

Full Length Research Paper

Use of isozyme analyses and PCR based methods RAPD and RFLP for assessment of biochemical and genetic diversity of morphologically similar ectomycorrhizal *Lactarius deliciosus* from India

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The present study was undertaken to assess the genetic diversity and phylogenetic relationship among the morphologically similar and ecologically variant isolates of *Lactarius deliciosus* using polymerase chain reaction (PCR) based techniques random amplification of polymorphic DNA (RAPD), rDNA analysis and isozyme patterns. Two major groups were defined amongst the total isolates by cluster analyses. Four bands of amylase isozymes viz; A_{232.4}, A_{342.7}, A_{461.5}, A_{672.0} and two bands each of pectinases (Pe_{364.18}, Pe_{261.11}), cellulases (C_{460.14}, C_{356.12}), glutamate dehydrogenases (GLD_{126.00}, GLD_{29.18}) and acid phosphatases (AP_{110.00}, AP_{214.18}) were common in all the morphologically similar isolates. The RAPD profile revealed that a total of 12 polymorphic bands were obtained, among these a locus of 550 bp was diversified in all strains except M221 and M222. Few common bands which is present in zymogram (isozymes) and electrogram (RAPD) exhibited genetic similarities amongst ecologically variant isolates. Amplifications of the ITS region resulted a single PCR product of approximately 560 bp for all the isolates. When amplified fragments were digested with restriction enzymes, identical banding patterns were observed, which showed that no inter-generic or intra-specific variation among the isolates.

Key words: Ectomycorrhizal, Isozyme, random amplification of polymorphic DNA, restriction fragment length polymorphism, zymography.

INTRODUCTION

The diversity and identification of ecologically variant isolates were previously determined on the basis of morphological and cultural characteristics. These traditional methods may be tedious and misleading which are sometime fails to recognize morphologically similar species. Therefore, some para-morphological criteria specially molecular markers such as isozyme patterns, random amplification of polymorphic DNA (RAPD) and

rDNA analyses were used for differentiation of morphologically similar or ecologically variant isolates or both (Cruickshank and Pitt, 1987; Huhtala et al., 1999; Stanosz et al., 1999; Upadhyay et al., 2003, 2010). During isozyme analyses, some common enzyme (protein) bands are diversified in all the isolates or in other terms it can be interpreted that loci or alleles, which code to this enzyme band is diversified in all the isolates (Banke et al., 1997). This concept gives a novel idea for phylogenetic analysis of not only for fungi but also for other organisms (Garbelotto et al., 1996). The basic concept of diversity of alleles or loci in electrogram of RAPD is same as in zymography of isozyme (Williams et al., 1990; Upadhyay et al., 2005). This method has been utilized for taxonomy, systematic studies, linkage analyses and genetic analyses for wide variety of organisms.

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Abbreviation: PCR, Polymerase chain reaction; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism.

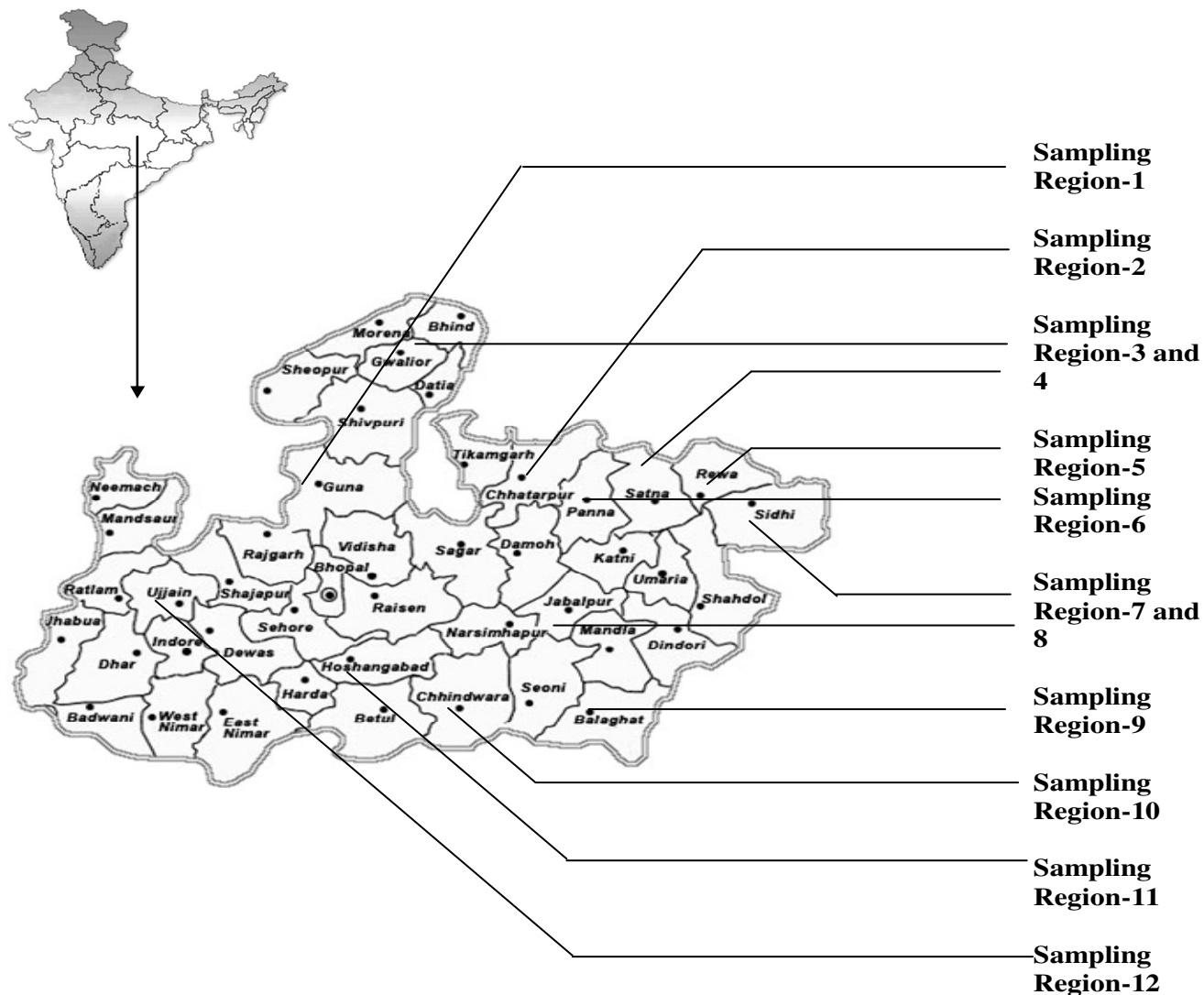


Figure 1. Sampling region of Indian deep forests from which isolates of *L. deliciosus* were obtained.

In case of basidiomycetes and other fungi, reports included identification of *Agaricus bisporus* (Khush et al., 1992), phylogenetic analyses of *Coprinus psychromorbidus* (Laroche et al., 1995), polymorphisms of *Russula* species by RAPD analyses (Upadhyay et al., 2010), analyses of homogeneous RAPD pattern among different isolates of *Serpula lacrymans* and polymorphisms for *Serpula himantioides* and *Coniophora puteana* (Schmidt and Moreth, 1998 a, b) etc. Beside RAPD analysis some workers more favored rDNA analyses (Witthuhn et al., 1999). Amplified ribosomal DNA restriction analysis of the internal transcribed spacer is using taxon- specific primers which were derived from sequence data. Therefore, this analysis is considered as most powerful molecular tool for fungal identification and phylogenetic analyses (Zaremski et al., 1998; Schulze and Bahnweg 1998; Kavousi et al., 2008). The aim of the present study is to characterize the genetic diversity of

Lactarius deliciosus using isozyme pattern of some extra cellular and intracellular enzymes, RAPD and rDNA analysis. The resulting binary matrices are analyzed by computer based programme and to find out whether isolates are evolutionarily similar or not.

MATERIALS AND METHODS

Fungal isolates

A total of only twenty two isolates of endangered ectomycorrhizal mushrooms *L. deliciosus* were isolated from different forests of Central India during survey conducted in rainy season of year 2000 to 2005 (Figure 1) and identified on the basis of morphological characteristics (Rinaldi and Tyndalo, 1985; Singer, 1986; Jordan, 1995). Detailed taxonomic studies were done in laboratory. Out of twenty two isolates, thirteen were associated with *Shorea robusta* (Sal) and rest with *Tectona grandis* (Teak). Pure culture of *L. deliciosus* were maintained on malt extract slant and a set isolates

were stored at 4°C in refrigerator for further use. These cultures have deposited in Fungal Germplasm Collection Center, R.D. University, Jabalpur, India for further references.

Extraction of extra-cellular isozyme

Enzyme extraction was done as per Upadhyay et al. (2005, 2010). 500 mg solid substrate (starch for amylases, cellulose for cellulases, pectin for pectinases) was autoclaved with 10 ml of basal salt solution (KH₂PO₄, 1.5 g; MgSO₄·7H₂O, 0.025 g; CaCl₂, 0.025 g; FeSO₄·7H₂O, 0.015 g; ZnSO₄, 0.005 g; D/W, 1000 ml) in 100 ml of Erlenmeyer flasks which were seeded with 5 mm disc separated from seven days old culture mycelium and incubated at 28°C for 8 days. Crude enzyme was pipette out and centrifuged at 10,000 rpm at 4°C for 1 min. Supernatant was taken as enzyme extract and stored at -40°C until used for electrophoresis.

Extraction of intra-cellular isozyme

Biomass of the fungal mycelium was harvested from the cultures grown as in modified complete medium (Sucrose, 15 g; Dextrose, 5.0 g; MgSO₄·7H₂O, 0.5 g; K₂HPO₄, 1.0 g; KH₂PO₄, 0.46 g; Yeast extract, 5.0 g; Bacto-peptone, 4.0 g; D/W, 1000 ml) (Choi et al., 1987). Crude intra-cellular enzyme was extracted from harvested biomass by centrifugation, which had been washed 4 times in 50 mM acetate buffer to remove extra-cellular enzyme. The mycelia were grinded in precooled mortar and pestle with grinding buffer (Skovgaard and Rosendahl, 1998). The extract was centrifuged at 20,000 rpm at 4°C, supernatant (crude intracellular enzyme) was collected and transferred to new 1.5 ml Eppendorf tube and stored at -40°C until use for electrophoresis.

Zymography

The enzymes were separated by SDS polyacrylamide gel electrophoresis in a discontinuous buffer system. The resolving gel contained 10% acryl amide with substrate and stacking gel was made 5% acrylamide (Sambrook et al., 1989; Hames, 1990). 15 µl enzyme samples were loaded in the wells along with gel loading dye. The gel was run at 80 V and 15 mA current at 4°C until the dye front reaches up to the bottom. The gel was washed for 10 min each with 1st washing buffer (50 mM Tris buffer, 49 ml; Triton-X-100, 1 ml) and 2nd washing buffer (50 mM Tris buffer) in shaking condition, and then it was incubated in recommended substrate buffer of individual enzyme (Upadhyay et al., 2005, 2010). The zymograms were prepared and relative positions of bands were calculated as per Upadhyay et al. (2003).

DNA extraction and RAPD

Twenty two isolates of *L. deliciosus* were grown in potato dextrose broth on a rotary shaker for five days. Mycelium was filtered through Whatman No. 1 filter paper, washed twice with TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) and DNA was extracted as described by Williams et al. (1990). PCR reactions were performed in 25 µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 µM each of dNTP, 5 picomoles of primer, 100 ng of genomic DNA and one unit of Taq polymerase. Selection of primers was based on the generation of relatively larger numbers of bands (whether monomorphic or polymorphic). Amplification was performed on a thermal cycler according to the following program: 95°C for 3 min followed by 40 cycles of 95°C for 1 min, 44°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. The amplification products (25 µl) were separated in 0.8% agarose gels

with TBE running buffer, stained with ethidium bromide and RAPD data were analyzed considering the presence or the absence of bands. A similarity matrix was analyzed by the unweighted pair group method using Jaccard's coefficient. The similarity matrix and dendrogram were calculated using both the monomorphic as well as polymorphic fragments.

Amplification of ITS region and RFLP

For amplification of ITS region, 50 µl PCR mixtures contained a final concentration of 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 200 µM of each dNTP, each 400 nM of universal ITS 1 (TCCGTAGGTGAACCTCGCGG) and ITS (TCCTCCGCTTATTGATATGC) primers, 0.6 unit of polymerase and 100 ng DNA solution. Ten microlitre of amplified fragments were digested with four randomly selected restricted endonucleases viz; Bam II, Hae III, EcoR I, Hind III under the condition recommended by manufacturer. The restricted fragments were separated in 2% agarose gel and size of fragment was estimated by comparison with 1 kb DNA ladder.

RESULTS AND DISCUSSION

The survey conducted during rainy seasons of year 2000 to 2005, only 22 endangered isolates of *L. deliciosus* were isolated from different deep forest of Central India. All the ecologically variant isolates were morphologically similar and their biodiversity were determined on the basis of external feature of basidiocarp which may sometime be tedious and misleading, therefore, paramorphological criteria specially molecular markers such as isozyme patterns and PCR based techniques RAPD and rDNA analyses were used for analyses of biochemical and genetic diversity of ecologically variant isolates. The biochemical diversity amongst all the isolates was analyzed for their electrophoretic profile of extra and intra-cellular isozyme pattern. Similarities and differences among the enzyme pattern with respect to isozyme numbers and electrophoretic mobility are summarized in Table 1 and the designation of enzyme bands were done as per Upadhyay et al. (2003).

Isozyme analyses

Amylases

A total of 107 bands were obtained in amylase isozyme patterns. There are four fractions viz; A₂^{32.4}, A₃^{42.7}, A₄^{61.5} and A₆^{72.0} were diversified in all the isolates whereas fraction A₇^{80.4} present only in M254, M256, M257 and M260 (Figure 2A).

Pectinases

A total of four polymorphic bands were obtained where fractions with relative mobility Pe₂^{61.11} and Pe₃^{64.18} were common in all the isolates. Rf Pe₄^{78.82} was absent in

Table 1. Zymogram pattern of extra-cellular and intra-cellular isozymes with their relative mobility of 21 isolates of *L. deliciosus*

Isolates Rf ↓	Isolates																					
	M193	M195	M196	M197	M254	M256	M257	M260	M221	M222	M229	M462	M463	M464	M498	M499	M288	M139	M220	M198	M615	M618
A1 _{12.6}	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1
A2 _{24.4}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A3 _{42.7}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A4 _{54.8}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A5 _{60.6}	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A6 _{72.0}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A7 _{80.4}	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A8 _{83.1}	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A9 _{88.6}	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P1 _{40.66}	1	1	1	1	0	0	0	0	1	0	1	0	0	1	1	1	1	1	0	0	0	0
P2 _{46.81}	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
P3 _{59.11}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pe1 _{10.12}	0	1	0	1	0	1	1	0	0	1	1	1	1	1	0	0	0	1	0	1	1	1
Pe2 _{61.11}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pe3 _{64.18}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pe4 _{78.82}	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1
C1 _{34.16}	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1
C2 _{50.00}	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
C3 _{56.12}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C4 _{60.14}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AP1 _{10.00}	1	1	0	1	0	0	1	1	1	0	1	1	0	1	1	1	0	0	1	1	1	1
AP2 _{14.18}	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
AP3 _{60.16}	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1
AP4 _{72.18}	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
GLD1 _{26.0}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
GLD2 _{29.18}	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
GLD3 _{63.11}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
GLD4 _{76.18}	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

1 = Band present, 0 = band absent, A = amylase band, P = protease band, Pe = pectinase band, C = cellulase band, GLD = glutamate dehydrogenase band and AP = acid phosphatase band.

M254, M257, M221, M464 and M139 whereas the bands with Rf Pe1_{10.12} was present in most of the isolates (Figure 2B).

Cellulases

A total of 68 bands were obtained in 4

polymorphic loci in cellulase isozymes. Fractions with Rf C3_{56.12} and C4_{60.11} were diversified in all isolates whereas Rf C2_{50.00} was present only in

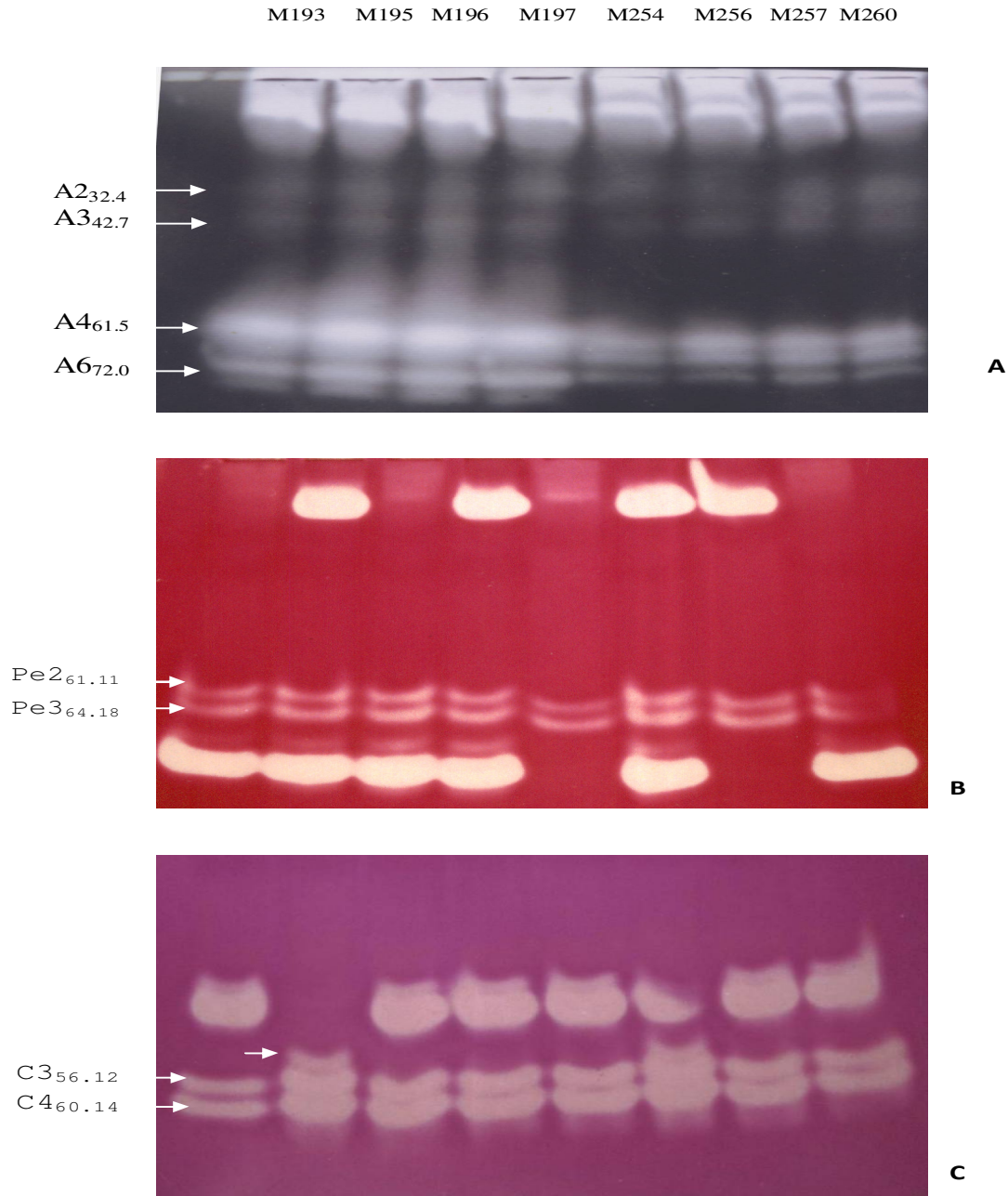


Figure 2. Polyacrylamidegel stained for (A) Amylases (B) Pectinases (C) Cellulases. Lane 1 to 8 is ecologically variant isolates of *L. deliciosus*.

five strains M195, M 254, M222, M 462 and M 220, similarly bands with Rf C1_{34.16} was common in most of the isolates except M195, M463, M464, M139 (Figure 2C).

Proteases

Three polymorphic bands were obtained in protease zymography. The fraction with Rf P3_{59.11} was present in all the isolates whereas the band with Rf P2_{46.81} was

absent only in M195, M196, M229 and M462

Acid phosphatases

Acid phosphatases had given 4 polymorphic bands. Rf AP4_{62.18} was common in most of the ecologically variant strains where as AP2_{44.18} was absent only in M196, M254, M256 and M139. Similarly AP1_{40.0} was present only in few isolates. No any band was common in all the

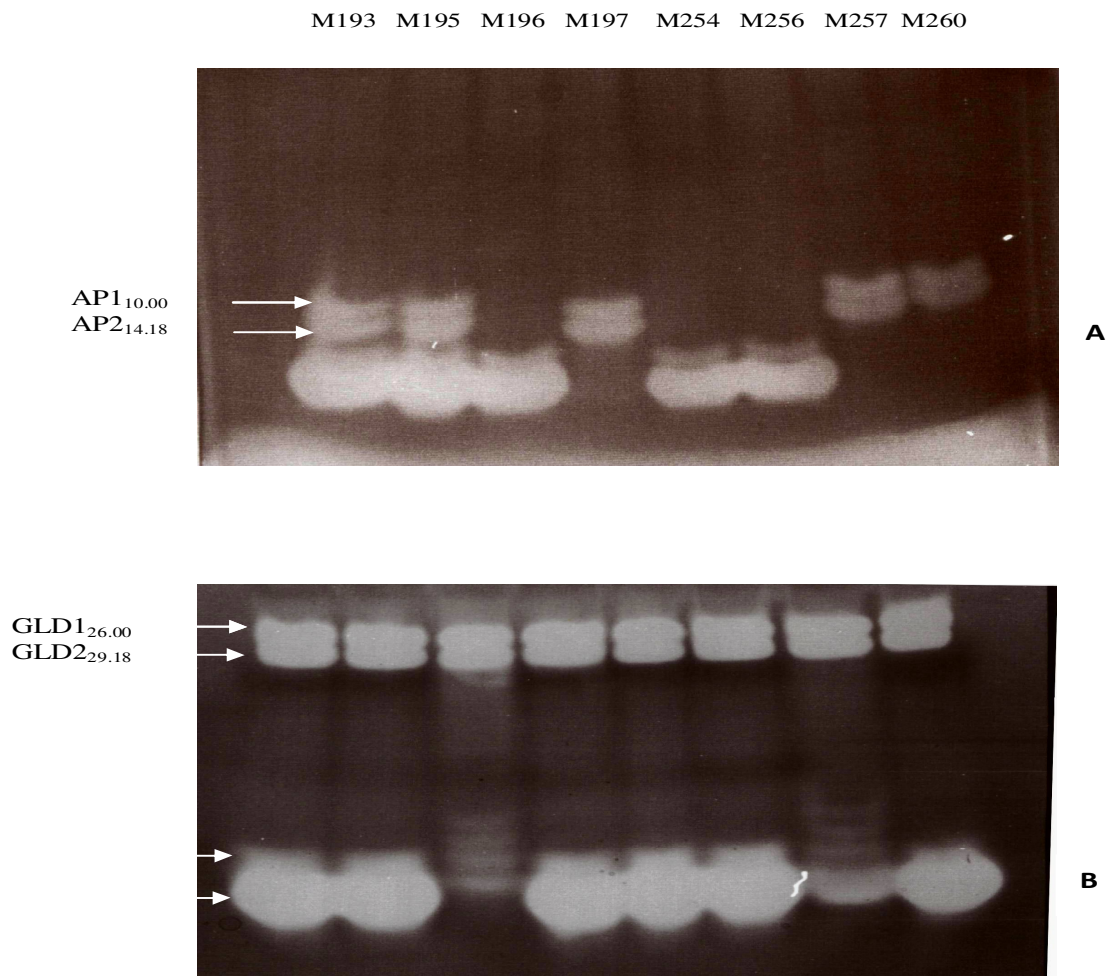


Figure 3. Polyacrylamide gel stained for (A) Acid phosphatases (negative mode) (B) Glutamate dehydrogenases (negative mode). Lane 1 to 8 is ecologically variant isolates of *L. deliciosus*.

isolates (Figures 3A and B).

Glutamate dehydrogenases

A total of 4 polymorphic bands of glutamate dehydrogenases were obtained. Fractions with Rf GLD1_{26.00}, GLD3_{63.11} and GLD4_{76.18} were common in all isolates while GLD2_{29.18} was absent only in M221 (Figure 3B).

RAPD and amplification of ITS region

Random amplified polymorphic DNA (RAPD), which is detected by use of polymerase chain reaction with arbitrary 10-mer primers for molecular characterization of morphologically similar and ecologically variant isolates of mushrooms. RAPD analysis of mushrooms used the following 10 base primers (Table 2) during the characterization at genetic level (Operon Technologies Inc.

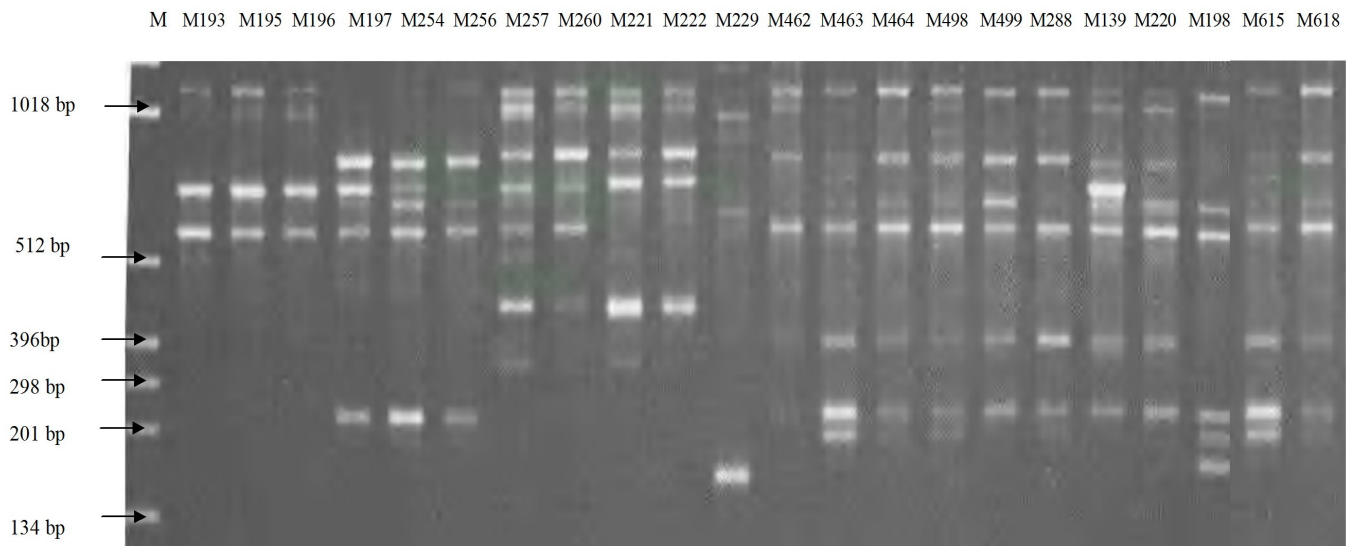
USA).

These primers were selected from preliminary screening and on the basis of their ability to generate highly reproducible RAPD fingerprints that could be used to differentiate the morphological similar mushrooms. Out of these eight primers, OPA-19 has given maximum of 12 bands. OPY-02, OPY-03, OPA-14 and OPA-17 had given only two bands where as OPA-02, OPF-8 and OPF-10 had not amplified with the genomic DNA of tested mushroom isolates. A total of 12 polymorphic bands (loci) were produced by primer OPA-19 (Figure 4).

Maximum number of loci that is eight were produced by M198, M220 and M257 followed by 7 bands revealed by M615, M618, M498 and M499 isolates. Rest of strains produced minimum number of bands (4 loci). The matrix was used to construct the dendrogram with help of isozyme analyses and RAPD bands by using NTSYSpc2.02i computer programme to establish the level of relatedness among the 22 ecologically variant isolates of *L. deliciosus*. The dendrogram (Figure 5)

Table 2. Primers sequences.

S/No.	Sequences	Primers
1	5'TGCCGAGCTG-3'	OPA-02
2	5' TCTGTGCTGG- 3'	OPA-14
3	5' GACCGCTTGT - 3'	OPA-17
4	5' CAAACGTCGG-3'	OPA-19
5	5' GGGATATCGG -3'	OPF-8
6	5' GGAAGCTTGG -3'	OPF-10
7	5'CATCGCCGCA-3'	OPY-02
8	5'ACAGCCTGCT-3'	OPY-03

**Figure 4.** RAPD pattern of 21 isolates of *L. deliciosus*. 12 polymorphic loci were produced by OPA-19.

showed the hierarchical clustering separation of the isolates into three groups according to their similarity coefficient. The first group consists of 8 isolates where as group 2 and 3 have 13 and 1 isolates respectively. Two isolates viz., M498 and M499 of group 1 have >0.99 similarity index this implies that these isolates are genetically near to each other. It further showed that all ecologically variant isolates could be divided into two closely related lines, in which group 2 and 3 as one line and group 1 as the other line. Similarly Ito et al. (1998), Ito and Yanagi (1999) and Kavousi et al. (2008) used RAPD analysis for differentiation of basidiomycete species viz. *Coprinus*, *Tricholoma* and *Agaricus* respectively and also determine the diversity of particular locus among studied isolates.

Amplifications of the ITS region using the primer pair ITS1/ITS4 resulted in a single PCR product of approximately 560 bp for all the isolates. Similarly, when amplified fragments were digested with four restriction enzymes, identical banding patterns were observed for all the isolates. Hind III showed two bands of approximate

size of 300 bp and another of 150 bp where as EcoR I showed only one band of 280 bp (probably a double restriction fragment), whereas Bam II and HaeIII showed a single band of 560 bp indicating that the fragments were not digested. Thus the amplification of ITS region revealed no inter-generic or intra-specific variation. Example of restriction patterns obtained from digestion is shown in Figure 6.

Although the genetic diversity is determined indirectly by isozyme analysis (Skovgaard and Rosendahl, 1998). There are four common alleles/loci of amylase isozymes viz; A_{232.4}, A_{342.7}, A_{461.5}, A_{672.0} and two bands each of pectinases (Pe_{364.18}, Pe_{261.11}), cellulases (C_{460.14}, C_{356.12}), glutamate dehydrogenases (GLD_{126.00}, GLD_{229.18}) and acid phosphatases (AP_{110.00}, AP_{214.18}) were diversified in all the morphologically similar isolates therefore, this might be clear that all the isolates are evolutionary related from same ancestor (Cruickshank, 1990). The grouping of all the isolates derived from numerical analysis of zymogram data demonstrated high degree of concordance and the result of isozyme analysis

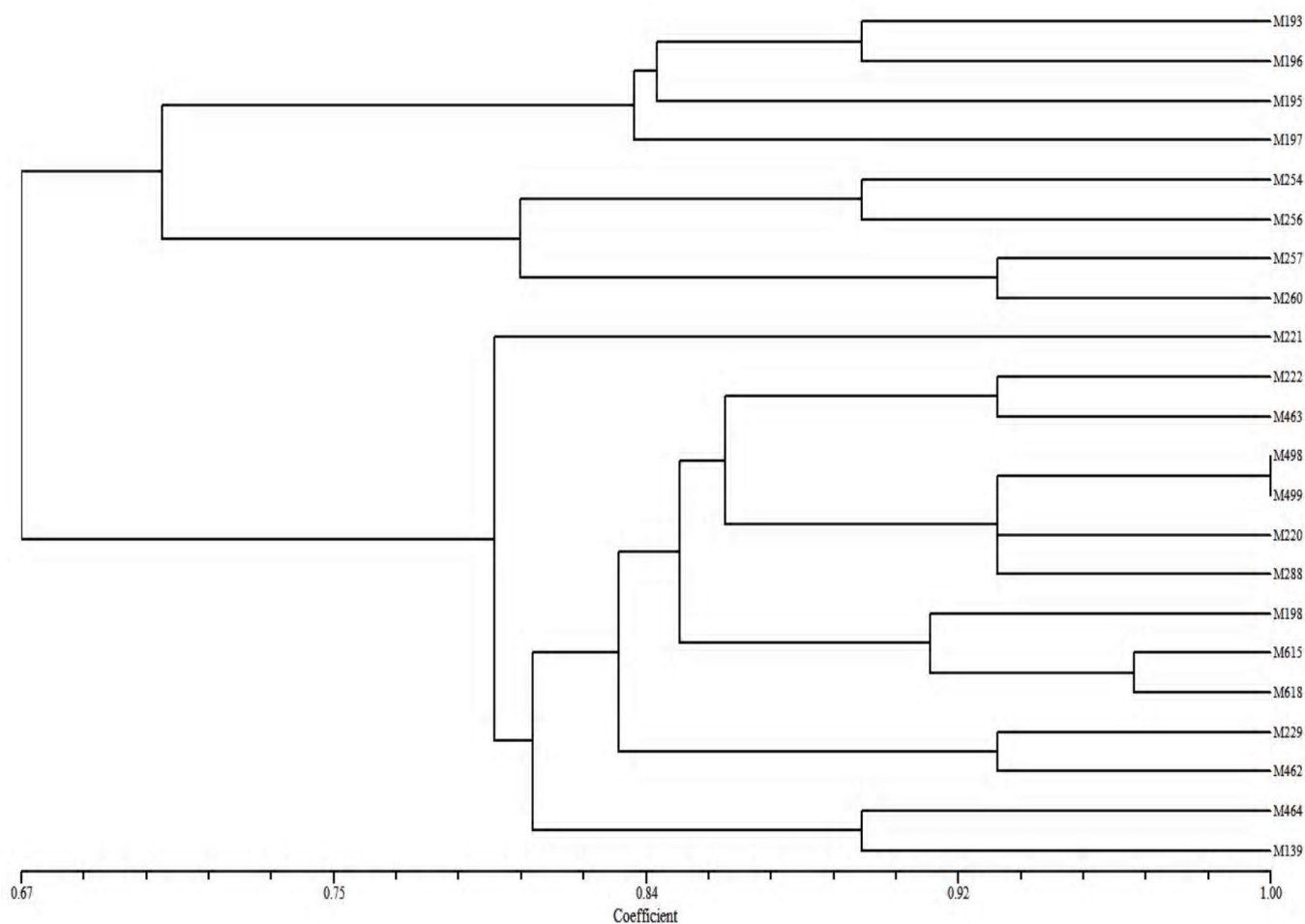


Figure 5. Dendrogram based on euclidian distance analysis of amylases, cellulases, pectinasea, glutamate dehydrogenases, acid phosphatases zymogram and RAPD of *L. deliciosus*.

showed that there was diversity of some enzyme (protein) bands which are obtained by electrophoretic separation which may be common in all the isolates. The common band (enzyme band) reveals the loci or alleles which coded to these enzyme band are diversified within the species which otherwise could not be detected on the basis of morphological characters (Banke et al., 1997). Sometime isozyme analyses fail to explain the difference between alleles and loci (Micales et al., 1986) therefore, a simple method is needed to describe the diversity at genetic/molecular level. It could be possible by random amplified polymorphic DNA (Wittuhn et al., 1999). This method not only explains the diversity of particular loci band(s) but also discriminate the genus, species or strains at molecular level for taxonomic description and systematic studies. Beside this some other tools such as analysis of ITS region and its RFLP etc. are also use for determination of inter-generic or intra-specific variation among the isolates/strains (Kavousi et al., 2008)

The present study showed that the extra and intra-cellular isozymes, RAPD and RFLP of ITS might be

specific for analyses of genetic diversity of ecologically variant isolates. The result arises from RFLP analyses showed that all the isolates (either associated with *Shorea robusta* or *Tectona grandis*) produced similar banding pattern therefore, it comprises only single group (>0.99 similarity index) and not differentiated among the isolates. It also implies that the conserved sequences are diversified, hence Hind III recognized its restriction site in all the ecologically variant isolates. In contrast to this, the UPGMA (Figure 5) exhibited that RAPD and isozyme distinguished the total isolates in to two groups, the isolates of group 1 associated with *T. grandis* where as all the isolates (thirteen) of group 2 were coupled with *S. robusta*, the clear-cut demarcation of isolates on the basis of attachment of the host which revealed that the biochemical relationship of specific plants with fungi (Liang et al., 2004). This result is also supported by banding pattern (Isozyme and RAPD) analyses that all the isolates associated with *S. robusta* are reside in one clad which makes the homogeneous groups and that is genetically dissimilar from the rest isolates that resides

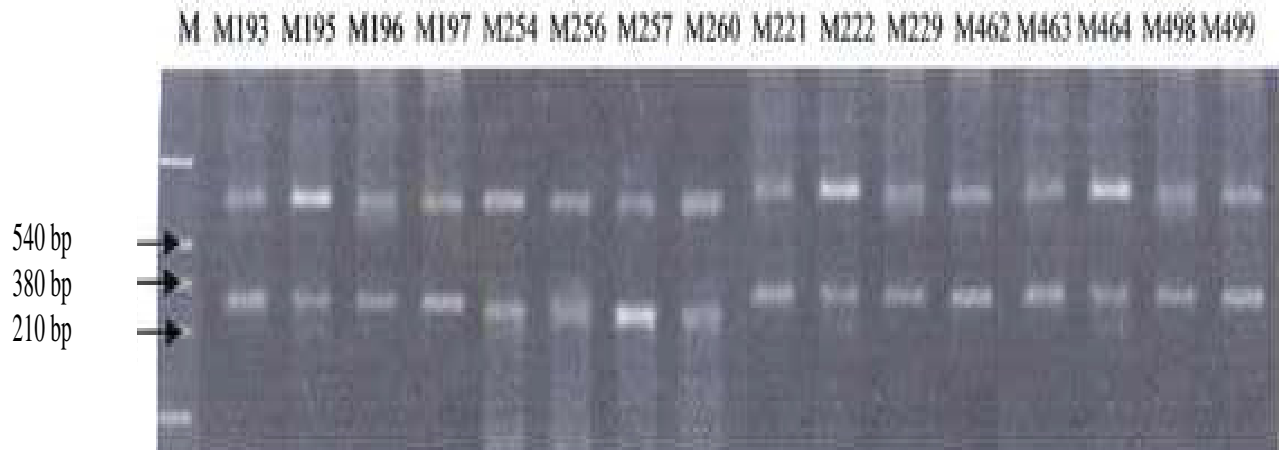


Figure 6. A simple gel of digested amplified ITS region by *Hind* III restriction enzyme.

on other clad. Similar observations were recorded by Liang et al. (2004) and Upadhyay et al. (2010) while studying the genetic diversity of *Fusarium oxysporum* and *Russula* spp. respectively on the basis of RAPD analyses and their association with host. Thus, it is difficult to determine the taxonomic significance of the observed dissimilarity (according to isozyme, RAPD and RFLP analyses) because of the heterogeneity among the ecologically variant and morphologically similar isolates with respect to host. Further work is needed to clarify the taxonomic relationship among the strains of ectomycorrhizal *L. deliciosus*.

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