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Advanced Immunoelectrophoresis: A promising approach to systematic research[1]

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진보된 면역학적 전기영동법 : 계통학 연구에의 유망한 접근

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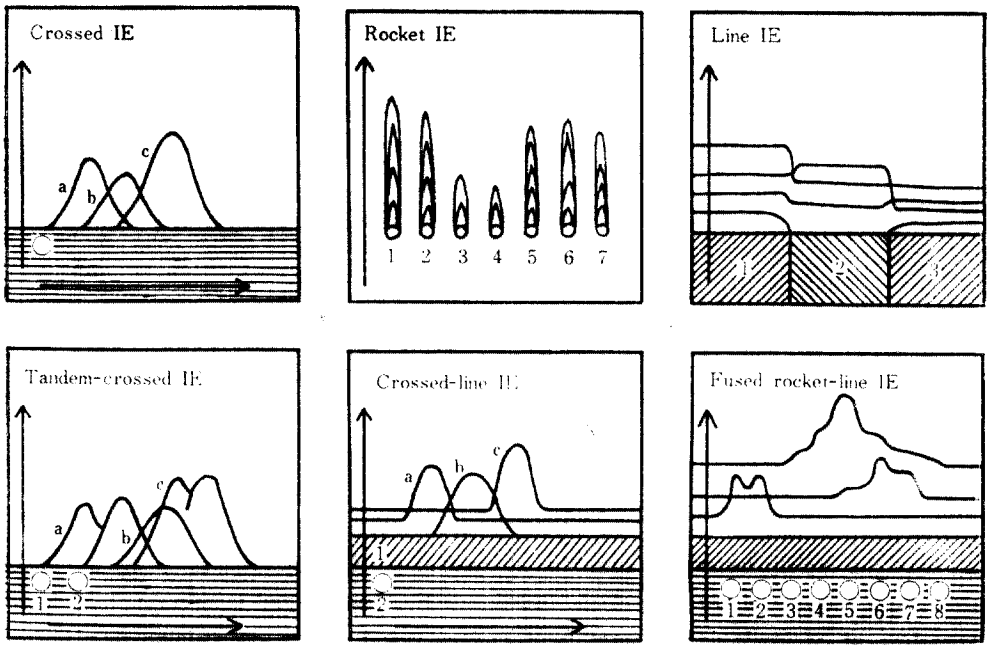
Along with the rapidly increasing molecular approaches to plant systematics we have witnessed also an equally rapid increase of the kind of the techniques. Systematists and taxonomists have learned that the comparison of antigenic determinants from various taxa helps in determining protein similarities which aid our understanding of relationships. It is expressed by the specific reactions between antigenic determinants and antideterminants which are valuable because they provide a means for the measurement of protein similarities. Various types of precipitin reactions have been used in plant comparative serological investgations. Particularly immunoelectrophoresis, or IE in short, first devised by Grabar and Williams had much attention by many researchers. This is based on two of their properties: ability to precipitate in agarose gel with specific antigen and antibody respectively and electrophoretic mobility in an electric field.

Last decade was a rich period of methodological development, having now immunoelectrophoretic techniques extremely useful for all protein researchers. Some outstanding modification of immunoelectrophoresis have appeared recently, but very few systematists or taxonomists have attempted to apply to plant taxa for systematic purpose. Now I am going to try to discuss here a practical application of some modified and advanced immunoelectrophoresis to obtain relatively fast and highly resolved serological data, concerning the degree of structural relatedness between homologous protein molecules of different taxa. Like all other methods, the serological methods have their own advantages and limitations. The basic defect of serological approaches is connected with the fact that we do not really know what we are measuring as indicated by Cronquist. And serological test is that they exist only as one to one comparisons gathered at the expense of much time and effort. In terms of those problems advanced

[1] (編輯者註) 위의 寄稿는 第13次 國際植物學會議 (XIII International Botanical Congress)의 Symposium 8·9 Macromolecular approaches to systematics (Symp. 8 Systematic and evolutionary botany의 한 Section)에서 施行된 lecture의 줄거리이다.
이는 Australia의 University of Sydney (Main quadrangle, General lecture theature 1)에서 1981년 8월 26일 (2:45—3:15 p.m.)에 施行되었다.

(MODIFIED IMMUNOELECTROPHORESIS)



PRESATURATION TECHNIQUE

reference antigen	(A) taxon	a, b, c, d, e, f,		
antiserum (antibody)	a', b', c', d', e', f',			
antigen for presaturation	(A) taxon a, b, c, d, e, f.	(B) taxon b, c, d, e, f, g.	(C) taxon d, e, f, g, h, i.	(D) taxon g, h, i, j, k, l.
antiserum content of presaturated antiserum	(none)	a'	a', b', c'.	a',b',c',d',e',f',
reaction with reference antigen	—	±	+	+ +
interpretation	idnntical ↓ identical	greatly similar ↓ closely related	partly similar ↓ related	dissimilar ↓ distantly related

Fig. 1. Modified immunoelectrophoresis and presaturation technique.

immunoelectrophoresis found to be more improved. Here are some illustrations on your hand-out (See Fig. 1).

1. Crossed immunoelectrophoresis (2-dimensional immunoelectrophoresis): After the first electrophoretic separation of antigens in one dimension, the separated antigens are forced into an antibody-containing

gel at righ tangles to the movement in the first dimension. The resulting precipitin patterns provide semi-quantitative data as well as high resolution of components. Qualitatively, three basic types of reaction may show up: (a) identity, (b) partial identity, and (c) nonidentity. In crossed immunoelectrophoresis, it was found that a dense population of antigen-antibody molecules must be present in the media to show sharp, well separated immunoprecipitin systems. So high antibody titer and strong specificity for the antigens are necessary for successfully adapting this technique to plant experiments. A major consideation to plant extracts is obtaining the proper ratio of antigen-antibody for crossed immunoelectrophoresis.

2. Rocket immunoelectrophoresis: The name was derived from the rocket-shaped precipitin bands that appeared after electro-immunodiffusion in antibody-containing gel. This is used for quantitative determination of a protein in a protein mixture. The rocket height originated at the antigen well is directly proportional to the antigen concentration. Both this technique and radial immunodiffusion can measure antigen concentrations. The great advantages of this technique are its speed as compared to diffusion methods and its specificity. Individual antigens can be quantitated without being isolated from the other constituents. And many samples can be run simultaneously within as little as 2 hrs. Rockets are very economical to analyze. Agarose containing monovalent antiserum is used in clinical serology, immunochemistry, and biochemistry. For the systematic purpose polyvalent antiserum is also used.

3. Line immunoelectrophoresis: In this technique the antigen is incorporated in a gel strip 1, 2 and 3. Electrophoresis at right angle to the strip in antibody-containing gel results in a distict precipitin line. This line is parallel to the origin after a distance of antigen migration which is proportional to the antigen-antibody ratio. Line-immunoelectrophoretic spectra developed from these adjoining samples against the same antiserum can be directly compared as a consquence of the continuity of individual precipitin lines between adjoining patterns. It appears that sometimes precipitin lines change their relative positions, and sometimes some lines are faint to observe. It is therefore imporant not only to apply a standardized antigen extract but also to establish a reference antiserum. This technique can be combined with crossed immunoelectrophoresis and fused rocket immunoelectrophoresis to detect particular protein fractions.

4. Tandem-crossed immunoelectrophoresis: In this techniqun two wells are punched in the same track in the first dimemsional electrophoresis gel. This also allows one to compare qualitatively and quantitatively two complex antigen systems. By producing fusion of related immunoprecipitin systems, starting from two adjacent sample wells, one can compare an antigen system with others.

5. Crossed-Line immunoelectrophoresis: Crossed immunoelectrophoresis and line immunoelectrophoresis are combined.

6. Fused rocket-Line immunoelectrophoresis: Fused rocket immunoelectrophoresis and line immunoelectrophoresis are combined.

In the serological systematic research aiming at comparing the total protein stock of plant taxa, I would prefer to use the first three techniques rather than last three ones, because the result we need is a total expression of the serological similarity. But last three techniques are particularly useful if certain protein fractions are considered as significant taxonomic markers.

Another technique which can be combined with advanced immunoelectrophoresis is the "presaturation". With presaturation an inverse relation is obtained. This is because with presaturation serologically similar

antigens and antibodies are removed by a prior exposure of the antiserum to antigens from another experimental taxon. The antiserum after presaturation contains only those antibodies not shared by the antigen used for presaturation. This modification aims at decreasing the specificity of antiserum in order to simplify immunoprecipitin systems. If such antiserum is reacted against its initial reference antigen (which was used to generate the antiserum), a decrease in the number of immunoprecipitin systems previously detected with the non-presaturated reference reaction is to be noted. The amount of reduction in the number will depend on the similarity of the antigen used for presaturation and antigen used to raise the antiserum being tested. If the taxa are serologically similar, few systems will remain. If they lack similarities, little or no change in the number will be detected (See Fig. 1).

Now I want to show practical application of these techniques to my selected plant taxa, *Ambrosia* and *Quercus* for systematic purpose. The first group is *Ambrosia* and relatives. Recent serological characterization of pollen antigens from *Ambrosia* has begun to elucidate the fine protein spectra using immunoelectrophoretic techniques and suggests that such data are taxonomically useful. The genus *Ambrosia* was recently revised and *Franseria* was included. In this serological investigation seven *Ambrosia* species and six putatively related genera were included.

Figure 2 shows the radial immunodiffusion using the antiserum against *Ambrosia artemisiifolia*. Pollen extract from various taxa was allowed to diffuse radially from a well. The precipitin rings formed are due to the gradient of polyvalent antiserum. With this antiserum all *Ambrosia* taxa including three species formerly classified as *Franseria* possessed high titers of antigens that bound strongly to the antibodies. In each experiment the numerous precipitin rings overlapped, producing precipitin zones of varying intensities. This technique is good for the pilot survey particularly when the genus or upper taxonomic category is compared.

Figure 3 illustrates the crossed immunoelectrophoresis of *A. artemisiifolia* reference reaction alone on the left, and in tandem with purified antigen E from the same species on the right. The first dimension consisted of electrophoresis in the buffered, plain agarose, and the anode was to the right. The second dimension consisted of electrophoresis into agarose charged with the antiserum against *A. artemisiifolia*, and the anode was at the top. This reference reaction produced at least 16 bands, 8 pronounced bands

Table 1. Numbers of RID rings (with diameters in mm) and 2-D IE bands formed with *Ambrosia artemisiifolia* reference reaction after antiserum (As) was presaturated with pollen proteins (Ag) from various species. Ag E: antigen E, *: most pronounced ring, As-Ag: antiserum presaturated with antigen mixture, See Fig. 2 for the abbreviations.

Ag / As-Ag	RID rings		2-D IE bands	
	Ag E	aa	Ag E	aa
aa-0 (non-presaturated)	2-3	7(14.0, 11.4, 9.2, 7.5*, 6.6, 5.8, 5.2)	2	7-8
aa-aa	0	0	0	0
aa-at	0	4 (9.0, 7.3, 6.2, 5.0*)	0	4
aa-ab	0	2 (6.9, 5.9*)	0	2
aa-ap	0	2 (7.0, 5.8*)	0	2
aa-fc	0	5 (11.2, 8.3, 7.5*, 6.4, 6.1)	0	1
aa-fm	0	2 (6.1, 6.2*)	0	1
aa-ft	0	4 (9.5, 7.7, 6.7, 5.9*)	0	2

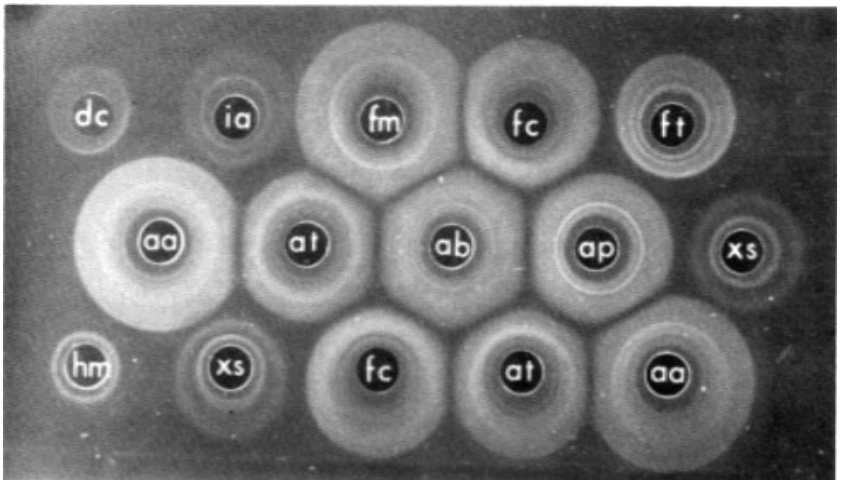


Fig. 2. Radial immunodiffusion plates showing comparison of various *Ambrosia* spp. and relatives by *A. artemisiifolia*. Antigens : aa = *A. artemisiifolia*, fm = *A. ambrosioides*, ft=*A. tenuifolia*, dc=*Dicoria canescens*, hm=*Hymenoclea monogyra*, ia=*Iva angustifolia*, xs=*Xanthium spinosum*.

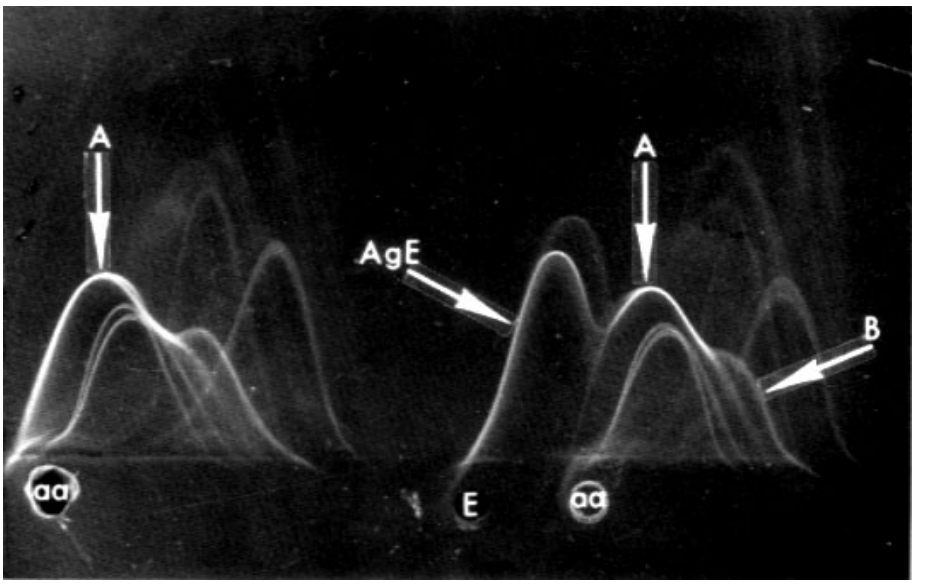


Fig. 3. Crossed immunoe lectrophoresis of *A. artemisiifolia* reference reaction(the left) and in tandem with purified antigen E from the same species(the right).

and 8 faint, relatively fast-moving bands. These 8 pronounced bands were used with combination of presaturation technique to detect shared immunoprecipitin systems between two samples. For example, when *A. artemisiifolia* antiserum was presaturated with pollen extract from *A, trifida,* the reference reaction shared 4 bands out of 8 bands. The observed bands here are due to the lack of corresponding antigenic determinants of cross antigen used for presaturation.

When *A. artemisiifolia* antiserum was presaturated with various antigens from different *Ambrosia* species, the reference reaction indicated that *A. bidentata, A. psilostachya,* and *A. ambrosioides* were more similar than *A. acanthicarpa* and *A. tenuifolia* to *A. artemisiifolia* (Table 1). Because of some technical difficulties, crossed immunoelecIrophoretic technique needs to be incorporated with results obtained by radial immunodiffusion for the better evaluation.

When *A. acanthicarpa* antiserum was presaturated (Table 2), the reference reaction showed that *A. ambrosioides* was highly similar in antigenic determinants. Three *Ambrosia* species, which were not formerly placed in the genus *Frvnseria,* were the next most similar grouping with *A. acanthicarpa.* The

Table 2. Numbers from RID rings (with diameters in mm) and 2-D IE bands formed with *Ambrosia* (*Franseria*) *acanthicarpa* reference reaction after antiserum (As) was presaturated with pollen proteins (Ag) from various species. See Fig. 2 for the abbreviations.

Ag / As-Ag	RID rings		2-D IE bands	
	Ag E	fc	Ag E	fc
fc-0 (non-presaturated)	1(7.5)	7 (13.7, 9.6, 8.3, 6.8, 6.5*, 5.8, 4.9)	1	11
fc-fc	0	0	0	0
fc-fm	0	2 (6.7*, 5.0)	0	2
fc-aa	0	4 (8.1, 6.6, 5.7*, 4.9)	0	2
fc-ab	0	4 (10.8, 7.1, 5.7*, 4.6)	0	3
fc-ap	0	4 (7.0, 5.8, 5.4, 5.0)	0	3
fc-ha	1(8.7)	5 (11.7, 9.8, 6.0*, 5.0, 4.3)	0	4
fc-hm	1(7.7)	7 (10.5, 9.9, 9.0, 7.2, 6.0*, 5.1, 4.6)	0	6

Table 3. Numbers from RID rings (with diameters in mm) and 2-D IE bands formed with *Helianthus annuus* (cv. Giant Gray Stripe) reference reaction after antiserum (As) was presaturated with pollen proteins (Ag) from various species. See Fig. 2 for the abbreviations.

Ag / As-Ag	RID rings		2-D IE bands	
	Ag E	ha	Ag E	ha
ha-0 (non-presaturated)	1(faint; not measurable)	7(13.2, 12.8, 11.1, 8.6, 6.8*, 6.1, 5.6)	0	12
ha-ha	0	0	0	0
ha-ha	0	5 (13.2, 11.7, 10.5, 9.4, 7.5*)	0	5
ha-at	0	5 (12.6, 10.8, 9.4, 6.7*, 5.9)	1	6
ha-ab	0	4 (13.0, 8.5, 7.5*, 6.0)	0	4
ha-ap	0	3 (14.4, 7.9*, 7.0)	0	5
ha-fc	0	4 (14.9, 8.3, 7.2*, 6.8)	1	3
ha-ft	0	3 (16.0, 7.5*, 7.6)	1	4
ha-hm	0	4 (15.9, 12.4, 7.1*, 6.7)	1	5

Table 4. Numbers from RID rings (with diameters in mm) and 2-DIE bands formed with *Ambrosia trifida* reference reaction after autiserum (As) was predsaturated with pollen proteins (Ag) from various species. See. Fig. 2 for the abbreviations.

Ag / As-Ag	RID rings		2-D IE bands	
	Ag E	at	Ag E	at
at-0 (non-presaturated)	1 (5.1)	6(15.9, 9.5, 7.4, 6.6, 5.7*, 5.0)	1	7
at-at	0	0	0	0
at-aa	0	3 (7.3, 6.0*, 5.0)	0	3
at-ab	0	3 (7.3, 5.8*, 4.8)	0	3
at-ap	1(faint to measure)	3 (7.0, 6.3*, 5.1)	0	3
at-fc	1	3 (6.5, 5.4*, 4.6)	0	3
at-fm	1 (4.8)	3 (6.4, 5.6*, 4.8)	1	1
at-ft	1 (5.1)	4 (8.4, 7.2, 5.8*, 5.0)	1	4
at-ha	1(faint to measure)	4 (7.1, 6.1, 5.4*, 4.9)	0	4
at-hm	1 (5.3)	6 (15.1, 8.0, 6.8, 5.7, 5.3*, 4.7)	0	7

similarity of *Helianthns annuus* and *Hymenoclea monogyra* to *A. acanthicarpa* was the least detected, because of the higher number of bands.

Table 3 illustrates the numbers of immunoprecipitin systems formed with *Helianthus annuus* reference reaction after presaturation. These data showed that no particular species were very similar.

When *A. trifida* antiserum was presaturated (Table 4), the reference reaction indicated almost equal similarity to the five *Ambrosia* species (*A. artemisiifolia, A. bidentata, A. psilostachya, A. acanthicarpa,* and *A. ambrosioides*). Last three, *A. tenuifolia, H. annuus,* and *H. monogyra,* revealed the amount of dissimilarity. So, evidence stems from the removal of bands from the reference reactions after presaturation with any of the species compared.

Overall serological similarity between *Ambrosia* and taxa formerly included in *Franseria* indicates a close relationship confirming congeneric nature of these taxa. Such data support the placement of all the taxa in one genus.

Advanced immunoelectrophoresis including crossed and rocket proved to be valuable new techniques for systematic serology to obtain the degree of protein similarity. However, it is becoming obvious that no single method can provide completely confident information on serological relationships. The complementary nature of different data from different technique helps to support the validity of systematic interpretations. Therefore, I would say, at least one or more serological techniques in addition to advanced immunoelectrophoresis such as Ouchterlony or radial immunodiffusion are still necessary to get the sum of serological information.

References

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