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https://doi.org/10.5109/22766

出版情報：九州大学大学院農学研究院紀要. 14 (3), pp.341-348, 1968-03. Kyushu University
バージョン：
権利関係：

KYUSHU UNIVERSITY

Journal of the Faculty of Agriculture, Kyushu University, Vol. 14, No. 3
March 30, 1968

Phylogenetic studies on *Brassica* species by means of serological method[1]

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Differentiation of cultivated plants is remarkably complex on complicated geographical, historical, as well as economical background. The precise systematics of these plants have not yet been established so far in spite of prevailing demand for the progress of breeding, and the existing classification systems can not offer in turn, necessarily reliable criteria. On the other hand it has been recently known in the biochemical investigations that the protein structure is controled by the gene,[1] and the genetic differences can be studied through the comparison of protein constituents among different genetic lines. In this point of view serological analyses of proteins have been utilized to the advantage of phylogenetic investigations in cultivated plants.[2,3,4,5]

This paper deals with the comparison of leaf proteins in several *Brassica* species by means of serological method. These plants are all regarded as distinct species on the cytogenetical ground, of which the genome analyses have been performed already, so that they are taken as the convenient materials to study relationships between the genome constitution and protein composition. It may be duly expected to obtain certain criteria to be applicable to phylogenetic investigation of the cultivated plants, of which genome analyses have not yet been fully performed, and also expected to analyze certain minor differentiations in those plants, which can be studied no more in advance by the usual cytogenetical methods.

Materials and methods

A hundred gm of foliage leaves set at the 5-7th nodes, sampled from

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the seedlings at 7-8 leaf stage, was frozen at -20°C and homogenized in 100 ml of M/30 phosphate buffer of pH 7.0. The homogenate was ultracentrifuged at 145,000×g to remove cell-debris and unclei. The supernatant was lyophilized and stored for use as antigen. The protein concentration in the extract was determined by micro-Kjeldahl analysis of trichloroacetic acid precipitate assuming the nitrogen content of 16 per cent.

Two ml of 1.5 per cent protein solution was emulsified with an equal volume of Freund's adjuvant and injected intramusculary to rabbits weighing about 2 kg. The second injection was followed at interval of 2 weeks by the same method as the first. The rabbits were exanguinated 4 weeks after the second injection. Serum was separated from blood and stored in refrigerator.

Ouchterlonys, method[1] was employed for the double diffusion test. Gel medium for the the immuno diffusion contained 1.3 gm of agar, 0.9 gm of sodium chloride and 0.1 gm of sodium azide in 100 ml of distilled water. The agar-gel plates were prepared in 9 cm petri dishes and were 2 mm in thickness. Basins were cut on the gel plate. Antiserum (0.2 ml) was poured into a central basin and each antigen (0.2 ml of 1 per cent protein solution) was simultaneously poured into circumferential basins on an agar-gel plate. After incubation (at 37°C for 3 weeks), precipitin lines were examined.

Results

Figs. 1 and 2 illustrate the comparison of precipitin lines among different basic mono genomes. When anti-*B. nigra* serum was used, precipitin (β) shown in Fig. 1 was found in *B. nigra* (*bb* genome) only. When anti-B. oleracea serum was used, precipitn line (α) shown in Fig. 2 was found in *B. oleracea* (*cc* genome) and *B. chinensis* (*aa* genome). These facts interpreted that *B. nigra* possesses a component which is not con-

Table 1. *Brassica* species used in the experiment and their genome constitutions.

Brassica species	Chromosome number (n)	Genome
B. nigra Koch "California brown"	8	*bb*
B. oleracea L. var capitata D. C. "Yoshin"	9	*cc*
B. chinensis L. "Seppaku-taisai'	10	*aa*
B. carinata Braun. "Harron"	17	*bbcc*
B. juncea Hemsel. "Miike-takana"	18	*aabb*
B. napus L.	19	*aacc*

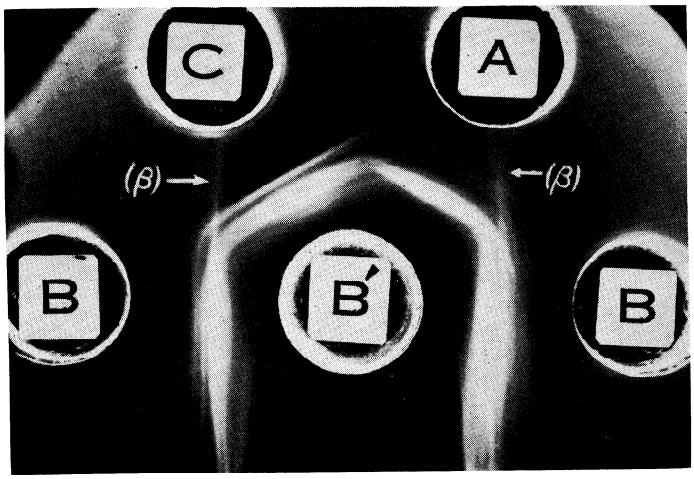

Fig. 1. Photograph of precipitin pattern showing the comparison of different monogenomic forms, obtained by using anti-*B. nigra* serum. (B), *B. nigra* (*bb* genome) antigen. (C), *B. oleracea* (*cc* genome) antigen. (A), *B. chinensis* (*aa* genome) antigen. (B'), anti-*B. nigra* serum.

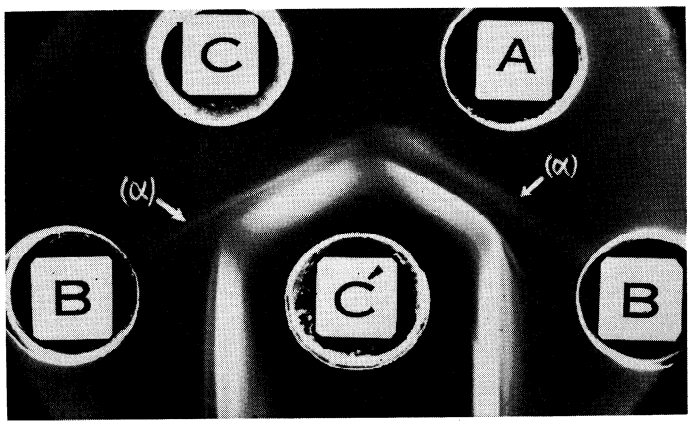

Fig. 2. Photograph of precipitin pattern showing the comparison of different monogenomic forms, obtained with anti-*B. oleracea* serum. (B), *B. nigra* antigen. (A), *B. chinensis* antigen. (C), *B. oleracea* antigen. (C'), anti- *B. oleracea* serum.

tained in *B. oleracea* and *B. chinensis*, in contrast to the fact that *B. oleracea* and *B. chinensis* possess another component in common, which is not contained in *B.* nigra. On the other hand, the differences in the precipitin lines between *B. oleracea* and *B.chinensis* were no more than "reaction of imperfect identity."[5] These findings suggest that *B. oleracea* and *B. chinensis* seem to be closer to each other than either one is to *B. nigra.*

In comparison between mono genomic forms and certain amphidiploid forms, as shown in Fig. 3, *B. nigra* was different from *B. carinata* in the precipitin line (γ) obtained with anti-*B. nigra* serum. This precipitin line (γ) showed that *B. nigra* (*bb* genome) possesses a component which *B. carinata* (*bbcc* genome) does not. On the other hand the precipitin line (δ) obtained with anti-*B. oleracea* serum, as shown in Fig. 4 was detected in *B. carinata* and was common to *B. oleracea*, but not detected in *B. nigra.* This component forming the precipitin line (δ) seems to be contained in both of *B. carinata* and *B. oleracea*, but not in *B. nigra.* The differences between *B. carinata* and *B. oleracea* could be found only in the precipitin line (ω) obtained with anti-*B. oleracea* serum. This precipitin line (ω) would not appear to show "reaction of non-identity,"[7] but to show "reaction of imperfect identity," revealing the appearance of this

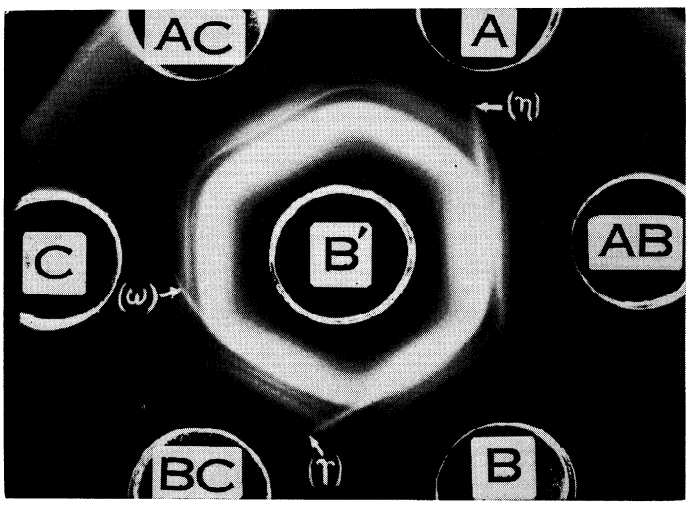

Fig. 3. Photograph of precipitin pattern obtained with anti-*B. nigra* serum. (A), *B. chinensis* (*aa* genome) antigen. (AB), *B. juncea* (*aabb* genome) antigen. (B), *B. nigra* (*bb* genome) antigen. (BC), *B. carinata* (*bbcc* genome) antigen. (C), *B. oleracea* (*cc* genome) antigen. (AC), *B. napus* (*aacc* genome) antigen. (B'), anti-*B. nigra* serum.

precipitin line as shown in Fig. 3. Accordingly *B. carinata* was different from *B. oleracea* in the quantity of the component forming this precipitin line (ω). These facts suggest *B. carinata* seems to be closer to *B. oleracea* than to *B. nigra.*

B. juncea (*aabb* genome) appeared to possess all the precipitin lines which have been contianed in *B. nigra,* when anti-*B. nigra* serum was used as shown in Fig. 3. In one of these precipitin lines, *B. juncea* was quite different from *B. chinensis.* That is, the precipitin line (η) showed distinct "reaction of non-identity" between *B. juncea* and *B. chinensis,* and was necessarily common to *B. nigra.* These facts suggest that the precipitin lines (η) and (γ) obtained with anti-*B. nigra* serum, seem to correspond to the precipitin line (β) obtained with the same antiserum. That is, the precipitin line (β), (γ) and (η) seem to be formed by the same antigen and antibody system, and in consequence they are characteristic of *b* genome. The fact that *B. carinata* (*bbcc* genome) does not possess this component which is characteristic of *b* genome, duly suggest that the *b* genome of *B. carinata* has differed somewhat from that of *B. nigra* (*bb* genome) and also from that of *B. juncea* (*aabb* genome).

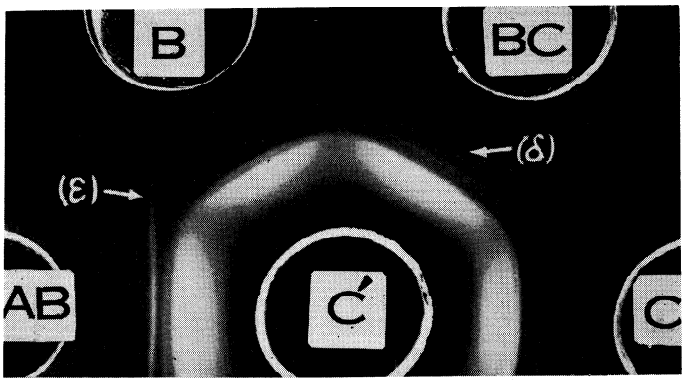

Fig. 4. Photograph of precipitin pattern showing the comparison between the monogenomic and the amphidiploid forms, obtained with anti-*B. oleracea* serum. (AB), *B. juncea* antigen. (B), *B. nigra* antigen. (C), *B. oleracea* antigen. (C'), anti-*B. oleracea* serum.

The precipitin line (ξ) obtained with anti-*B. oleracea* serum was found in *B. chinensis* and *B. juncea,* but not in *B. nigra.* That is, *B. juncea* and *B. chinensis* hold the component in common, which has formed the precipitin line (ξ), while *B. nigra* does not as shown in Figs. 4 and 5. This precipitin line (ξ) seems to have been formed by the same component as the precipitin line (α) in Fig. 2, according to the fact that precipitin lines (α) and (ξ) have obtained with same antiserum (anti-*B.*

oleracea serum) and have been detected in *B. chinensis*, but not in *B. nigra*.

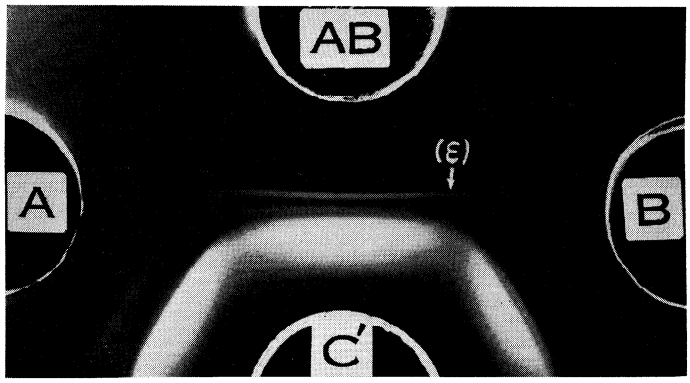

Fig. 5. Photograph of precipitin pattern obtained with anti-*B. oleracea* serum (A), *B. chinensis* antigen. (AB), *B. juncea* antigen. (B), *B. nigra* antigen. (C'), anti-*B. oleracea* serum.

When anti-*B. juncea* serum was used, *B. juncea* appeared to be different in the precipitin line (μ) from *B. nigra*, and also in the precipitin line (ν) from *B. chinensis* as shown in Fig. 6. That is, the precipitin line (μ) was held in common by *B. juncea* and *B. chinensis* but not in *B. nigra*. On the other hand the precipitin line (ν) was common to *B. nigra*, *B. juncea* and *B. carinata*, but not found in *B. chinensis*. In other words,

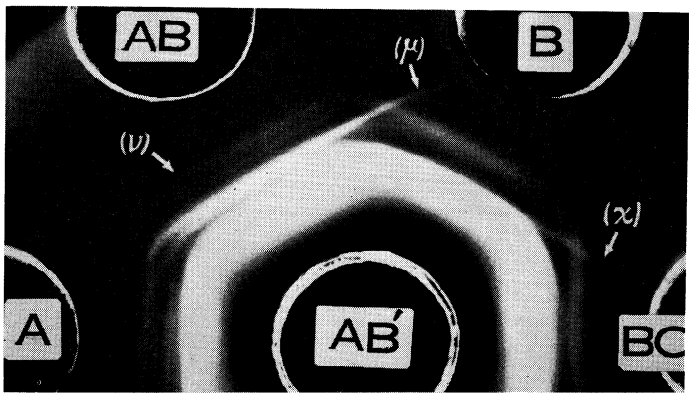

Fig. 6. Photograph of precipitin pattern obtained with anti-*B. juncea* serum. (A), *B. chinensis* (*aa* genome) antigen. (AB), *B. juncea* (*aabb* genome) antigen. (B), *B. nigra* (*bb* genome) antigen. (BC), *B, carinata* (*bbcc* genome) antigen. (AB'), anti-*B. juncea* serum.

the components in *B. juncea* (*aabb* genome) seem to have been composed of all the components in both of the two forms with basic genomes, *B. chinensis* (*aa* genome) and *B. nigra* (*bb* genome). These findings suggest that *a* and *b* genomes of *B. juacea* have individually the same function of the protein synthesis as in each of the mono genomic forms, *B. chinensis* (*aa*) and *B. nigra* (*bb*).

The precipitin line (χ) in Fig. 6, showed the difference between *B. nigra* and *B. carinata*. The similar difference has been found in precipitin line (γ) in Fig. 3 as mentioned above. However it is uncertain whether this precipitin line (χ) is corresponding to the precipitin line (γ) or not, because the (χ) was obtained with anti-*B. juncea* serum, while the (γ) was obtained with anti-*B. nigra* serum.

The precipitin line (ν), obtained with anti-*B. juncea* serum, was common to *B. nigra* and the amphidiploid forms having *b* genome, and was quite characteristic of *b* genome.

In *B. napus* any specific precipitin line was not obtained with anti-*B. nigra* serum as shown in Fig. 3.

All the specific precipitin lines detected in the present experiments were listed in Table 2, showing the comparison between the genome constitutions and those precipitin lines. There are certain results from those serological analyses, such as, 1) there exists a closer affinity between *a* and *c* genomes than between either one of these genomes and *b* genome; 2) *a* and *b* genomes composing an amphidiploid form, *B. juncea* (*aabb*), have individually the same function in the protein syntheses as each genome of primary species with the basic genome, *B. chinensis* (*aa*) and *B, nigra* (*bb*); 3) the *b* genome of *B. carinata* (*bbcc*) appears to have differed somewhat from that of *B. nigra* (*bb*) over long period of evolutionary history. Furthermore it may be conceivable that amphidiploid form, *B. carinata* had been naturally synthesized with

Table 2. Specific precipitin lines detected, and genome constitutions.

Brassica species	Genome constitution	Specific precipitin lines obtained with		
		anti-*B. nigra* serum	anti-*B. oleracea* serum	anti-*B. juncea* serum
B. chinensis	*aa*		α, $\varepsilon(=\alpha)$†	μ
B. juncea	*aabb*	$\eta(=\beta)$*, $\gamma(=\beta)$*,	$\varepsilon(=\alpha)$†	ν, μ, x
B. nigra	*bb*	β, $\eta(=\beta)$*, $\gamma(=\beta)$*,		ν, x
B. carinata	*bbcc*		δ, ω(?)	ν,
B. oleracea	*cc*		α, δ, ω	

*, precipitin lines (η) and (γ) seem to be corresponding to precipitin line (β).
†, precipitin line (ε) seems to be corresponding to precipitin line (α).

the b genome differing from that of existing *B. nigra.*

Summary

Serological analyses have been utliized to advantage in the phylogenetic studies. This paper deals with the comparison of leaf proteins of several *Brassica* species by means of the Ouchterlony's method. These plants are regarded as distinct species on the cytogenetical ground, and are convenient to study the relationships between the genome constitution and protein composition.

In each comparison of precipitin lines among the different basic monogenomes, *B. nigra* appeared to possess a component which is not contained in *B. oleracea* and *B. chinensis*, and in contrast, *B. oleracea* and *B. chinensis* have held another component in common which is not contained in *B. nigra.* On the other hand, the differences in the precipitin lines between *B. oleracea* and *B. chinensis* were no more than the "reaction of imperfect identity." These facts suggest that there is a closer affinity between *a* and *c* genomes than between the either one of these genomes and *b* genome.

From the comparison between these species having basic mono genome and amphidiploid species, *B. jnncea* (*aabb* genome), it appeared that the latter possesses all the components which are contained in each species with the basic genome, *B. chinensis* (*aa* genome) and *B. nigra* (*bb* genome). That is,, *a* and *b* genomes of *B. juncea* seem to have individually the same function of protein syntheses as *a* genome of *B. chinensis* and *b* genome of *B. nigra.* On the other hand, at least one of the components which are contained in *B. nigra* (*bb* genome), was not detected in *B. carinata* (*bbcc* genome). This fact suggests that the *b* genome of *B. carinata* appears to have differed from that of *B. nigra.*

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