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Establishing genetic diversity among indigenous cultivated and wild rice species of Manipur using isozyme analysis

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ABSTRACT

Isozyme studies provide preliminary indications that genetic variation may be differently apportioned in some crops compared to their wild relatives. The present investigation was undertaken to study the Isozyme banding patterns and its inheritance in rice with an aim to establish the genetic diversity among the 9 cultivated and wild rice species of Manipur. The indigenous rice collections were subjected to analysis of Isozyme polymorphism at three loci by Polyacrylamide gel electrophoresis. Zymograms of all the tested enzyme system (esterase, alcohol dehydrogenase, glutamate dehydrogenase) were then constructed, genetic relationships between the nine varieties on the number of loci at which Isozyme pattern differs were determined. In all the enzyme systems studied, only one zone of isozymatic activity defined by the degree of mobility could be observed. Maximum number of bands was observed in Taothabi and Wainuchara (O. rufipogon) local collection I in comparison to other germplasms, the number of bands varied from 1-2. The relationship between these germplasms had been assessed from electrophoresis data and it was observed that Taothabi and Wainuchara i.e., O. rufipogon local collection I showed close relationship in comparison to other germplasms. There is possibility to identify rice species or cultivars available in Manipur using Isozyme analysis and obtain first hand information regarding variability of rice germplasms and also to trace the phylogenetic relationship between the indigenous rice varieties and their wild progenitors.

Keywords: Isozyme, *Oryza sativa*, genetic marker, Zymogram, Relationship, Manipur

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INTRODUCTION

Rice (*Oryza sativa* Linn) belonging to Gramineae (Poaceae), is one of the major staple food crop of the world. Manipur, a north eastern state of India bordering Myanmar, has a large variability of indigenous rice germplasms which range their adaptation from low lying lake areas to rain fed uplands of Manipur hills. The local rice germplasms have many desirable characters e.g. tolerance to cold temperature and drought conditions, adaptability to local environment such as low light intensity, various soil nutrient deficiencies, low organic contents etc. Biochemical techniques such as Isozyme analysis can ideally be used as genetic marker. Isozyme are variant molecular forms of the same gene family that are responsible for governing the same character and usually exhibit co-dominant segregation patterns since allelic expression of Isozyme's are usually co-dominant and free from epistatic difference in each allelic expression product can be easily traced as mobility difference which is independent of functional role, less susceptible to phenocopy responses and modification by environmental varieties. Rice varieties have received careful study [1]. Second [2,3] combining information from Isozyme's, biogeography and hybridization study proposed a model for evolution of cultivated rice. Chu [4] studied the variation in

peroxidase of *O. perenis* and *O. sativa*. The changes in peroxidase banding pattern in highly regenerative callus of selected and non selected lines of rice. Two strong peroxidase bands which showed fast mobility and one with slow mobility were detected only in the calli of selected line. Glaszmann (1985) classified varieties of *O. sativa* based on Isozyme polymorphism into I and V groups. Moore and Collins[5] have suggested the use of Isozyme in plants for characterization and selection of desired variability, assaying, purity of seed lot, identification of somatic hybrids and mapping of chromosomes. Enzymes in different locations of plants are differentially expressed. An early study of esterase Zymogram pattern demonstrated that there were Zymogram differences between spices as well as between tissues[6]. In rice tissue specificity has been detected for thirteen Isozyme's [7,8] and Shahiet al. [9] studied peroxidase and esterase variations in a number of cultivated rice varieties. Ishikawa [10] investigated 10 Isozyme systems including 17 loci in 60 Asia varieties which had been classified into *indica* and *japonica* types. Significant differentiation was observed between *indica* and *japonica* types at 11 loci. Six Isozyme loci were examined to assess the extent of differentiation into the two types of Asia rice in its diversity centre[11]. Romero et al. [12] used Isozyme electrophoresis to compare the Isozyme phenotypes of *Oryza sativa* and *O. minuta* accessions for 10 enzyme systems. Between two species, two systems were monomorphic and eight were polymorphic. Nakagahra [13] reported geographical distribution of the esterase Isozyme's based on a survey indicates the genetic diversity of *O. sativa* centers around Indo-China and Burma.

MATERIALS AND METHODS

Plant Materials

Nine indigenous rice collections were subjected to analysis of Isozyme polymorphism at three loci (Table 1) viz., *Changlei* (Acc. No. 47), *Chakhao poireiton* (Acc. No. 33), *Langmanbi* (Acc.no. 129), *Murshi* (Acc. No. 35), *Moirangphou kokngangbi* (Acc. No. 167), *Sangsungba* (Acc.no. 218), *Taothabi* (Acc. No.236), *Phouren* (Acc.no.198) and *Wainuchara* (*O. rufipogon*, Acc.no.33)

Table 1: Enzymes used in the Analysis

Sl. No.	Enzyme	Abbreviation	Enzyme number
1	Esterase	EST	E.C.3.1.1.1
2	Alcohol dehydrogenase	ADH	E. C 1.1.1.1
3	Glutamate dehydrogenase	GDH	E.C.1.4.1.2

Extraction Buffer

Different extraction buffer were tried and tested to select the appropriate buffer for the three enzymes. They are (1). 10% w/v sucrose solution, (2). 20% w/v sucrose solution + 10 mM $MgCl_2$ + 10 mM dimethyl dithiocarbamate + 0.1% BSA, (3). 20 % sucrose solution + 0.1% pvp + 0.1% BSA + 10mM $MgCl_2$, (4). 20% w/v sucrose solution, (5.) 30% w/v sucrose solution, (6). 0.8% NaCl + 0.2 % $NaNO_3$, (7). 0.2 M phosphate buffer (PH, 7.5) + 0.1 % pvp + 0.1% BSA + 10M $MgCl_2$. (8). 0. 2M phoshate buffer (PH, 7.5) + 0.1% pvp + 0.1% BSA + 10mM $MgCl_2$ +14mM b – mercaptoethanol (0.1 % v/v), (9). 100 mM Tris –HCl (PH,7.5) + 50mM ascorbic acid, sodium salt + 0.1% BSA + 0.1% pvp, (10). 100mM Tris- HCl (PH,7.5) + 0.1% pvp + 0.1% BSA + 10mM $MgCl_2$ + 10mM dimethyl dithiocarbamate. Out of all these extraction buffers, 20% (w/v) sucrose solution gave the best result for all the enzymes and subsequently it was used for the extraction of samples.

Extraction

Young mature leaves were collected in an ice bucket; one gram of leaves from each sample was weighed. The leaves were gently homogenized with the extraction buffer using mortar and pestle at 4 ^{0}C. The extract was filtered and the filtrate was centrifuged at 15000 rpm for 5 minutes at 4 ^{0}C using 1379 rotor in Hitachi Biofuge (17 RS). 500 ml of the supernatant was then mixed with 250ml of V/V glycerol. Bromophenol blue (0.05 mg/ml) was added to the extract.

Electrophoresis

The gel mold was prepared for the vertical zone electrophoresis apparatus (LKB, Broma, 2001) system. 21.30ml of solution A containing 4.2 M acrylamide and 0.065 M n-methylbisacrylamide, 10 ml of solution B containing 0.3 M Tris HCl, pH 8.9, 40 ml of solution C consisting of 1.4 M acrylamide and 0.16 M n-methylbisacrylamide, 0.6 ml of 10 mg/ml riboflavin, 8.75 ml of d.H_2O, 40 ml TEMED was prepared. The mixture of solutions was quickly dispersed into gel mold with a Pasteur pipette, comb was inserted and the gel was allowed to polymerize within 30 min. 50 µl of sample was loaded in each well. The upper and lower reservoir was filled with 0.005 M Tris and 0.038

M glycine (pH 8.3). The run was carried out at 250 volts and 50 mA using power supply system (Pharmacia electrophoresis power supply EPS 500/400) for 3 hours. Bromophenol blue was used as tracking dye.

Staining and Post Staining Treatment of Gel

The gel was carefully removed from the apparatus and carefully washed at 4^0C with wash buffer. The gel was stained for 1-6 hr or until the bands appeared. Separate staining solution was used for different Isozyme's analyzed. Staining reaction was stopped by washing the gel 3-5 times with d. H_2O. Gels were fixed with 7% acetic acid solution. Banding patterns were recorded on plain paper and Zymogram was drawn to scale and intensity of each band was scored. Relative mobility (RM) values were calculated for each band based on the migration of the band relative to the front or tracking dye.

Preparation and scanning of the zymograms were done both qualitatively and quantatively. Qualitative variation describes the polymorphic iszymatic patterns exist among different taxa where as quantitative variation exists when a particular band is found in zymograms of two different taxa but differs in staining intensity[14]. For the present study, however, quantitative variations were not taken into consideration

RESULTS

In all the three enzymatic analyzed as shown in Table 2, the number of bands observed were 1-3 bands (EST), 1-2 (GDH) and 1 (ADH). The method proposed by TORRES et al. (1978) was followed for the nomenclature of alleles and genotypes of enzymes. In all the enzyme systems studied here, only one zone of isozymatic activity defined by the degree of mobility could be observed. The slower migration set, i.e. nearest to the origin of the gel was represented as zone1(EST-1)and the faster migrating set i.e. the zone far from the origin of the gel as zone(III)(EST-III).In between these two zones there was one zone designated as EST-II. Alleles were designated as F for fast M for medium and S for slow relative to the rate of migration of the enzymes.

Table 2: Composition of staining reagent and gel incubation conditions (Scandalions, 1969)

Sl. No.	Enzyme	Staining reagent	Gel incubation condition
1	Esterase (EST)	Esterase buffer (pH 6.4)-150ml, Sodium Phosphate, monobasic (13.9g/L) Sodium Phosphate dibasic (5.3g/L), Fast Blue RR salt (160mg) 1% µ - Naphthyl acetate solution (2ml)	Incubated at room temperature (1-6 hrs) or untill the bands appear
2	Alcohol dehydrogenase (ADH)	ADH buffer (P.H.8.0) 150 ml, (0.05M Tris Hcl), NAD (20mg/ 2 ml d. H2O, Nitroblue tetrazolium (NBT)-20mg/2ml d. H2O, Phenazine methosulphate (PMS)- 5mg/ml d.H2O, 95% ethyl alcohol = 2ml.	Incubated in dark (1-6 hrs) or until the bands appear
3	Glutamate dehydrogenase (GDH)	GDH buffer (0.1M Tris Hcl,pH 8.0) - 150ml, L- Glutamic acid (4g), NAD (20mg/2ml d.H2O), NBT (20 mg/ 2 ml d.H2O, Phenazine (PMS)- 5mg/ ml d.H2O methosulphate.	Incubated in dark (1-6 hrs) or until the bands appear

In EST enzyme system, the bands were observed in three zones as shown in Figure 1. The zone nearest to the origin of the gel (EST-I), a middle zone (EST-II) and a zone farthest from the origin EST-III. *O. sativa* cultivar *Changlei* showed presence of three bands i.e. one F-band, one M-band and one S-band. *O*. sativa cultivar *Chakhao Poireiton* also showed presence of same band as that of *Changlei*. *Langmanbi* showed one M and one F band. In *Murshi* (*O. rufigon)* local collection III, only one band i.e. F-band was observed. O. sativa cultivar *Moirangphou Khokngang*bi showed same band as that of *Changlei* and *Chakhao Poireiton.* The band found in *O. sativa* cultivar *Phouren* was found similar to that of Moirangphou Khokngangbi. *O. sativa* cultivar *Sangsungba* and *Wainuchara* (*O. rufipogon*) local collection I showed presence of only one F-band. For *O. sativa* cultivar *Taothab*i and *Wainuchara* (*O. rufipogon* local collection I), bands were observed in three zones one in each zone i.e. EST-I, EST-II and EST-III. The possible genotypes and their frequencies were FS (3), FM (2) and FF (4) in EST III.

Figure 1 PAGE studies of Rice species using Esterase enzymes

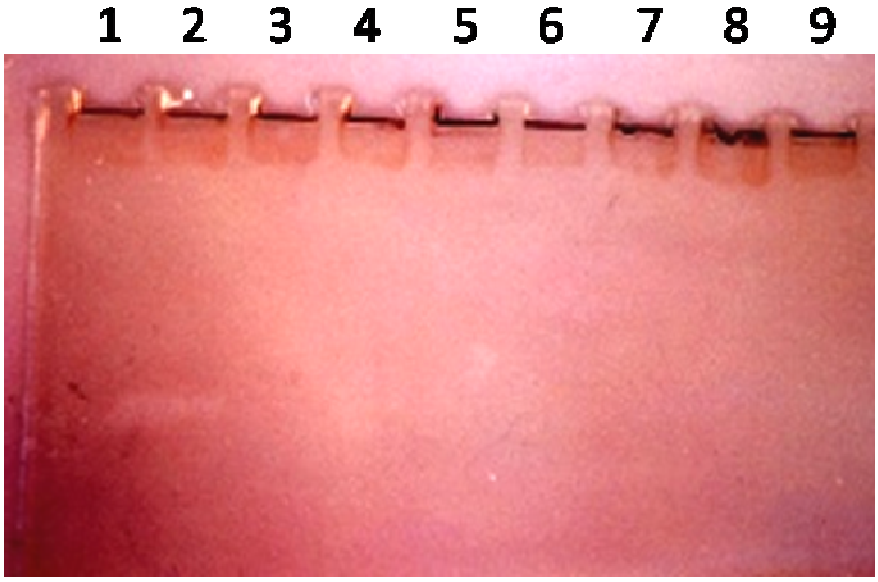
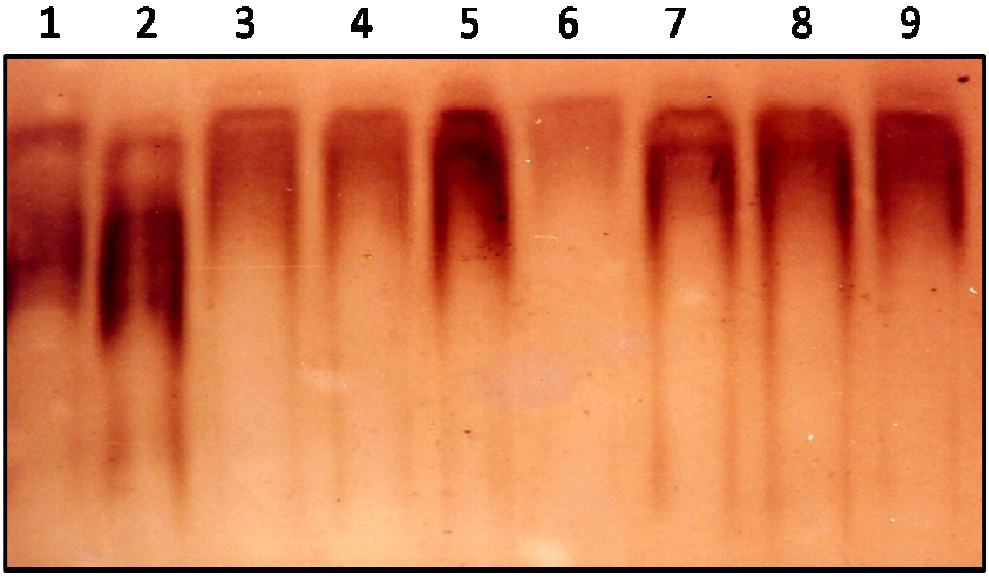


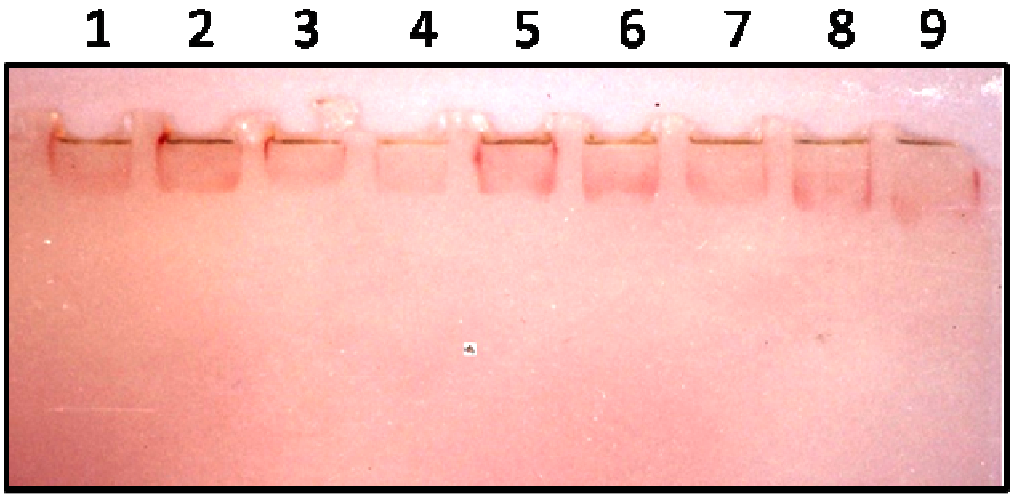
Figure 2 PAGE studies of Rice species in Alcohol dehydrogenase enzymes



All ADH bands were observed only in one zone as show in Figure 2. Each variety showed only one band. According to mobility, the bands were classified into three type's viz. F, M and S. The M band was present in all the varieties except in *Taothab*i and *Wainuchara* (*O. rufipogon* local collection I). S-band was present in *Wainuchara* (*O. rufipogon* local collection I) and F-band was observed in *Taothabi*. The possible genotypes and their frequencies were SS (1), FM (7) and FF (1).

In GDH, the resolution of bands was found to be very poor as seen in Figure 3. Here the number of bands was also very limited and could be observed only in one zone which consisted of 1-2 bands. The M band was found to be present in all the cultivars except in *Taothabi* and *Wainuchara* (*O. rufipogon* local collection I). The F and S bands were present only in *Taothabi* and *Wainuchara* (*O. rufipogon* local collection I). The genotype and their frequencies are MM (7) FS (2).

Figure 3 PAGE studies of Rice species in Glutamate dehydrogenase enzymes



Lane No	Rice Variety
1	Changlei
2	Chakhao poireiton
3	Langmanbi
4	Murshi
5	Moirangphou
6	Phouren
7	Sangsungba
8	Taothabi
9	Wainuchara

Table 3: Isozyme genotypes of nine varieties of Rice

Sl. No.	Variety	ADH	EST-(III)	GDH
1	Changlei	MM	FS	MM
2	Chakhao poireiton	MM	FS	MM
3	Langmanbi	MM	FM	MM
4	Murshi	MM	FF	MM
5	Moirangphou	MM	FM	MM
6	Phouren	MM	FM	MM
7	Sangsungba	MM	FF	FS
8	Taothabi	FM	FF	FS
9	Wainuchara	SS	FF	FS

FF = Fast, SS = Slow, MM = Medium

DISCUSSION

More than 15 enzyme systems have been detected in rice[1], more than 40 polymorphic genes encoding Isozyme's have been reported in the sativa group of *Oryza* [13,7]. Polyacrylamide gel electrophoresis of the three enzyme systems viz., esterase, glutamate dehydrogenase and malate dehydrogenase were carried out utilizing leaf samples from nine varieties of rice. From the observation of characteristic isozymatic patterns, it was noticed that there was not much considerable variation within these varieties as shown in Table 3. The resolution of bands was found to be very poor in glutamate dehydrogenase and malate dehydrogenase. The bands were observed to be prominent in esterase. In esterase, bands were observed in three zones. In this enzyme system all the varieties showed different banding patterns. Apart from the study of rice diversity study, Habarurema et al., reported Population diversity of rice bacterial leaf blight isolates in Uganda [16].

Table 4: Genetic relationships between the nine varieties on the number of loci at which Isozyme pattern differs

Sl. No	Variety	1	2	3	4	5	6	7	8	9
1	Changlei	0	0	1	1	0	1	1	3	3
2	Chakhao poireiton		0	1	1	0	1	1	3	3
3	Langmanbi			0	1	1	0	1	3	3
4	Murshi				0	1	1	0	2	3
5	Moirangphou					0	1	1	3	3
6	Phouren						0	1	3	3
7	Sangsungba							0	2	2
8	Taothabi								0	1
9	Wainuphou									0

The number of bands varied from 1-3. In alcohol dehydrogenase bands were observed to be present only in one zone. Similarly, in glutamate dehydrogenase bands were observed only in one zone. The relationship between these germplasms had been assessed from electrophoresis data and it was observed that *Taothab*i the cultivated rice and *Wainuchara* i.e., *O. rufipogon* local collection I, the wild rice showed close relationship in comparison to other germplasms and correlate the indication of phylogeny from wild to cultivated rice in Table 4. Isozyme analysis has successfully been used as markers of important agronomic traits. The technique is useful in studies of the systematic and evolution of crop plants because Isozyme's reflects discrete changes in the hereditary materials. The results will form the basis for breeding works of popular rice varieties.

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