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Level in Allozyme Variations of Malaysian Isolates of *Trichoderma harzianum* and its Taxonomic Implications

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Abstract: Electrophoretic variation of nine allozyme systems encoded by 14 gene loci were studied on 47 isolates from 3 species of *Trichoderma* namely, *T. harzianum*, *T. aureoviride* and *T. longibrachiatum*. Polyacrylamide gel electrophoresis was used to investigate the taxonomic circumscription of *T. harzianum* populations and to evaluate the levels of genetic variations and the population structure. The level of genetic variations in *T. harzianum* populations were moderately high (P = 57.10%, A = 0.7857, Ap = 0.60714 and H_E = 0.1542) compared to *T. aureoviride* and *T. longibrachiatum*. The genetic variation attributable to differences among populations was 7.857%. The mean gene flow among populations was Nm = 1.3351. Genetic identities (I) ranged from 0.9397 to 0.9642 with a mean of 0.94846. Outcrossing rates based on fixation indices average (t) was 0.2334. Nevertheless, the alleles for α-EST-b showed a very low frequency of 0.0400. The polymorphic locus of MDH1 was of the fast allele of *T. harzianum*, MD1-a, was prevalent in *T. aureoviride* and *T. longibrachiatum* populations. Using a UPGMA cluster analysis, *T. harzianum* and *T. longibrachiatum* populations were totally separated in these cluster except *T. aureoviride* populations. *Trichoderma harzianum* populations found high levels of genetic variations comparing with others populations of *Trichoderma* species.

Key words: Trichoderma, polyacrylamide gel electrophoresis, allozymes, genetic variations, identification of fungi

INTRODUCTION

The genus *Trichoderma* has been extensively studied for their abilities to rapidly colonize substrates (Grondona *et al*., 1997) for the induction of acquired systemic resistance in plants (Enkerly *et al*., 1999), for the growth inhibitory effect of their metabolites on other pathogens (Keszler *et al*., 2000; Sivasithamparam and Ghisalberti, 1998) and for the production of cell wall-degrading enzymes against many plant pathogens (Lorito, 1998). Species of *Trichoderma* have been used as agents of biological control of plant diseases (Howell, 2003) whether in the form of whole cell or protein formulations or used for expressed genes in transgenic plants (Kubicek *et al*., 2001; Monte, 2001).

Avise (1974) was conducting many procedures that had become standard in population genetics but unnecessary limitations to systematic. These included (1) using only the allozymic component of enzymatic variation, (2) summarizing particulate allozymic data into distance or similarity coefficients and (3) employing only phenetic methods to estimate relationships. After that, all three of these points were discussed in Buth's (1984) updated: (1) that allozyme characters could be of systematic value, (2) that allozyme data could be treated in a particulate manner, although the coding of such data remains controversial (Avise, 1983; Murphy, 1993) and (3) obviously both allozymic and isozymic data can be analyzed cladistically.

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The taxonomic position of *Trichoderma* species based on their morphological characteristic is still unsolved. In view of this, many researchers used other characteristics such as isozyme profiles as additional taxonomic criteria in the classification and identification of *Trichoderma* species. A wide range of molecular techniques has been introduced to obtain new characters for the classification of *Trichoderma* techniques include DNA sequencing (Gherbawy *et al*., 2004; Chaverri *et al*., 2003), random amplified polymorphic DNA (RAPD) (Muthumeenakshi *et al*., 1998; Gomez *et al*., 1997), restriction fragment length polymorphism (Bowen *et al*., 1996; Meyer *et al*., 1993) and isozyme analysis (Samuels *et al*., 1994; Stasz *et al*., 1988).

Isozyme analysis is a powerful method for taxonomic, genetic and population studies. The first characterization of *Trichoderma* species by isozyme patterns was done by Zamir and Chet (1985). Twenty-three geographically diverse isolates of *T. harzianum* were grouped into five types according to the isozyme profiles. Results indicated that enzyme electrophoresis is useful for distinguishing *Trichoderma* at the intra species level. Stasz *et al*. (1988) used 16 enzyme loci that resolved 109 alleles in a study of 71 strains that were distributed between five morphological species (Rifai, 1969), namely *T. harzianum*, *T. hamatum*, *T. koningii*, *T. pseudokoningii* and *T. viride*. Samuels *et al*. (1994) showed that 23 enzyme analyses on starch gels were used to detect of *T. longibrachiatum*, *T. pseudokoningii* and *T. reesei*. Leuchtmann *et al*. (1996) studied the enzyme subgroups within *Trichoderma* section *Longibrachiatum*, using 78 strains and ten isozyme systems. Szekeres *et al*. (2006) also found seven appropriate for identifying the species as *T. pseudokoningii*, *T. koningii* or *T. citrinoviride*. The results of this study supported Bissetts (1992) morphology based taxonomic scheme for this section. Grondona *et al*. (1997) characterized 15 isolates of *T. harzianum* on the basis of morphological, physiological, molecular and biochemical features, including 99 isozyme bands revealed by PAGE based on six isozyme systems.

PAGE is a promising alternative technique for isozyme analysis and has been applied successfully for the identification of fungal species within the genera *Ganoderma* (Smith and Sivasithamparam, 2000), *Fusarium* (Laday and Szecsi, 2001) and *Phytophthora* (Oudemans and Coffey, 1991; Goodwin *et al*., 1995). PAGE based multilocus enzyme electrophoresis is used for the investigation of isozyme polymorphisms among *Trichoderma* isolates.

In this study, allozymes were typed from 3 known *Trichoderma* genera. The aims of this study were to (a) determine the profile of 3 *Trichoderma* genera based on 14 allozyme; (b) valuate the levels of genetic variations and population's structure and gene flow taking into explanation of all populations; (c) contrast the results and conclusions with others species of *Trichoderma* populations and (d) examine the genetic differentiation by the cluster analyses based on allele frequencies of the gene loci.

MATERIALS AND METHODS

Isolates of *Trichoderma* Samples

A total of forty- seven *Trichoderma* samples were used in the isozyme analysis of which 31 were from the Mycology Laboratory, Department of Biology, UPM and 16 others were freshly isolated and identified by morphological means in this study (Table 1). The isolates were cultured in liquid

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

Species	Code	Reference	Locality
T. harzianum	FA2, FA4, FA7, FA8, FA15, FA17, FA24, FA26, FA29, FA30, FA31, FA34, FA36, FA38, FA40, FA44,	Present study	Sedenak, Johor baru
T. harzianum	T32, T60, T66, T71, T79, T80, T100, T101, T102, T121, T124,	Illias (2000)	Negeri Sembilan
T. longibrachiatum	T28, T76, T82, T87, T90, T91, T99, T104, T118, T120,	Illias (2000)	Negeri Sembilan
T. aureoviride	T29, T45, T47, T55, T58, T65, T86, T106, T126, T127	Illias (2000)	Negeri Sembilan

Table 2: Allozyme systems resolved in this study

Allozyme	Abbreviation	EC No.	Buffer*
α-Esterase	α-EST	EC 3.1.1.1	TCB/P
Malate dehydrogenase	MDH	EC 1.1.1.37	TCB/P
Acid phosphatase	ACP	EC 3.1.3.2	TBE
Glucose-6-phosphate dehydrogenase	G6PDH	EC 1.1.1.49	TCB/P
General protein	GP	-	TBE
Superoxide dismutase	SOD	EC 1.15.1.1	TBE
Sorbitol dehydrogenase	SORDH	EC 1.1.1.14	TCE
Malic enzyme	ME	EC 1.1.1.40	TCB/P
Alcohol dehydrogenase	ADH	EC 1.1.1.1	TCB/P

Nomenclature and abbreviations follow Wendel and Weeden (1989), based on IUBNC (Nomenclature Committee of the International Union of Biochemists). EC No. = Enzyme Commission No. *Buffer, TCB/P = Tris- Citrate- Borate/Poulik (pH 8.0), TBE = Tris-Borate- EDTA (pH 8.0), TCE = Tris-Citrate- EDTA (pH 6.90)

basal medium, as that described by Nawawi and Ho (1990). These experiments carried out into Mycology and Plant Pathology Laboratory, UPM, Malaysia.

Electrophoresis

Approximately 100 mg of the ground mycelia powder was added 600 μL extraction buffer of centrifuge tubes by Manwell and Baker (1968) method. The samples were then centrifuged at 13,000 rpm for 10 min at 4°C. After that, the supernatants were transferred into 1.5 microcentrifuge tubes which were stored on filter paper wicks at -20°C for further allozyme analysis. These allozyme extractions were prepared every one month until end in this experiment. *Trichoderma* populations were obtained through horizontal gel electrophoresis (8% w/v). For each individual population, allozymic variations were scored for 14 loci (Table 2). Electrophoresis was carried out at 4°C for 3 h (current of 50 mA and voltage of 250 V).

Gel Scoring and Analysis

Loci were numbered consecutively and alleles at each locus were labeled alphabetically, beginning from the anodal form in both cases. Allozyme frequencies at each locus were calculated for each population. Data were analysed using the POPGENE 32 version 1.31 (Yeh and Yang, 1999) software based on the computer program. Percentage of polymorphic loci (P), mean number of alleles per locus (A) and mean number of alleles per polymorphic locus (A_P), effective number of alleles (A_E), observed heterozygosity (H_O) and expected mean heterozygosity (H_E) with respect to the Hardy-Weinberg equilibrium (Hedrick, 2000) were calculated. Loci were considered polymorphic if more than one allele was detected. Levels of genetic variation were calculated for individual populations and for all individuals pooled across all populations.

Fixation indices (F), reflecting deviations from Hardy-Weinberg equilibrium, were calculated and outcrossing rates (t) were estimated using $t = (1 - F)/(1 + F)$ (Weir, 1990). The partitioning of genetic diversity within and among all populations were analysed using F-statistics (Nei, 1973) according to the equations of Weir and Cockerham (1984).

The average gene flow among populations (Nm) was estimated from Fst values according equation such as $Fst = 0.25(1 - Fst)/Fst$ (Nei, 1978). Nei's unbiased genetic distance and genetic identity value were calculated for pairwise comparisons of populations. A cluster analysis was performed using the unweighted pair-group method using arithmetic average (UPGMA) dendrogram (Sneath and Sokal, 1973), depicting the genetic relationships among the population of *Trichoderma* species. A dendrograms was constructed using the computer software package NTSYS-pc version 1.80 by Rohlf (1997).

RESULTS

Loci and Alleles Scored

Allozyme electrophoresis resulted in clear and consistent staining for 9 allozymes encoded by 14 putative loci: EST, MD1, MD2, AP, G6PD, GP1, GP2, GP3, SORD, SOD1, SOD2, SOD3, ME and AD1. All alllozymes were migrated anodal zone. Eleven loci were polymorphic into one population (78.37%, Table 3). A total of 28 alleles were detected in all populations of *Trichoderma* species. Nevertheless the allele lacking in these population EST-b was found of *T. harzianum* only with a very low frequency (0.0400) (Table 4). In the other polymorphic locus found (MDH1) the faster allele.

Genetic Variation Within the Species

Genetic variations were quantified with populations of three *Trichoderma* species (Table 3). Seven (50.00 %) of the 14 loci, 8 (57.10%) of the 14 loci and 6 (42.86%) of 14 loci (P) resolved for populations of *T. harzianum*, *T. aureoviride* and *T. longibrachiatum*, respectively. Mean number of alleles per locus (A) were 0.7857, 0.7857 and 0.7500, respectively and mean numbers of alleles per polymorphic locus (A_P) were 0.60714, 0.57142 and 0.50000, respectively. Observed mean heterozygosity were 0.0350, 0.0666 and 0.0957, respectively and Expected mean heterozygosity were 0.1242, 0.1466 and 0.1425, respectively with all populations of three *Trichoderma* species. Values of observed heterozygosity were lower than those of expected heterozygosity in all populations.

Table 3: Summery of genetic variation for 14 loci in three populations of *Trichoderma* species

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 No. of alleles per locus; A_p = Mean No. of alleles per polymorphic locus; A_O = Mean observed No. of alleles; A_E = Mean effective No. of alleles; H_o= Mean observed heterozygosity; H_E = Mean expected heterozygosity; F = Fixation Index; t = Outcrossing Rate

Table 4: Allele frequencies of polymorphic loci in the three species of *Trichoderma*

Locus	Allele	*T. harzianum*	*T. aureoviride*	*T. longibrachiatum*
EST	a	0.9600	1.0000	1.0000
	b	0.0400	0.0000	0.0000
MD1	a	0.9615	0.8889	0.9286
	b	0.0385	0.1111	0.0714
MD2	a	1.0000	0.9000	1.0000
	c	0.0000	0.1000	0.0000
AP	a	0.8519	0.6667	0.8333
	b	0.0000	0.3333	0.1667
	c	0.1481	0.0000	0.0000
G6PD	a	1.0000	1.0000	1.0000
GP1	a	1.0000	1.0000	0.2500
	b	0.0000	0.0000	0.2500
	c	0.0000	0.0000	0.5000
GP2	a	0.8519	0.8500	0.8333
	b	0.1481	0.1500	0.1667
GP3	a	0.8519	0.9500	0.7143
	b	0.1481	0.0500	0.2857
SORD	a	0.5000	0.9167	1.0000
	b	0.3889	0.0833	0.0000
	c	0.1111	0.0000	0.0000
SOD1	a	1.0000	1.0000	1.0000
SOD2	a	1.0000	0.6000	1.0000
	c	0.0000	0.4000	0.0000
SOD3	a	0.8800	1.0000	0.9375
	b	0.1200	0.0000	0.0625
ME	a	1.0000	0.9286	1.0000
	b	0.0000	0.0714	0.0000
AD1	a	1.0000	1.0000	1.0000

Fixation Indices and Outcrossing Rates

Mean fixation indices (F) was ranged from 0.16495 to 0.62141 with a mean of 0.3654, indicating an overall conformance to Hardy-Weinberg equilibriums (Table 1). F-value was significantly greater than zero and positive, indicating excess of homozygotes. Twenty one of 42 fixation indices for individual loci (ranging from F = -0.0400 to F = 1.00) are significant. Outcrossing rates (t) based on fixation indics range from 0.2334 to 0.7168 (mean t = 0.4923). Outcrossing rates (t) were low and far (mean t = 0.4923) of all populations.

Genetic Structure

Genetic divergence among populations was qualified by computing the Fst parameter, which measures differentiation among populations (Table 5). The mean value of Fst was considerably low (Fst = 0.1577; 95% confidence interval: 0.3472- 0.9636) with respect to the mean value of Fit (0.5469) but significantly different from zero ($p<0.01$). The Mean Fis, representing average deviation from Hardy-Weinberg expectations within populations to be positive and significantly different from zero (Fis = 0.4620).

Gene Flow

Gene flow (Nm) for all loci indicated an average of 1.3351 among all populations of *Trichoderma* species. The lowest and highest Nm values were obtained from 0.2679 to 471.7541 (Table 5).

Genetic Distance

The genetic distance of 47 taxa examined using allozyme patterns and distance similarity equation was made for 3 groups of *Trichoderma* populations. The distance similarity coefficients of 3 species of *Trichoderma* were ranged from 0.9397 to 0.9642 (Table 6), with the lowest distance value obtained for *T. aureoviride* to *T. harzianum* populations. *T. aureoviride* populations were shown the highest similarity value with *T. harzianum* populations. The highest similarity value was 96.42%. In the species of *T. longibrachiatum* populations, a larger similarity coefficient was obtained from 93.97-94.15% with *T. aureoviride* and *T. harzianum* populations.

Table 5: Summary of F-statistics and gene flow for all loci among three populations of *Trichoderma* species (Nei, 1978)

Locus	Fis	Fit	Fst	Nm*
EST	-0.0417	-0.0135	0.0270	9.0000
MD1	-0.0937	-0.0795	0.0129	19.0888
MD2	1.0000	1.0000	0.0690	3.3750
AP	1.0000	1.0000	0.0854	2.6778
G6PD	-	-	0.0000	-
GP1	0.7333	0.8621	0.4828	0.2679
GP2	0.1934	0.1938	0.0005	471.7541
GP3	0.1114	0.1728	0.0691	3.3683
SORD	0.3236	0.4871	0.2417	0.7844
SOD1	-	-	0.0000	-
SOD2	1.0000	1.0000	0.3077	0.5625
SOD3	0.6194	0.6354	0.0420	5.6979
ME	-0.0769	-0.0244	0.0488	4.8750
AD1	-	-	0.0000	-
Mean	0.4620	0.5469	0.1577	1.3351

*Nm = Gene flow estimated from Fst = 0.25 (1-Fst)/Fst

Table 6: Genetic identity (above diagonal) and genetic distance (below diagonal) value of 3 species of *Trichoderma* according to the index of Nei (1973)

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

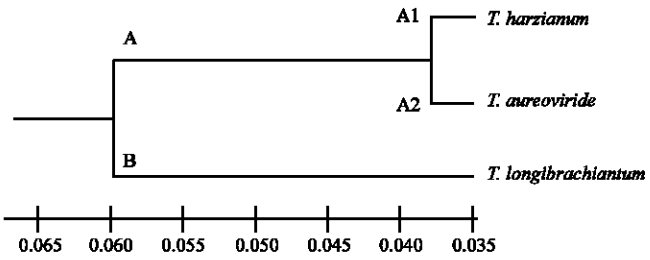

Fig. 1: Dendrogram based on UPGMA analysis of genetic similarity obtained from isozyme data, showing relationships among 3 species of *Trichoderma*

Cluster Analysis

Cluster analyses were valuable for determining relationships among 3 different species of *Trichoderma* populations. The cluster analysis included all nine isozyme systems, thus giving more completed information than if each system was analyzed separately. Pairwise genetic similarity according to the distance similarity coefficient were calculated between taxa and used in the cluster analysis.

The dendrogram obtained by the UPGMA clustering method revealed the genetic relationship of 3 different species of *Trichoderma* populations from a total of 47 *Trichoderma* samples tested in this study (Fig. 1). The genera examined were divided into two groups, A and B. According to the dendrogram the first major group was divided into two subgroups, A1 and A2. Whereas, the second group was showed of *T. longibrachiatum* populations belonging to the genus of *Trichoderma*.

Figure 1 showed that the two major groups were closely contracted into the cluster analysis. The isolates of samples in the first major group were dispersed. They split into two distinct subgroups. The subgroups first and second were included *T. harzianum* and *T. aureoviride* from all *Trichoderma* populations. This subgroup was clearly distinct from those populations of *T. harzianum* and *T. aureoviride*, the genetic distance similarity value was highly similar each others species populations (similarity value of 96.42%). Another major group was formed by the species of *T. longibrachiatum* populations.

DISCUSSION

There is a need for simple, rapid, inexpensive and reliable diagnostic methods for the identification of *Trichoderma* fungi. Identification of *Trichoderma* species on the basis of morphological and physiological characteristics are 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 (Bissetts, 1984).

Allozyme polymorphism has a simple genetic basis with the allozyme bands that denote polymorphism at a single locus often termed the allozymes (Prakash *et al*., 1969). Allozyme display the codominant expression so that heterozygous. The allozyme locus may be distinguished from either homozygote. When heterozygous at a locus for a monomeric enzyme, two allozyme bands will be produced if the enzyme is dimeric because the allelic product at demeric loci can associate at random producing both homodimer and heterodimer (Moore and Durham, 1996).

Allozyme polymorphism data obtained from this study provided good resolution to support the taxonomical identification of *Trichoderma* populations. Although SOD are allozymes involving in the defense mechanism of mycelium (Weeden and Wendel, 1989), the allozyme levels and allozyme patterns are depending on various environmental factors (Karpinska *et al*., 2001). The two allozyme

systems (GP and SOD) examined are more than suitable to assess genetic variability in *T. harzianum, T. aureoviride* and *T. longibrachiatum* populations. In these methods show different patterns which these patterns are useful for taxonomy identification. MDH did not reveal any variation with same species populations but its displayed variation among three species populations.

T. harzianum populations exhibit moderately high levels of genetic variation, especially at the taxon level. Standard measures of genetic variation are considerably higher (P = 0.571, A = 0.7857, H_E = 0.1542) than the mean of *T. longibrachiatum* species in general (P = 0.428, A = 0.7500, H_E = 0.1425) (Table 3). However, the population level of *T. aureoviride* (P = 0.562, A = 0.7857, HE=0.1466) are slightly difference when comparing with the mean value of *T. harzianum* populations. The mean percentage of polymorphic loci in *T. harzianum* populations is slightly higher comparing to other populations of *T. aureoviride* (P = 0.562) and *T. longibrachiatum* (42.80%). The mean of expected heterozygosity within populations for *T. harzianum* is also slightly higher (H_E = 0.1542) than those values reported for other populations of *T. aureoviride* and *T. longibrachiatum*. The moderately high levels of genetic variation are reported here for *T. harzianum* populations to be closely related to the *Trichoderma* characteristic and reproductive system.

High levels of genetic diversity are not normally expected in a species that underwent founder events. For example, the dioecy in *D. angustifolium* may ameliorate the effects of local inbreeding (Berg and Hamrick, 1997). High genetic diversity has been reported in other cycads that have narrow distributions and relatively low population densities (cf. *Macrozamia riedlei*, Byrne and James, 1991; and *D. edule* (Gonzalez-Astorga *et al*., 2003a).

The morphological data and genetic polymorphisms are revealed by our allozyme analysis of the taxa studied corresponding very well of each other populations. The genetic similarity and cluster analysis among 47 taxa between three species of *Trichoderma* populations showed that *T. harzianum* populations are closer to *T. aureoviride* populations than to *T. longibrachiatum* populations (Table 6). *T. harzianum* and *T. aureoviride* populations show greater genetic similarity than *T. longibrachiatum* and *T. harzianum*. The dendrogram also displays two major groups (Fig. 1) the first major group contains *T. harzianum* populations and *T. aureoviride* populations, whereas *T. longibrachiatum* populations are in the second group. The results are supported the taxonomic character, which those *T. aureoviride* populations are closely related to *T. harzianum* populations.

CONCLUSION

The allozyme technique has proved to be more successful which supports the taxonomical identification and provides the cluster analysis of *T. harzianum* populations and its related taxa. Levels of genetic variation are moderately high in *T. harzianum* populations, especially considering differentiates status to other *Trichoderma* species populations. It appears to be a slightly difference character to *T. aureoviride* populations. Whether *T. longibrachiatum* populations are fully distinguished different character comparing to *T. harzianum* populations. So investigation of genomic DNA technique using *T. harzianum* populations such as allozyme and morphological data, should be removed any confusion for further identify of *T. harzianum* populations.

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