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論　文

Electrophoretic Comparison of Soluble Pollen Proteins of *Alnus* in relation to Inter- and Intraspecific Identification

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ハンノキ属花粉の可溶性蛋白質溶液の電気泳動による種の同定について

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要　旨: 植物体の各組織から抽出される可溶性蛋白質溶液の電気泳動による泳動パターンは，その系統ならびに発育時期によっていろいろな特徴をもっている。

ハンノキ属5種（ケヤマハンノキ3個体，ハンノキ2個体，カワラハンノキ1個体，シナハンノキ1個体，ヤシャブシ1個体）の花粉の可溶性蛋白質溶液の電気泳動パターンによる種の同定の可能性についてしらべた。

その結果，同一種内の個体間では，ほとんどその泳動パターンにちがいが認められず，種間では明らかなちがいがみとめられた。

Summary: Application of the technique of acrylamide gel electrophoresis to the separation of components of soluble pollen proteins of *Alnus* trees is described.

Five *Alnus* species involving 8 individuals (*A. hirsuta*—3, *A. japonica* var. *genuina*—2, *A. serrulatoides*—1, *A. cremastogyne*—1 and *A. firma*—1) were chosen as test materials.

It was demonstrated that pollens collected from different trees within one species showed similar electrophoretic patterns. But, the electrophoretic patterns in a certain specific zone of more highly charged and low molecular weight protein components showed considerable differences among species.

Introduction

The *Alnus* species can contribute to the fertility of forest soil. These hardwoods show the most rapid growth in northern Japan. Studies on the improvement of the species are carried out by controlled crossing at present.[1)]

By the use of disc-electrophoresis using polyacrylamide gel as a supporting medium, the electrophoretic properties of soluble pollen protein complements obtained from these *Alnus* trees were investigated in this paper.

Observations were made on the soluble protein preparations obtained from the mature pollens. The mature pollen serve as the experimental material in this work, because the immature pollen enzyme pattern may be changed as the pollen matures.

Application of the disc-electrophoresis using polyacrylamide gel as the supporting medium to the separation of cytoplasmic protein components of plants is perhaps the most sensitive technique at present. Therefore, this means has been succeeding in separations of the soluble proteins of various plant tissues recently.[2),3)]

Materials and Methods

A. Collection of materials and preparation of extracts

The pollen materials used for this work were collected from five *Alnus* MILL. species involving eight planted and wild individuals (about eight years old); *A. hirsuta* TURCZANINOW (4×, 2n=28)—3, *A. japonica* SIEB. et ZUCC. var. *genuina* CALL. (4×, 2n=28)—2, *A. serrulatoides* CALL. (2×, 2n=14)—1, *A. cremastogyne* BURKILL (2×, 2n=14)—1 and *A. firma* SIEB. et ZUCC. (4×, 2n=28)—1 individual grown in the experiment forests or the neighboring fields of both Shikoku Branch of Government Forest Experiment Station and Kansai Forest Tree Breeding Station, Kochi, Japan.

The sampling was made from late January to middle April in 1969.

Several twigs with male flowers were removed from every individual before the dissemination of pollen, covered over by paper bag, transferred to water culture in glass pot and grown in doors until the natural falling of pollen.

The collected pollens were stored in a freezer at

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0℃ until they were examined electrophoretically.

The pollen grains were washed by ethyl-ether, homogenized in cold phosphate buffer (M/30, pH 7.5) with the homogenizer designed and manufactured by Takashima-Shoten Co., and the resulting homogenates were incubated for one night in a freezer at 0℃. The homogenates were centrifuged at 3,000×g for 15 minutes to remove cell debris at room temperature. These supernatant fractions were centrifuged again at 10,000×g for 30 minutes to remove cell organelles at 0℃. The supernatant materials were then dialyzed for two days at 0℃ against the cold phosphate buffer (M/30, pH 7.5) through 3 changes of buffer. The dialyzed protein suspensions were lyophilized, preserved in some ampoules and stored in a freezer at 0℃. They were dissolved again in deionized water before electrophoretic separations. That is, the lyophilized protein samples were prepared with deionized water to a final concentration of approximately 6.0 mg of protein per m*l*, following their protein content determined by the micro-KJELDAHL method.

B. Preparation of the gel and electrophoresis

Electrophoresis was carried out according to some modified method of the procedures outlined by STEWARD et al.[3] The method was as follows:

(1) Stock solutions

1	1N-hydrochloric acid	24.0 m*l*
	Tris (hydroxymethyl) amino-methane (TRIS)	15.85 g
	N,N,N′,N′-tetramethylethylenediamine (TEMED)	0.32 m*l*
	Deionized water to	100 m*l*
2	1N-hydrochloric acid	24.0 m*l*
	TRIS	2.99 g
	TEMED	0.46 m*l*
	Deionized water to	100 m*l*
3	Acrylamide	30.0 g
	N, N′-methylenebisacrylamide (BIS)	0.8 g
	Deionized water to	100 m*l*
4	Acrylamide	10.5 g
	BIS	2.5 g
	Deionized water to	100 m*l*
5	Riboflavin	4.0 mg
	Deionized water to	100 m*l*
6	Riboflavin	1.0 mg
	Deionized water to	100 m*l*
7	Electrode buffer	
	TRIS	6.0 g
	Glycine	28.8 g
	Deionized water to	10,000 m*l*

The stock solutions except the 7 solution were stored in brown bottles in the cold.

(2) Working solutions

8 For the lower gel
1 Solution—1 part
3 Solution—1 part
6 Solution—2 parts

9 For the middle layer gel
2 Solution—1 part
4 Solution—2 parts
5 Solution—1 part
Deionized water—4 parts

10 For the upper gel
2 Solution—1 part
4 Solution—2 parts
5 Solution—1 part
Sample solution—4 parts

11 For staining solution

Amidoschwarz 10 B	10 g
Deionized water	400 m*l*
Methanol	500 m*l*
Acetic acid	100 m*l*

12 7% acetic acid solution

The working solutions were prepared immediately before use.

(3) Polymerization of gel

The polymerization of gel was carried out according to the procedure used by STEWARD et al.,[3] that is., the method of light polymerization using riboflavin instead of ammonium persulfas (oxidizing agent).

(4) Electrophoresis

The electrophoretic runs after the polymerization of the gel were made at 1.67 ma per tube for 2～4 hours, or until the tracking dye by bromophenol blue approached at a distance 10 mm to the lower end of the tube.

(5) Staining and destaining the gels

After the electrophoretic run, each gel was loosened from their tubes in deionized water. The gels were fixed and stained by Amidoschwarz 10 B (11 Solution) for 60 minutes immediately after their removal from the tubes, and then washed by deionized water through several changes of water. The washed gels were incubated to 7% acetic acid solution (12 Solution). The acetic acid solution was exchanged several times.

When the part of the gel which contained no protein was completely clear of dye, the gels were stored at 0℃ in 7% acetic acid solution and analyzed by densitometer.

(6) Recording of results

The gels were analyzed densitometrically using a Model DMU-2 Densitorol (439 mμ Filter) designed and manufactured by Toyo Kagakusangyo Co.

Some of the very fine and faint bands in the gels did not appear well on photographs. Therefore, diagrammatic interpretations were made under the densitometric traces for each gel.

During the electrophoresis, it was very difficult to arrange the electrophoretic runs to the same length from the origin to the front (Fig. 1-1～1-5). Therefore, the diagrammatic interpretations with relative movement (comparable to the Rf values) to the front of indivdual bands (movement of the front =100) in the diagrammatic interpretations (Fig. 1-1 ～1-5) obtained from all the gels are represented again in Fig. 2 so that the protein constituents of the various samples are directly compared. The peaks on the densitometric traces of all the gels are marked with arrows. Four shades of stippling in the diagrammatic interpretations, that is, the broad dense, narrow dense, dotted and linear bands were used to indicate various standards of the staining portions on the gels. The former three shades mean visible bands on the gels, but the third is a less dense band. The forth means invisible, very fine and faint band.

Results

A. Intraspecific differences in electrophoretic separations

Before taking the information on the interspecific differences, it was necessary to find out how the densitometric traces of the electrophoretic separations varied among different individuals within one species.

The electrophoretic separations for this observation were carried out among 3 individuals in *Alnus hirsuta* (Fig. 1-1) and 2 individuals in *A. japonica* var. *genuina* (Fig. 1-2).

The schematic diagrams of the general banding patterns of the gel columns are shown in Fig. 1～2. The bands represent anodic moving proteins at a pH maximum of 8.3.

From Fig. 1-1 and 2, it can be seen that all the samples from *A. hirsuta* held in common 1 strong (broad dense) band (Rf value; 46) as well as the mobile material which constituted the front, 5 narrow dense bands (Rf values; 7, 25, 37, 54～56 and 72) and 4 weak dense (less dense) bands (Rf values; 11～14, 64～66, 80 and 92～94). In addition, several very fine and faint (linear) bands were found. The variations in the number, position and relative intensity of broad dense, narrow dense and weak dense bands except linear bands were not found among individuals within *A. hirsuta* species. But, there were marked differences in the number and position of the linear bands.

It was recognized that the samples from *A. japonica* var. *genuina* held in common 3 strong bands (in addition to a front band) (Rf values; 46, 53 and 90～94), 4 narrow dense bands (Rf values; 7, 25～28, 37～50 and 70～72) and 1 dotted band (Rf value; 79～80) in Fig. 1-2 and 2. There were not marked differences among individuals within this species in the number, position and intensity of the bands except several linear bands.

According to these facts, it was confirmed that there were no variations among individuals within one species in the number, position and intensity of the visible bands on the gels.

B. Interspecific differences in electrophoretic separations

The electrophoretic separation of soluble pollen protein of *Alnus serrulatoides* CALL. individual is shown in Fig. 1-3 and 2. In these Figures, 4 broad dense (Rf values; 47, 52, 67 and 85), 3 narrow dense (Rf values; 7, 14 and 35) and 2 weak dense (Rf values; 11 and 15) bands are observed on the gel.

The diagrammatic interpretation of *Alnus crematogyne* individual is shown in Fig. 1-4 and 2. The 1 broad dense (Rf value; 46), 8 narrow dense (Rf values; 7, 14, 25, 35, 49, 67, 76 and 85) and 1 weak dense (Rf value; 58) bands were presented. The schematic diagrams shown in Fig. 1-5 and 2 represent the electrophoretic pattern of the soluble protein from *Alnus firma* individual. This species had 2 broad dense (Rf values; 50 and 80), 5 narrow dense (Rf values; 7, 26, 42, 60 and 68) and 4 weak dense (Rf values; 15, 33, 37 and 40) bands.

From the electrophoretic separations in Fig. 2, from 8 to 11 visible bands, excepting the front, are observed on the gels, and the number, location and intensity of the bands vary among species. The most striking difference in the electrophoretic patterns of the protein complements of the pollens in *Alnus* species was the electrophoretic patterns in a certain specific zone (Rf value; 75～95) of more highly charged and low molecular weight protein components. The most specific pattern among *Alnus* species was the diagrammatic pattern of *Alnus firma* individual.

Discussion

It was shown that the acrylamide gel electrophoresis technique was sensitive and useful when applied to soluble pollen proteins of *Alnus* individuals.

The study on pollen grains from every individual will preclude errors in evaluating species special characteristics. The observed differences in Rf values of the protein bands and in their relative intensities should reflect differences among individuals in properties of the soluble protein extracts.

The electrophoretic patterns of the soluble pollen protein components of *Alnus* species showed the general banding pattern of the gels. The marked differences in a certain specific zone (Rf value; 75

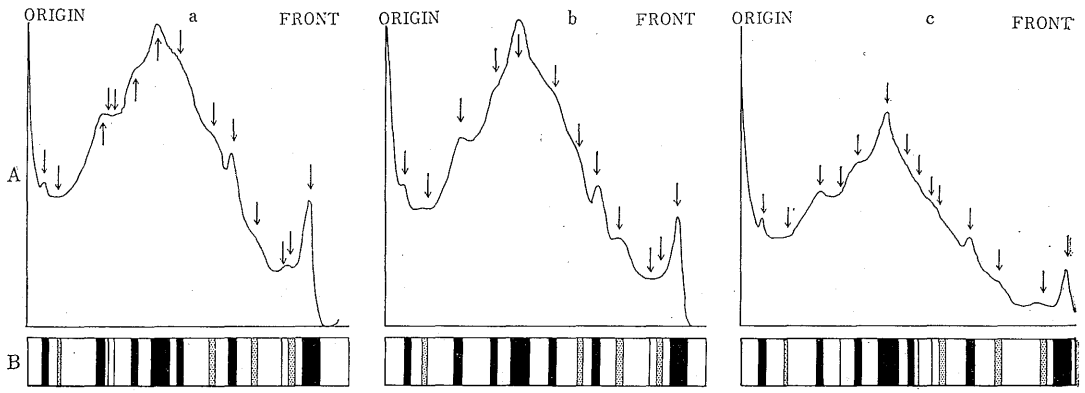

Fig. 1-1. *Alnus hirsuta* TURCZANINOW

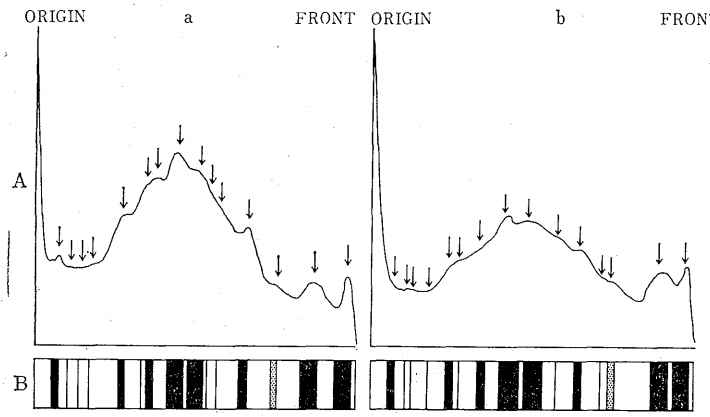

Fig. 1-2. *Alnus japonica* SIEB. et ZUCC. var. *genuina* CALL

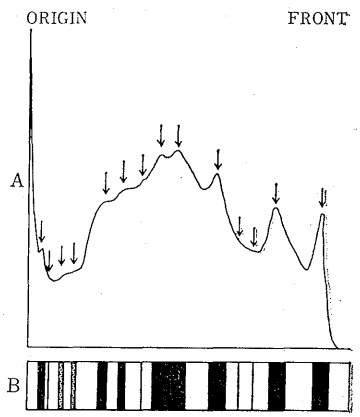

Fig. 1-3. *Alnus serrulatoides* CALL

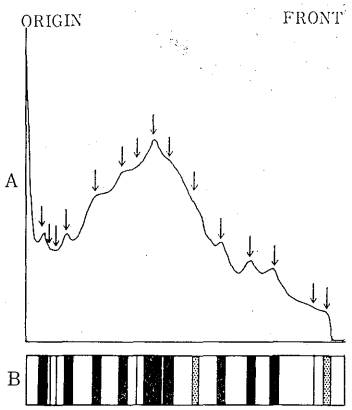

Fig. 1-4. *Alnus cremastogyne* BURKILL

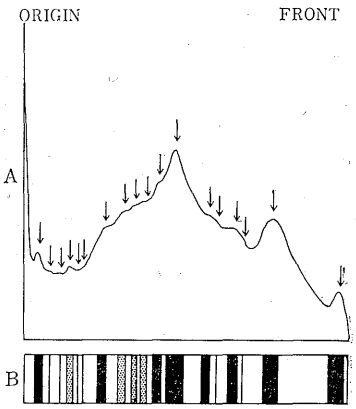

Fig. 1-5. *Alnus firma* SIEB. et ZUCC.

Fig. 1 (1~5). The electrophoretic separations on acrylamide gel of the soluble protein of five *Alnus* species pollens extracted by phosphate buffer (M/30, pH 7.5)
A is a densitometric trace of the gel; B is a diagrammatic interpretation of the gel.

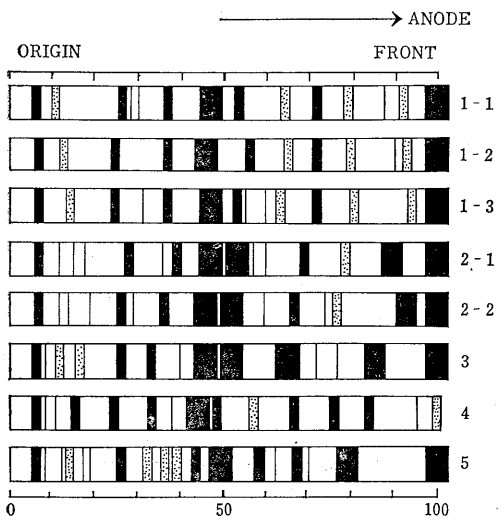

Fig. 2. Diagrammatic interpretations of the electrophoretic separations on acrylamide gel of the soluble pollen proteins of *Alnus* species extracted by phosphate buffer (M/30, pH 7.5): Diagrammatic interpretations by relative movement to front (movement of the front=100) of individual bands from *Alnus hirsuta* (1–1, 1–2, 1–3), *A. japonica var. genuina* (2–1, 2–2), *A. serrulatoides* (3), *A. cremastogyne* (4) and *A. firma* (5)

~95) of more highly charged and low molecular weight protein components were found among species. The electrophoretic patterns in this zone showed a specific one in each species. It seems that the protein components presented in this region of the electrophoretic pattern reflect species speciality. That is, the 1 broad dense (Rf value; 90~94) and 1 weak dense (Rf value; 79~80) bands were specific in *Alnus japonica* var. *genuina* species. *Alnus serrulatoides* had a species specific broad dense band (Rf value; 85). In *A. cremastogyne*, 2 narrow dense bands (Rf values; 76 and 85) were specific in this region. In addition, 1 broad dense band (Rf value; 80) was peculiar to *A. firma*.

In the studies on sunflower pollen by immuno-electrophoresis, IWANAMI[4] found out some changes among forms in the protein complement. He was able to assume the breed names of unknown sunflowers by observing the characteristic precipitation lines between the extracts of various pollens and the antiserum taken from the pollen of a form. Thus, these protein components investigated by him showed constant difference among forms. Therefore, it is probable that the information obtained in this paper provide an evidence that the soluble protein components of *Alnus* species pollens may vary among species.

The relations between the schematic diagrams of the general banding patterns of the gels and the morphological characteristics or the degree of polyploidy in *Alnus* species were not confirmed in this work.

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Literature Cited

1) CHIBA, S.: Studies on tree improvement by means of artificial hybridization and polyploidy in *Alnus* and *Poplus* species. Bull. Oji Inst. For. Tree Improv. **1**, 1~165, (Oji Paper Co., Ltd.), Japan, 1966
2) COLEMAN, E.A., BULA, R.J. and DAVIS, R.L.: Electrophoretic and immunological comparisons of soluble root proteins of *Medicago sativa L.* Genotypes in the cold hardened and non-hardened condition. Plant Physiol., **41**, 1681~1685, 1966
3) STEWARD, F.C., LYNDON, R.F.and BARBER, J.T.: Acrylamide gel electrophoresis of soluble plant proteins, A study on pea seedling in relation to development. Amer. J. Bot. **52**, 155~164, 1965
4) IWANAMI, Y.: Physiological researches of pollen XVIII. Studies on sunflower pollen by immuno-electrophoresis. Bot. Mag. Tokyo, **81**, 25, 582~589, 1968

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