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Rubiaceae: Three-dimensional model for affinity relationships within the family by serological quantitation

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꼭두서니(蒨草)科 : 血淸學的 計量에 依한 親疎關係의 立體模型 製作硏究

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Abstract

The three- dimensional model for affinity relationships within the Rubiaceae was constructed by the relative amounts of serological correspondence among taxa. The serological correspondence was determined by quantitative testing of immunoprecipitin formed between antiserum and antigenic materials (seed proteins). Although the results by photronreflectometer analyses were used for the serological correspondence, the new method of rocket immunoelectrophoresis was examined for its application and proved to be suitable. Serological groupings shown in the model were briefly discussed with taxonomic interpretations of the current classification of the Rubiaceae.

Introduction

Comparative analyses of the protein-spectra of living organisms has proven to be a useful procedure to obtain information indirectly about the degree of similarity of genetic material and therewith of relationships. Even without resource to analyses of amino acid sequence, data concerning the degree of structural relatedness between the homologous protein molecules of different species can be obtained by serological methods. (Sparich & Wilson 1966)

Boyden (1932) studied the multidimensional relationships in an attempt to plot the results of the ring-test comparisons of representative antisera from animals belonging to several orders. For the in vestigation of plant systematics, Hammond (1955) constructed a three-dimensional model of the serological relationships of twelve genera of the Ranunculaceae, and Hillebrand and Fairbrothers (1970)

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discussed the affinity relationships of caprifoliaceous tribes to *Cornus* with a three-dimensional model illustrating serological correspondence.

Although the Rubiaceae was one of the earliest families to be recognized by many botanists, a considerable difference of opinion presently exists as to the correct taxonomic treatment of the family. De Candolle (1830) subdivided the Rubiaceae into 13 tribes; Bentham and Hooker (1862~83) 25 tribes (3 series) ; Lindley (1846) 11 tribes; Schumann (1891)21 tribes (2 subfamilies); Verdcourt (1958) 27 tribes (3 subfamilies); Wagenitz (1964) 21 tribes (2 subfamilies); and Bremekamp (1966) 41 tribes (8 subfamilies). The delimitation of taxa within the family remains to be completed.

An attempt in this research was made to construct a three-dimensional model of serological affinity relationships among selected taxa of the Rubiaceae. In additioe to the traditional methods, the rocket immunoelectrophoresis, another possible procedure to determine the amounts of serological correspondence among taxa, was tested and it appeared to be a suitable method.

Materials and Methods

Plant materials: seed materials were obtained from D. E. Fairbrothers' seed collection stored in the cold at the Department of Botany, Rutgers University. These seeds were washed in 0.1N $NaHCO_3$, rinsed in distilled water, air-dried, sealed in evacuated jars and stored at 4° C. Seeds were ground to fine powder in liquid nitrogen, delipified in petroleum ether for 30 min at room temperature, and further delipified in acetone (4° C) for 18 hr, and subsequently in a modified Raab extractor. The delipified meal was stored under vacuum conditions at 4°C until used.

Antigenic material: protein antigenic material was extracted by suspending the seed meal in a 0.068 M sodium-potassium phosphate buffer, pH 7.0, for 24 hr at 4°C (10ml of buffer/g meal), then centrifuged at 11,000×g for 20 min at 1° C. The clear supernatant fluid was decanted and stored frozen for use as the antigenic material.

Antiserum: Antisera were produced in New Zealand white rabbits by injection of antigenic materials. The buffered emulsion of meal and Freund's incomplete adjuvant (0.1 g crude meal and 3 ml of adjuvant) was used for the initial injection. Each booster series consisted of an int ramuscular injection of 2 ml of antige nic material for each of 4 consecutive days followed by a 3 ml injection for each of the next 3 consecutive days. Bleedings were made by cardiac puncture 7 days after the last injection. The serum was prepared by standard procedures.

Boyden procedure: The precipitin tests were made according to the method deseribed by Boyden and DeFalco (1943) and Lee (1976). The Libby photronreflectometer was employed to measure the turbidity formed upon reactions of antigenic materials and antibodies. Mixtures of antigenic material and phosphate buffered (pH 7) 2.5% of NaCl were prepared in a series of 12 (or more) doubling dilutions of known concentration. Aliquots of 0.85 ml from a double dilution series were mixed in cuvettes with 0.15 ml antiserum and incubated for 40 min at 37° C. Using this technique, the reactions measured are not affected by the protein concentrations. Thus the data obtained using this technique are an index of protein similarity of serological correspondence. Serological correspondence is expressed as a percentage of the curve area of the reference reaction (cross reaction/reference reaction×100).

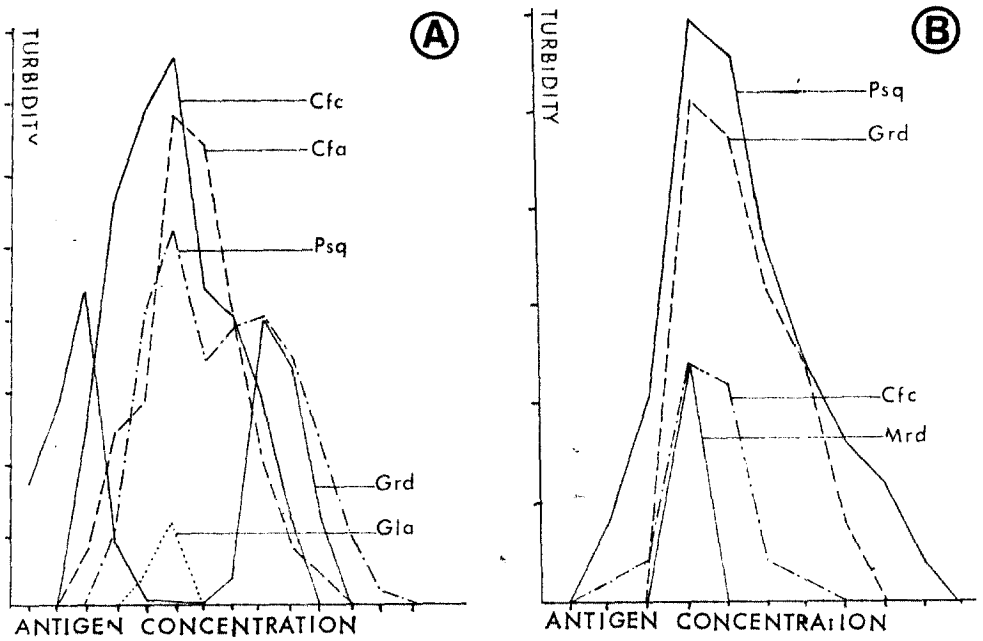

Fig. 1. Graphs showing amount of immunoprecipitin as measured by turbidity when antiserum was reacted with antigenic materials from various other species.

A. *Coffea canephora* antiserum was used. Note that no reactions were detected with antigenic materials from *Asperula humifusa* and *Galium aparine.*

B. *Posoqueria latifolia* antiserum was used. No reactions were detected with *Asperula* and *Galium.* See Table 1 for abbreviations.

Rocket immunoelectrophoresis (I.E): The name was derived from the rocketshaped immunoprecipitin bands which appear after electro-immunodiffusion (Crowle, 1973) in agarose gel charged with antiserum. For rocket IE, 14 ml of antibody-containing agarose is poured onto the plate. This volume of agarose solution contains 3 ml of antiserum which is added after cooling the agarose to 60° C or less. The wells for the samples are punched directly on the antibody-containing agarose gel. The wells are 7 mm apart, and the first and last wells are at least 14 mm from the edge of the gel slab. Approximately 7 μ l of each sample is placed in each well. The electrophoresis is performed with 50V·35mA for 14hrs. Several observations on the immunoprecipitin bands are made at 10–12hr intervals. The heights of the rocketshaped immunoprecipitin bands were measured to the nearest 0.1mm with a calibrated viewer (Kallestad Co., Chaska, MN).

Results and Discussion

Determination of serological correspondence: The precipitin reaction is based on the chemical combination of antigenic material and antibody in multiple proportions. Thus, with a constant volume of antiserum in the Boyden procedure, the amount of precipitate will increase with increasing concentrations in constant volumes of antigenic material until a maximum reaction is obtained, after which, there will be a decrease, even to zero, if the antigen excess is great enough (Fig.1). Thesefacts would warrant the conclusion that any complete titration of a given antiserum with some reacting antigenic

materials would require that the reaction be carried out over the entire antigen reaction range producing what we have called a normal titration curve.

The resulting turbidity, measured with the photronreflectometer, was plotted and the sum total of the readings from a series gave a value proportional to the area under the curve which was designated as serological correspondence. The serological comparisons among taxa obtained by turbidimetric analyses are presented in Table 1. Curves representing the amount of reaction (turbidity) over the entire activity range from antigenic excess to antibody excess using each antisera of *Coffea canephora* and *Posoqueria latifolia* against selected rubiaceous taxa presented in Fig. 1.

Table. 1. Serological comparisons among selected taxa of the Rubiaceae as determined by turbidimetric analysis. The numbers represent percentage area of the reference reaction, which is expressed as 100%.

Ab[1] / Ag[2]	Cfa	Cfc	Gla	Psq
Asperulahumifusa Bess. (Asp)	0	0	43	
Coffea arabica L. var. arabica (Cfa)	100	92	0	0
C. canephora Pirre cv. Laurentii (Cfc)	91	100	0	24
C. robusta Linden (Cfr)	81	72		0
Galium aparine L. (Gla)	0	4	100	0
G. parisiense L. (Glp)	100			
Gardenia grandifolia L. (Grd)	64	50	0	69
Morinda citrifolia L. (Mrd)	15	0	0	10
Mussaenda mutabilis (Msd)		10	0	
Posoqueria latifolia Roam. & Schult (Psq)	58	79	0	100
Psychotria berteriana DC (Psc)		0		

1 Antibody; 2 Antigenic materia

Antiserum produced to *Coffea canephora* gave strong reactions with other species of *Coffea, Gardenia, Posoqueria,* and essentially produced weak or nonreactions with *Galium* and *Asperula. Coffea canephora* antiserum produced two peaks in the curve when it reacted with *Gardenia* antigenic material. *Posoqueria latifolia* antiserum produced the strongest reactions with *Gardenia* and less with *Coffea,* and least with *Morinda.* No reactions were detected with *Galium.* In the Table 1, the numbers represent percentage area of the reference reaction, which is expressed as 100%.

New approach to determination of serological correspondence: The recent development of immunoelectrophoresis for conducting precipitin reactions on agarose-gel plates has brought us to our goal of obtaining comparable testing procedures. Particularly, the two-dimensional (crossed) immunoe-lectrophoresis and the rocket immunoelectrophoresis procedures were adapted and demonstrated by Lee (1977) and Hejgaard (1976) with plant seed proteins.

Boyden (1964) indicated that the difficulties of measuring the amounts of precipitate formed in the arcs of reaction were considerable and, therefore, the comparisons would remain qualitative only. This problem was overcome by development of rocket IE, in which the quantitative detertmination could be made with measurement of rocket height on the antibody containing agarose gel. In this particular experiment, the quantitation was made with all available rocket heights in *Coffea* obtained by rocket

IE and then the amounts of serological correspondence were compared to those obtained by Boyden procedure. Serological correspondence was determined by percentage value of total rocket heights in the reference reaction (cross reaction/reference reaction × 100). In this technique, qualitative differences between taxa were not considered and total precipitin systems were measured without indentifying the systems.

Table é. Quantitation of all available rocket heights in *Coffea* obtained by rocket immunoelectrophoresis. See Table 1 for abbreviations.

Ab	Ag	rocket heights[1] of individual bands							total	SC[2](%)
Cfc	Cfa	68.7	31.8	23.6	20.1	20.1	18.7	—	183.0	99.5
	Cfc	66.0	32.5	17.6	18.9	20.9	13.2	14.9	184.0	100.00
	Cfr	56.2	29.9	10.8	16.1	9.8	7.5	—	130.1	70.7

1 Rocket heights are arbitrary values obtained with the Kallestad viewer;
2 Serological correspondeenc

Tablə 2 shows that the amounts of serological correspondence are 99.5% in *Coffea arabica*, 70.7% in *C. robusta* and 100% in *C. canephora*, when *C. canephora* antiserum is used. These values substantiate the results obtained by photronreflectometer and it would appear to be useful to develop this advanced immunoelectrophoretic technique for determination of serological correspondence. In the present recearch, data obtained by the Boyden procedure were used for construction of the three-dimensional model.

Three-dimensiomal model: Figure 2 diagrammatically shows the relative curves obtained by testing *Coffea arabica* antiserum with the rubiaceous species. The successive curves are placed from the reference locus at distances which are proportional to their differences from the reference area, and they are arranged linearly in the absence of any data to determine their proper direction from *C. arabica* and from each other. The second step is to prepare and test another antiserum, viz., *Galium aparine* antiserum. The relative sizes of the curves obtained are not shown in Fig. 2, but their distances are, and the directions are fixed by the coincidence of their arcs. Thus, *Gardenia* is located 36 units from *C. arabica*, 31 units from *Posoqueria*, and 64 units from *Galium*. Similarly, with the other species, each should serve as a locus for determining both the direction and the distance of each species from every other. Studies involving eigat principal loci lead regularly to three-dimensional placements which satisfy the specifications derived from the tests.

These measurements are based upon biochemical similarities. The differences are not measured directly, but the distances are plotted as the reciprocal of 100 — — the percent relationship value. Data are, therefore, in a sense complementary to those obtained by measuring differences directly, as is done in some numerical taxonomy studies. These measurements may also reveal biochemical homology because antibodies against certain proteins will combine only with antigens which have serological correspondence with the reference antigen in regard to their reactive sites (antigenic determinants). Figure 3 shows the three-dimensional model made of the serological correspondence and, therefore, it shows the serological affinity relationships within the Rubiaceae.

Systematic treatments: The relative amounts of serological correspondence shown in the precipitin testing of protein antigens have systematic significance because these correspondences are systematically

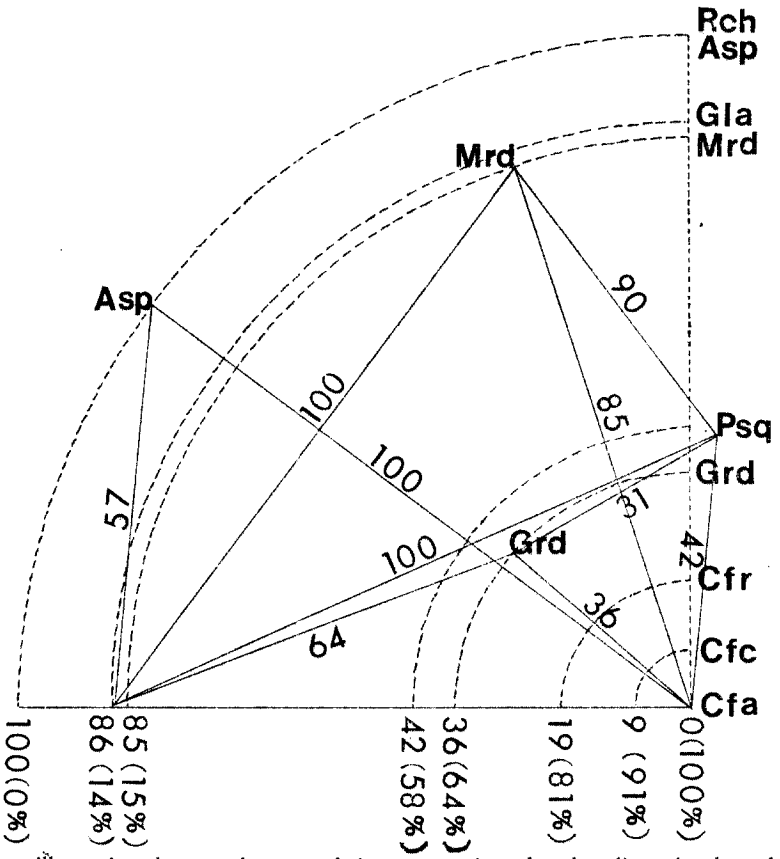

Fig. 2 Diagram illustrating the procedures used in constructing the threedimensional model from values of serological correspondence. The linear placement series for 9 taxa of the Rubiaceae with *Coffea arabica* antiserum were obtained by relative distance according to serological correspondence. Next the relative distance and direction of all the species were determined by additional reference points resulting from comparisonswith other antisera, *Galium aparine* and *Posoqueria*. See Table 1 for abbreviations.

distributed (Boyden, 1964). The serological groupings of Rubiaceae detected from this three-dimensional model (Fig.3) show the great similarity to Bremekamp's (1966) taxonomic treatment with 8 subfamilies and 41 tribes. Bremekamp placed the tribes Ixoreae (Coffeeae) including *Coffea* and Gardenieae, including *Gardenia* and *Posoqueria*, in the subfamily Ixoroideae. He characterized this subfamiyl as possessing simple interpetiolar stipules, having stamens interted in the corolla throat, and lacking raphides. This subfamily comprises all tribes in which the upper part of the style acts as a receptaculum pollinis, which means that stigmatic hairs collect the pollen from the anther before the flower opens. Cytologically, the tribes in this subfamily have a consistent basic chromosome number of x=11.

The classification proposed by Verdcourt (1958) is the one most similar to Bremekamp's of the other attempts at taxonomic treatment of the family. He had less subdivisions (3 subfamilies, 27 tribes) than did Bremekamp, and included Bremekamp's entire Ixoroideae in the subfamily Cinchonoideae. He characterized this subfamily as lacking raphides and having both albuminous seeds and nonseptate hairs.

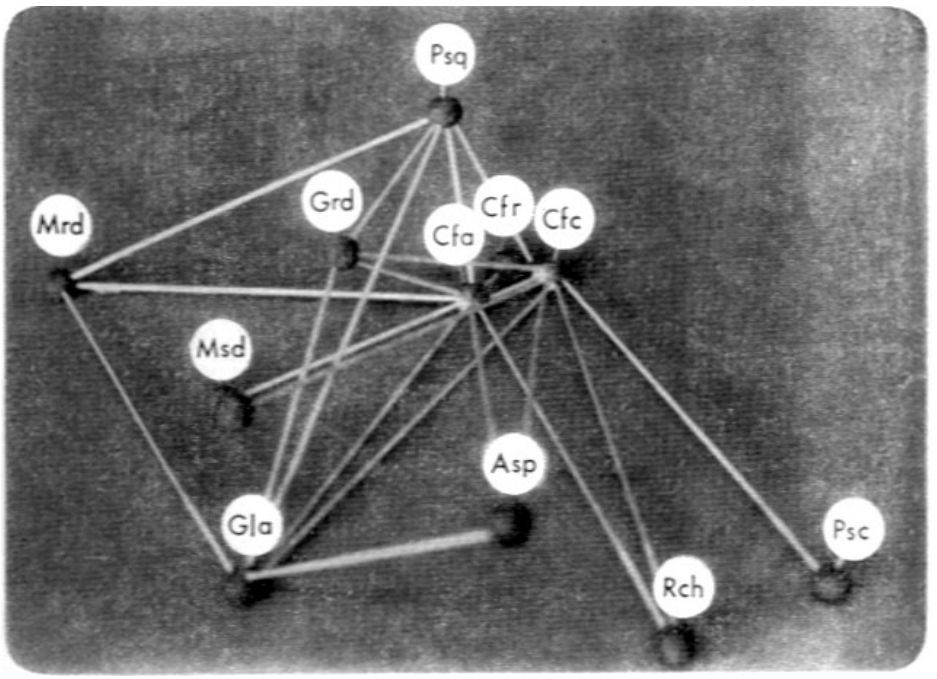

Fig. 3 Three- dimensional model for serological affinity relationships of rubiaceous taxa in this study. See Table 1 for abbreviations.

wagenitz's (1964) classification with only 2 subfamilies and 21 tribes is more similar to the older classifications such as those of Lindley (1846) and De Candolle (1830). He placed the tribe Gardenieae in his subfamily Cinchonoideae which was characterized by having a pluri-ovular locule, and the tribes Ixoreae (Coffeeae) in the Rubioideae which was characterized by a uni-ovular locule.

The serological grouping which includes *Galium* and *Asperula* is the most distinct group detected in the Rubiaceae. That these two genera are serologically far removed from other rubiaceous taxa is indicated by the strong reactions with each other. Both *Asperula* and *Galium* have always been placed in tribe Rubieae of the subfamily Rubioideae in all classifications, showing that serological data can be added to morphological data in classifying these two genera.

The Rubieae is a tribe with a polyploid chromosome series based on X=11. It is one of the few tribes of Rubiaceae which has successfully occupied diverse geographicalareas from the tropics to the arctic. The diverse fruit type which occur in the Rubieae may reflect many dispersal adaptations. It is possible that the serological distinctness of the living Rubieae is associated with the tribe being an end of long evolutionary processes, and thus it has become genetically distinct from other tribes of Rubiaceae.

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摘 要

꼭두서니(茜草)科內의 몇몇 taxa 間의 親疎關係를 나타내는 立體模型이 血淸學的 類似度의 量的比較로 製作되었다. 血淸學的 類似度는 種子蛋白質을 抗原物質로 하여 其抗體間에 形成되는 沈澱度의 量的 測定으로 決定되었다. Photronreflectometer로 마련된 結果가 血淸學的 類似度로 利用되었지만 rocket immunoelectrophoresis의 새 方法도 試圖되었고, 그의 應用이 類似度測定에 適當함이 立證되었다. 立體模型에 나타난 taxa 間의 血淸學的 grouping이 最新 꼭두서니科의 分類體制에 依據하여 簡單히 論議되었다.

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