식물학회지 제 28권 제 2호
Korean J. Bot. 28(2): 95-104 (1985)

Further Systematic Studies on *Cornus* and Relatives by Immunoelectrophoresis

Lee, Yoo Sung

(Department of Biology, Chungbuk National University, Chongju)

免疫學的 電氣泳動에 의한 층층나무屬과 그 近緣群의 系統學的 追加硏究

李 惟 性

(忠北大學校 自然科學大學 生物學科)

ABSTRACT

Additional serological data from radial immunodiffusion and conventional immunoelectrophoresis with combination of presaturation of antibody were obtained for further interpretation of *Cornus* (and relatives) systematics. Pollen proteins were compared by qualitative and quantitative means. *Cornus drummondi* and *C. stolonifera* are very similar to *C. florida*, and *C. amomum* and *C. recemosa* are less similar. *Nyssa* constitutes the next distinct and most similar family, Nyssaceae to the Cornaceae. The serological affinities indicate that *Corokia* and *Griselinia* should not be included within the Cornaceae. Their taxonomic treatment to elevate to the family level is now awaiting until more data are accumulated.

INTRODUCTION

The *Cornus* and related taxa have long been the subject of diverse studies which have incorporated the evaluation of many kinds of taxonomic characteristics.

This present paper includes an immunoelectrorhoretic comparison among species of the following genera: *Cornus*, *Corokia*, *Griselinia*, and *Nyssa*. This is an addendum to serological investigation of Cornaceae which have continued intermittently for last several years.

Depending upon the classification adopted, 7 to 16 genera have been ascribed to the Cornaceae (Ferguson, 1966). The genera have been placed in different subfamilies: Wagarin (1910) used 3, and Ferguson (1966) 8. Some taxonomists have elevated selected genera to family, while other genera are transferred to other families, e.g. *Cornus* (Willis, 1972; Gibbs, 1974) is placed in Hederaceae (Araliaceae).

The genus *Cornus* has been divided into several genera or into subgenera based on both

This work was supported by the 1984 research grant from Ministry of Education.

morphological and serological data (Wagarin, 1910; Fairbrothers, 1966; Brunner and Fairbrothers, 1978). There is still dispute about the taxonomic rank of these taxa, but their circumscription into grouping is possible.

Nyssa was placed in the separate family Nyssaceae by Dumortier (1829). However, taxonomists essentially continued to regard the genus as a member of the Cornaceae for over 100 years. In the last 20 years, virtually all published classifications recognized the separation of the Nyssaceae (Melchior, 1964; Cronquist, 1968; 1981; Takhtajan, 1980; Dahlgren, 1983; Thorne, 1983). *Corokia*, a genus originally included as part of the Cornaceae, has been shifted among various families and subfamilies, and the proper taxonomic position is still in question. Among the taxonomists retaining the genus in the Cornaceae are Kubitzki (1963) and Melchior (1964). Others relate the genus to the Saxigragaceae or Escalloniaceae (Smith, 1958; Philipson, 1967), and Takhtajan (1980) adopted a similar placement. Cronquist (1968) considered *Corokia* to be a genus intermediate between the Saxifragaceae and Cornaceae.

Mature seeds are a good protein-source since they represent a definite developmental state (maturity) which is easily recognized and readily available (Lee, 1978). In this research seed proteins from *Cornus* related taxa was analyzed and compared using serological techniques experienced in author's earlier works (Lee, 1977, 1981, 1983a, b, 1984; Lee and Fairbrothers, 1978).

MATERIALS AND METHODS

In order to prepare the protein extracts, mature seeds were harvested from natural populations or obtained from D. E. Fairbrother's (Rutgers University, NJ, USA) seed collection stored in jars under vacuum at 4 C. The plant taxa used in this research include 5 *Cornus* (Cornaceae) species, 1 *Corokia* (Cornaceae or Saxifragaceae) species, 2 *Nyssa* (Nyssaceae) species, and 2 *Griselinia* (Cornaceae or Araliaceae) species (See Table 1 for full names). For serological tests antisera were prepared for *C. amomum*, *C. drummondi*, *C. florida*, and *N. aquatica*.

Dry seeds were ground to a fine powder with a Prolabo Microbroyeur Quantitatif Dangoumau for 3-5 min, and passed through a 60-mesh sieve. Extraction of fat was carried out with petroleum ether at room temperature and constant stirring, followed by air-drying and passing through the 60-mesh sieve to assure uniformity of particle size. The second extraction was performed with acetone in a Raab extractor for 20 hr at 15 C. After fat extraction the meals were dried and stored at 4 C.

Protein antigenic material was extracted by suspending the prepared seed meal in a 0.068 M sodium-potassium phosphate buffered 2.5% saline solution, pH 7.0 (KH_2PO_4 3.815 g; Na_2HPO_4 14.912g; NaCl 500 g; Merthiolate 2 g; QS to 20 l) for 24 hr at 4 C (10 ml of buffered solution/g meal), then centrifuged at 11,000×*g* for 20 min at 4 C.

Antisera were produced in New Zealand White rabbits by injection of antigenic material made from seed meal slurry mixed with a phosphate buffer. The antigenic material plus Freund's incomplete adjuvant (0.1 g meal and 3 ml of adjuvant) was used for the first injection. Each booster series consisted of an intramuscular injection of 2 ml of antigenic material followed by a 3 ml injection for the next 3 consecutive days. Blood samples were obtained by cardiac puncture and antisera obtained were stored in vials at −25 C. Before the first injection, a blood sample was obtained from each rabbit to be used as control for the detection of any non-specific reactions in the normal rabbit serum (Clausen, 1969).

Immunoelectrophoretic analyses were performed according to the method of Gravey *et al* (1977) using an LKB apparatus and 0.1 % agarose in phosphate-buffered (pH 7.0) 2.5% NaCl. Protein extracts (antigenic materials) in the wells were electrophoresed at 2 V/cm for 2 hr. After electrophoresed, the antiserum was placed in the trough and prepared plates were incubated for 48 hr at room temp. and 100% relative humidity. Precipitin arcs (bands) were photographed Polaroid type 55 film with dark field lighting by using a Polaroid MP-4 camera equipped with a 105 mm lens and deep yellow filter. Stainining was not required in order to observe the immunoprecipitin bands, rings, or arcs. Presaturation of antibody (Lee, 1977) refer to the selective removal of antibodies from an antiserum by its previous exposure to a meal containing the corresponding antigenic materials. In theory the closer presaturating seed meal is to the seed meal that produced the antiserum, the greater will be the loss of antibody activity from that antiserum. Presaturation thus allows for the ranking of taxa as regards their effectiveness as absorbing agents on antisera prepared from injections of seed meal. A precipitate was obtained by incubating 0.3 g of powdered delipified seed meal with 3.0 ml of appropriate antiserum for 3 days at 4 C. After 3 days the mixture was centrifuged (11,000×*g*) for 20 min at 4 C. This presaturated antiserum (supernatant) was used in electrophoresis to react against selected cross-reacting antigenic material.

RESULTS AND DISCUSSION

Present research has further substantiated serological data obtained in the previous investigation (Lee, 1983b).

Double immunodiffusion (DID) and conventional immunoelectrophoresis (CID). *Cornus drummondi* and *C. stolonifera* are serologically very similar to *C. florida*. Except one apparent band showing partial identity (TYPE III, Ouchterlony and Nilsson, 1978), all the band was identical (TYPE I) (Fig. 1). Only one band of *C. florida* spectrum was not directly equivalent in *C. amomum* or *C. stolonifera*, which had 2 immunoprecipitin systems (IPS) fusing with one *C. florida* IPS. Such a pattern can result from slightly different distribution of determinant sites on the antigenic molecules from *C. amomum* or *C. stolonifera*, or the one band in *C. florida* with which the *C. drummondi* or *C. stolonifera* united

actually represents 2 very similar systems which are indistinguishable using DID (Fig. 1). In this case the latter may be true because 2 unique arcs in *C. florida* was distinguishable using CID (Fig. 5 and Table 1). *Cornus amomum* and *C. recemosa* antigenic materials also strongly react with *C. florida* antiserum (Fig. 1 and Table 1). In the previous report (Lee, 1983b) *C. amomum and C. drummondi* were placed in the same serological grouping. These representative *Cornus* species were reacted with the *C. florida* antiserum to establish the degree of intrageneric similarity expressed by the IPS (bands or arcs) which could be compared with the selected other genera.

Of all the cross-reacting non-*Cornus* antigenic materials tested, *Nyssa* was most similar, and it shares 1 strong identity band with DID (Fig. 1). Another strong band was not consistently distinguishable with DID, however it was observed as an arc in all CIE (Fig. 5). In addition to these 2 IPS, one more faint but apparent arc was observed at cathode side in *N. aquatica* and *N. sylvatica* (Fig. 5).

The genus *Corokia* also reacts with *C. florida* and forms 1 distinct non-identity precipitatin band with DID (Fig. 1) and 3 faint arcs with CID (Fig. 5). It revealed less similarity with *Cornus* than did *Nyssa*. However, *Nyssa* is placed in a separate family, while *Corokia* is often maintained within the Cornaceae by various authors. Results obtained from this research indicate that the retention of *Corokia* in the Cornaceae is not appropriate. The *Griselinia* revealed 1 non-identity band with the *Cornus* antiserum (Fig. 1). Those were not clear enough in CIE for photograph (Fig. 5). Thus it revealed more less serological affinity with *Cornus*.

Antisera against *C. amomum* and *C. drummondi* were used for precipitin reaction without presaturation (Table 1). They showed the similar tendency of serological affinities among and within *Cornus* mentioned above.

Figs. 1-4. Illustration of immunoprecipitin systems obtained from double immunodiffusion (Fig. 1) and radial immunodiffusion (Figs. 2-4) for *Corokia*, *Griselinia*, and *Nissa*.

In Fig. 1, the well with letter received antiserum and well with number antigenic material. Antisera: (A) *C. florida*; (B) *C. florida* antiserum presaturated with antigenic material from *C. drummondi* (*C. florida—C. drummondi*); (D) *C. florida—N. aquatica*; (F) *C. florida—Co. cotoneaster*; (F) *C. florida—G. scandens*; (G) *C. florida—C. amomum*; (H) *C. drummondi*. Antigenic materials: (1) *C. amomum*; (2) *C. drummondi*; (3) *C. florida*; (4) *C. stolonifera*; (5) *Co. cotoneaster*; (6) *G. scandens*; (7) *N. aquatica*; (8) *N. sylvatica*.

In Fig. 2, the agarose contained rabbit antiserum against antigenic material from *C. florida*. The well received the following antigenic materials: (the first raw from the left) *C. amomum*, *C. drummondi*, *C. florida*, and *C. stolonifera*; (the second raw) *Co. cotoneaster*, *G. scandens*, *N. aquatica*, and *N. sylvatica*; (the third raw) *C. florida*, *N. aquatica*, *G. scandens*, and *C. amomum*.

In Fig. 3, the agarose contained antiserum against *C. drummondi*. Antigenic materials: (the first and second raw) the same as in Fig. 2; (the third raw) *C. drummondi*, *N. aquatica*, *G. scandens*, and *C. amomum*.

In Fig. 4, the agarose contained antiserum against *N. aquatica*. Antigenic materials: (the first raw) the same as in Fig. 2; (the secod raw) *Co. cotoneaster*, blank, *N. aquatica*, and *N. sylvatica*; (the third raw) blank, *N. aquatica*, *G. scandens*, and *C. amomum*.

Radial immunodiffusion (RID). Each agarose plate containing antisera against *C. florida* (Fig. 2), *C. drummondi* (Fig. 3), and *N. aquatica* (Fig. 4) included 12 wells for antigenic materials from various taxa tested. In Fig. 2, 4 species of *Cornus* and 2 species of *Nyssa* revealed the fairly good sizes of RID rings with strong intensity. *Corokia cotoneaster*, however, appeared with 1 faint ring only, and *G. scandens* showed nothing at least.

In Fig. 3, when *C. drummondi* antiserum was used, each well has relatively faint IPS (rings). RID rings with cross antigenic materials are very similar to those in Fig. 2. Reaction with *Co. cotoneaster* was even weaker than that in Fig. 2. Of all the non-*Cornus* antigenic material tested, *Nyssa* was the most similar to *Cornus*, and *Griselinia* was the least. In Fig. 4, when *N. aquatica* antiserum was used, the similarity of *Nyssa* to *Cornus* was confirmed by particularly strong intensity of IPS formed in both genera.

RID technique seems to be good for primary screening of IPS, but not good for the

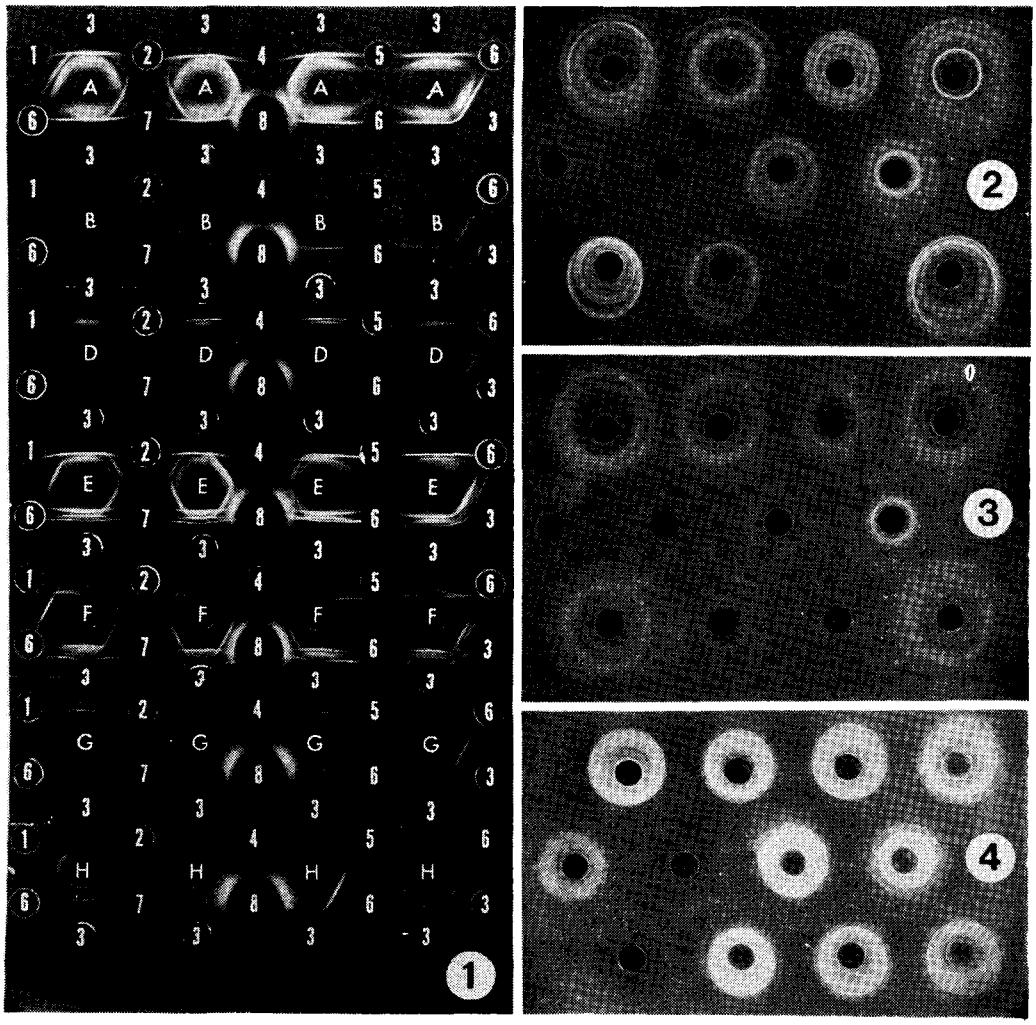


Table 1. Number of immunoprecipitin systems (bands or arcs) obtained with antisera of *Cornus amomum*, *C. drummondi*, and *C. florida*. *Cornus florida* antiserum was presaturated with *Cornus*, *Corokia*, *Griselinia*, and *Nyssa*, and subsequently reacted with these antigenic materials (f: faint)

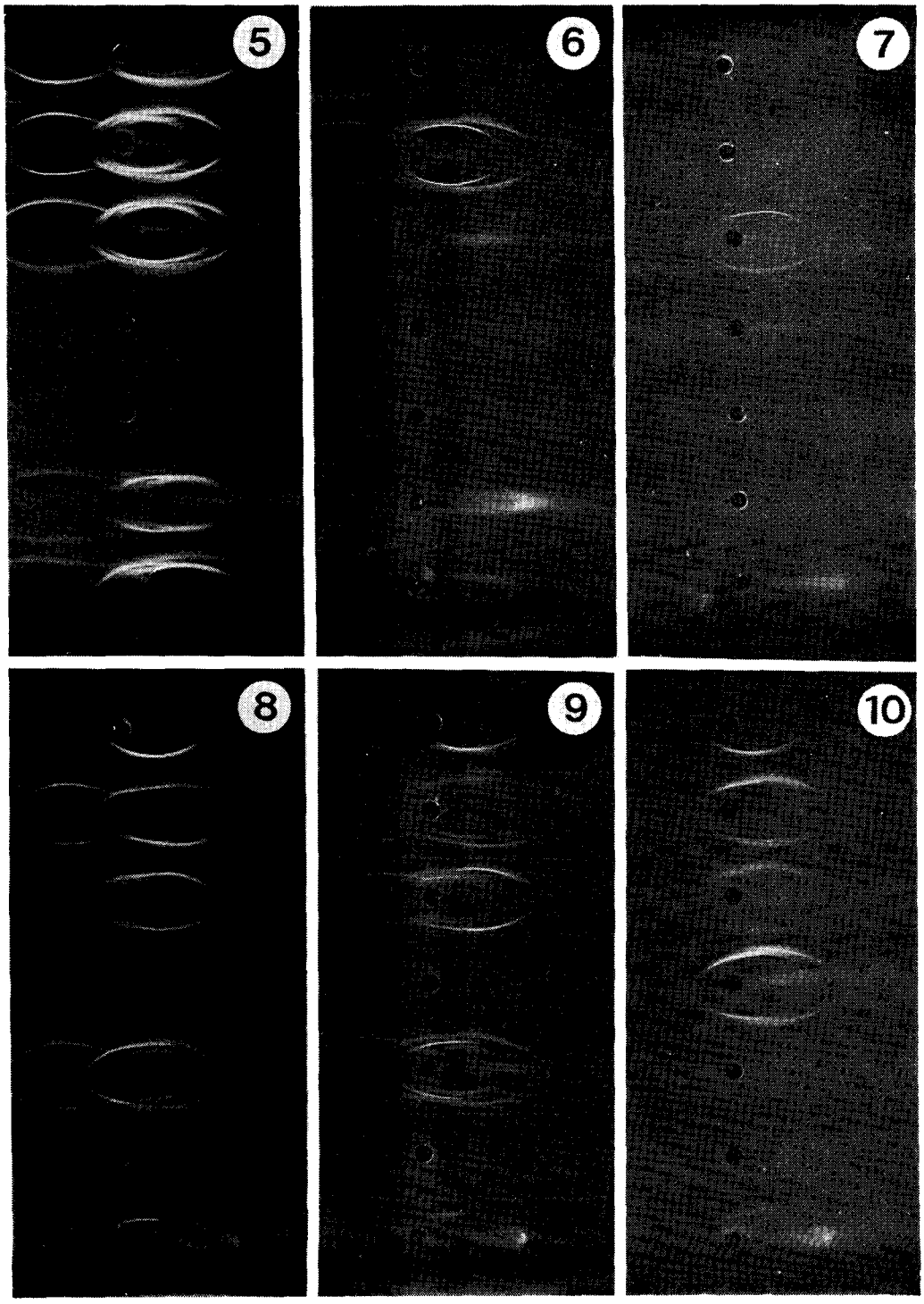
Antiserum / Antigenic materials	Non-presaturated			Antigenic materials with which *C. florida* antiserum was presaturated							
	C. amomum	*C. drummondi*	*C. florida*	*C. florida*	*C. amomum*	*C. drummondi*	*C. racemosa*	*C. stolonifera*	*Co. cotoneaster*	*N. aquatica*	*G. scandens*
	Number of IPS										
C. florida	4	3	5	0	2~3	0	3	2~3	4	3	4
C. amomum	5~6	3	3	0	0	0	0	1	3	2	3
C. drummondi	4	3	4	0	0	0	—	1	3	2	3
C. racemosa	—	3	3	0	0	0	0	1	—	—	3
C. stolonifera	—	3	4	0	0	0	0	0	4	2	3
Co. cotoneaster	1	0	3f	0	0	0	0	0	0	0	0
N. aquatica	3	1	4	0	0	0	0	1	1	0	1
N. sylvatica	—	2	4	0	1	0	0	1	2	0~1	2
G. ruscifolia	—	0	2	0	0	0	0	0	0	0	0
G. scandens	1	0	0~1f	0	0	0	0	0	0	0	0

analysis of IPS.

Presaturation of antibody. Results from presaturation of antibody is also shown in DID (Fig. 1) and CID (Figs. 6-10). Experiments in Fig. 1 and Fig. 7 further substantiate the similarity of *C. amomum*, *C. drummondi*, and *C. stolonifera*, when compared to *C. florida*. The *C. florida* antiserum, presaturated with antigenic materials from one of 3 species above followed by reaction with *C. florida* antigenic material (reference antigenic material), reveals 2 arcs (Figs. 1, 7). This indicates that 2 or 3 have been removed by the presaturating antigenic material and thus shared by 3 *Cornus* species above. The remaining ≈3 IPS are unique to *C. florida*.

When *N. aquatica* antigenic material was combined with *Cornus* antiserum 2 or 3 IPS were removed from *C. florida*, 1 or 2 IPS from other *Cornus* species, and all the IPS from non-*Cornus* species compared. From these experiments it is not definite that the 2 IPS of

Figs. 5-10. Conventional immunoelectrophoretic patterns with *C. florida* antiserum non-presaturated (Fig. 5) or presaturated (Figs. 6-10) with antigenic materials from *Cornus*, *Corokia*, *Griselinia*, and *Nyssa*. Antisera: (5) *C. florida*; (6) *C. florida* antiserum presaturated with antigenic material from *N. aquatica* (*C. florida—N. aquatica*); (7) *C. florida—C. drummondi*; (8) *C. florida —Co. cotoneaster*; (9) *C. florida—G. scandens*; (10) *C. florida—C. stolonifera*. Antigenic materials from the top: (5~6) *C. drummondi*, *C. florida*, *C. stolonifera*, *Co. cotoneaster*, *G. scandens*, *N. aquatica*, and *N. sylvatica*; (7~10) *C. amomum*, *C. drummondi*, *C. florida*, *C. stolonifera*, *Co. cotoneaster*, *G. scandens*, and *N. aquatica*.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Nyssa is unique to that genus. Results obtained from CIE showed only reduced intensity of IPS in the reference reaction of *C. florida* after presaturated with *N. aquatica* (Fig. 6). When *C. florida* was presaturated with *Co. cotoneaster* and subsequently reacted with *C. florida*, 4 IPS appeared. The reactions with other *Cornus* species yield 3 IPS (4 with *C. stolonifera*) indicating that *Co. cotoneaster* did not remove any IPS from *C. florida* antiserum, thus it was unaffected by presaturation with *Co. cotoneaster* (Figs. 1, 8, and Table 1). *Nyssa*, on the other hand, retained 1 distinct IPS.

Cornus florida antiserum was presaturated with the *G. scandens* and reacted against all the cross-reacting materials. *Griselinia scandens* removed 1 IPS from *C. florida*, and either none or 1 from the other *Cornus* species (Fig. 9 and Table 1). This indicates that *Griselinia* had no more serological affinity to *Cornus* than *Nyssa*. *Cornus florida* antiserum, presaturated with either *Co. cotoneaster* or *G. scandens* antigenic material followed by reaction (combination) with non-*Cornus* antigenic materials, reveals the same number of IPS (Table 1).

This indicates that the same number of rest IPS have been removed by the presaturating antigenic materials and thus shared in the serological distance to *C. florida* by *Co. cotoneaster* and *G. scandens* (Fig. 8, 9).

Finally, *C. florida* antiserum was presaturated with *C. stolonifera* and related against *C. florida*, 1-2 IPS appeared. When reacted against other *Cornus* species or *Nyssa* species, 1 IPS was yielded (Fig. 10 and Table 1).

Significantly more information than that obtained by DID was detected in CIE experiments using presaturation of antibody. In all *Cornus* species number of IPS was the same in both DID and CIE, but CIE separated IPS more efficiently.

CONCLUSION

From the foregoing results the following patterns emerge: *C. drummondi* and *C. stolonifera* are serologically very similar to *C. florida; C. amomum* and *C. recemosa* are less similar, and *C. florida* antiserum does not distinguish between them. *Nyssa* constitutes the next distinct and most similar family, Nyssaceae, to the Cornaceae. *Corokia* follows, but is less like *Cornus* than is *Nyssa* of the Nyssaceae. *Griselinia* has also a little affinity with *Cornus*, but is rather distinct from both *Corokia* and *Griselinia*. The serological affinities indicate that *Corokia* and *Griselinia* are not likely to be included within the Cornaceae. All genera tested revealed some affinity as serological relatives. The placement of them into the Cornaceae and Nyssaceae and in the order Cornales is an appropriate taxonomic arrangement. *Corokia* and *Griselinia* have distinct serological affinities with the above families, and they are serologically distinct from each other. Additional information is awaiting to indicate that the placement of these genera in others would best reflect the taxonomic position for them.

摘 要

층층나무屬(*Cornus*) 植物 數種과 그 近緣群의 系統學的 類緣關係를 밝히기 爲하여 種子蛋白質을 二重免疫擴散法, 事前飽和處理法, 放射狀免疫擴散法, 免疫學的 電氣泳動法 等으로 分析함으로 血淸學的 追加資料를 얻었다. *Cornus florida* 免疫血淸을 使用한 沈澱反應에서 *C. drummondi*와 *C. stolonifera*는 血淸學的 類似度가 매우 높았고, *C. amomum*과 *C. recemosa*는 다음으로 높았다. *Cornus*와의 近緣群에 該當하는 分類群 중에서 *Nyssa* (Nyssaceae)가 가장 가까운 近緣性을 나타냈고, *Corokia* (Cornaceae 또는 Saxifragaceae)와 *Griselinia*는 매우 낮은 近緣性을 보여서 Cornaceae에의 所屬을 支持하고 있지 않다. 그러나 그들의 獨立된 科로서 區分하기 爲해서는 많은 다른 分類學的 證據가 必要할 것으로 본다. 免疫學的 電氣泳動法과 事前飽和處理法의 組合된 技術은 比較的 精巧한 種子蛋白質 分析이 可能했다.

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(Received November 17, 1984)