

Evaluation of Affinities in Mulberry and Its Relatives by Peroxidase Isozyme Technique

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Isozyme criterion for taxonomy may be superior to morphological one in respect that a more direct estimate of gene homology is provided by isozyme patterns, leading to the assessment of taxonomic relationship at the gene level. The use of isozyme techniques has become widely accepted as an adjunct to the conventional taxonomic traits. The results so far obtained in various plant species (Frydenberg and Nielsen 1965, Johnson and Hall 1965) suggest that isozyme technique is possible to be a useful tool for research of mulberry systematics. Based on this suggestion the work concerning relationships in mulberry and its relatives was commenced in 1973. First, the affinity in mulberry relatives was evaluated using peroxidase isozymes by Hirano and Nakajima (1976), followed by the affinity in mulberry varieties by Hirano (1977). In the present paper, results and conclusions of the works will be outlined.

The use of isozymes in various fields of plant science has been already reviewed by Shannon (1968), Scandalios (1969) and Feret and Bergmann (1976).

Isoelectric focusing and peroxidase staining

Details of techniques for detecting isozymes vary depending on researchers and plant materials used. Following is a brief description about the technique which was established by the author and has been extensively applied to mulberry and its relatives these years.

Leaf blade material was manually ground in deionized water using 0.5 g fresh weight of leaf blade per 0.5 ml of water and the extract was centrifuged for 5 min at $10,000\times g$ at $-5^{\circ}C$. After centrifugation the supernatant solution was subjected to electrophoresis. Thin-layer gel isoelectric focusing was run at a constant voltage of 40 V per cm during 4 hrs at $5^{\circ}C$ to separate peroxidase isozymes (pH 5.0–7.0). Staining of peroxidase was performed according to the method described by Endo (1972). Peroxidase isozyme bands detected in mulberry and its relatives were numbered from 1 to 18 in regard to their migration rate towards anode.

Peroxidase phenotype in mulberry

A large-scale experiment on the isoelectric focusing was performed using 240 mulberry varieties to find out the possibility to classify them into 2 or more groups by peroxidase isozyme patterns.

A total of 10 different peroxidase isozyme bands from leaf blades were found in the varieties used. The varieties might be broadly classified into 5 types designated as Type I, II, III, IV and V with regard to peroxidase isozyme patterns by visual inspection where the existence or absence and the staining intensity of such 6 bands as peroxidase-8, -10, -11, -12, -13 and -14 were used as criteria (Fig. 1). Type I, II, III, IV and V were composed of 38, 46, 13, 2 and 1% of the total number of the varieties used, respectively. Some examples for the varieties belonging to

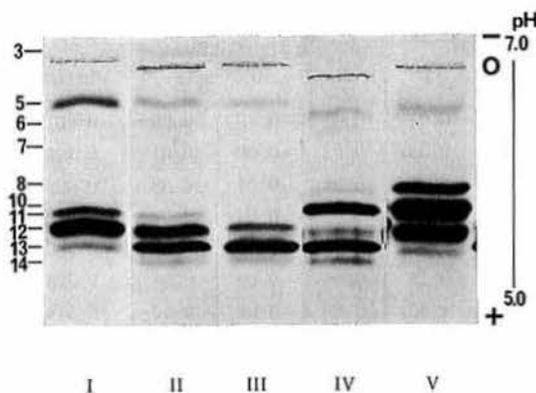


Fig. 1. Zymogram showing 5 types of peroxidase isozyme patterns of leaf blades in mulberry varieties. Varieties of Type I, II, III, IV and V illustrated are Daishukaku, Kasasagiso, Kokokuso, Kumonryu and Hosoe, respectively. 0 represents position of sample inserted.

each type were given as follows. Type I: Daishukaku, Kairyoichinose, Ozekijumonji, Roso, Takinokawa and Tsukasaso, Type II: Fukushimaoha, Ichinose, Kasasagiso, Kenmochi, Shimanouchi and Shinichinose, Type III: Atsubamidori, Kokokuso, Kokuso No. 21 and Tanakaoshu, Type IV: Kumonryu and Yamanishiki, Type V: Hosoe and Kairyokinbei.

A few problems need to be solved to make the above grouping more accurate, since the experiment was carried out using leaf blades with varying leaf age, tree age and planting location. In this connection, Feret and Bergmann (1976) reviewed that electrophoretic patterns are frequently modified by growth, developmental and environmental processes. Therefore, an additional experiment was performed concerning these points and it proved that they had no effect on isozyme variation as far as the experiment is concerned.

Varietal affinities assessed by PCA

Similarity of peroxidase isozyme patterns between varieties was evaluated by PCA (principal component analysis), which proved useful in classifying varieties of maize (Mochizuki and Okuno 1967), and rice (Kamijima 1974), etc.

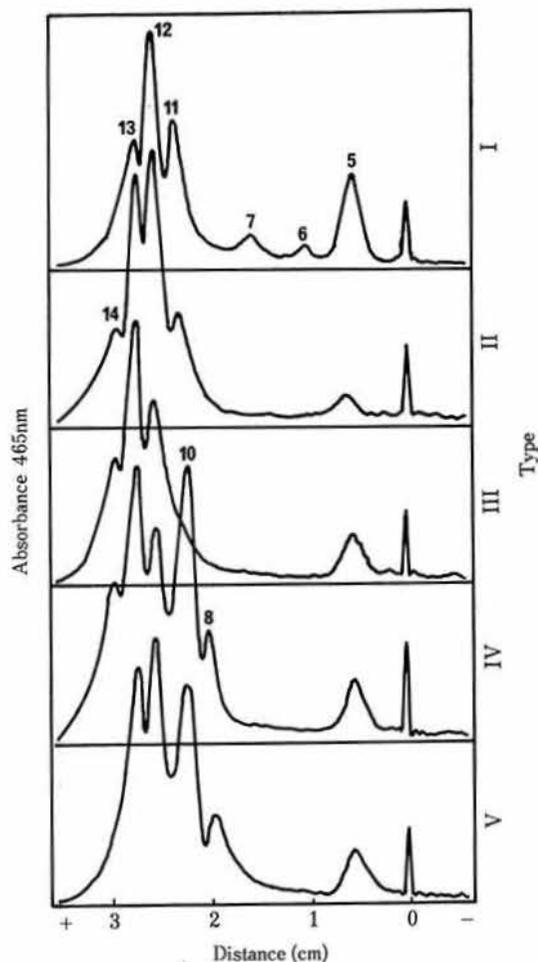


Fig. 2. Densitometric traces of peroxidase isozyme stained-gel illustrated in Fig. 1. 0 represents position of sample inserted.

The staining intensities of the 10 peroxidase isozyme bands were determined by the use of densitometer (Fig. 2). It was indicated that the variances of peroxidase-3, -6 and -7 were very small and peroxidase-8 was highly correlated with peroxidase-10 (Table 1). Consequently, these 4 isozyme bands were considered to be unimportant for PCA, so that PCA was carried out by the use of correlation matrix of peroxidase-5, -10, -11, -12, 13 and -14 except peroxidase-3, -6, -7 and -8.

The sum of the 1st and the 2nd principal components consisted of only 59% of the whole of the information (Table 2). Consequently,

Table 1. Correlation matrix and variances computed from peroxidase isozyme patterns of leaf blades in 240 mulberry varieties

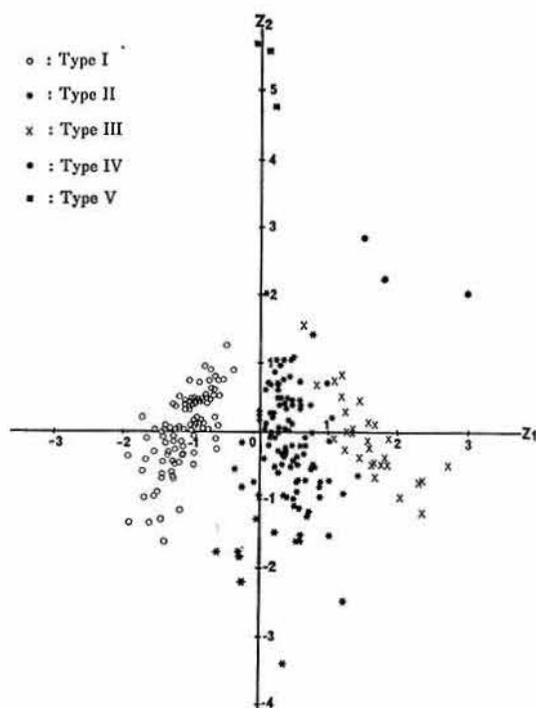
Band No.	3	5	6	7	8	10	11	12	13	14	Variance
3	1.00	-0.02	-0.01	-0.03	-0.02	-0.02	0.01	0.01	-0.13	-0.09	0.001
5	-0.02	1.00	0.27	0.43	-0.01	0.02	-0.03	-0.03	-0.06	0.01	0.172
6	-0.01	0.27	1.00	0.31	-0.05	-0.04	-0.02	0.01	-0.01	-0.03	0.005
7	-0.03	0.43	0.31	1.00	-0.03	-0.01	0.10	0.05	-0.06	0.04	0.002
8	-0.02	-0.01	-0.05	-0.03	1.00	0.93	-0.17	-0.03	-0.05	-0.02	0.043
10	-0.02	0.02	-0.04	-0.01	0.93	1.00	-0.18	-0.06	-0.02	0.01	0.308
11	0.01	-0.03	-0.02	0.10	-0.17	-0.18	1.00	0.63	-0.32	-0.19	0.514
12	0.01	-0.03	0.01	0.05	-0.03	-0.06	0.63	1.00	-0.49	-0.35	1.444
13	-0.13	-0.06	-0.01	-0.06	-0.05	-0.02	-0.32	-0.49	1.00	-0.71	1.465
14	-0.09	0.01	0.03	0.04	-0.02	0.01	-0.19	-0.35	0.71	1.00	0.152

Table 2. Eigen values, contributions and factor loadings of the 1st, 2nd, 3rd and 4th principal components computed from peroxidase isozyme patterns of leaf blades in the mulberry varieties

Band No.	Component No.			
	1	2	3	4
5	0.003	0.265	0.953	0.124
10	0.079	0.641	-0.272	0.702
11	-0.445	-0.478	0.084	0.387
12	-0.524	-0.213	-0.008	0.359
13	0.541	-0.331	0.007	0.195
14	0.478	-0.393	0.100	0.417
Eigen value	2.372	1.163	0.994	0.870
Contribution	0.395	0.194	0.166	0.145
Cumulative contribution	0.395	0.589	0.755	0.900

relationships of peroxidase isozyme patterns between varieties are not perfectly indicated by the scatter diagram of scores of their 2 components. The scatter diagram, however, suggests that the nearer the positions for the varieties the more similar is their isozyme patterns for them. In other words, it suggests that the nearer the positions for them the higher is the affinity for them, because resemblance of isozyme patterns indicates that of genic composition coding for isozyme syntheses.

Fig. 3 shows the scatter diagram of scores of the 1st and the 2nd principal components

**Fig. 3. Scatter diagram of 240 mulberry varieties in Z_1 - Z_2 plane obtained by principal component analysis.**

Z_1 : 1st principal component
 Z_2 : 2nd principal component

calculated by PCA. It is estimated by distribution of the scores that the varieties of the same type resemble most closely each other, that the varieties of Type II show closer affinity with those of both Type I and III, and that

the varieties of Type IV and V show a rather low affinity with those of the other types.

Most taxonomists agree that the majority of 240 mulberry varieties examined belong to one of 3 *Morus* species, namely, *M. bombycis* KOIDZ., *M. alba* L. and *M. latifolia* POIRET, depending on the differences in morphological characteristics in regard to flower, fruit, bud, leaf and shoot (Koidzumi 1917, Hotta 1954). Within limits of the experiment, the relationship between those 3 species and the 5 types of the peroxidase isozyme patterns is not yet clarified.

Peroxidase phenotype in mulberry relatives

Darlington and Wylie (1955) described that Moraceae consists of 75 species, some of which, e.g. fig, India-rubber, mulberry, paper mulberry, are familiar to human beings.

A question arises about how the mulberry is related to other Moraceae plants at the gene level. This question may be settled by comparing isozyme patterns of them. It is, accordingly, to be a prerequisite to examine the specificity of isozyme patterns for Moraceae plant species. In this connection, leaf blade peroxidase isozymes in 2 *Broussonetia* species, a *Cudrania* species and 3 *Ficus* species were

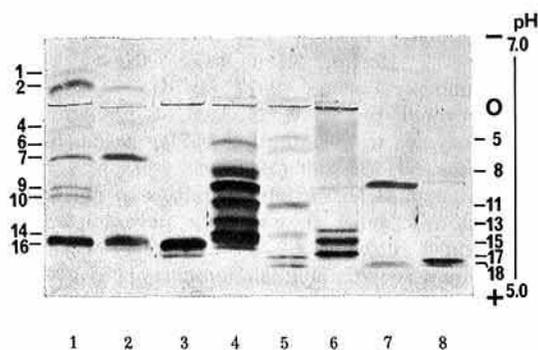


Fig. 4. Zymogram showing peroxidase isozymes from leaf blades in Moraceae.

1: *B. kazinoki*, 2-3: *B. papyrifera*, 4: *C. tricuspidata*. 5: *F. carica*, 6: *F. erecta*, 7-8: *F. retusa*. 0 represents position of sample inserted.

analyzed.

A total of 8, 7, 8, 6, 4 and 3 peroxidase isozyme bands were detected in *B. kazinoki* SIEB., *B. papyrifera* VENT. (paper mulberry), *C. tricuspidata* BUREAU (silkworm tree), *F. carica* L. (fig), *F. erecta* THUMB. and *F. retusa* L. (India laurel), respectively. The peroxidase isozyme patterns were considerably characteristic to species (Fig. 4). Additionally, there were some intraspecific differences of the enzyme activity of peroxidase-7 in *B. papyrifera* and peroxidase-9 and 17 in *F. retusa*.

Affinities between mulberry relatives

The close similarity between different species belonging to the same genus and the conspicuous dissimilarity between species belonging to different genera in peroxidase isozyme patterns are demonstrated in Fig. 4. This is substantiated by *s* values (similarity index values), by which resemblance of peroxidase zymograms between species is indicated (Table 3). The *s* values are calculated by the equations, $s = \{ N_c / (N_c + N_d) \} \times 100$ (%) where *N_c* is the number of bands common to 2 species and *N_d* is the number of bands appeared in either species.

Table 3. Similarity index values (%) of peroxidase zymograms between species in Moraceae

Species	(1)	(2)	(3)	(4)	(5)	(6)
(1) <i>B. kazinoki</i>	—	50.0	23.1	0	9.1	10.0
(2) <i>B. papyrifera</i>		—	25.0	18.2	10.0	11.1
(3) <i>C. tricuspidata</i>			—	27.3	33.3	22.2
(4) <i>F. carica</i>				—	25.0	28.6
(5) <i>F. erecta</i>					—	40.0
(6) <i>F. retusa</i>						—

The conclusion to be drawn from these results is that different species belonging to the same genus show greater affinity each other, and species belonging to the different genera are not closely related. These affinities

evaluated by s values are in close agreement with those in conventional taxonomy.

Relationship between mulberry and its relatives

It may be rather difficult to make strict comparison between mulberry and its relatives concerning peroxidase isozyme patterns, since the materials used differ slightly in growth, developmental and environmental processes and, therefore, resultant variations of the patterns are only feasible. It may be, however, pointed out that homology of the isozyme patterns between mulberry varieties tends to be greater than that between mulberry and its relatives. This means that affinities between mulberry varieties are closer than those between mulberry and its relatives. These affinities deduced from the peroxidase zymogram coincide with those in conventional taxonomy.

Conclusions

As noted above, it was recognized that, in mulberry and its relatives, affinities evaluated by peroxidase zymogram are in fair agreement with those in conventional taxonomy at higher taxonomic level such as species and genus. While, at low taxonomic level such as mulberry variety, relationship between the affinities assessed by the peroxidase zymogram and those in the conventional taxonomy is not well understood.

In the present study electrophoretic variants of peroxidase were examined in order to assess the affinities in mulberry and its relatives. However, the gene or genes coding for peroxidase isozyme syntheses are only a little part of numerous genes in a given plant. To find out the genetic relationships, homology of compositions of as many genes as possible between plants is necessary to be investigated. Therefore, not merely peroxidase isozymes but isozymes of many kinds of enzymes are required to be electrophoretically detected.

It seems impossible to judge taxonomic

affinities of plants simply on the basis of their morphology. Baker (1970) pointed out that, whenever possible, information about the physiology, the ecology, the geographical distribution, the cytology, the genetics and the biochemistry of the plants should be considered along with their morphological traits. Plant taxonomy will be given the genetical as well as the biochemical information by isozyme techniques, not excepting the taxonomy of mulberry and its relatives.

Acknowledgement

The author is grateful to Dr. K. Nakajima who read the manuscript and made valuable suggestions.

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