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Isozyme Analysis and Relationships Among Three Species in Malaysian *Trichoderma* Isolates

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Isozyme and protein electrophoresis data from mycelial extracts of 27 isolates of *Trichoderma harzianum*, 10 isolates of *T. aureoviride*, and 10 isolates of *T. longibrachiatum* from Southern Peninsular Malaysia were investigated. The eight enzyme and a single protein pattern systems were analyzed. Three isozyme and total protein patterns were shown to be useful for the detection of three *Trichoderma* species. The isozyme and protein data were analyzed using the Nei and Li Dice similarity coefficient for pairwise comparison between individual isolates, species isolate group, and for generating a distance matrix. The UPGMA cluster analysis showed a higher degree of relationship between *T. harzianum* and *T. aureoviride* than to *T. longibrachiatum*. These results suggested that the *T. harzianum* isolates had high levels of genetic variation compared with the other isolates of *Trichoderma* species.

Keywords: *Trichoderma harzianum*, isozyme and allozyme activities, protein patterns, cluster analysis

The genus *Trichoderma* contains filamentous fungi. Teleomorphs belonging to the Hypocreales order of the division Ascomycota have great economic importance as producers of enzymes and antibiotics, cell wall lysing enzymes, plant growth promoters, xenobiotic decomposers, and biofungicides for greenhouse and field plants [12]. *Trichoderma* species are free-living fungi that are highly interactive in root, soil, and foliar environments, and have successfully been used in field trials to control crop pathogens. They are opportunistic avirulent plant symbionts and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from diseases.

To date, *Trichoderma* species are the most extensively studied biological control agents (BCAs), and are commercially marketed as biopesticides, biofertilizers, and soil additives [12, 25]. Depending on the strain, *Trichoderma* have numerous advantages as biocontrol agents. For example, (i) colonization of the rhizosphere by the BCA ("rhizosphere competence"), resulting in rapid establishment within the stable microbial communities of the rhizosphere; (ii) control of pathogenic and competitive/deleterious microflora by a variety of mechanisms; (iii) improvement of plant health and (iv) enhanced root growth [12].

Accurate identification of *Trichoderma* species based on their morphological characteristics is still problematic. For this reason, many researchers have used other characteristics as aids to *Trichoderma* systematics, the earliest of which was isozyme analysis [35, 41]. Markert and Moller [26] proposed the term isozyme to describe "the different molecular forms in which proteins may exist with the same enzymatic specificity." This functional definition is intended to be broad, covering all molecular forms of enzymes. The expression of various enzymes differs both temporally (ontogenetic differentiation) and spatially (different tissues) [47]. Isozyme markers can correctly identify several levels of taxa, accessions, and individuals, since the assumption of homology can be more accurate than for some genomic DNA markers [19].

Polyacrylamide gel electrophoresis (PAGE) has been used by geneticists since 1957, when Hunter and Markert [16] used the catalytic properties of enzymes to reveal their presence with histochemical methods. Two properties have made protein-coding genes the preferred tool of geneticists: (i) A substantial proportion of these genes are polymorphic; that is, they have two or more alleles. This property was shown by Lewontin and Hubby [24] in *Drosophila*, and by Harris and Hopkinson [13] in humans. (ii) The alleles of protein-coding genes are generally codominant; that is both alleles are expressed in heterozygous organisms.

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Table 1. List of species, code number, references, and locality for *Trichoderma* isolates analyzed in this study.

Species name	Code	Reference	Locality
T. harzianum	FA2, FA4, FA7, FA8, FA15, FA17, FA24, FA26, FA29, FA30, FA31, FA34, FA36, FA38, FA40, and FA44	This author	Sedenak, Johor Baru
T. harzianum	T32, T60, T66, T71, T79, T80, T100, T101, T102, T121, and T124	Ilias [17]	Negeri Sembilan
T. longibrachiatum	T28, T76, T82, T87, T90, T91, T99, T104, T118, and T120	Ilias [17]	Negeri Sembilan
T. aureoviride	T29, T45, T49, T55, T58, T65, T86, T106, T126, and T127	Ilias [17]	Negeri Sembilan

Isozyme analysis is a powerful technique for taxonomic, genetic, and population studies, and has been successfully applied for identifying fungal species in the genera *Ganoderma* [39], *Fusarium* [20], and *Phytophthora* [11]. In *Trichoderma*, the first characterization was done by Zamir and Chet [50], who reported that the 23 geographically diverse isolates of *T. harzianum* were grouped into 5 types according to their isozyme profiles. The results indicated that enzyme electrophoresis was useful for distinguishing *Trichoderma* at the intraspecies level. In a similar study by Stasz *et al*. [41], 16 enzyme loci showed 109 alleles in a study of 71 strains that were distributed between 5 morphological species; namely, *T. harzianum, T. hamatum, T. koningii, T. pseudokoningii*, and *T. viride*. Szekeres *et al*. [42] reported that the cellulose-acetate electrophoresis (CAE) technique detected several species; namely, *T. pseudokoningii*, *T. koningii*, *T. citrinoviride*, *T. longibrachiatum*, *T. virens*, and *T. harzianum*. These results were also supported by Bissett's [6] morphology-based classification.

Isozymes have been applied as molecular-genetic markers for studying genetic diversity and phylogenetic affinities among three species of *Trichoderma* isolates and for distinguishing among them at the species levels. The aims of this study were to describe the electrophoretic isozyme and protein phenotypes and their variation patterns, and to examine genetic differences by cluster analysis using allele frequencies of the gene loci and the variation in the banding patterns of the different total protein zones.

Materials and Methods

Trichoderma Isolates

A total of 47 *Trichoderma* isolates were used; 31 were from the collection of the Mycology and Plant Pathology Laboratory, Department of Biology, Universiti Putra Malaysia, and 16 were fresh isolations morphologically identified as *T. harzianum* (Table 1). In total, 27 isolates were putatively identified as *T. harzianum*, 10 as *T. aureoviride*, and 10 as *T. longibrachiatum*. All isolates were cultured in potato dextrose agar (PDA) (Difco; U.S.A.).

Sample Preparation

For isozyme and total protein analysis, isolates were grown in 250-ml Erlenmeyer flasks containing 100 ml of basal medium (peptone 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l, KH_2PO_4 0.5 g/l, glucose 2.0 g/l, asparagine 2.68 g/l, trace elements 10.0 ml/l, thiamine 0.002 g/l, in water). Actively growing hyphal tips of 4-day-old isolates were cut with a 5-mm diameter cork borer and transferred into flasks. Each inoculated flask was incubated at 28±2°C for 9 days, and the resulting mycelial mass was harvested by filtering through a double-layered muslin cloth before being washed twice with 100 ml of ddH_2O and lyophilizing. The mycelia from each isolate were placed into individual plastic bags and excess liquid drained out, before labeling and storing at −20°C until enzyme extraction. Frozen mycelia were removed and immediately ground to powder by using a mortar and pestle that had been sterilized by swabbing with ethanol. The samples were then placed in microcentrifuge tubes and kept at −20°C until used.

Enzyme Extraction and PAGE

Ground mycelial powder (1 g) from individual isolates was added to 600 µl of extraction buffer (Trizma base 6.06 g/l, $CaCl_2$ 1.40 g/l, NAD 0.07 g/l, NADP 0.07 g/l, glycylglycine 1.32 g/l, in water, pH 8.00) in microcentrifuge tubes. Isolates were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was transferred into a sterilized microcentrifuge tube for isozyme assay. Isozyme extractions were prepared every 3 months throughout the experiment. The supernatants from the isozyme and protein extractions were soaked onto 4.0×1.0 mm pieces of Whatmann No. 17 filter paper and inserted into the wells of discontinuous polyacrylamide gels (containing acrylamide, N,N-methylene-*bis*-acrylamide, 10% ammonium persulfate, TEMED, and ddH_2O). One filter paper piece was inserted into 1% (w/v) bromophenol blue for use as a migration marker.

The gel plates were placed on a horizontal cooling plate at 4°C on top of two custom-made buffer tanks (approximately 20 cm×5 cm×

Table 2. Allozyme and protein systems utilized in this study.

Allozyme	Abbreviation	E.C. No.	Buffer[a]
α-Esterase	α-EST	E.C. 3.1.1.1	PEB
Malate dehydrogenase	MDH	E.C. 1.1.1.37	PEB
Acid phosphatase	ACP	E.C. 3.1.3.2	TBE
Glucose-6-phosphate dehydrogenase	G6PDH	E.C. 1.1.1.49	PEB
Total protein	TP	-	TBE
Superoxide dismutase	SOD	E.C. 1.15.1.1	TBE
Sorbitol dehydrogenase	SORDH	E.C. 1.1.1.14	TC
Malic enzyme	ME	E.C. 1.1.1.40	PEB
Alcohol dehydrogenase	ADH	E.C. 1.1.1.1	PEB

Nomenclature and abbreviations follow Weeden and Wendel [44], based on IUBNC (Nomenclature Committee of the International Union of Biochemists). E.C. No.=Enzyme Commission number.

[a]Buffer: PEB= Poulik gel/electrode buffer (pH 8.0); TBE=Tris-borate gel/electrode buffer (pH 8.2); TC=gel/electrode buffer (pH 7.5).

5 cm), containing electrode buffer. U-Tork tissue paper soaked in electrode buffer (Table 2) was used as a wick to transport the ionic charges between the gel and buffer. The gel was covered with a polythene sheet to prevent desiccation during electrophoresis, which was carried out at 4°C for 3 h at a constant current of 50 mA and voltage of 250 V. The electrophoresis was stopped when the bromophenol blue marker had migrated to the edge of the gel.

Isozyme and Total Protein Staining

The gels were stained for 8 enzyme systems, and the enzymatic activities were detected using agar overlays. The staining protocols were according to Pasteur *et al.* [31] for acid phosphatase (AP) (modified: 0.2 g of Na-α-napthylphosphate, 0.2 g of fast blue RR, and 200 ml of 0.5 M acetate buffer, pH 5.0), sorbitol dehydrogenase (SODH) [modified: 0.5 g of sorbitol, 0.4 ml of 0.5 M $MgCl_2$, 4 ml of NAD (1%), 2 ml of NBT (1%), 0.6 ml of MTT (1%), 1 ml of PMS (1%), and 80 ml of Tris-HCl buffer, pH 8.0], and alcohol dehydrogenase (ADH) [modified: 6 ml of ethanol (95%), 0.4 ml of 0.5 M $MgCl_2$, 4 ml of NAD (1%), 2 ml of NBT (1%), 0.6 ml of MTT (1%), 1 ml of PMS (1%), and 80 ml of 0.5 M Tris-HCl, pH 8.0]; according to Harris and Hopkinson [13] for α-esterase (α-EST) [modified: 0.06 g of α-napthyl acetate, 0.12 g of fast blue RR, 180 ml of esterase buffer (22 g/l $NaH_2PO_4 \cdot 2H_2O$+28.55 g/l $Na_2HPO_4 \cdot 2H_2O$, pH 8.0), and 6 ml of acetone], glucose-6-phosphate dehydrogenase (G6PDH) [modified: 60 mg of NADP, 4 mg of NBT, 40 mg of PMS, 50 ml of Tris-buffer (0.5 M Tris-HCl, pH 7.10), 40 mg of Na_2 glucose-6-phosphate, and 90 ml of distilled water], and superoxide dismutase (SOD) [modified: 0.40 ml of 0.5 M $MgCl_2$, 2 ml of NAD (1%), 2 ml of NBT (1%), 1 ml of PMS (1%), and 80 ml of Tris-buffer, pH 7.10], and according to Shaw and Prasad [37] for malate dehydrogenase (MDH) [modified: 1.2 g of DL-malic acid, 0.1 g of NAD, 0.02 g of PMS, 0.3 g of NBT, 50 ml of 0.5 M Tris buffer (pH 8.7), and 170 ml of distilled water] and malic enzyme (ME) [modified: 4 ml of NADP (1%), 4 ml of NAD (1%), 2 ml of PMS (1%), 3 ml of MTT (1%), 120 mg of DL-malic acid, 5 ml of 0.5 M Tris-HCl (pH 8.0), and 17 ml of distilled water]. For total protein (TP), the gel stain protocols (modified: 1 g of Coomassie blue, 45 ml of methanol, 10 ml of acetic acid, and 45 ml of distilled water) were according to Harris and Hopkinson [13].

All isolates were extracted and analyzed on three occasions in separate runs. The most common allele (for isozymes) at each locus was assigned a relative mobility (R_m) value. The values were unified for the selected standard electrophoresis system with the extraction method used to normalize the reference isolates on the same gel slabs in different combinations. The bands were ordered alphabetically, based on their relative mobilities, with the band closest to the anode designated as "A" for each enzyme. Photographs of the visible bands on the gels were taken. Schematic zymograms of the average R_m value were drawn from the bands. The relative mobility value was calculated as

$$R_m = \frac{\text{Distance to which the isozyme band migrated}}{\text{Distance to which the marker dye migrated}}$$

Gel Scoring and Data Analysis

The data were collected immediately after the isozyme and total protein activity staining. Only clear and consistent bands were scored. Loci and protein patterns were numbered consecutively and alleles labeled as described above. Different patterns that occurred in each zone of activity were scored as discrete variables, using "1" to indicate the presence and "0" to indicate the absence of a unique pattern. The isozyme and total protein variations at each locus/zone were calculated using the POPGENE 32 version 1.31 software based on the computer program of Yeh and Yang [49]. The percentages of polymorphic loci (P), and the mean number of alleles per locus (A), and per polymorphic locus (A_P), were calculated, along with the effective numbers of alleles (A_E), observed heterozygosity (H_O), and expected mean heterozygosity (H_E) with respect to the Hardy–Weinberg equilibrium [14, 15]. Loci were considered polymorphic if more than one allele was detected. The levels of genetic variation were calculated for individual isolates, and for all the individuals pooled across all isolates.

Heterozygosity (H_E) was defined as the probability of an individual containing two alleles of different sizes at a particular locus, when sampled at random. Heterozygosity at a locus was calculated as

$$H = 1 - \sum x_i^2,$$

where x_i=the frequency of the *i*th allele [23, 29].

Fixation indices (F) reflecting deviations from Hardy–Weinberg equilibrium [48], and outcrossing rates (t) were estimated using $t = (1-F)/(1+F)$ [45]. The partitioning of genetic diversity within and among all isolates was analyzed using F-statistics [28] according to the equations of Weir and Cockerham [46].

The relationships among individual isolates were determined by the distance matrix method. The data were entered into the genetic distance matrix software RAPDistance 104 (Exeter Software, Setauket, NY, U.S.A.; http://www.anu.edu.au/BoZo/software/index.html). The

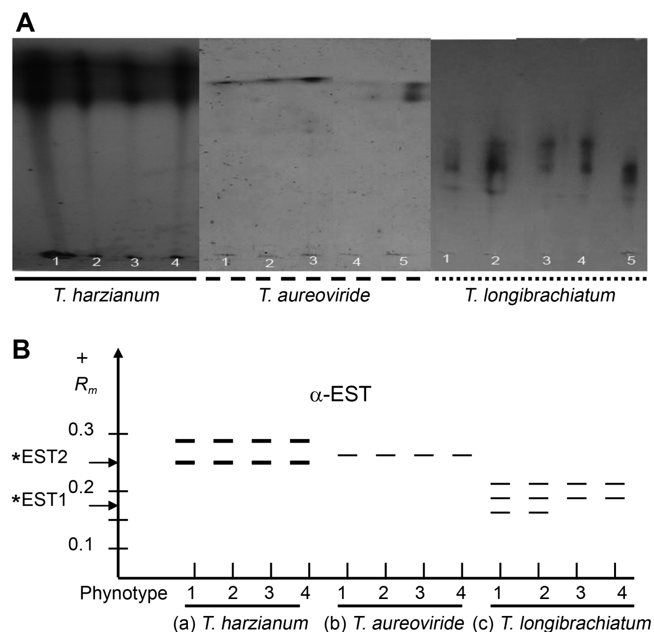

Fig. 1. Gel electrophoresis patterns of α-esterase.
A. The gel photograph representing α-esterase banding patterns in isolates of *T. harzianum* (Lanes left to right: FA2, FA4, FA7, and FA8), *T. aureoviride* (Lanes left to right: T29, T45, T49, T55, and T67), and *T. longibrachiatum* (Lanes left to right: T28, T76, T82, T90, and T99). **B**. Schematic representation of electrophoretic patterns of α-esterase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c). The stars (*) and arrows (→) indicate locus and alleles.

Nei and Lei [30] similarity coefficients were calculated for all pairwise comparisons between individual and group isolates in order to generate a distance matrix based on them. Cluster analysis was performed based on the unweighted pair-group method with arithmetic averaging (UPGMA), as in Sneath and Sokal [40], to generate a dendrogram using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) software package version 1.80 [33] to depict the genetic relationships among the individual and group isolates of three *Trichoderma* species.

Results

Isozyme Banding Pattern

High enzyme activity and informative electrophoretic bands were obtained from *Trichoderma* isolate mycelia. The mycelia were used immediately, owing to the detrimental effect on enzymatic activity of any storage, even at −20°C. The electrophoretic patterns of *Trichoderma* isolate enzymes appeared to be controlled by 10 loci. Allele frequencies were directly calculated from observed genotypes at these loci.

R_m values were calculated using references of isolates in different combinations, on the same gel slab. Mobility identities and differences in closely migrating bands were assessed on gel slabs, using two or more alternative gel buffers or electrode buffers, to find the best method for resolving each enzyme.

α-Esterase (α-EST, E.C. 3.1.1.1)

T. harzianum isolates showed two bands (EST1 and EST2) from a single EST locus with relative R_m values of 0.260 and 0.300, respectively (Fig. 1A). Fig. 1B, the diagrammatic representations of α-EST electrophoretic phenotypes shows the different banding patterns between *T. harzianum* isolates and those of the other two species. Isolates 1 to 8 of *T. aureoviride* showed monomorphic bands, whereas two isolates (T126 and T127) showed heterozygosity. Ten isolates of *T. longibrachiatum* showed two high-mobility bands, depicting heterozygosity.

Malate Dehydrogenase (MDH, E.C. 1.1.1.37)

The MDH zymogram showed two bands (MDH1 and MDH2) at two loci (MDH-A and MDH-B) for all isolates (Fig. 2A). Twenty-four isolates of *T. harzianum*, five of *T. aureoviride*, and six of *T. longibrachiatum* showed a monomorphic band each, whereas three isolates of *T. harzianum* (T66, T60, and FA44) and four isolates of *T. aureoviride* (T86, T49, T45, and T29) showed heterozygosity. As is shown in the diagrammatic representation of MDH phenotypes (Fig. 2B), for each locus, the band closest to the cathode and that closest to the anode showed R_m values

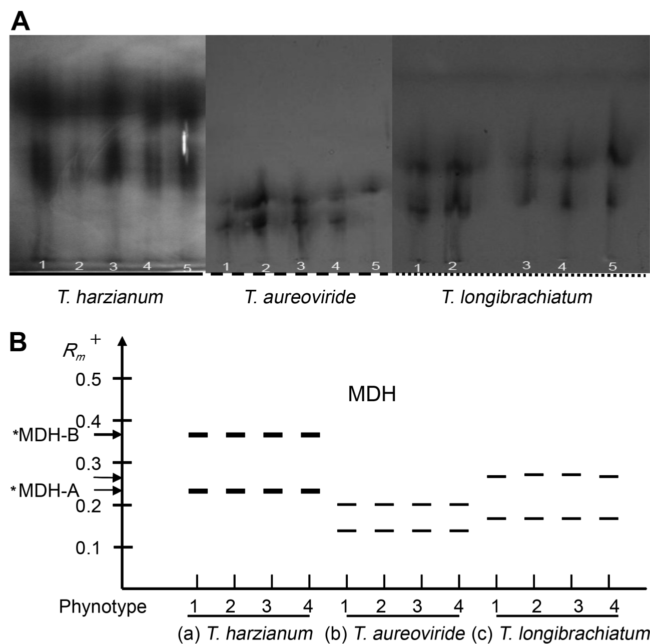

Fig. 2. Gel electrophoresis patterns of malate dehydrogenase.
A. The gel photograph representing malate dehydrogenase banding patterns in isolates of *T. harzianum* (Lanes left to right: FA2, FA4, FA7, FA8, and FA15), *T. aureoviride* (Lanes left to right: T127, T126, T106, T86, and T67), and *T. longibrachiatum* (Lanes left to right: T28, T76, T82, T90, and T99). **B**. Schematic representation of electrophoretic patterns of malate dehydrogenase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c). The stars (*) and arrows (→) indicate locus and alleles.

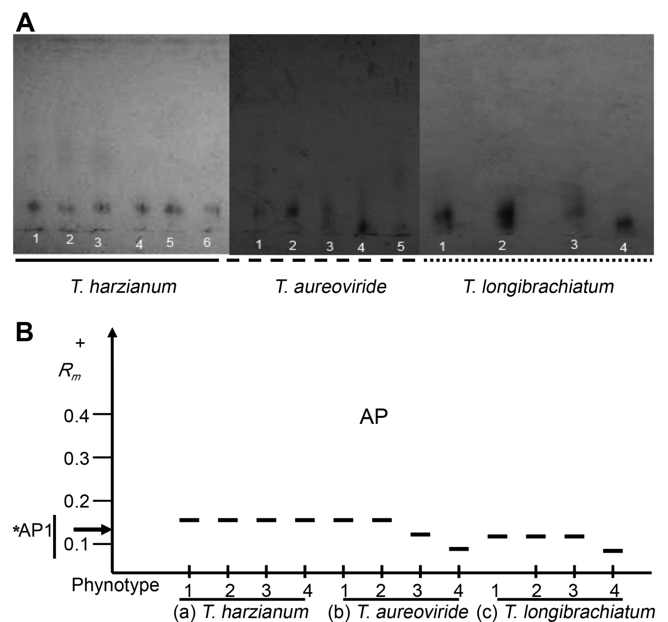

Fig. 3. Gel electrophoresis patterns of acid phosphatase.
A. The gel photograph representing acid phosphatase banding patterns in isolates of *T. harzianum* (Lanes left to right: FA2, FA4, FA7, FA8, FA15, and FA24), *T. aureoviride* (Lanes left to right: T127, T126, T106, T65, and T58), and *T. longibrachiatum* (Lanes left to right: T120, T118, T99, and T90). **B**. Schematic representation of electrophoretic patterns of acid phosphatase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c). The star (*) and arrow (→) indicate locus and alleles.

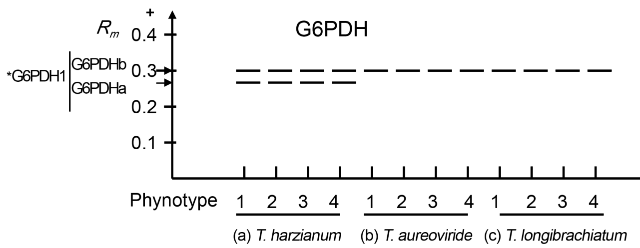

Fig. 4. Schematic representation of electrophoretic patterns of glucose-6-phosphate dehydrogenase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c).
The star (*) and arrows (→) indicate locus and alleles.

of 0.220 and 0.380 for *T. harzianum*, 0.160 and 0.200 for *T. aureoviride*, and 0.180 and 0.300 for *T. longibrachiatum* isolates.

Acid Phosphatase (ACP, E.C. 3.1.3.2)

ACP was monomeric and controlled by a single locus (AP1) with a single band (Fig. 3A) that was strongly expressed in 27 *T. harzianum*, 10 *T. aureoviride*, and 10 *T. longibrachiatum* isolates. All of the isolates were homozygous as shown in the diagrammatic representation in Fig. 3B.

Glucose-6-Phosphate Dehydrogenase (G6PD, E.C. 1.1.1.49)

G6PD was monomeric, controlled by a single locus, G6PDH1, with the *T. harzianum* isolates showing one band of G6PDHa (R_m=0.275) for low mobility and one of G6PDHb (R_m=0.300) for high mobility, indicating the isozyme activity of two alleles (Fig. 4). Isolates of *T. aureoviride* and *T. longibrachiatum* produced a single band from a single locus (G6PDH1) with an R_m value of 0.300.

Total Protein (TP)

TP was expressed by three different zones in all isolates, such as TPA, TPB, and TPC, respectively, which ran in the fast, slow, and medium zones. The slow zone of *T. harzianum* and *T. aureoviride* isolates consisted of a single focused monomorphic band, except for three isolates (T66, T71, and T121) of *T. harzianum* that showed two bands (Fig. 5). The faster zone, closest to the anode of *T. harzianum* and *T. aureoviride* isolates, consisted of a single locus possessing two bands, depicting heterozygosity. Similarly, the faster zone of *T. longibrachiatum* consisted of a single locus possessing three bands, showing heterozygosity. The medium-mobility zone of all isolates displayed a single locus with two bands representing heterozygosity.

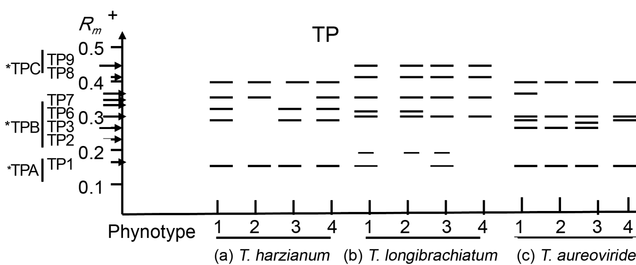

Fig. 5. Schematic representation of electrophoretic patterns of total protein in isolates of *T. harzianum* (a), *T. longibrachiatum* (b), and *T. aureoviride* (c).
The stars (*) and arrows (→) indicate zones and bands.

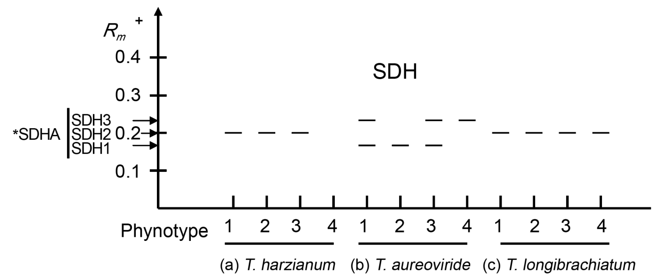

Fig. 6. Schematic representation of electrophoretic patterns of sorbitol dehydrogenase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c).
The star (*) and arrows (→) indicate locus and alleles.

Sorbitol Dehydrogenase (SORDH, E.C. 1.1.1.14)

SORDH was controlled by a single locus, SDHA, which was strongly expressed in the mycelia of all isolates (Fig. 6). *T. harzianum* isolates showed a single banded pattern in the gel, showing monomorphism for this isozyme. *T. aureoviride* isolates showed polymorphism for two bands, SDH1 and SDH2 in the gel, with the presence of heterozygosity, but *T. longibrachiatum* isolates showed a single monomorphic band on the gel.

Superoxide Dismutase (SOD, E.C. 1.15.1.1)

SOD plays a prime role in the defense mechanism against oxygen toxicity. Various polymorphisms were found in the SOD isozyme electrophoretic pattern. It showed three loci, SODA, SODB, and SODC, in all isolates (Fig. 7). The

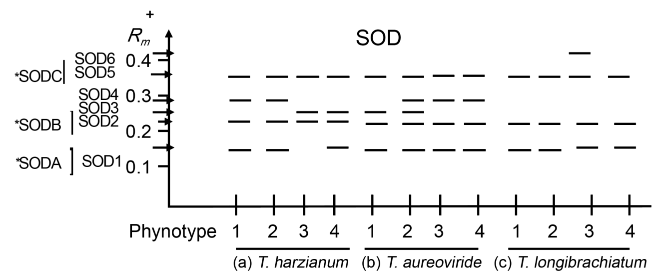

Fig. 7. Schematic representation of electrophoretic patterns of superoxide dismutase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c).
The stars (*) and arrows (→) indicate locus and alleles.

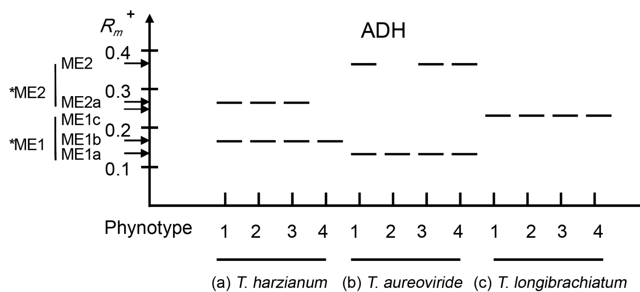

Fig. 8. Schematic representation of electrophoretic patterns of malic enzyme in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c).
The stars (*) and arrows (→) indicate locus and alleles.

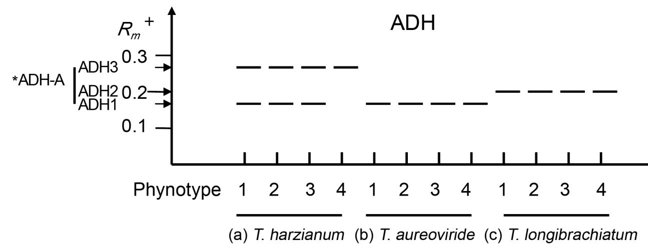

Fig. 9. Schematic representation of electrophoretic patterns of alcohol dehydrogenase in isolates of *T. harzianum* (a), *T. aureoviride* (b), and *T. longibrachiatum* (c).
The star (*) and arrows (→) indicate locus and alleles.

SODA locus displayed a single band (SOD1) in *T. harzianum* and *T. aureoviride* isolates, illustrating homozygosity, and *T. longibrachiatum* isolates showed two bands. Isolates of *T. harzianum* and *T. aureoviride* showed two bands of locus SODB; only *T. longibrachiatum* isolates showed a single band. Locus SODC consisted of a single band in all isolates.

Malic Enzyme (ME, E.C. 1.1.1.40)

ME was controlled by a single locus, ME-A. Isolates of *T. harzianum* and *T. aureoviride* showed bands of ME1 and ME2 on gels at pH 8.00 (Fig. 8). *T. longibrachiatum* isolates showed a single band.

Alcohol Dehydrogenase (ADH, E.C. 1.1.1.1)

ADH was controlled by a single locus, ADH-A, and displayed two bands patterns in *T. harzianum* isolates (Fig. 9). Isolates of *T. aureoviride* and *T. longibrachiatum* demonstrated a single band each.

Loci, Alleles, and Protein Pattern Scoring

Isozyme and protein electrophoresis resulted in clear and consistent staining for 10 loci for eight isozymes, and 3 loci for total protein (TP); namely EST, MDHA, AP, G6PD, SORDH, SOD1, SOD2, SOD3, ME, AD1, TP1, TP2, and TP3. All isozymes and proteins migrated toward the anode. Nine loci were polymorphic in *T. harzianum* (78.57%, Table 3). A total of 25 alleles were detected in all isolates. One allele that was lacking in most isolates, EST-b, was found only in *T. harzianum* isolates at a very low frequency (data not shown). The polymorphic locus MDHA had a faster allele that was prevalent in *T. aureoviride* and *T. longibrachiatum* isolates, whereas the faster allele of MDHA-a was the most frequent in isolates of *T. harzianum* and *T. longibrachiatum*. In all, unusual alleles were found in less than 0.05% from all isolates.

Genetic Similarity

The genetic similarity values among the 47 fungal isolates, based on the scoring data of the isozyme and total protein patterns and the genetic distances calculated among the three groups of *Trichoderma* isolates, are shown in Table 4. The similarity values among the three species ranged from 0.9397 to 0.9642, with the highest value being between isolates of *T. aureoviride* and *T. harzianum*.

Cluster Analysis

Cluster analyses were valuable for determining relationships among the isolates. The cluster analyses were based on the data from all the eight isozymes and the total protein pattern. The combined data give more complete information than if each system were to be analyzed separately. Genetic distances calculated based on the similarity coefficient matrix values among all the different isolates were used in the cluster analysis.

On the basis of the UPGMA cluster analysis, all isolates fell into two major clusters, labeled as I and II (Fig. 10). Major cluster II consisted entirely of 10 isolates of *T. longibrachiatum*, which could be considered as outgroup isolates. Major cluster I was larger and split into two

Table 3. Summary of genetic variation for 10 loci in isolates of three species of *Trichoderma*.

Population	P	A	A_p	A_O	A_E	H_O	H_E	F	t
T. harzianum	0.571	0.7857	0.60714	1.5714	1.2044	0.0350	0.1542	0.62141	0.2334
T. aureoviride	0.562	0.7857	0.57142	0.5136	1.2121	0.0666	0.1466	0.30976	0.5269
T. longibrachiatum	0.428	0.7500	0.50000	1.5000	1.2437	0.0957	0.1425	0.16495	0.7168

P=Percentage of polymorphic loci; A=Mean number of alleles per locus; A_p=Mean number of alleles per polymorphic locus; A_O=Mean observed number of alleles; A_E=Mean effective number of alleles; H_o=Mean observed heterozygosity; H_e=Mean expected heterozygosity; F=Fixation index; t=Outcrossing rate.

Table 4. Nei and Lei [30] similarity (above diagonal) and genetic distances (below diagonal) values for three species of *Trichoderma*.

Species of *Trichoderma*	*T. harzianum*	*T. aureoviride*	*T. longibrachiatum*
T. harzianum	0.0000	0.9642	0.9415
T. aureoviride	0.0358	0.0000	0.9397
T. longibrachiatum	0.0585	0.0603	0.0000

subclusters, GIA and GIB. Subcluster GIB consisted of 10 isolates that were primarily morphologically identified as *T. aureoviride*. Subcluster GIA consisted of 27 isolates that were putatively identified as *T. harzianum*. Subclusters GIA and GIB included isolates of *T. harzianum* and *T. aureoviride*. These subclusters were closely related to *T. harzianum* and *T. aureoviride* isolates, and highly similar to each other (similarity value of 0.9642). Another element of major cluster II was formed by *T. longibrachiatum* isolates, which was clearly separated from major cluster I.

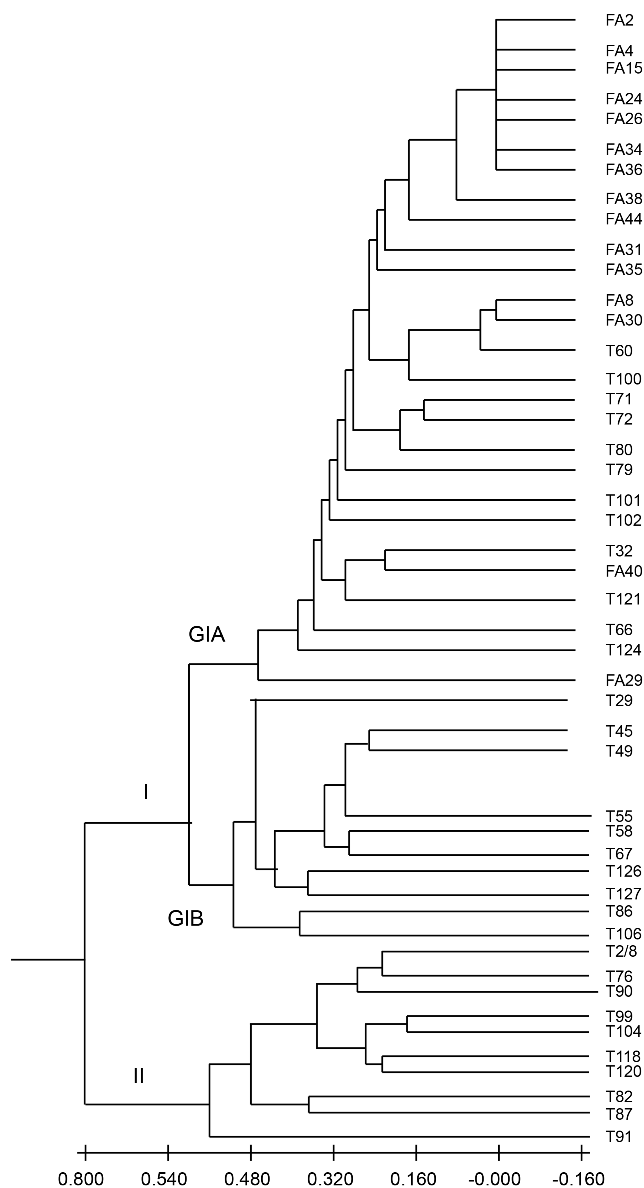

Fig. 10. Dendrogram from UPGMA analysis using genetic distances derived from the simple matching coefficients of Nei and Li [30], based on isozyme and protein pattern data of all *Trichoderma* isolates.

Another cluster analysis was done to ascertain the relationships among the three different fungal species. The genetic distances were calculated based on the similarity coefficient matrix among the isolates of the three different species. The dendrogram obtained using the UPGMA clustering method divided the species into two major clusters, A and B (Fig. 11). Major cluster A consisted of two groups, A1 and A2. Major cluster B contained only one species, *T. longibrachiatum*. Fig. 11 shows two major clusters that were in close agreement with the isolate cluster analysis shown in Fig. 10. The members in major cluster A were dispersed, and split into two distinct groups, A1 and A2, which consisted of *T. harzianum* and *T. aureoviride*, respectively. Major cluster B contained *T. longibrachiatum*, which was clearly separate from major cluster A.

DISCUSSION

Simple, rapid, inexpensive, and reliable diagnostic methods for the identification of *Trichoderma* fungi in microbiology laboratories are needed. The Identification of *Trichoderma* species based on morphological and physiological

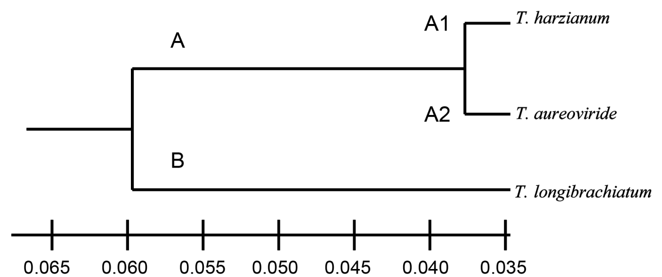

Fig. 11. Dendrogram based on the UPGMA analysis of genetic distances derived from the simple matching coefficient of Nei and Li [30], based on isozyme and protein data, showing the relationships among three species of *Trichoderma*.

characteristics is difficult, as these traits exhibit variations on a continuous scale that may overlap between the species. Although a detailed taxonomic key based on these characteristics is available for the identification of *Trichoderma* species [2–5], molecular techniques can be used for species-specific detection of *Trichoderma*. Amplification of polymorphic DNA, using random primers, was used successfully [43], but the results were not always reproducible, and the profiles might be unstable or sensitive to reaction conditions, as was demonstrated with *Aspergillus fumigatus* [22].

Isozyme and protein patterns analyzed using PAGE are an inexpensive and useful alternative for the identification of *Trichoderma* isolates. PAGE is highly likely to detect markers that can be used to differentiate other relevant fungal species, such as in the genus *Fusarium* [20]. The best resolution of isozymes occurs with pre-cast gels, a short run time (20 min), and minimal equipment requirements, making PAGE a promising and alternative method for culture-based diagnosis that can be applied for the identification of *Trichoderma* isolates, and at a time used more than 100 independent characteristics [34].

Enzyme polymorphism has a simple genetic basis, with the isozyme bands that denote polymorphism at a single locus often termed as allozymes [32]. Allozymes display codominant expression, so heterozygosity can be detected since the heterozygotes can be distinguished from either homozygotes. Heterozygosity at a locus for a monomeric enzyme produces two allozyme bands, whereas three bands are produced if the enzyme is dimeric, because the allelic products of a locus for a dimeric enzyme can associate as both homodimers and heterodimers [27].

The isozyme polymorphism data obtained from this study was at a high enough resolution to support taxonomical identification at the species level. Since SOD isozymes are involved in the defence mechanisms of mycelia [8, 21, 44], their isozyme levels and patterns depend on various environmental factors [18, 38]. A single isozyme (SOD) together with the protein patterns at the three zones that were examined were sufficient for assessing the genetic variability and for taxonomic identification of the isolates of *T. harzianum*, *T. aureoviride*, and *T. longibrachiatum*.

T. harzianum isolates exhibited moderately high levels of genetic variation, especially at the taxon level. The standard mean measure for genetic variation was considerably higher ($P=0.571$, $A=0.7857$, $H_E=0.1542$) for the *T. longibrachiatum* isolates, since the values overall were in general $P=0.428$, $A=0.7500$, and $H_E=0.1425$ (Table 3). However, the population level variation of *T. aureoviride* ($P=0.562$, $A=0.7857$, $H_E=0.1466$) was slightly different from the mean values of the *T. harzianum* isolates. The mean percentage of polymorphic loci in the *T. harzianum* isolates was slightly higher than those for the isolates of *T. aureoviride* ($P=0.562$) and *T. longibrachiatum* (0.428). The mean expected heterozygosity among the isolates of *T. harzianum* was slightly higher ($H_E=0.1542$) than those for isolates of *T. aureoviride* and *T. longibrachiatum*. Moderately high levels of genetic variation were found for the *T. harzianum* isolates.

High levels of genetic diversity are not normally expected in a species that has undergone founder events. For example, the dioecy in *D. angustifolium* may ameliorate the effects of local inbreeding [1]. High genetic diversity has been reported in other cycads that have narrow distributions and relatively low population densities (cf. *Macrozamia riedlei* [7]; and *D. edule* [10]).

The morphological data and genetic polymorphisms revealed by isozyme analysis of the isolates studied corresponded well. The genetic similarity values and the cluster analysis of the 47 isolates from the three *Trichoderma* species showed that the *T. harzianum* isolates were closer to *T. aureoviride* than to *T. longibrachiatum*. The *T. harzianum* and *T. aureoviride* isolates showed greater genetic similarity than the isolates of *T. longibrachiatum* and *T. harzianum*. The UPGMA dendrogram also displayed two major clusters, the first containing isolates of *T. harzianum* and *T. aureoviride*, with the *T. aureoviride* isolates being closely related to *T. harzianum*. Hence, the *T. aureoviride* isolates could not be determined from isozyme analysis alone. Shalini *et al*. [36] reported some identification of strains of *T. aureoviride* within complex collections from Raipur and Bilaspur in India, which were morphologically similar to *T. harzianum* strains. Gams and Bissett [9] stated that *T. harzianum* and *T. aureoviride* differ only in spore morphology, which is a difficult criterion to observe, and easily prone to errors. *T. longibrachiatum* isolates were clearly in the second cluster. The isozyme and protein pattern technique have proven to be useful for the classification of the *Trichoderma* isolates. The levels of genetic variation were moderately high in the *T. harzianum* isolates, especially considering its differentiated status relative to the other isolates. It appears to have slightly different characteristics from the *T. aureoviride* isolates. The *T. longibrachiatum* isolates have completely distinguishing characteristics when compared with the *T. harzianum* isolates. The utilization of genetic markers such as allozymes and isozymes together with morphological analysis to study *T. harzianum* isolates should alleviate confusion in the identification of *Trichoderma* isolates at the species level.

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