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VARIETAL DIFFERENCES OF LEAF PROTEIN PROFILES IN MULBERRY

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Key Word Index—*Morus* spp.; Moraceae; mulberry; leaf protein; gel electrophoresis; genetic variation.

Abstract—Varietal differences in the leaf protein profiles of 17 diploid ($2n = 28$) varieties of mulberry (*Morus* spp.) were demonstrated by 2D-gel electrophoresis and silver staining; similarity was statistically evaluated by means of principal component analysis. A close correlation was found between inter-varietal similarity, as estimated by the electrophoretic banding patterns of certain enzymes, and the leaf protein profiles obtained by 2D-gel electrophoresis. Little apparent varietal difference in the amount of ribulose-1,5-bisphosphate carboxylase was detected immunoelectrophoretically in the leaves. Varietal differences in crude protein content were obtained with leaves and this was discussed in relation to the observed 2D-gel electrophoretic patterns.

INTRODUCTION

The nutritive value of the mulberry leaf has a considerable influence on the growth of the silkworm, which feeds on it. Improvement of the food value, which has been a breeding objective in mulberry is probably mainly determined by the protein quantity and quality [1]. However, the genetic variation of the leaf protein profile is poorly understood in mulberry, and so far only a few reports on varietal differences in the total nitrogen content [2, 3] and amino acid content [3] of the leaves have been published.

In the present study, the varietal differences in the leaf protein profiles were investigated using 2D-polyacrylamide gel electrophoresis (2D-PAGE), and silver staining with 17 varieties of mulberry; similarity of the profiles among the varieties was statistically evaluated by means of principal component analysis.

RESULTS AND DISCUSSION

Protein subunits were separated from the 20-day-old leaves of 17 mulberry varieties, A–Q (Table 1) by means of SDS–urea PAGE and stained with Coomassie brilliant blue. These varieties had the same leaf protein profile in which two major bands with MWs *ca* 50000 and 20000, and at least ten minor bands were identified. The similarity of the profiles suggested that more sensitive electrophoretic and protein staining methods would be required if varietal differences of leaf protein profiles in mulberry were to be detected.

Leaf protein subunits were analysed by the combined method of 2D-PAGE and silver staining, instead of SDS–urea PAGE with Coomassie blue staining. This method was extremely sensitive and revealed varietal differences in the protein profiles.

Before determining the varietal differences of leaf protein profiles, any developmental changes in the profiles were examined using the variety Ichinose (K). Little differences of protein profiles between 10, 20 and 40-day-old leaves were found. Differences could be demonstrated only between 2-day-old and 10–40-day-old leaves (Fig. 1). More than 250 subunits were identified in the profiles of 2-day-old leaves, but less than 200 subunits in those of 10–40-day-old ones. Although many subunits common to both 2-day-old and 10–40-day-old leaves were detected, there were some differences in staining intensity between the 2-day-old and 10–40-day-old leaves. It was concluded that 10–40-day-old leaves should be used as source materials when protein profiles were compared among varieties.

In each of the 17 varieties, A–Q, 20-day-old leaves were collected and their protein profile was examined by 2D-PAGE. *Ca* 200 spots of leaf protein subunits were detected in all the varieties. Most of the subunits detected ranged from MW 20 000 to 100 000 and pI 6–10. The subunits with MW *ca* 50 000 and pI *ca* 7 were more heavily stained. Some of the more heavily stained subunits possibly correspond to the large subunits of ribulose-1,5-bisphosphate carboxylase (RuBPCase). The electrophoretic patterns of leaf protein subunits in the two varieties Sozanguwa (M) and Kumonryu (Q), are represented in Fig. 2. Among the 17 varieties, the differences in electrophoretic patterns which prove to be reproducible in more than two experiments were identified as 23 major subunits of leaf proteins (Fig. 2). Table 2 shows the presence or absence of the 23 subunits and their staining intensity in the 17 varieties.

The similarity of the protein profiles between the 17 varieties was examined by means of principal component analysis based on the presence or absence of the 23 subunits and their staining intensity (Table 2). Correlation coefficients as to the electrophoretic patterns of the 23 subunits were calculated among the varieties and principal component analysis was performed based on the resultant correlation matrix. Table 3 shows the eigen vector, eigen value and con-

Table 1. The 17 mulberry varieties used

Variety	Varietal group*	Species†
A. Ginbasho	I	c
B. Sugutate	I	a
C. Takinokawa	II	b
D. Jinguwa	III	c
E. Obata	III	b
F. Rohachi	III	c
G. Shoji	III	c
H. Canada A	IV	d
I. Hikojiro	IV	a
J. Homare	IV	b
K. Ichinose	IV	a
L. Nakazawaso	IV	b
M. Sozanguwa	IV	d
N. Tsuruguwa	IV	d
O. Ruinashi	V	b
P. Hironiwase	VI	c
Q. Kumonryu	VII	c

*Classification based on the homology of electrophoretic banding patterns of enzymes and protein [1].

†Morphological classification [5]: a, *Morus alba* L.; b, *M. bombycis* Koidz.; c, *M. latifolia* Poiret; d, unidentified.

tribution of the first, second and third principal components obtained in principal component analysis. Fig. 3 shows a scatter diagram of the scores of the 17 varieties in the Z_1–Z_2 plane. The cumulative contribution of the first and second principal components was 41% (Table 3). The scatter diagram suggests that the nearer to each other the varieties are in the diagram, the more similar are their protein profiles. For example, the variety Ginbasho (A) is more similar in the protein profile to the variety Sugutate (B) than to the variety Shoji (G).

Recently the affinity has been assessed for 131 mulberry varieties by principal component analysis and cluster analysis based on the differences in electrophoretic patterns of peroxidase from leaf and latex, malate dehydrogenase, alkaline phosphatase, acid phosphatase, α-amylase and leucine amino peptidase from shoot bark, and IAA oxidase and protein from latex [1]. These varieties have been classified into seven varietal groups, Groups I–VII. It has been concluded that possibly the varieties of Groups I and II or Groups IV and V most closely resembled each other, and that the varieties of Groups I–V showed a closer affinity with each other, while the varieties of Groups VI and VII (but notably VII) showed a low affinity with those of the other groups. The electrophoretic variants of leaf peroxidase have been found to be controlled by two codominant alleles, Px_1^1 and Px_1^2, at a single locus, Px_1 [4]. Groups I–III were homozygous for Px_1^1 and Group VI was homozygous for Px_1^2, while Groups IV and V were heterozygous for Px_1^1 and Px_1^2. The varietal groups to which the 17 varieties belong are represented by different marks in Fig. 3. Figure 3 shows that the leaf protein profiles of the varieties within the same varietal group closely resemble each other, and those of the varieties between the varietal groups with closer affinity also resemble each other. Thus the

Table 2. Leaf protein subunits in the 17 mulberry varieties

Subunit	Variety																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	—	1	1	1	1	—	—	1	1	1	1	—	1	—	—	1	2
2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	1	1	2
3	1	1	1	1	—	—	—	1	—	1	1	—	1	1	1	1	—
4	1	1	2	2	1	—	—	—	—	—	—	—	—	—	—	1	1
5	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
6	—	1	1	1	1	1	—	1	1	1	1	—	1	1	—	1	1
7	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	2	1
8	1	2	—	—	2	1	1	—	1	—	2	1	2	1	1	2	2
9	1	1	1	1	1	—	1	—	1	1	1	1	1	1	—	1	1
10	1	—	1	—	1	—	1	1	—	1	1	—	1	—	1	—	1
11	1	1	1	1	1	1	—	1	1	1	—	1	1	1	—	—	—
12	1	1	1	1	1	1	1	1	1	1	—	1	1	1	1	1	—
13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
14	1	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1
15	—	—	—	—	1	—	—	1	—	—	—	—	—	—	1	1	1
16	1	—	1	1	1	—	1	1	1	1	1	—	1	1	1	1	1
17	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
18	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	1
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
20	1	—	1	—	1	—	—	—	—	—	—	—	—	—	—	1	1
21	1	1	1	1	1	1	1	2	1	1	2	2	1	1	1	2	1
22	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	1	1	1	1	1	—	1	—	—	1	1	1	1	—	—	—	—

1, Less heavily stained subunit; 2, more heavily stained subunit. For explanations of the subunit number and the varieties (A–Q), see Fig. 2 and Table 1, respectively.

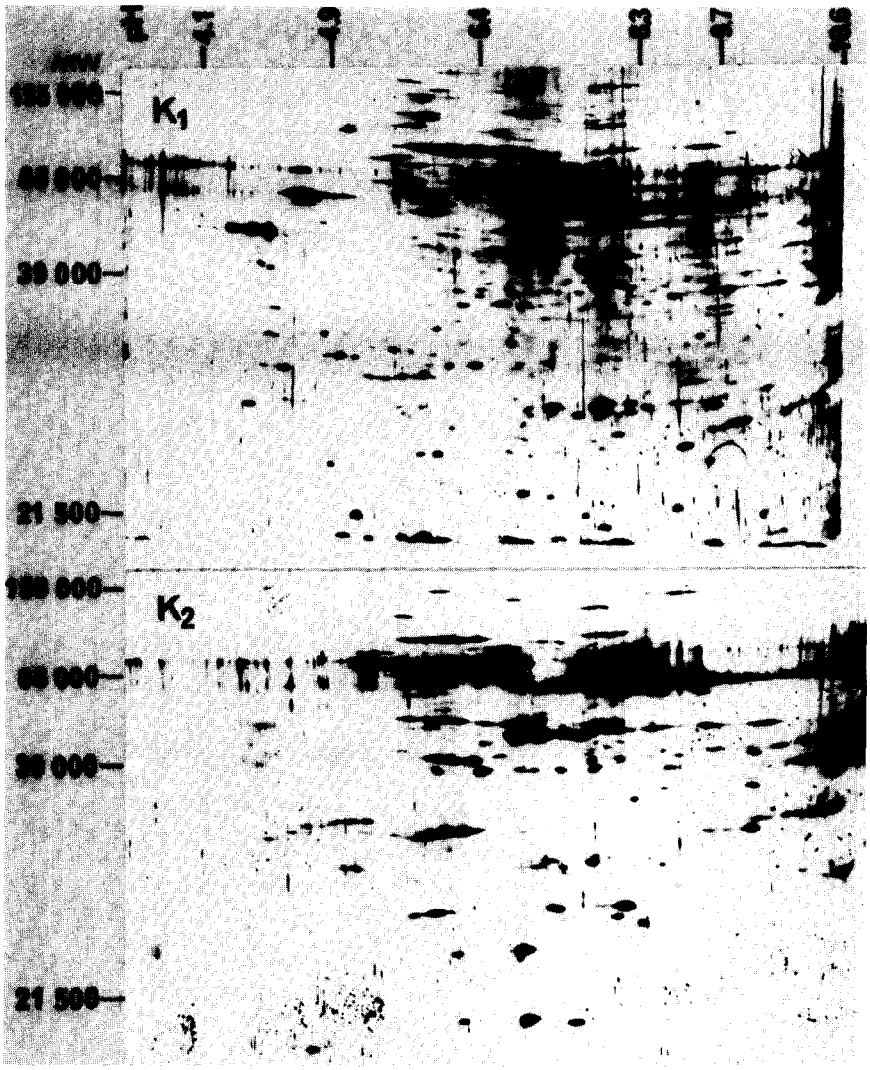

Fig. 1. 2D-PAGE of proteins from 2-day-old (K_1) and 20-day-old (K_2) leaves of the variety Ichinose. Proteins were separated in the horizontal dimension by isoelectric focusing and in the vertical dimension by SDS-PAGE. The gels were stained by silver staining.

similarity of leaf protein profiles is consistent with the affinity estimated previously by the electrophoretic banding patterns of enzymes from leaf, shoot bark and latex, and protein from latex.

According to the conventional morphological classification, most of the 17 varieties belong to either *Morus alba* L., *M. bombycis* K. or *M. latifolia* P. (Table 1) [5]. It is well known that the three species cross easily, that zygotes can be formed, and that F_1 hybrids are fertile [6]. Few differences have been found among these species in the frequency of the electrophoretic variants of enzymes from leaf, shoot bark and latex and of the protein from latex [1]. In the present study, no apparent differences of leaf protein profiles were found among these species. These results suggest that these three species should be 'lumped' into one.

There are many polyploidy varieties ($3x - 22x$) in mulberry [7]. The number of chromosomes of the 17 varieties used was examined and all the varieties were found to be diploid ($2n = 28$). This shows that the differences in protein profiles do not depend on polyploidy. The variety Takinokawa has been reported to be triploid [8]. However, the variety Takinokawa (C) used in the present study was diploid.

RuBPCase is major soluble chloroplast protein which can account for up to 50% total soluble protein in leaf extract [9]. In the present study, RuBPCase subunits were not specifically identified in 2D-PAGE patterns. The amount of RuBPCase present was examined immunoelectrophoretically in the 17 varieties, but little apparent varietal difference was found in the electrophoretic patterns (result not shown).

The leaf crude protein content varied, depending on the age of leaf in the variety Ichinose (K); the content tended to decrease as the leaf aged. However, the contents in leaves of similar ages were

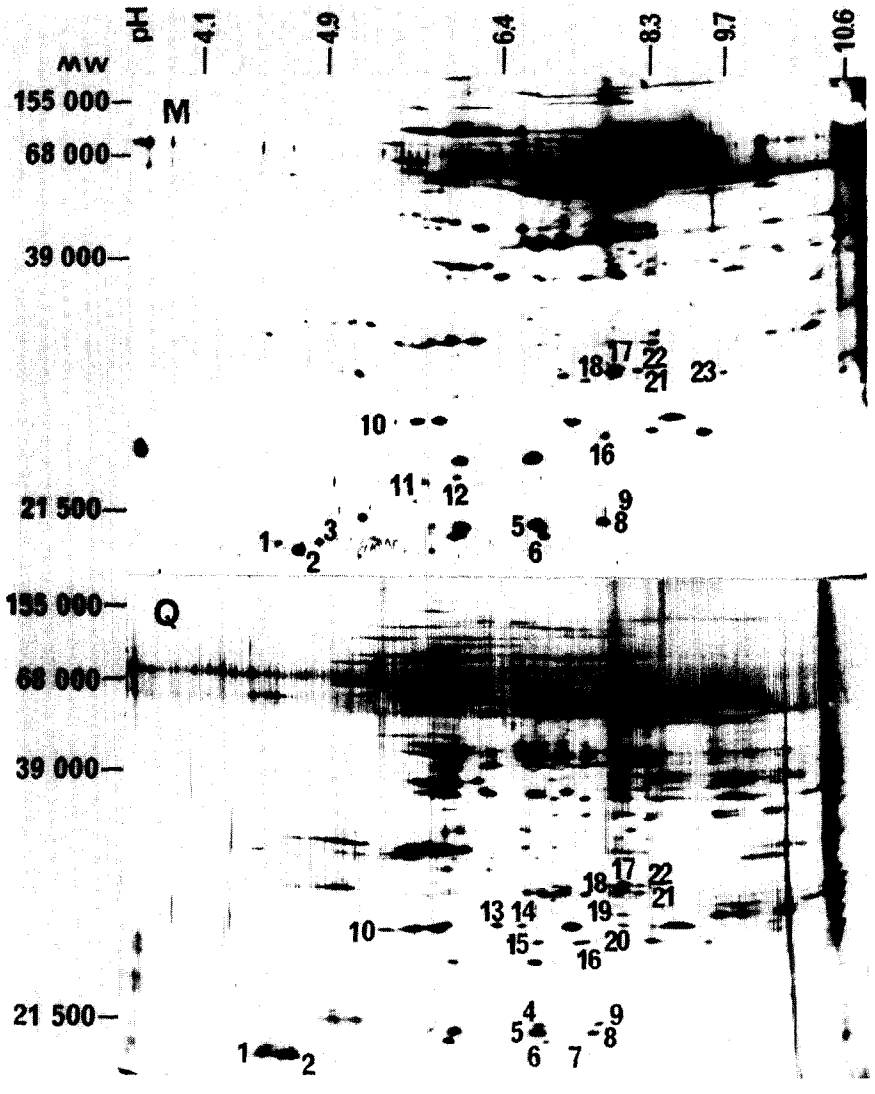

Fig. 2. 2D-PAGE of proteins from 20-day-old leaves of the varieties Sozanguwa (M) and Kumonryu (Q).

identical in three plants. Accordingly, it was considered possible to determine the varietal differences of the leaf crude protein contents when the ages of leaf samples were similar. Table 4 shows the crude protein contents of 20-day-old leaves in the 17 varieties. An additional experiment showed that the means of the crude protein contents in the 190 mulberry varieties including the 17 varieties were 27.0% (s.d. 1.78, CV 6.6%) in dry matter and 7.42% (s.d. 0.50, CV 6.7%) in fresh matter. There was a significant correlation between the contents in dry and fresh matter ($r = 0.550$, significant at the 1% level). The grouped distribution of the 190 varieties with nine classes on the basis of crude protein content in dry matter is represented in Fig. 4. Table 4 and Fig. 4 show that the variety Nakazawaso (L) had the highest content of crude protein of the 190 varieties, and the variety Hikojiro (I) the lowest. The crude protein content in the varieties Jinguwa (D), Canada A (H), Sozanguwa (M) and Tsuruguwa (N) is relatively high, and that of the varieties Sugutate (B) and Shoji (G) relatively low. The possibility exists that the varietal differences in crude protein content are brought about by specific subunit contents. In the present study, an attempt was made to determine whether or not any of the 23 protein subunits which differed between the varieties examined were related to high or low crude protein content, and it proved that no specific subunit content correlated with the crude protein contents.

EXPERIMENTAL

Plant materials. 17 field-grown mulberry varieties were used. More than two plants, 3 years old, were tested for each variety. 20-day-old leaves were collected *ca* 20 days after leaf expansion from the shoots which had developed from stumps following spring pruning. Besides these materials, 2, 10 and 40-day-old leaves were collected from the shoots of variety Ichinose (K) to examine the developmental changes in protein profile.

SDS–urea PAGE and Coomassie blue staining. Proteins from 50 mg ground leaves were extracted in 1 ml 0.0625 M Tris–HCl buffer at pH 6.8 containing 8 M urea, 2% SDS, 5%

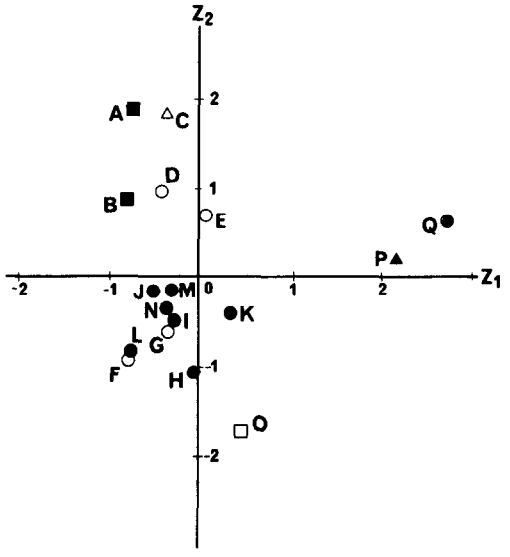

Fig. 3. Scatter diagram of the 17 mulberry varieties in Z_1–Z_2 plane obtained by principal component analysis. Z_1, First principal component; Z_2, second principal component. For an explanation of the varieties (A–Q), see Table 1. Varietal group: (■), I; (△), II; (○), III; (●), IV; (□), V; (▲), VI; (◉), VII.

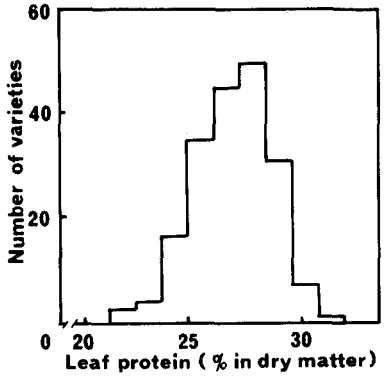

Fig. 4. Histogram of the grouped distribution of the 190 mulberry varieties with nine classes on the basis of the crude protein contents in dry matter.

β-mercaptoethanol and 10% sucrose, and heated at 90° for 5 min. After centrifugation at 15 000 *g* for 20 min. the proteins in 10 μl of supernatant were separated by SDS–urea PAGE. The separation gel contained 8 M urea, 0.1% SDS, 12.5% acrylamide (acrylamide–bisacrylamide, 15 : 0.4) in 0.375 M Tris–HCl buffer at pH 8.5. The stacking gel contained 8 M urea, 0.1% SDS, 4.5% acrylamide (acrylamide–bisacrylamide, 15 : 0.4) in 0.125 M Tris–HCl buffer at pH 6.8. The electrode soln was 0.025 M Tris–0.185 M glycine buffer containing 1% SDS. After electrophoresis for *ca* 3 hr at 2 mA/cm, the gels were stained in a soln containing 0.1% Coomassie brilliant blue R250, 50% MeOH and 10% HOAc for 30 min and destained in a soln containing 7% HOAc.

2D-PAGE and silver staining. Proteins from 30 mg ground leaves were extracted in 1 ml 8 M urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5–10), 5% β-mercaptoethanol and 5%

Table 3. Eigen vector, eigen value and contribution of the first, second and third principal components computed from the electrophoretic patterns of leaf proteins in the 17 mulberry varieties

Subunit	Component number		
	1	2	3
1	0.235	0.181	0.326
2	−0.154	0.180	0.291
3	−0.029	0.113	−0.008
4	0.067	0.435	0.053
5	0.081	−0.243	0.387
6	0.084	0.105	0.502
7	0.281	0.205	0.132
8	0.184	0.004	0.018
9	0.021	0.309	0.126
10	0.066	0.050	−0.300
11	−0.299	0.141	0.211
12	−0.241	−0.014	−0.045
13	0.387	0.075	0.047
14	0.106	0.169	−0.236
15	0.303	−0.097	−0.098
16	0.157	0.080	−0.141
17	−0.177	0.290	−0.113
18	−0.252	0.112	0.257
19	0.387	0.075	0.047
20	0.216	0.351	−0.147
21	0.105	−0.166	0.148
22	−0.125	0.371	−0.150
23	−0.203	0.248	−0.013
Eigen value	5.697	3.728	2.628
Contribution	0.248	0.162	0.114
Cumulative contribution	0.248	0.410	0.524

Table 4. Contents of protein in the 17 mulberry varieties

Variety	% in dry matter	% in fresh matter
A	25.04 ± 0.40	7.01 ± 0.12
B	22.59 ± 0.89	7.04 ± 0.45
C	27.00 ± 0.22	7.32 ± 0.23
D	31.23 ± 0.59	8.36 ± 0.06
E	27.42 ± 0.42	7.72 ± 0.08
F	27.15 ± 0.20	6.81 ± 0.22
G	22.31 ± 1.80	6.66 ± 0.27
H	30.83 ± 0.74	7.82 ± 0.40
I	21.61 ± 1.84	7.49 ± 0.46
J	27.13 ± 1.30	7.11 ± 0.39
K	26.81 ± 0.41	7.72 ± 0.58
L	32.71 ± 1.10	8.28 ± 0.29
M	30.42 ± 1.22	7.87 ± 0.08
N	29.73 ± 1.33	8.06 ± 0.04
O	25.83 ± 0.32	7.82 ± 0.08
P	26.27 ± 0.41	7.29 ± 0.11
Q	26.25 ± 1.28	6.95 ± 0.36

Values are given as the means ± s.d. of three experiments using different plants. For an explanation of the varieties (A–Q), see Table 1.

PVP, and heated at 90° for 5 min. After centrifugation at 15000 *g* for 20 min, the proteins in 10 μl of supernatant were separated by 2D-PAGE according to the procedure of ref. [10] modified as follows for isoelectric focussing in the first dimension: the gels contained 8 M urea, 3.5% acrylamide (acrylamide–bisacrylamide, 4.73 : 0.27) and 2% Ampholine (a 1 : 1 mixture at pH 3.5–10 :pH 4–6). After pre-electrophoresis of the gels at 2.4 W for 1 hr, 10 μl of protein sample soln was applied to the top of the gel. The proteins were run at 3.6 W for 4 hr, and the gel was stained with silver [11].

Calibration of pI and MW. PIs of proteins were inferred using the following pI marker proteins as standards: horse heart cytochrome *c* (pI 10.6) and its acetylated derivatives (*p*Is 9.7, 8.3, 6.4, 4.9 and 4.1). MWs were estimated using the following proteins as standards: soybean trypsin inhibitor (MW 21 500), bovine serum albumin (MW 68 000) and *E. coli* RNA-polymerase α-subunit (MW 39 000) and β-subunit (MW 155 000).

Principal component analysis. Similarity of leaf protein profiles among the 17 varieties was estimated by principal component analysis [12].

Determination of the number of chromosomes. The tips of young leaves were collected and the number of chromosomes was determined according to the Feulgen's squash technique.

Immunoelectrophoresis of RuBPCase. Proteins from 20 mg ground leaves were extracted in 1 ml 2.5 M Tris–HCl buffer at pH 7.4 containing 20 mM NaCl and 10 mM β-mercaptoethanol. After centrifigation at 5000 *g* for 10 min, RuBPCase in 5 μl of supernatant was quantitatively assayed by immunoelectrophoresis according to the 'rocket' technique [13]. The gel contained 1% agarose and 0.2% anti-RuBPCase-antiserum in 0.2 M Tris–HCl buffer at pH 8.6. The same buffer was used as electrode soln. Electrophoresis for 4 hr at 2 mA/cm was performed.

Evaluation of crude protein content. After the fr. and dry wt of leaves were measured, total nitrogen content in the dried powder of leaves was estimated by the Kjeldahl method. Crude protein content was calculated by multiplying the total nitrogen content by 6.25. As an additional expt the crude protein contents in leaves were examined in a further 173 varieties of mulberry according to the same procedure.

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