Clades were clearly separated and homogeneous, whereas the Harzianum Clade split into three different branches (I–III; Fig. 5), and strains in this clade identified as *T. harzianum* and *T. aggressivum* fell into different groups.

At the species level, *T. virens* formed a homogeneous group, with a minimum similarity of 77%, and was well separated from *T. crassum*. The *T. aggressivum* isolates could also be easily separated from *T. harzianum*. An undescribed strain (DIS 252e) was placed in cluster II, containing *T. harzianum* G.J.S. 97-106, G.J.S. 00-12, and G.J.S. 01-105. *T. harzianum* cluster I shared the same branch with *T. brevicompactum* isolates with 61% similarity (Fig. 5).The two representatives of clades I and II of *T. harzianum* (Fig. 8a) had identical dominating peaks in the *m/z* 6,000 and 8,000 mass range (*m/z* about 6,070, 6,095, 6,582, and 6,708). Some variations in the intensity of the peaks were observed within the *m/z* 3,000 and 6,000 mass range, but the major difference between these two groups concerned the number of detected masses.

Eight strains of *T. stromaticum* had at least 74.5% similarity, while only one (G.J.S. 04-331) was more divergent, with only 57.5% similarity to the others. Inspection of the MALDI-TOF MS spectra (Fig. 8b) revealed differences in the predominance of some masses between *m/z* 3,000 and 8,000, as well as the number of masses within the *m/z* 4,000 and 6,000 range.

In this group of clades, the phylogenetic trees produced using both ITS and *tef1* (Fig. 2a, b) differed from the MALDI-TOF MS dendrogram in three aspects (Figs. 2c and 5). Clade Brevicompactum was more distant from the other Clades by sequence analyses than by MALDI-TOF MS. *T. polysporum* was inserted into this group of Clades by phylogenetic analysis but not by MALDI-TOF MS. Finally, *T. stromaticum* CBS 101875 was clearly separated by *tef1* sequencing, but not by MALDI-TOF MS. In the ITS and *tef1* trees, *T. virens* strains were quite different from members of the Harzianum Clade. Moreover, this later also allowed a clear differentiation between *T. harzianum* and *T. aggressivum*, forming clearly separated groups. The undescribed strain DIS 252e was more similar to *T. virens* than to *T. harzianum* by ITS sequencing (Fig. 2a).

Discussion

One hundred and twenty nine morphologically and genetically well-characterized strains of *Hypocrea* and *Trichoderma* were studied. They belonged to 25 species in 8 phylogenetic clades (Brevicompactum, Hamatum, Harzianum, Longibrachiatum, Polysporum, Stromaticum, Virens and Viride).

Dominating peaks were observed in two different mass ranges (Fig. 1). The most important one was situated between *m/z* 6,000 and 8,000. Part of these proteins represented probably hydrophobins, as previously described (Neuhof et al. 2007b, c). The second, dominating peptides were situated at about *m/z* 2,000–5,000. These two dominating regions make the MALDI-TOF MS spectra quite characteristic for *Trichoderma* species, but one has to take into consideration that the intensity of the peaks depends on the physiological state of an organism, and could therefore vary according to external conditions. With the SARAMIS™ system, the problem is avoided, as only the presence or absence of the peptides is considered for strains identification and clustering analyses.

Kubicek et al. (2008a) reported that species of *Hypocrea/Trichoderma* contained many hydrophobin encoding genes (6 in *H. jecorina*, 9 in *H. virens* and 10 in *H. atroviridis*), which were suggested to evolve by a birth-and-death mechanism, followed by purifying selection. Moreover, Neuhof et al. (2007b) showed that, in intact-cell mass spectrometry, spectra of hydrophobins were the dominating protein masses. They also observed that sporulating and nonsporulating mycelia of several species of *Hypocrea/Trichoderma* differ in hydrophobin composition (Neuhof et al. 2007b). In our case, it was therefore crucial to adopt standardized growth conditions (temperature, type of incubation plates, time of incubation) to allow good comparison of the strains, as well as subsequent valid identifications with the SARAMIS™ SuperSpectra™. According to Neuhof et al. (2007b), 29 different species of *Hypocrea/Trichoderma* exhibited a unique combination of hydrophobin peaks with unique molecular masses. Another mass spectrometry study of intact-cell with

Sepedonium strains (Neuhof et al. 2007c) also confirmed that these signals could be used as specific biomarkers for the differentiation below the species level. Although the dominating peaks are suspected to be composed in part of hydrophobins (mass range *m/z* 6,000–8,000), we decided in our study to keep the whole mass range (*m/z* 3,000–20,000) to enable a more accurate discrimination between the species, based not only on the hydrophobin-mediated interactions with the environment. The use of hydrophobins as biomarkers for MALDI-TOF MS analyses will be further evaluated and used as an additional tool for more subtle classification at the subspecies or strain level, as previously proposed (Neuhof et al. 2007b, c).

The second major group of important peptides was detected between *m/z* 2,000 and 5,000 (Fig. 1). This suggests that not only hydrophobins, but also many other peptides and proteins can be detected by the MALDI-TOF MS system. This is not comparable with previous observations suggesting that hydrophobins were the only dominant peptides in the spectra (Neuhof et al. 2007b). According to our work, characterization of *Trichoderma* strains can be carried out using a much larger mass range.

Intact-cell MALDI-TOF MS analyses of *T. brevicompactum* strains have recently been published (Degenkolb et al. 2008c). When comparing the mass spectra of 2-day old (Fig. 1), respectively, 6-day old (Fig. 3 in Degenkolb et al. 2008c) cultures of *T. brevicompactum* CBS 112444, only the mass limit of presence of the peaks (*m/z* 12,000) was the same in both results. In general, in the 2-day old culture, the detected peptides were more numerous. Moreover, while in the 6-day old culture only one peak dominated (*m/z* 7,116), in the 2-day old material, many peptides were present at high intensities. These differences reflect the high activity detected in young cultures, in comparison to older ones, which probably have entered into a steady-state. This demonstrates the crucial importance of standardizing growth conditions to obtain comparable mass spectra. Moreover, the different hydrophobin patterns observed according to the age of the cultures confirm previous results showing that mycelia in differing physiologic states provide differential expression of the hydrophobin genes (Neuhof et al. 2007b).

As seen in Table 2, the SuperSpectra™ created and inserted into the SARAMIS™ database did not include peptaibiotics, which have masses smaller than *m/z* 2,000 (Neuhof et al. 2007a). Their wide mass range (*m/z* 3,000–20,000), however, included the hydrophobins and was representative enough to enable good comparisons between the species and further correct identifications.

The MALDI-TOF MS analysis accurately reflected the phylogenetic classification reported in Fig. 2 as well as in recent publications (e.g., Degenkolb and Brückner 2008) and presented in the ISTH web site (www.isth.info). Strains

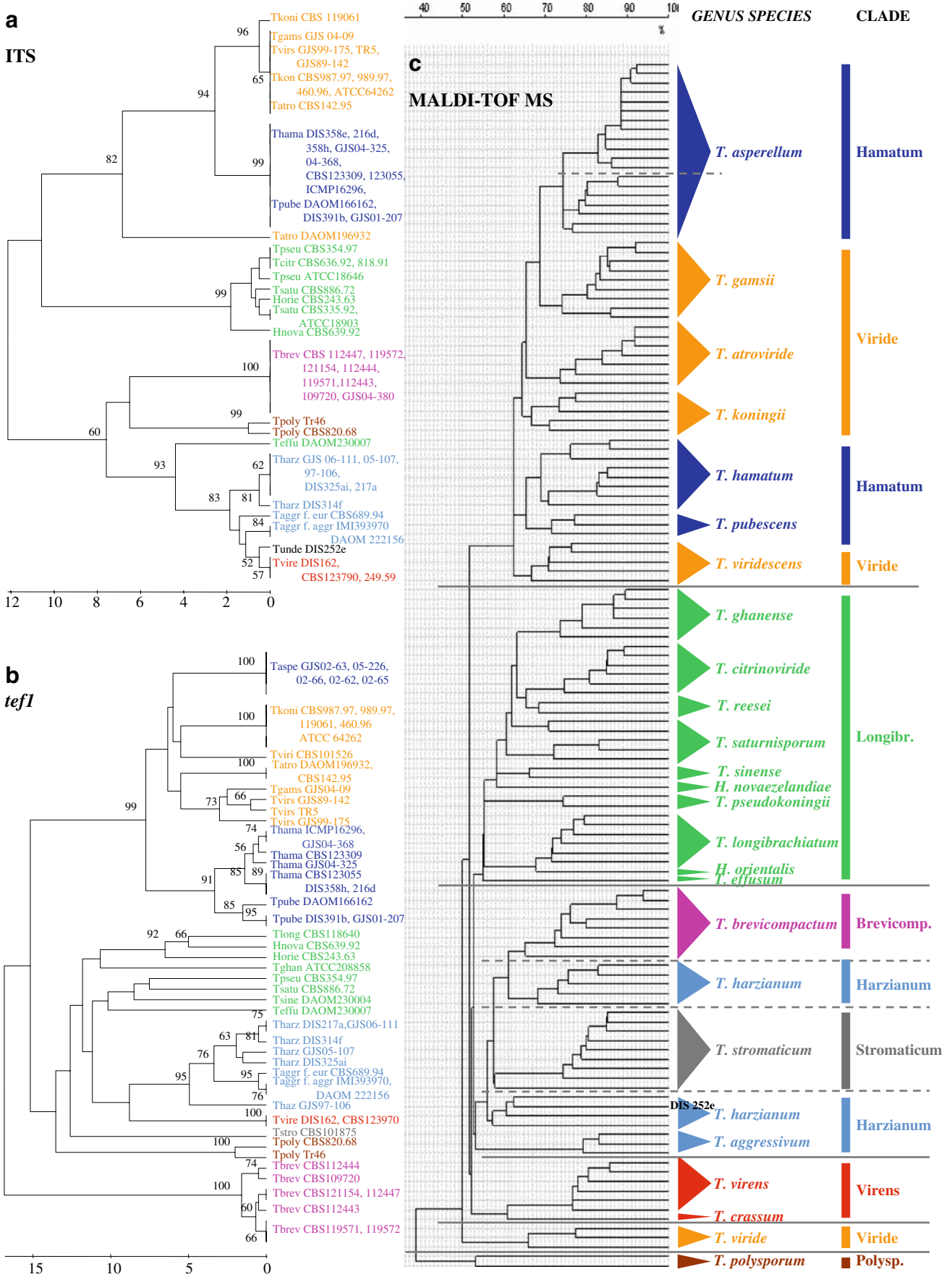
Fig. 2 Taxonomic trees after UPGMA clustering anaysis of **a** ITS sequences from GeneBank (bootstrap, 1,000 replicates; model, number of nucleotide differences), **b** *tef1* gene sequences from GeneBank (bootstrap, 1,000 replicates; model, number of nucleotide differences), and after single-linkage clustering analysis of **c** MALDI-TOF mass spectra (error 0.08%, range from *m/z* 3,000 to 20,000). Hnova *H. novaezelandiae*, Horien *H. orientalis*, Taggr *T. aggressivum*, Taspe *T. asperellum*, Tatro *T. atroviride*, Tbrev *T. brevicompactum*, Tcitr *T. citrinoviride*, Teffu *T. effusum*, Tgams *T. gamsii*, Tghan *T. ghanense*, Thama *T. hamatum*, Tharz *T. harzianum*, Tkoni *T. koningii*, Tlong *T. longibrachiatum*, Tpoly *T. polysporum*, Tpseu *T. pseudokoningii*, Tpube *T. pubescens*, Tsatu *T. saturnisporum*, Tsine *T. sinense*, Tstro *T. stromaticum*, Tunde *T.* undescribed, Tvire *T. virens*, Tviri *T. viride*, Tvirs *T. viridescens*, *Continuous lines* main clusters, *discontinuous lines* less relevant clusters

were mostly placed in their 'correct' clade and, in most cases, strains identified by DNA sequence analysis clustered together by MALDI-TOF MS (Fig. 2). The MALDI-TOF MS results confirmed several molecular phylogenetic studies (Druzhinina et al. 2005; Hermosa et al. 2004; Kullnig-Gradinger et al. 2002; Samuels et al. 2006) in the separation of the morphologically defined *Trichoderma* sect. *Pachybasium* Bissett (Bissett 1991) into sections A1, A2 (Viride Clade) and B (Fig. 2). In previous investigations (Druzhinina et al. 2005; Kullnig-Gradinger et al. 2002), the Longibrachiatum Clade was considered the phylogenetically most distant group compared to all other sections. Our results, however, place Longibrachiatum isolates in a more central position within the genus *Trichoderma*, although some differences of clustering were observed between ITS, *tef1* and MALDI-TOF MS analyses (Fig. 2).

Recent data based on multilocus phylogenetic analysis of clinical and wild-type isolates (Druzhinina et al. 2008) showed that *H. orientalis* is not the teleomorph of *T. longibrachiatum* as previously hypothesized (Samuels et al. 1998), but that both taxa represent individual phylogenetic species. MALDI-TOF MS and *tef1* sequencing confirmed their close relatedness, as well as a good separation of the two species (Figs. 2b, c and 3), thus supporting the hypothesis of Kubicek et al. (2008b) that they could have evolved in parallel from a common ancestor, forming two sympatric species.

Phylogenetic analysis indicated that the Brevicompactum Clade represents a unique lineage in *Trichoderma* (Degenkolb et al. 2008c; Kraus et al. 2004). These authors also reported that it was difficult to assess its closest relatives (see also Fig. 2), despite its 'Pachybasium' morphology. MALDI-TOF MS results grouped the Brevicompactum Clade with other species having 'Pachybasium' morphology.

The Polysporum Clade differs morphologically from all other *Trichoderma* considered in this study by its production of white conidia. This clade undoubtedly belongs to *Trichoderma/Hypocrea* (Kindermann et al. 1998; Lu et al.


a
ITS
Tkoni CBS 119061
Tgams GJS 04-09
Tvirs GJS99-175, TR5, GJS89-142
Tkon CBS987.97, 989.97, 460.96, ATCC64262
Tatro CBS142.95
Thama DIS358e, 216d, 358h, GJS04-325, 04-368, CBS123309, 123055, ICMP16296,
Tpube DAOM166162, DIS391b, GJS01-207
Tatro DAOM196932
Tpseu CBS354.97
Tcitr CBS636.92, 818.91
Tpseu ATCC18646
Tsatu CBS886.72
Horie CBS243.63
Tsatu CBS335.92, ATCC18903
Hnova CBS639.92
Tbrev CBS 112447, 119572, 121154, 112444, 119571,112443, 109720, GJS04-380
Tpoly Tr46
Tpoly CBS820.68
Teffu DAOM230007
Tharz GJS 06-111, 05-107, 97-106, DIS325ai, 217a
Tharz DIS314f
Taggr f. eur CBS689.94
Taggr f. aggr IMI393970
DAOM 222156
Tunde DIS252e
Tvire DIS162, CBS123790, 249.59
12 10 8 6 4 2 0
b
tef1
Taspe GJS02-63, 05-226, 02-66, 02-62, 02-65
Tkoni CBS987.97, 989.97, 119061, 460.96 ATCC 64262
Tviri CBS101526
Tatro DAOM196932, CBS142.95
Tgams GJS04-09
Tvirs GJS89-142
Tvirs TR5
Tvirs GJS99-175
Thama ICMP16296, GJS04-368
Thama CBS123309
Thama GJS04-325
Thama CBS123055
DIS358h, 216d
Tpube DAOM166162
Tpube DIS391b, GJS01-207
Tlong CBS118640
Hnova CBS639.92
Horie CBS243.63
Tghan ATCC208858
Tpseu CBS354.97
Tsatu CBS886.72
Tsine DAOM230004
Teffu DAOM230007
Tharz DIS217a,GJS06-111
Tharz DIS314f
Tharz GJS05-107
Tharz DIS325ai
Taggr f. eur CBS689.94
Taggr f. aggr IMI393970, DAOM 222156
Thaz GJS97-106
Tvire DIS162, CBS123970
Tstro CBS101875
Tpoly CBS820.68
Tpoly Tr46
Tbrev CBS112444
Tbrev CBS109720
Tbrev CBS121154, 112447
Tbrev CBS112443
Tbrev CBS119571, 119572
15 10 5 0
c
MALDI-TOF MS
40 50 60 70 80 90 100
%
GENUS SPECIES
CLADE
T. asperellum
Hamatum
T. gamsii
T. atroviride
Viride
T. koningii
T. hamatum
Hamatum
T. pubescens
T. viridescens
Viride
T. ghanense
T. citrinoviride
T. reesei
T. saturnisporum
T. sinense
H. novaezelandiae
T. pseudokoningii
T. longibrachiatum
H. orientalis
T. effusum
Longibr.
T. brevicompactum
Brevicomp.
T. harzianum
Harzianum
T. stromaticum
Stromaticum
DIS 252e
T. harzianum
T. aggressivum
Harzianum
T. virens
T. crassum
Virens
T. viride
Viride
T. polysporum
Polysp.

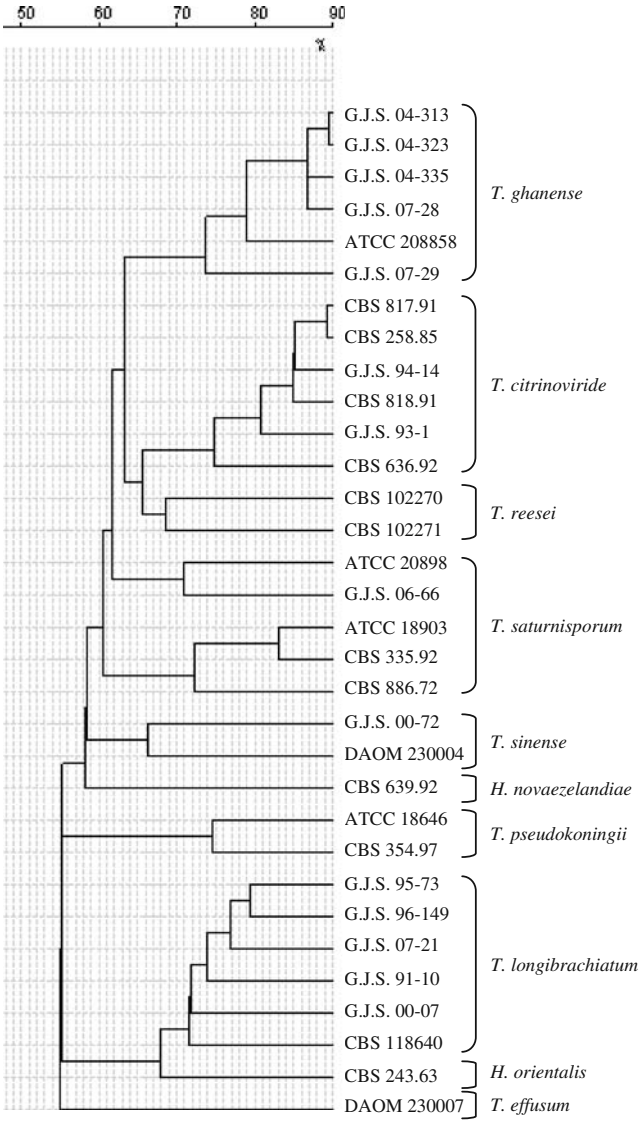


Fig. 3 Dendrogram resulting from single-linkage cluster analysis of MALDI-TOF mass spectra *Trichoderma/Hypocrea* strains, Clade Longibrachiatum. Error 0.08%; Mass range from *m/z* 3,000 to 20,000; Mass peaks mean/median: 102.71/97; Mass peaks min/max: 41/291

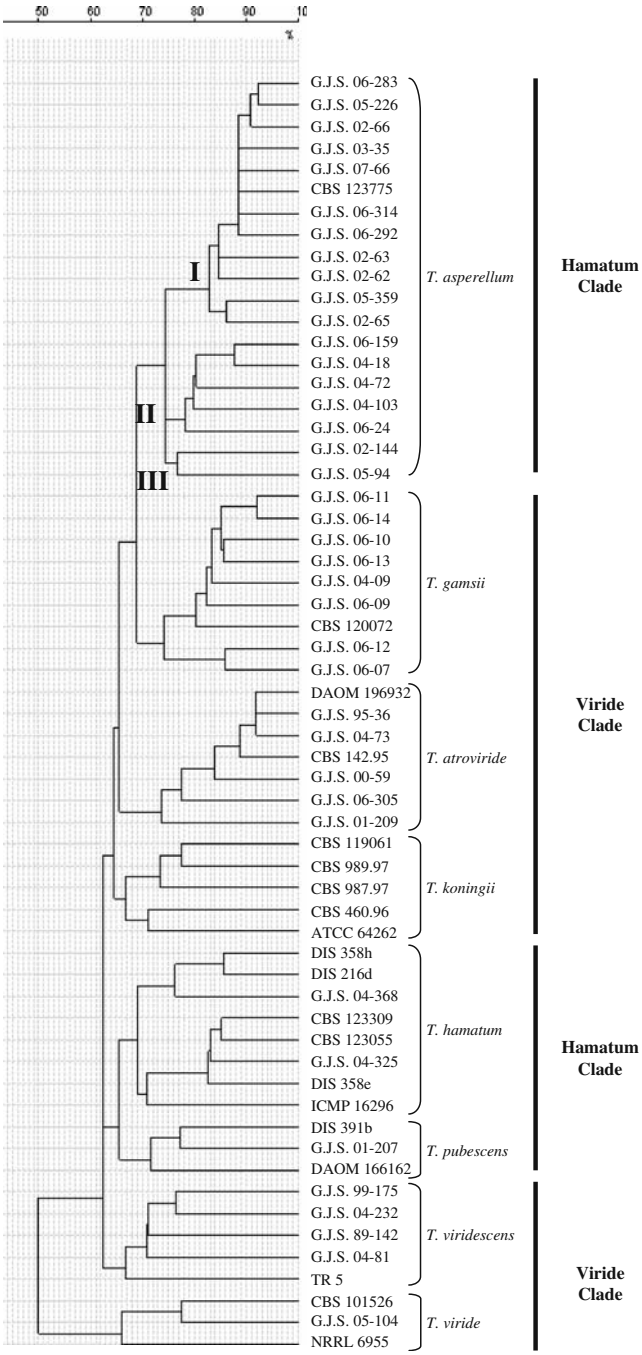


Fig. 4 Dendrogram resulting from single-linkage cluster analysis of MALDI-TOF mass spectra, *Trichoderma* strains, Clades Hamatum and Viride. Error 0.08%; mass range from *m/z* 3,000 to 20,000; mass peaks mean/median: 99.05/100; mass peaks min/max: 31/150

2004), and if also the MALDI-TOF MS data are considered (Fig. 2c), this makes *T. polysporum* the ideal "outgroup" for phylogenetic analyses of the genus.

Analysis of *tef1* sequences placed *T. viridescens* phylogenetically close to *T. viride* (Jaklitsch et al. 2006). Conversely, our MALDI-TOF MS analysis placed *T. viride* very distant from all other *Trichoderma* isolates (Fig. 2).

The separation of *T. asperellum* isolates into three clusters (Figs. 4 and 7) confirms unpublished results of ITS, *tef1*, and *rpb2* sequencing (Samuels et al., unpublished), where the isolates were grouped into two clades, the first one corresponding to MALDI-TOF MS clade I and the second comprising isolates in MALDI-TOF MS clades II and III. The representatives of these clades were further characterized by allele-specific PCR primers (Tondje et al. 2007; Bon et al., unpublished). Despite an absence of biogeographic or phenotypic differences between the two groups (Samuels et al., unpublished), the combined DNA sequence analyses and MALDI-TOF MS results lead to the conclusion that the morphological species *T. asperellum*

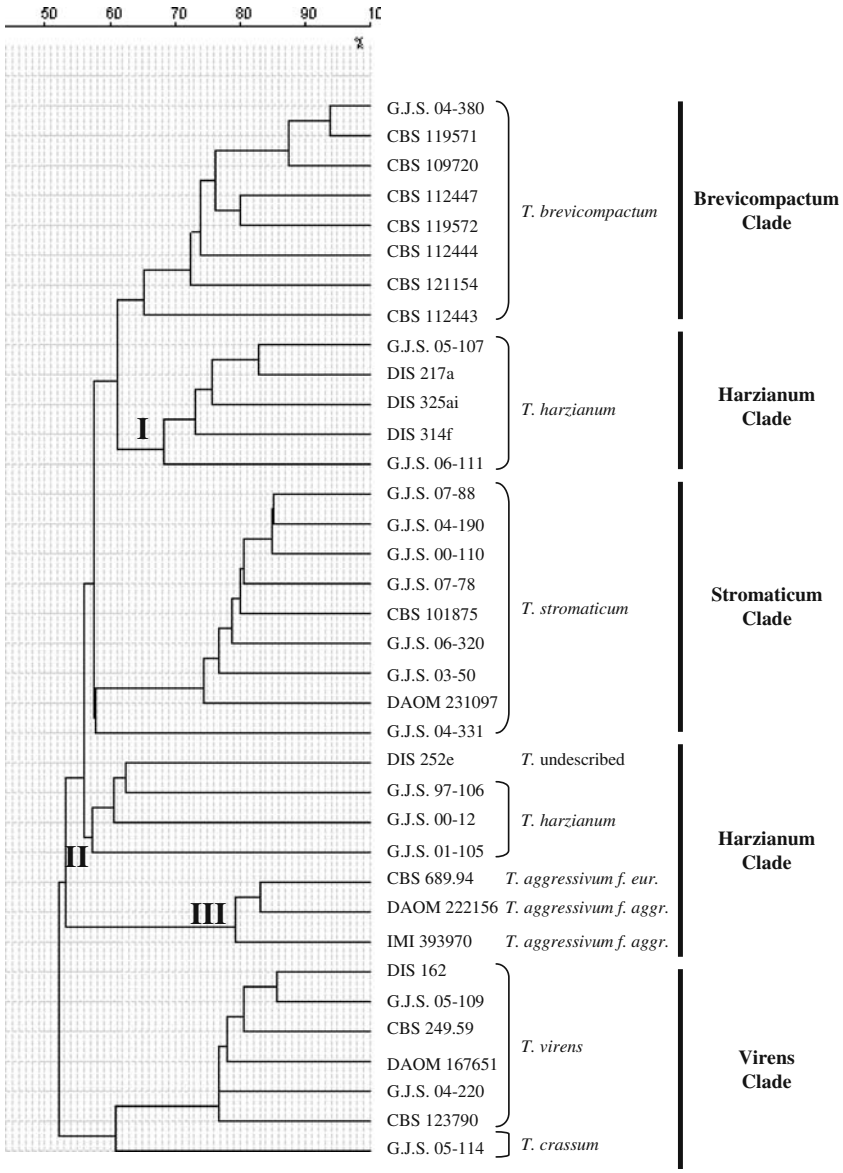


Fig. 5 Dendrogram resulting from single-linkage cluster analysis of MALDI-TOF mass spectra, *Trichoderma* strains, Clades Brevicompactum, Harzianum, Stromaticum, and Virens. Error 0.08%; mass range from *m/z* 3,000 to 20,000; mass peaks mean/median: 102.80/103; mass peaks min/max: 48/153

comprises two taxa. Moreover, this is confirmed by the different hydrophobins profiles observed in clade I and both clades II and III (Fig. 7), reinforcing the hypothesis by Neuhof et al. (2007b, c) that these proteins could be optimal biomarkers at the intraspecific level.

Trichoderma hamatum and *T. pubescens* grouped together (Figs. 2 and 4), as already proposed by molecular taxonomic studies of *Trichoderma* based on single or multigene approaches, which show them to be sister species (Chaverri and Samuels 2003; Degenkolb et al. 2008c; Druzhinina et al. 2005; Hermosa et al. 2004; Kindermann et al. 1998; Kullnig-Gradinger et al. 2002; Samuels 2006; Samuels et al. 2006; Samuels and Ismaiel 2009). *Trichoderma hamatum* is a common and cosmopolitan species with significant genetic variation (Samuels and Ismaiel 2009); this variation is also seen in the MALDI-TOF MS analysis although the present analysis does not agree with the genetic variation. The phylogenetic analysis of *T. hamatum* by Samuels and Ismaiel (2009) was based on three genes (*tef1*, *rbp2*, *act*) and showed several well-supported lineages. One might think that these lineages represent incipient speciation. However, the MALDI-TOF MS results show a different relationship among the reduced set of strains. If the MALDI-TOF MS results also represent the genome, then the phylogenetic lineages revealed by three protein-coding genes differ from the presumed phylogenetic lineages supported by MALDI-TOF MS: the various parts of the genome revealed by the different tools give different stories, but taken as a whole, from a taxonomic point of view, there is no sufficient reason for proposing a taxonomic separation. *T. asperellum,* a member of the Hamatum Clade, clustered distantly from *T. hamatum* and *T. pubescens* but close to *T. gamsii* (Fig. 4).

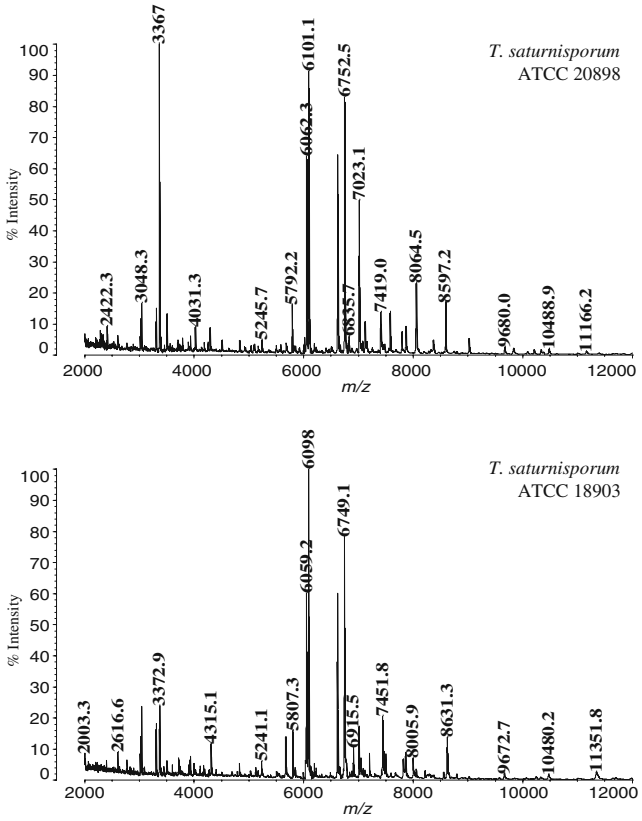


Fig. 6 MALDI-TOF mass spectra of strains representative of the two separated groups of *T. saturnisporum*

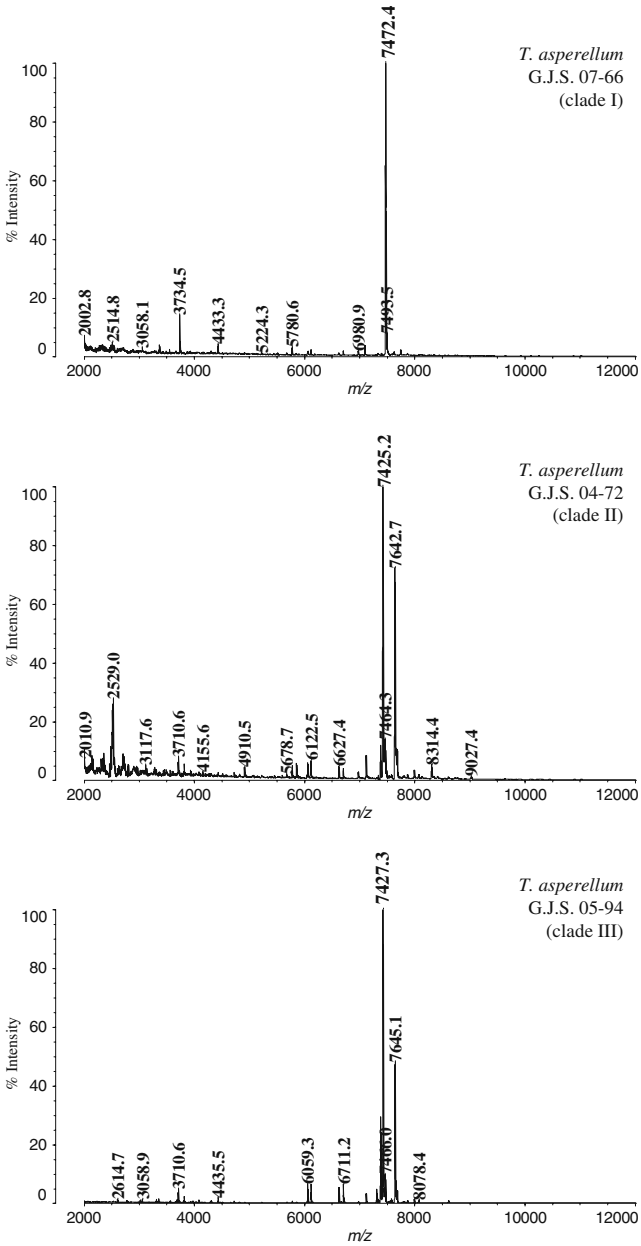


Fig. 7 MALDI-TOF mass spectra of strains from clusters I, II, and III of *T. asperellum*

However, based on *tef1* sequencing, *T. gamsii* is more similar phylogenetically to *T. viridescens* and *T. viride* than to *T. asperellum* (Fig. 2b; and Jaklitsch et al. 2006).

T. saturnisporum strains were separated into two distinct groups (Fig. 3). This reflects the small (1 bp) difference in ITS sequences between CBS 886.72 (South Africa) and three other collections from the USA (2) and Italy (1) reported previously (Kuhls et al. 1997). The two groups most likely represent two separate taxa.

Trichoderma harzianum is one of the most frequently reported species in the genus. However, it is known to be polyphyletic according to morphology and isonitrile antibiotics production, as well as by DNA sequence comparison, RFLP analysis of rDNA, and RAPD analysis (Chaverri et al. 2003; Fujimori and Okuda 1994; Kuhls et al. 1997; Muthumeenakshi et al. 1994). MALDI-TOF MS analysis (Figs. 5 and 8a) reinforced the diversity. Thus, it is likely that two or more taxonomic species will be eventually resolved. We analyzed only a small number of strains that are broadly representative of the *T. harzianum* complex. The analyzed members of the complex were distributed between two distinct clusters: cluster I, a relatively homogeneous group comprising five strains was grouped with the Brevicompactum Clade; and cluster II, consisting of three *T. harzianum* strains (G.J.S. 97-106, 00-12, 01-105) and one undescribed species close to *T. harzianum* (DIS 252e). The analysis showed that cluster II was relatively heterogeneous (57–63% similarity; Fig. 5). A separation of *T. harzianum* into different species or subspecies on the basis of morphological, biochemical, and molecular results is justified, but the MALDI-TOF MS groupings did not reflect phylogeny (Samuels, unpublished), because more distantly related strains (e.g., G.J.S. 05-107 and DIS 217a) clustered together by MALDI-TOF MS, while more closely related strains (e.g., DIS 325ai and GJS 97-106) were separated into different clusters.

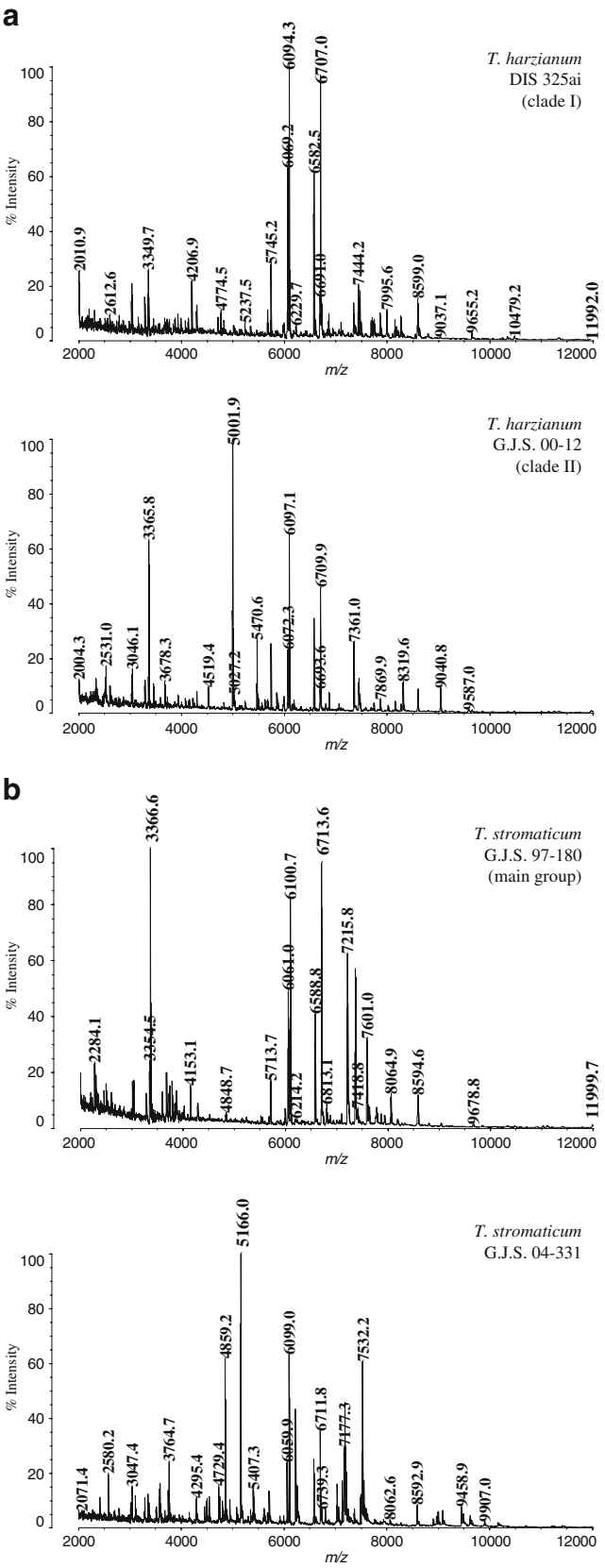


Fig. 8 MALDI-TOF mass spectra of **a** strains representing the two groups of *T. harzianum*, **b** one strain representative of the main group of *T. stromaticum*, and the more distant strain G.J.S. 04-331

The cause of the green mold disease of commercial mushrooms was originally identified as *T. harzianum* on the basis of its morphology. Samuels et al (2002) determined that the cause of the epidemic was the new species *T. aggressivum*. The MALDI-TOF MS results distinguish *T. harzianum* from *T. aggressivum*, but not *T. aggressivum* f. *aggressivum* from f. *europaeum*, the two forms reported by Samuels et al (2002) and Kullnig-Gradinger et al (2002). This difference at the subspecific level was observed by ITS and *tef1* clustering analyses (Fig. 2a, b). Strain DIS 252e represents an undescribed species that is known only as an endophyte of trunks of *Theobroma cacao* and is closely related to *H. epimyces* Pat. (Jaklitsch et al. 2008) and *T. aggressivum* (Samuels, unpublished). The MALDI-TOF MS results as well as ITS sequencing (Fig. 2a, c) indicate that this species is distinct from *T. aggressivum*.

Like *T. harzianum*, *T. stromaticum* is a species complex. De Souza et al. (2006) delimited two AFLP groups within the complex and observed a differential ability to sporulate on infected cacao material in vitro as well as a different growth rate on cornmeal agar. Group 2 included the ex type culture. Sequencing 4 protein-coding genes (*tef1*, *act, cal, rpb2*) and ITS rDNA supports a phylogenetic lineage for de Souza's group 1, but the members of Group 2 do not cluster into a resolved group (Samuels, unpublished). Although the MALDI-TOF MS analysis supports *T. stromaticum* s. lat., it does not show any pattern of clustering among the studied strains. The MALDI-TOF MS result that showed strain G.J.S. 04-331 to be somewhat different from the rest of the analyzed strains (Figs. 5 and 8b) was surprising, because there is no phylogenetic or phenotypic basis for this difference (Samuels, unpublished).

Much work has still to be done to unravel the phylogeny of this genus that has expanded from 9 morphologically defined aggregate species in 1969 (Rifai 1969) to more than 100 phylogenetic species recognized today (www.isth.info, Druzhinina et al. 2006). The phylogenetic species recognized to date are also characterized to a greater or lesser degree by phenotype. Nonetheless, unresolved species complexes still exist wherein there is no visible phenotype despite strongly supported phylogenetic lineages. Should these lineages be recognized with a classical taxonomy? In the case of *T. asperellum*, MALDI-TOF MS agrees with ITS analysis in defining two species, each of which is also supported by sequences of protein-coding genes. In the cases of *T. harzianum, T. hamatum*, and *T. stromaticum*, ITS rDNA is too highly conserved to distinguish groups that are delimited by protein coding genes, a situation that is mirrored by the MALDI-TOF MS results. The resolution

of MALDI-TOF MS, as performed here, is therefore roughly equivalent to ITS rDNA, although evidently more discriminant for some species of Clades Hamatum and Viride. Samuels and Ismaiel (2009) concluded that despite the existence of phylogenetic lineages within *T. hamatum*, they could not recognize the lineages. The conflicting MALDI-TOF MS and DNA sequence results justify that position. The taxonomic separation of *T. harzianum* and *T. stromaticum* has not yet been conclusively decided. The MALDI-TOF MS analysis, on the other hand, closely reflects the genome by representing peptide sequences as expression of genomic differences; therefore, one could conclude from the data presented here that further subdivision of the *harzianum* and *stromaticum* complexes is not warranted.

The analysis of mass ions spectra from intact cells provides a comprehensive picture of the metabolic products of a given sample, with, possibly, a good discrimination of isolates. The creation of SARAMIS™ SuperSpectra™ for *Trichoderma* species (Table 2), which is a by-product of this investigation, is based on this consideration and will allow a reliable identification of most *Trichoderma* strains for which SuperSpectra™ now exist in the database.

This is the first taxonomic analysis of a large number of strains and species of a species-rich genus of microfungi using MALDI-TOF MS. We demonstrate here an effective and quick method for diagnosing species. The MALDI-TOF MS results provide two important taxonomic tools. On the one hand, the technique analyzes peptides, which closely reflect the genome structure; it is therefore a rough equivalent to sequencing, making this method a useful adjunct for determination of species limits. On the other hand, and most importantly, MALDI-TOF MS allows simple, reliable, and quick species identification, thus representing a valid alternative to gene sequencing for species diagnosis of *Trichoderma* and other fungal taxa. The primary advantage of MALDI-TOF MS is the speed by which identifications can be made. The same amount of time as DNA sequence analysis is required to prepare samples (cultures), but the MALDI-TOF MS analysis itself can be completed in a few minutes as opposed to two or more days required for DNA sequence analysis.

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