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Glycine formosana Hosokawa in Taiwan: pod morphology, allozyme, and DNA polymorphism

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Abstract. *Glycine formosana* Hosokawa is distributed over the grassland along the riverside and roadside at Dahshi, Guanshi, and Herngshan in Taoyuan county, Taiwan. It is a twining annual herb. It was long considered to be of the same species as *G. soja*, distributed widely in East Asia. Because the plant showed a continuous variation with *G. soja* in appearance, Tateishi and Ohashi (1992) considered it a geographic subspecies and renamed it *G. max* subsp. *formosana* (Hosokawa) Tateishi et Ohashi. This study attempts to determine the differences between *G. formosana* and the *G. soja* collected in China, Korea, and Japan. The materials were planted at National Chung-Hsing University. The pods, allozymes, and DNA polymorphisms were investigated. *Glycine formosana* has small seeds which are significantly different from the *G. soja* in China, Korea, and Japan. *Lap1-d* — one of the 16 loci of 9 allozymes exists only in *G. formosana*. Twenty-five random sequence 10-mer primers were employed in RAPD analysis for all samples. Twenty-one produced bands, and 14 of those showed polymorphisms. One hundred and thirty-two bands were produced, and 84 bands (64%) showed polymorphisms. Based on the appearances of markers, the genetic similarity coefficients were calculated. Among different samples of *G. formosana*, few genetic variations were observed (0.885~0.887). However, *G. formosana* showed marked differences from *G. soja* in China and Korea and nested within the Japan accessions.

Keywords: Allozyme; DNA polymorphism; *Glycine formosana*; *G. soja*; Pod; Seed.

Abbreviation: RAPD, random amplified polymorphic DNA.

Introduction

Glycine consists of many species in two subgenera, *Soja* and *Glycine*. Subgenus *Soja* includes *G. max*, a cultivated form, and *G. soja*, a wild relative. Subgenus *Glycine* has at least 17 species including *G. albicans, G. arenaria, G. argyrea, G. canescens, G. clandestina, G. curvata, G. cytoloba, G. dolichocarpa, G. falcata, G. hirticaulis, G. lactovirens, G. latifolia, G. latrobeana, G. microphylla, G. pindanica, G. tabacina*, and *G. tomentella* (Tindale, 1984, 1986a,b; Tindale and Craven, 1988, 1993; Tateishi and Ohashi, 1992).

Glycine soja is generally distributed in China, Japan, Korea, and Taiwan (Hymowitz and Newell, 1981). In 1924, Shimada collected a wild relative in Hsinchu and Dahshi, Taiwan, and recognized it as *G. ussuriensis* Regal et Maack (cf: Tang and Lin, 1962). Soon after, another wild relative was collected in Hsinchu, Chutong, and Herngshan. In 1932, Hosokawa identified and classified the latter as *G. formosana* Hosokawa. Not until 1962 did Tang, Lin, and Hermann identify the two wild relatives as the same species as *G. soja*. Huang and Ohashi (1977) replaced the name *G. ussuriensis* with *G. soja* Sieb. et Zucc.

In the mid-1980s, Ohashi et al. (1984) again replaced it with *G. max* subsp. *soja* (Sieb. et Zucc.) Ohashi. Recently, considering the characteristics of its leaf, flower, pod, and seed, Tateishi and Ohashi (1992) named this wild relative found in Taiwan as *G. max* subsp. *formosana* (Hosokawa) Tateishi et Ohashi. However, all of the above were based on the result of plant morphology investigations. In contrast, this experiment is based on DNA and allozyme investigations.

Materials and Methods

Plant Materials

Three accessions (accession numbers 1~3) of *G. formosana* were collected from three counties in Taiwan, including Dahshi, Guanshi, and Hernshan. Each accession collected ten plants as a population from each county. Two accessions of *G. soja* were collected from Korea (accession numbers 4~5), four from China (accession numbers 6~9), and nineteen from Japan (accession numbers 10~28). Twenty seeds were randomly selected from each accession. The seeds served as base materials in this experiment. First, their coatings were pierced, and then the seeds were planted on plastic plates. When the seedlings had grown to about three centimeters, they were moved to pots; two in each pot and ten pots for each

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accession. In the flowering stage, for each accession, we took five pots and collected their leaves for DNA extraction; the pods and seeds of the other five pots were harvested when matured.

Extraction of DNA

DNA was extracted using a modified version of the method of Doyle et al. (1990). Leaf materials (1 g fresh weight) were ground to fine powder in liquid nitrogen. The powdered leaf tissue was transferred to a beaker, and 5 ml of pre-heated extraction buffer [2% CTAB (Hexadecyltrimethyl-ammonium bromide, $C_{19}H_{42}BrN$); 1.4 M NaCl; 0.2% 2-mercaptoethanol; 20 mM EDTA; and 1,000 mM Tris-HCl pH=0.8] was added. After 20 min at 60°C, 5 ml of chloroform-isoamyl alcohol was added, and the upper clear part of the solution was collected by centrifugation at 6,000 *g* (4°C, 10 min). Then, 5 ml of chloroform-isoamyl alcohol was again added, and the cell debris was removed by another 10 min of centrifugation at 4°C. The DNA was precipitated by the addition of 3.3 ml of isopropanol and recovered by centrifugation for 5 min at 10,000 *g* after incubation in a freezer (-20°C) overnight. The pellet was dried and re-dissolved in 2 ml TE [10 mM Tris-HCl pH=7.4, 1 mM EDTA], 0.2 ml 2 M NaCl, and 5 ml alcohol (95%). Then, the precipitate was collected by centrifugation at 10,000 *g* for 10 min at 4°C. The pellet was re-dissolved in 1 ml TE. Contaminating RNA was removed by digestion with 1 µg RNase (10 mg/ml) for 30 min at 37°C. 100 µl of 4.4 M ammonium acetate and 2.5 ml alcohol (95%) were then added. After 30 min at -20°C, the sample was centrifuged for 10 min. The final pellet was dissolved in 0.2 ml TE buffer, and the DNA concentration was determined using a fluorometer and following the procedures supplied by the manufacturer. The extracted DNA was stored at 4°C in a cooler.

RAPD Amplification

A set of 25 10-mer primers (#1~#25) obtained from the University of British Columbia were used in the reactions with *G. soja* and *G. formosana*. Each of them was then reacted with COY TempCycler 2 (COY Corporation). Components for PCR reaction and PCR reaction cycles are shown in Table 1 and 2. Fragments generated by amplification were separated according to size on 2% agarose gels run in 0.5×TBE buffer [0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA], stained with ethidium bromide, examined optically with ultraviolet illumination, and photographed with Polaroid film 667.

Table 1. Reaction mixture used in RAPD analysis.

Components	Volume (µl)	Concentration
Sterile water	15.2	
10× buffer	2.5	1×
2.5 mM dNTP	2.5	0.25 mM
1.5 µM primer	3.3	0.2 µM
50 ng/µl Genomic DNA	1	2 ng/µl
2 U/µl Taq	0.5	0.04 µ/µl
(Dynazyme TM, Finnzymes Inc.)		

Table 2. PCR reaction cycles.

Step	Temperature (°C)	Time (min)	Cycles
1	94	2	
	42	2	1
	72	2	
2	94	1	
	42	1	40
	72	1	
3	94	1	
	42	1	1
	72	2	

Data Analysis

Fragments generated by amplification were separated in 2% agarose gel and photo-recorded. Clearly identifiable bands were analyzed. Using each individual plant as an OTU (operational taxonomic unit), the similarity coefficients of 28 samples of *G. soja* and *G. formosana* were generated. Data were scored for computer analysis on the basis of the presence or absence of the amplified products. If a product was present in genotype, it was designated "1". If absent, it was designated "0". Jaccard's coefficients were generated based on Jaccard's definition (1908). Using the NT-SYS (Rohlf et al., 1971) computer program, the similarity coefficients were then used to construct a dendrogram by UPGMA (unweighted pair group method with arithmetic mean).

Enzyme System Analysis

Seeds from each accession (three seeds for each) were chosen to perform the following nine enzyme system examination: *Aco1, Aco2, Aco3, Aco4,* and *Aco5* (aconitase); *Ap* (acid phosphatase); *Dia1* (diaphorase); *Lap1* (leucine aminopetidasw); *Enp* (endopeptidase); *Est1* (esterase); *Idh1* and *Idh2* (isocitrare dehydrogenase); *Mpi* (mannose phosphate isomersae); *Pgm1, Pgm2,* and *Pgm3* (phosphoglucomutase). Isozyme assays were adapted from Griffin and Palmer (1987). Two electrophoretic buffer systems were used. One was the Histidine-citrate (pH= 6.5, D buffer by Cardy and Beversdorf, 1984), and the other was a modification of the method by Second (1982), which consists of 5 mM histidine HCl, 16 mM Tris-Histidne pH= 7.0 (gel buffer), 400 mM Tris, 132 mM citric acid HCl pH= 7.0 (electrode buffer). Electrodphoresis was conducted for five hours under a constant voltage of 250V in the first system, and for six hours under a constant current of 25mA in the second system. The enzymes visualized using the first system were ACO, APH, DIA, and IDH. The enzymes visualized using the second system were PGM, MPI, ENP, EST, and LAP. The staining procedure for these enzymes was adapted from Griffin and Palmer (1987) and Bult and Kiang (1989).

Results and Discussion

The pods are shown in Figure 1. Pods from Taiwan are the smallest, with those from China somewhat larger and those from Korea and Japan the largest. A maximum of three seeds are in each pod of the four populations, and the seeds are oval in shape. The smallest seeds are found in the Taiwanese population while the seeds of the others are larger. This result resembles that of Abe et al. (1994) and Tateishi and Ohashi (1992). *Glycine formosana* from Taiwan doesn't show a continuous variation with the accessions from other locations in seeds sizes.

Sixteen loci of the allozymes of *G. formosana* were tested. All plants tested exhibited the same genotype, and no within-population or between-population genetic variation in accessions was detected. This is consistent with the results of Yiu (1993), which found no variations within-population (thirty accessions in each population) or between-population (three populations) in *G. formosana* for five allozymes. However, these results are in contrast with the findings obtained for *G. soja* distributed in China, Korea, and Japan, where most populations were an aggregation of different homozygotes for several loci. The paucity of allozyme variation in *G. formosana* suggests a severe genetic bottleneck it might have passed during evolution. The alleles observed in *G. formosana* are as follows: *Aco1-a, Aco2-1, Aco3-a, Aco4-a, Ac05-a, Ap-a, Dial-a, Enp-a, Est1-b, Idh1-b, Idh2-b, Lap1-d, Mpi-b, Pam1-a, Pam2-b*, and *Pam3-b*. Comparing this result with the different homozygotes shown in *G. soja* distributed in China, Korea, and Japan on some alleles, all have been detected in *G. soja* besides *Lap1-d* (Abe et al., 1992). This allozyme found on the *Lap1-d* allele of *G. formosana* codes for the slowest moving mobility variant (Figure 2). The *Lap1-d* allele has not been detected in those populations of subsp. *soja* and subsp. *max* originating in China, Korea, and Japan so far (Kiang et al., 1987; Perry et al., 1991; Abe et al., 1992; Hirata et al., 1994). As a result, the *Lap1-d* allele may be an important marker in determining the taxonomy and origin of *G. soja* while at the same time suggesting an allied relationship among *G. formosana*, subsp. *max*, and subsp. *soja*.

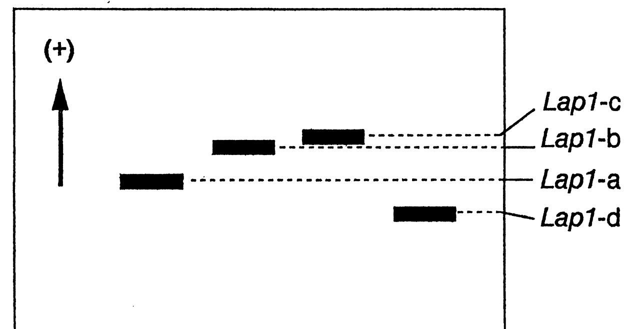

Figure 2. Mobility variant of the leucine aminopetidase Isozyme in *G. formosana*.

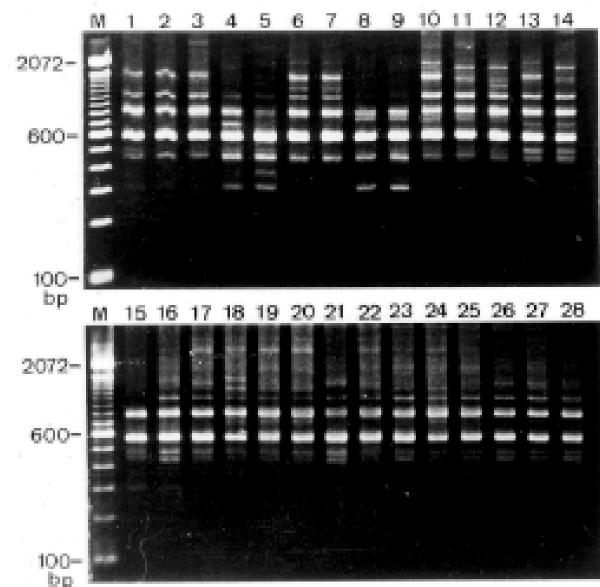

Figure 3. RAPD profile of DNA from 28 samples of *G. formosana* and *G. soja* using primer #4. M, DNA marker. Note: Numbers indicated in this figure are the same as in Table 4.

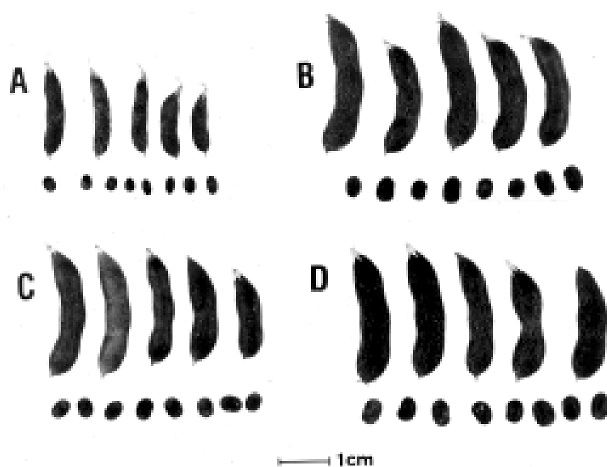

Figure 1. Seed and podmorphology of *G. formosana* and *G. soja*. A, *G. formosana* from Taiwan; B, *G. soja* from Korea; C, *G. soja* from China; D, *G. soja* from Japan.

Twenty-five primers were used in the RAPD analysis performed on three *G. formosana* samples and twenty five *G. soja* samples. The results of primer #4 are shown in Figure 3. Among thirteen bands, nine of them showed polymorphism. All samples produced 450 bp, 480 bp, 580 bp, and 800 bp bands. A 300 bp band was present in the Korea and China accessions and was absent in the other samples. Three samples from Taiwan had the same bands and had at least one DNA cluster different from the other twenty-five samples. Figure 4 presented the result of primer #25. Twelve out of thirteen bands showed polymorphism. A 380 bp band is present in all samples. Three samples from Taiwan again had the same bands, and all produced thirteen bands. Nine bands were different

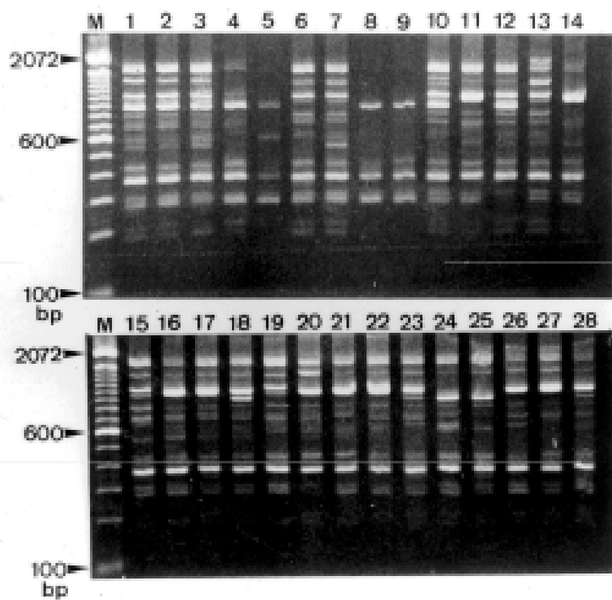

Figure 4. RAPD profile of DNA from 28 samples of *G. formosana* and *G. soja* using primer #25. M, DNA marker. Note: Numbers indicated in this figure are the same as in Table 4.

among the Taiwanese accessions and two Korean samples, two among the Taiwanese accessions and two of the four Chinese samples, eight among the Taiwanese accessions and the other two Chinese samples, and two to six among the Taiwanese accessions and the Japanese accessions.

Of the twenty-five primer reactions, four had no products and twenty-one had amplified DNA products. Among those twenty-one primers, fourteen were polymorphic. One hundred and thirty-two DNA products were amplified in those fourteen primers, which showed polymorphism. As shown in Table 3, 84 products (64%) showed polymorphism. Those 84 products were used in the construction of a matrix in which “1” and “0” were inserted according to the presence or absence of amplified products. Similarity coefficients were then generated according to Jaccard’s definitions (Table 4). The degree of similarity is between 0.176 and 0.887. Sample 8 (the Chinese accession) and sample 19 (the Japanese accession) had the lowest degree of similarity (0.176), and the three Taiwanese samples had the highest degree of similarity (0.887). Based on the matrix, cluster analysis was performed using UPGMA. The results are shown in Figure 5. Apparently, two clusters were identified. The first cluster contained twenty-four accessions including all those from Japan (acc. No. 10~28, except 15) and Taiwan (acc. No. 1~3). Another cluster with four accessions included accessions 4 and 5 collected from Korea and accessions 8 an 9 collected from northeastern China. These results indicated that *G. formosana* nested well within all the *G. soja* accessions from Japan and that results of accessions from Korea and northeastern area of China were close.

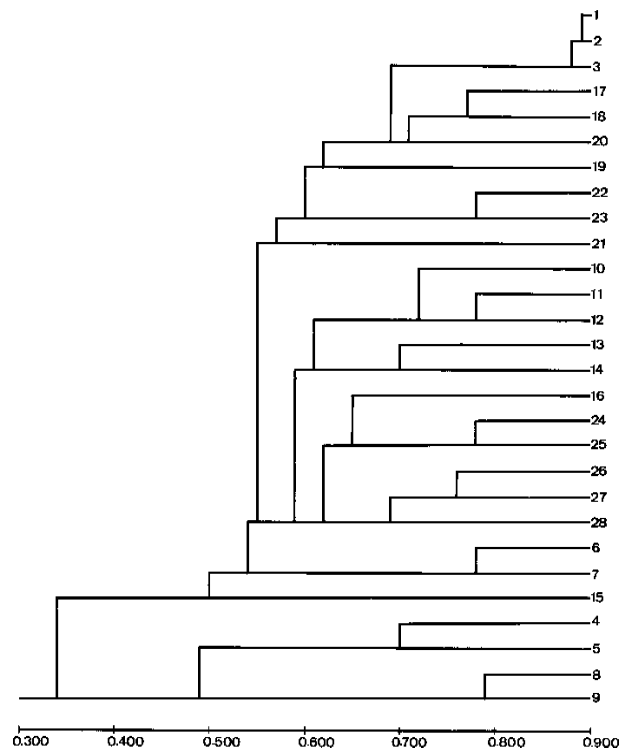

Figure 5. Dendrogram of *G. formosana* and *G. soja* constructed based on Jaccard’s similarity coefficients by using UPGMA method. Note: Numbers indicated in this figure are the same as in Table 4.

Table 3. Primers utilized in RAPD analysis of *G. formosana* and *G. soja.* The total number of DNA fragments (bands) amplified and the number that polymorphic are given for each primer.

U.B.C. Sequence primers (5’ to 3’)	Total bands (no. polymorphic)	U.B.C. Sequence primers (5’ to 3’)	Total bands (no. polymorphic)
#1 CCTGGGCTTC	9 (6)	#9 GGTGGCGGGA	6 (4)
#2 CCTGGGCTTG	12 (9)	#11 CCTGGGCCTC	8 (4)
#3 CCTGGGCTGG	11 (8)	#12 GGGCCGTTTA	9 (6)
#4 CCTGGGTTCC	13 (9)	#13 GCCCGGTTTA	8 (5)
#6 CCTGCGCTTA	6 (3)	#15 CCTGGGTTTG	8 (4)
#7 CCTGGGGGTT	9 (5)	#21 TCCGGGTTTG	11 (5)
#8 CCTGGGTTTG	9 (4)	#25 ACCGGGTTTC	13 (12)
Total			132 (84)

Table 4. Similarity matrix for 28 samples of *G. formosana* and *G. soja* Jaccard's coefficient range of values from 0 to 1.0, with values closer to 1.0 indication increasing similarity.

Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1.00																											
2	0.89	1.00																										
3	0.89	0.87	1.00																									
4	0.39	0.40	0.41	1.00																								
5	0.42	0.42	0.41	0.71	1.00																							
6	0.60	0.59	0.61	0.49	0.52	1.00																						
7	0.60	0.57	0.61	0.46	0.46	0.78	1.00																					
8	0.24	0.25	0.28	0.46	0.45	0.32	0.24	1.00																				
9	0.27	0.27	0.31	0.54	0.54	0.30	0.27	0.79	1.00																			
10	0.52	0.49	0.55	0.43	0.36	0.55	0.48	0.35	0.32	1.00																		
11	0.56	0.53	0.59	0.42	0.37	0.55	0.48	0.26	0.27	0.73	1.00																	
12	0.62	0.59	0.65	0.42	0.33	0.58	0.53	0.31	0.30	0.71	0.78	1.00																
13	0.57	0.56	0.55	0.37	0.32	0.48	0.43	0.31	0.32	0.52	0.64	0.71	1.00															
14	0.58	0.55	0.57	0.48	0.45	0.54	0.47	0.30	0.34	0.59	0.60	0.61	0.70	1.00														
15	0.59	0.56	0.57	0.42	0.33	0.39	0.35	0.32	0.33	0.43	0.47	0.50	0.61	0.58	1.00													
16	0.62	0.59	0.61	0.43	0.43	0.59	0.52	0.28	0.31	0.55	0.64	0.58	0.58	0.67	0.59	1.00												
17	0.74	0.68	0.72	0.39	0.35	0.61	0.56	0.27	0.28	0.60	0.66	0.67	0.60	0.64	0.64	0.75	1.00											
18	0.68	0.65	0.69	0.33	0.31	0.52	0.52	0.18	0.21	0.57	0.66	0.64	0.54	0.63	0.51	0.64	0.77	1.00										
19	0.57	0.56	0.58	0.36	0.26	0.46	0.44	0.18	0.21	0.48	0.52	0.53	0.46	0.49	0.48	0.53	0.64	0.69	1.00									
20	0.69	0.68	0.68	0.39	0.35	0.49	0.49	0.26	0.29	0.51	0.57	0.58	0.58	0.54	0.59	0.61	0.72	0.69	0.70	1.00								
21	0.54	0.51	0.57	0.38	0.31	0.56	0.52	0.26	0.29	0.46	0.53	0.56	0.53	0.57	0.55	0.62	0.64	0.60	0.61	0.64	1.00							
22	0.56	0.51	0.57	0.35	0.32	0.52	0.52	0.19	0.21	0.54	0.51	0.52	0.39	0.50	0.40	0.55	0.62	0.70	0.59	0.62	0.53	1.00						
23	0.59	0.55	0.59	0.38	0.33	0.49	0.57	0.19	0.22	0.53	0.49	0.52	0.44	0.53	0.32	0.50	0.57	0.69	0.61	0.59	0.53	0.78	1.00					
24	0.59	0.58	0.59	0.46	0.46	0.60	0.46	0.30	0.31	0.62	0.63	0.64	0.49	0.63	0.47	0.67	0.61	0.61	0.46	0.53	0.55	0.51	0.49	1.00				
25	0.62	0.61	0.65	0.47	0.44	0.58	0.56	0.33	0.34	0.60	0.64	0.65	0.55	0.61	0.48	0.63	0.70	0.67	0.47	0.54	0.52	0.52	0.57	0.78	1.00			
26	0.55	0.52	0.59	0.49	0.41	0.61	0.59	0.37	0.38	0.67	0.60	0.66	0.55	0.62	0.48	0.64	0.66	0.57	0.44	0.54	0.52	0.57	0.54	0.65	0.69	1.00		
27	0.55	0.54	0.55	0.51	0.38	0.64	0.58	0.28	0.32	0.69	0.59	0.66	0.52	0.59	0.73	0.60	0.63	0.57	0.48	0.55	0.51	0.59	0.59	0.67	0.66	0.77	1.00	
28	0.44	0.43	0.43	0.48	0.35	0.56	0.56	0.29	0.30	0.46	0.47	0.53	0.47	0.48	0.36	0.58	0.51	0.49	0.43	0.46	0.48	0.51	0.54	0.52	0.55	0.71	0.67	1.00

Note: Acc. No. 1~3: *formosana* from Taiwan; 4~5: *soja* form Korea; 6~9: *soja* from China; 10~28: *soja* from Japan.

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台灣野生大豆 *Glycine formosana* 種子形態、同功異構酶及 DNA 多形性之研究

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Glycine formosana Hosokawa 分布於台灣桃園縣關西、橫山、大溪一帶之路旁及河旁之灌木草原地，為一攀緣纏繞性強之一年生植物。過去一直被認為與中國及日本的 *G. soja* 同種。最近，1992 年 Tateishi and Ohashi 根據植物形態，因與 *G. soja* 呈連續變異，而認為是地理上的亞種稱為 *G. max* subsp. *formosana* (Hosokawa) Tateishi et Ohashi。本試驗為瞭解這種野生大豆與中國、韓國及日本之 *G. soja* 的差異，將材料種於中興大學，進行莢果、同功異構酶及 DNA 多形性之探討。*Glycine formosana* 之種子較小，與中國、韓國及日本的 *G. soja* 有顯著差異。 9 種同功異構酶 16 基因座中，一個基因座 *Lap1-d* 僅存於 *G. formosana*。以 25 條引子對所有的樣本進行 RAPD 分析，有 21 條引子產生 DNA 放大產物，其中 14 條引子之產物具有多形性。共產生 132 條 DNA 的放大產物，有 84 (64%) 條放大產物具多形性。依條帶之出現計算各樣品間之相似度得：*G. formosana* 種內為 0.885~0.887，幾乎無遺傳變異，而與中國及韓國有差異，但與日本的 *G. soja* 間較類似。

關鍵詞：莢果；同功酶；DNA 多形性；*Glycine formosana*；*G. soja*。