Aliso: A Journal of Systematic and Evolutionary Botany

Volume 7 | Issue 2 Article 12

1970

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Jean-Pierre Simon
Rancho Santa Ana Botanic Garden

ALISO

VOL. 7, No. 2, pp. 243-261 JUNE 22, 1970

COMPARATIVE SEROLOGY OF THE ORDER NYMPHAEALES

I. PRELIMINARY SURVEY ON THE RELATIONSHIPS OF *NELUMBO*

JEAN-PIERRE SIMON

Rancho Santa Ana Botanic Garden
Claremont, California

INTRODUCTION

The order Nymphaeales as considered by Cronquist (1968) and Thorne (1968), is a small order of aquatic vesselless plants numbering less than 100 species. Taxonomists differ widely in their evaluation of the hierarchical position and relationships of these primitive taxa. Earlier classifications (e.g., Caspari, 1888) had considered these taxa as belonging to one family Nymphaeaceae, which was divided into three subfamilies, Nelumbonoideae, Cabomboideae and Nymphoideae. Later these were upranked as separate families and placed in the Ranales (Small, 1933; Fernald, 1950), or Magnoliales (Gundersen, 1950). Earlier, Bessey (1915) had retained Cabombaceae and Nelumbonaceae in Ranales but removed Nymphaeaceae to Rhoedales. More recent treatments isolate these families in the Order Nymphaeales (Cronquist, 1968) or even in the superorder Nymphaeiflorae (Thorne, 1968) but, while a close relationship among the taxa is implied from these treatments, others stress fundamental morphological and anatomical differences betwen Nelumbonaceae (with one genus: *Nelumbo*, and two species) and the remaining two families, and have even taken the Nelumbonales as a separate order (Li, 1955; Takhtajan, 1959, 1969). Furthermore Li (1955) has also proposed that the tropical genera *Euryale* (Euryalaceae) and *Barclaya* (Barclayaceae) be segregated from the Nymphaeaceae and placed in a new order, Euryalales, on the basis of the distinct flower morphology. A synopsis of the recent treatments by Li (1955), Takhtajan (1969), Cronquist (1968) and Thorne (1968) are included in Table 1 for the purpose of comparison.

The reason for the discrepancies in the taxonomic ranking of the taxa may be due to the similarities of morphological and anatomical characters which apparently are the result of the specialized aquatic habitat of this group of primitive plants and may or may not reflect true phylogenetic relationships. On the other hand, the component genera show extreme morphological diversification in all diagnostic features (Li, 1955). Aquatic plants in the Angiosperms are thought to be specialized derivatives of in-

dependent and widely separated terrestrial stocks. Li (1955) claims that such diversification in the Nymphaeales clearly indicates the heterogeneous nature of the whole group. He proposes that the genera, or groups of genera of the Nymphaeales, are not only polyphyletic in origin but also are derived from unrelated ancestors. According to Li (1955), the affinities of the taxa would be as follows:

TAXON:	AFFINITIES:
Cabombaceae	to Ranunculaceae.
Nymphaeaceae	to Ranunculaceae and Berberidaceae.
Nelumbonales	to herbaceous Berberidaceae, Magnoliales and also to Monocotyledons, but very isolated.
Euryalales	
Euryalaceae	to Papaveraceae, Aristolochiaceae.
Barclayaceae	to Euryalaceae, but isolated.

According to Takhtajan (1959, 1969), any direct link between Nelumbonales and Nymphaeales would be unlikely and *Nelumbo* would probably be more close to the Illiciales–Ranunculales stock than to the Magnoliales. Cronquist (1968) while recognizing the isolated position of Nelumbonaceae still considers them in the Nymphaeales. His main argument is that there are too many similarities in habitat, habit, flower morphology and placentation to consider segregating this taxon to a separate order. He accepts on purely phenetic grounds that the Nymphaeales might be treated as a suborder of the Ranunculales, but suggests that, on phylogenetic grounds, this order must have been derived much earlier and that the hypothesized ancestor would be a "now extinct, highly primitive member of the Magnoliales."

While a number of morphological and anatomical characters point towards a relationship between certain genera of the Nymphaeales and members of the Magnoliales, Ranales or Papaverales, several taxa also exhibit a combination of characters similar to those present in the Monocots (Cronquist, 1968). In fact, the Nymphaeales have often been regarded as near-ancestral to the monocots and at least one taxonomist proposed that the Nymphaeales should be reclassified in the Monocotyledoneae as a subseries of the Helobiae (Lyon, 1901). Recently, Takhtajan (1969) reviewed evidence pointing towards the close relationships of primitive taxa belonging to the monocot orders Alismales, Liliales and the Nymphaeales. Takhtajan (1969) emphasizes that "the immediate ancestors of the monocots were most likely some extinct vesselless herbaceous plants with apocarpous gynoecia and monocolpate pollen which probably had much in common with modern Nymphaeales."

The available information based mainly on external morphology and anatomy has not been sufficient to clarify the relationships of the taxa assigned to the Nymphaeales, nor the position of this order *vis-à-vis* other major groups of primitive angiosperms. Evidence concerning these relationships based on approaches other than those of traditional morphologi-

cal taxonomy needs to be evaluated and a biochemical study using antigenic protein characters is in progress at this institution. The field of systematic serology has developed considerably in the last two decades and recent reports indicate that this approach may be very useful in clarifying the taxonomic position of selected taxa (Fairbrothers, 1968). Serological comparisons have been carried out at intrageneric (e.g., Kloz, 1966), intergeneric (Simon, 1969a) and interfamily levels (Jensen, 1968) and appears to be useful as an adjunct approach in clarifying taxonomic problems at all these levels.

As part of a comprehensive study of the Order Nymphaeales, I am reporting here comparative serological data of a preliminary nature concerning the relationships of *Nelumbo* with other members of the Nymphaeales and with representative taxa of the Annonales, Ranunculales (Berberidales) and monocots.

TABLE 1. *Comparison of treatments of the Nymphaeales.*

LI (1955)

Order.—Ranales.
Family.—Cabombaceae: 1, 2*
Family.—Nymphaeaceae: 3, 4
Order.—Nelumbonales.
Family.—Nelumbonaceae: 5
Order.—Euryalales.
Family.—Euryalaceae: 6, 7
Family.—Barclayaceae: 8

TAKHTAJAN (1969)

Order.—Nymphaeales.
Family.—Cabombaceae: 1, 2
Family.—Nymphaeaceae: 3, 4, 6, 7
Family.—Barclayaceae: 8
Family.—Ceratophyllaceae: 9
Order.—Nelumbonales.
Family.—Nelumbonaceae: 5

CRONQUIST (1968)

Order.—Nymphaeales.
Family.—Nymphaeaceae: 1, 2, 3, 4, 6, 7, 8
Family.—Nelumbonaceae: 5
Family.—Ceratophyllaceae: 9

THORNE (1968)

Superorder.—Nymphaeiflorae.
Order.—Nymphaeales.
Family.—Nymphaeaceae.
Subfamily.—Nymphaeoideae: 3, 4, 6, 7, 8
Subfamily.—Nelumbonoideae: 5
Subfamily.—Cabomboideae: 1, 2
Family.—Ceratophyllaceae: 9

*Numbers refer to genera as follows: 1. *Cabomba*; 2. *Brasenia*; 3. *Nymphaea* (including *Castalia*); 4. *Nuphar*; 5. *Nelumbo*; 6. *Euryale*; 7. *Victoria*; 8. *Barclaya*; 9. *Ceratophyllum*.

MATERIALS AND METHODS

Seeds of representative species of the Nymphaeales, Ranales, Annonales and monocots available for this study were received from various sources and are listed in Tables 2 and 3. Detailed information on the origin and history of each accession may be obtained from the author.

PREPARATION OF ANTIGENS

Protein extracts were prepared from seed flour. The seeds were first washed in several changes of a 0.1 N solution of $NaHCO_3$, thoroughly rinsed in distilled water and air dried at 5 C. A refrigerated micro-mill (Chemical Rubber Company, Cleveland, Ohio) was used to grind the

seeds. The proteins were extracted in an aqueous extracting solution (5 ml/g flour; NaCl, 12 g/liter; $NaH_2PO_4 \cdot H_2O$, 0.22 g/liter; $Na_2HPO_4 \cdot 12 H_2O$, 3 g/liter; pH 7.6) for 18 hours at 5 C. The slurry produced was pressed through two layers of cheesecloth and the extracts centrifuged (3,000 × g) for 15 minutes. The supernatant was then dialyzed at 5 C for 24 hours against several changes of the phosphate buffer solution, the dialyzed extract was centrifuged for 20 minutes (3,000 × g) and the protein content of the supernatant was estimated by the method of Lowry *et al.* (1951). Due to the great number of extracts to be processed, protein analyses were not carried out for all the extracts produced. About 50% of all the Nymphaeales samples representing, with the exception of *Cabomba*, at least one species per genus were estimated but, an average of only two species from each of the other orders were analyzed for protein content. Each extract was adjusted to contain approximately 30 mg of protein/cc by evaporation through a dialysis membrane at 5 C. The protein content of nonanalyzed extracts was arbitrarily equated to that of the closely related species tested.

Parallel tests using dialyzed and undialyzed extracts disclosed that the latter produced essentially similar reactions in the Ouchterlony plates. Undialyzed extracts have been used to test taxa of families other than the Nymphaeales for which the seed samples received were often quite small. However, those crude extracts giving no reactions were dialyzed and retested.

Comparative tests using antigen extracts prepared from defatted and nondefatted seed meals disclosed differences in reactivity for some of the Nymphaeales taxa but not for members of other families of Angiosperms. Therefore, parallel serological tests were also made with protein extracts prepared from defatted seed flour. The flour was delipified by soaking it for 30 minutes in petroleum ether at –20 C and subsequently washing the flour by centrifugation and resuspension in cold acetone. This was done three times, each wash lasting for 15 minutes and protein extraction proceeded as indicated above.

PREPARATION OF ANTISERA

Protein extracts of one accession of *Nelumbo nucifera* (NU-1; received from Longwood Botanical Gardens, Kennett Square, Pennsylvania) and *N. lutea* (LU-1; from Tallahassee, Florida) were each injected into two female New Zealand rabbits; the four rabbits being from the same litter. The rabbits were bled before being injected and the normal sera did not react with any of the antigen extracts prepared for this study. The animals received one intramuscular injection of a mixture of 1 cc of complete Freund adjuvant (Difco) and 1 cc of antigen (nondelipified, ca. 10 mg protein/cc). After a rest period of two weeks, 1 cc of antigen was injected intravenously three times on alternate days. Ten days following the last injection, 40 cc of blood were withdrawn from the marginal vein of the ear. The blood from each rabbit was allowed to clot for three hours at room temperature and was kept in the refrigerator at 5 C for 12 hours. It was then centrifuged and the serum stored in small aliquots at –20 C

following the addition of sodium azide to a final concentration of 0.1%. Subsequently, each rabbit was reinjected at three-week intervals and bleedings were made 10 days after injections. All the rabbits produced antisera with a titer of 1/512 or higher after the third bleeding and no additional precipitin bands were observed in Ouchterlony and immunoelectrophoresis tests with antisera drawn from the six subsequent bleedings.

SEROLOGICAL TECHNIQUES

Gel immunodiffusion (ID): gels for the Ouchterlony plates (Ouchterlony, 1964) were prepared using ionagar No 2 (1%) in barbital buffer (pH 8.2) according to the method of Lester, Alston and Turner (1965). Conditions for the characterization and interpretation of the precipitin reactions were as described by Simon (1969 a, b).

Parallel immunoelectrophoresis (IE): These tests were carried out on 4 × 5 in. lantern slide cover glasses using ionagar (0.75%) in barbital buffer (pH 8.2) according to the method of Crowle (1961) under the conditions reported by Simon (1969 b).

Absorption of antisera: Absorption tests were carried out according to the procedure outlined earlier (Simon 1969 a, b).

RESULTS

SEROLOGICAL RELATIONSHIPS OF *Nelumbo lutea* AND *N. nucifera*

Seven accessions of *N. lutea* and five of *N. nucifera* were available for this investigation and the ID tests indicated that, irrespective of their area of collection, these extracts produced similar patterns in the Ouchterlony plates when reacted against any of the four antisera produced (Fig. 1–4). These tests showed that there was a complete fusion of all the precipitin bands in the zone of overlap between the different reactions. This indicates that the accessions of the two species are serologically similar. The number of bands formed in the plates depended on the antiserum and kind of antigen extract used but two major bands having very similar diffusion rates were always present in all the reactions. In some of the reactions there is an overlapping of these two bands but their presence can be inferred from the splitting occurring in adjacent reactions in the same plate (Fig. 3). With nondelipified extracts, anti-LU-1 sera from both rabbits gave reactions with three additional minor (faint) bands (Fig. 1, 2, 4) while anti-Nu-1 from rabbit 3 produced two such bands (Fig. 3) and anti-Nu-1 from the fourth rabbit only one additional such band. In some cases, the relative position of each band in the different reactions is variable and produces deviations and crossings of the bands in the zone of overlap between the reactions. These changes in position are probably due to differences in concentration among homologous antigens in the various reactions, the rate of diffusion being known to be affected by the concentration of antigens (Ouchterlony, 1964).

Reactions obtained from delipified extracts produced fainter bands and some of the minor bands were lacking. It appears that delipification may have an adverse effect on the antigenicity of these protein extracts and suggests that the affected antigens may be lipoproteins.

IE analyses of the reaction between seven *N. lutea* extracts (nondelipified) and anti-Lu-1 sera consistently disclosed a similar pattern formed of two major arcs covering a substantial length of the anodal (+) reactive zone and merging over most of their length, and three additional minor faint arcs positioned close to the origin as shown in Fig. 13a. The five accessions of *N. nucifera* showed similar patterns except that the two major arcs did not extend to the origin as in *N. lutea.* In addition there was a shift of the minor arcs further toward the anode (Fig. 13 b). The elongated, and in the case of *N. lutea,* wavy shape of the two major bands probably indicates electrophoretic heterogeneity of the proteins carrying homologous antigenic sites. Equal mixtures of LU and NU antigen extracts produced a spectrum with the same two bands and minor arcs which were located in an intermediate position relative to those of LU or NU. The fact that the differences in net electric charge among these antigens appear to be compensated in mixtures would suggest that although changes in aminoacid composition and/or their sequences in the proteins of the two species might have occurred, these have not drastically altered the antigenicity of the proteins. IE spectra obtained with anti-NU-1 sera were similar to those of anti-Lu sera except that only one or two minor arcs were observed. IE spectra obtained from the reaction of anti-Lu-1 sera and delipified extracts confirmed the adverse effect observed in the ID plates. Here, the spectra showed two major arcs but only one of the three minor arcs present in reactions with nondelipified extracts could be observed (Fig. 13 c).

Absorption tests confirmed the serological similarities between the two species since no reactions occurred when either antisera were absorbed with heterologous antigens and subsequently reacted against homologous antigens. This indicates that all the corresponding antibodies obtained against one species' antigens were completely removed by absorption with antigens from the other species.

CROSS REACTIONS WITH TAXA OF THE NYMPHAEALES

In initial tests, nondelipified antigen extracts of all species of Nymphaeales assembled for this survey (Table 2) failed to react with antisera from the four rabbits bled at six different intervals. In these tests the ID plates were incubated and observed for 15 days to insure complete diffusion of all the reactants. In addition, parallel tests were carried out in which the antigen extracts were concentrated up to five times the original protein concentration (up to 150 mg protein/cc). A series of ID tests was also performed with antigens which had been partially purified by precipitation with saturated $(NH_4)_2SO_4$ as described by Hillebrand and Fairbrothers (1969), but all these tests were negative. However, when it was discovered that taxa of a number of families of Angiosperms reacted with anti-*Nelumbo* sera, a more involved analysis of various protein extraction procedures of Nymphaeales seeds was deemed necessary. Extracts from defatted seed meal reacted with anti-*Nelumbo* sera, all the reactions are characterized as reactions of partial identity (type II reaction of Ouchterlony, 1964) resulting in heavy spur formation when compared side by

side with an homologous reaction in the Ouchterlony plates (Fig. 6, 7). This type of reaction indicates that while all the antigens involved in these reactions are serologically related to those of *Nelumbo*, they do not carry identical determinant sites. The ID patterns showed the presence of only one precipitin band. The intensity and position of the bands present in

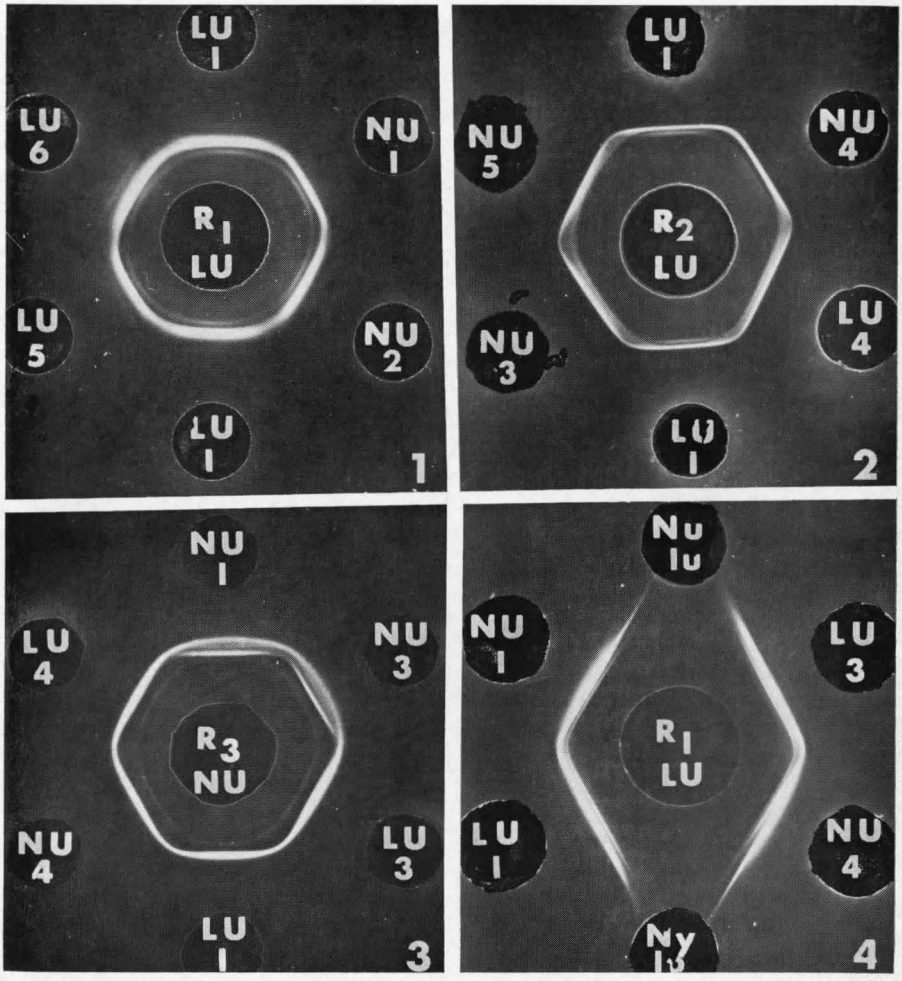

Fig. 1–4. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Nelumbo* sera (R_1, R_2) or anti-*N. nucifera* serum (R_3) and antigen extracts of *Nelumbo* and other Nymphaeales taxa.—KEY TO SYMBOLS: NU, antigen extracts of *Nelumbo nucifera*; LU, antigen extracts of *N. lutea*. Numbers following symbols refer to accession analysed. Nu lu, *Nuphar luteum* subsp. *macrophyllum*; Ny lo, *Nymphaea lotus*. (All extracts from nondelipified seed meals.)

these reactions varied according to the species involved and the presence of spurs in the zone of overlap between some of the reactions indicate that the antigens present in the taxa are not identical (Fig. 7). IE tests confirmed the heterogeneity of the antigens involved in these reactions since the position of the arcs did not match exactly those produced in reactions with *Nelumbo* antigens and there were slight variations in their electrophoretic positions with respect to each other. (Fig. 13 e–g). Delipified extracts of *Nuphar polysepalum, Victoria cruziana* and *Euryale ferox* were used to absorb anti-*Nelumbo* sera. The ID patterns were similar to those obtained with unabsorbed antisera except that the intensity of the two major arcs was greatly reduced.

CROSS REACTIONS WITH OTHER FAMILIES OF ANGIOSPERMS

Extracts from species of a number of families reacted with the anti-*Nelumbo* sera (Table 2, Fig. 8–12). Delipified and nondelipified extracts produced similar patterns in the ID and IE plates, although the former tended in some cases to produce less intense spectra. Both kind of extracts were tested for all available taxa giving negative tests with nondelipified extracts.

Among the Dicotyledoneae, positive reactions were obtained with all the tested taxa of the Magnoliaceae, Annonaceae, Degeneriaceae, Calycanthaceae, Ranunculaceae and Papaveraceae. However, only one of the three species of Lauraceae (Umbellularia) reacted with the antisera. Among the Monocotyledoneae, reactions were recorded with several taxa of the Alismales (*Sagittaria, Butomus, Ottelia*), with *Veratrum* (Liliaceae) and with all the tested members of the Agavaceae. All these reactions are also characterized as reactions of partial identity and with the exception of reactions involving *Magnolia* species, the ID patterns showed the presence of only one precipitin band. The intensity and position of the bands present in these reactions varied according to the species involved. The *Magnolia* extracts produced two strong bands of intermediate position in the agar arena between the antigen and antisera wells; most of the other species produced rather weak bands of variable position but *Agave* species were unusual in producing one heavy diffuse band positioned against the antiserum well (Fig. 12). The diffusion of the *Agave* antigen occurred very rapidly and the band was already observable within 6–8 hours after the setting of the plates, well in advance of the homologous reaction. Changes in the antigen concentration did not alter the position of the band in the Ouchterlony plates. Reactions of a different type were obtained with extracts of the other Monocotyledoneae taxa such as *Veratrum, Sagittaria, Butomus* and *Ottelia*; these were characterized by the formation of a very weak and diffuse banding pattern of low stainability.

A more restricted number of IE tests confirmed the heterogeneity of the antigens involved in these reactions since the position of the arcs did not match exactly those produced in reactions with *Nelumbo* antigens (Fig. 14). IE spectra of *Magnolia* species produced two arcs with a similar anodal mobility (Fig. 14 b). Extracts of *Degeneria vitiensis* produced one arc of higher mobility towards the anode (Fig. 14 c), while one arc of

TABLE 2. *Species giving positive reactions against anti-*Nelumbo *sera.**

SPECIES GIVING IDENTITY REACTIONS.

Nymphaeales**
- Nelumbonaceae
  - *Nelumbo lutea* (Wild.) Pers. (7)
  - *Nelumbo nucifera* Gaertn. (5)

SPECIES GIVING POSITIVE REACTIONS OF PARTIAL IDENTITY BUT ONLY WITH DELIPIFIED EXTRACTS.

Nymphaeales
- Cabombaceae
  - *Brasenia schreberi* Gmel. (2)
- Nymphaeaceae
  - *Euryale ferox* Salis. (3)
  - *Nuphar luteum* (L.) Sibth & Smith subsp. *macrophyllum* (Small) Beal (3)
  - *Nuphar luteum* (L.) Sibth & Smith subsp. *polysepalum* (Engelm.) Beal (3)
  - *Nuphar luteum* (L.) Sibth & Smith subsp. *variegatum* (Engelm.) Beal
  - *Nymphaea alba* L. (3)
  - *Nymphaea capensis* Thunb. subsp. *zanzibariensis* Casp.
  - *Nymphaea gigantea* L. (2)
  - *Nymphaea lotus* L. (2)
  - *Nymphaea nucholi* L.
  - *Nymphaea tetragona* Georgi
  - *Nymphaea* hybrid (*Lotus* subgenera)
  - *Nymphaea* hybrid (Brachyceras subgenera)
  - *Victoria amazonica* Sow. (3)
  - *Victoria cruziana* D'Orb. (2)
  - *Victoria* 'Longwood hybrid'

SPECIES GIVING REACTIONS OF PARTIAL IDENTITY WITH BOTH DELIPIFIED AND NON-DELIPIFIED EXTRACTS.

DICOTYLEDONEAE
- Magnoliaceae
  - *Magnolia acuminata* L.
  - *Magnolia campbellii* Hook. f. et Thoms.
  - *Magnolia delavayi* Franch.
  - *Magnolia grandiflora* L. (2)
  - *Magnolia kobus* D.C.
  - *Magnolia soulangeana* Soul.
  - *Magnolia sprengeri* Pamp.
  - *Liriodendron tulipifera* L. (2)
- Annonaceae
  - *Annona cherimola* Mill.
  - *Asimina triloba* Dunal (2)
- Degeneriaceae
  - *Degeneria vitiensis* I. W. Bailey & A. C. Smith
- Calycanthaceae
  - *Calycanthus occidentalis* Hook. & Arn.
- Lauraceae
  - *Umbellularia californica* Nutt.
- Ranunculaceae
  - *Aquilegia pubescens* Cov.
  - *Caltha howellii* (Huth) Greene
  - *Clematis lasiantha* Nutt.
  - *Clematis ligusticifolia* Nutt.
  - *Delphinium parryi* Gray
  - *Delphinium variegatum* T. & G.
  - *Helleborus niger* L. (2)
  - *Ranunculus californica* Benth.
  - *Thalictrum polycarpum* (Torr.) Wats.
- Papaveraceae
  - *Argemone munita* Dur. & Hilg.
  - *Eschscholzia californica* Cham.
  - *Eschscholzia lobbii* Greene
  - *Papaver californicum* Gray
  - *Stylomecon heterophylla* (Benth.) G. Tayl.

MONOCOTYLEDONAE
- Agavaceae
  - *Agave deserti* Engelm.
  - *Agave shawii* Engelm.
  - *Agave utahensis* Engelm.
  - *Nolina parryi* Wats.
  - *Yucca brevifolia* Engelm. in Wats.
  - *Yucca whipplei* Torr.

Species giving positive reactions of partial identity, but weak and diffuse pattern of banding in Ouchterlony plates.

MONOCOTYLEDONEAE
- Alismataceae
  - *Sagittaria lancifolia* L.
  - *Sagittaria latifolia* Willd.
  - *Sagittaria platyphylla* (Engelm.) Smith
- Butomaceae
  - *Butomus umbellatus* L.
- Hydrocharitaceae
  - *Ottelia alismoides* (L.) Pers.
- Liliaceae
  - *Veratrum album* L. (2)
  - *Veratrum nigrum* L.

*If more than one accession was studied, number is indicated in parentheses.

**A small sample of nondelipified extract of *Cabomba caroliniana* Gray did not react with anti-*Nelumbo* sera; unfortunately there were not enough seeds to prepare delipified extracts.

variable intensity and usually positioned around or very close to the origin, but always towards the anode, was produced in reactions with taxa of the Ranunculaceae and Papaveraceae (Fig. 14 d, e). Extracts of *Agave* and *Yucca*, however, were unusual in producing one arc in the cathodal (−) zone of the spectrum (Fig. 14 f, g).

Extracts of *Magnolia grandiflora, Caltha howellii, Argemone munita* and *Agave shawii* were used to absorb anti-*Nelumbo* sera. When the antisera were absorbed with any of the first three extracts and was then reacted against *Nelumbo* antigens, the ID patterns were similar to those obtained with unabsorbed antisera except that the intensity of the two major bands was greatly reduced. Absorption with *Agave* produced patterns with only one band remaining. These tests indicate that the antigens present in these taxa have determinant sites which are able to combine with and remove antibodies produced against *Nelumbo* antigens.

DISCUSSION

SEROLOGICAL IDENTITY OF *Nelumbo nucifera* AND *N. lutea*

The serological identity of the two *Nelumbo* species appears to correlate with their anatomical and morphological similarities (Li, 1955; Wood, 1959). *Nelumbo lutea* and *N. nucifera* are differentiated mainly by the colour of the flower and the shape of their fruits. The former species has sulfur-yellow to white flowers and nearly spherical fruits, the latter pink to white flowers and somewhat ellipsoidal fruits. The present geographical distribution shows a striking disjunction between the two species. *Nelumbo lutea* is distributed from eastern North America to Colombia, while *N. nucifera* is found in warmer and tropical parts of Asia to North Eastern Australia (Li, 1955). Although the fossil evidence indicates that in the past the two species had a more or less continuous distribution in the Northern Hemisphere, their isolation appears to have been completed since at least the early Pleistocene (Good, 1964). The serological identity of the two species clearly indicates the conservative nature of the seed proteins involved, for these have remained essentially unchanged over a period of at least one million years and possibly as long as five million years. Both species, and particularly *N. nucifera*, are reported to be morphologically quite variable and there is need for a more comprehensive survey of additional populations of these species over their entire range of distribution.

SEROLOGICAL RELATIONSHIPS OF *Nelumbo* WITH TAXA OF THE NYMPHAEALES

The difference in results obtained with delipified and nondelipified extracts is a puzzling one. Clearly, delipification appears to affect the antigenicity of native seed proteins and may remove specific lipoproteins. The adverse effect is observed in this study from the comparison of ID and IE spectra obtained in homologous reactions with both kind of extracts. The positive reactions obtained with taxa of the Nymphaeaceae only after delipification suggest that in this case, delipification "releases" some antigenic sites, possibly from lipoproteins. Another possibility may be that preextraction with solvents such as petroleum ether and acetone may inhibit pro-

teases which may be more active in denaturing antigens from the various Nymphaeales taxa, with the exception of *Nelumbo*. However, whatever

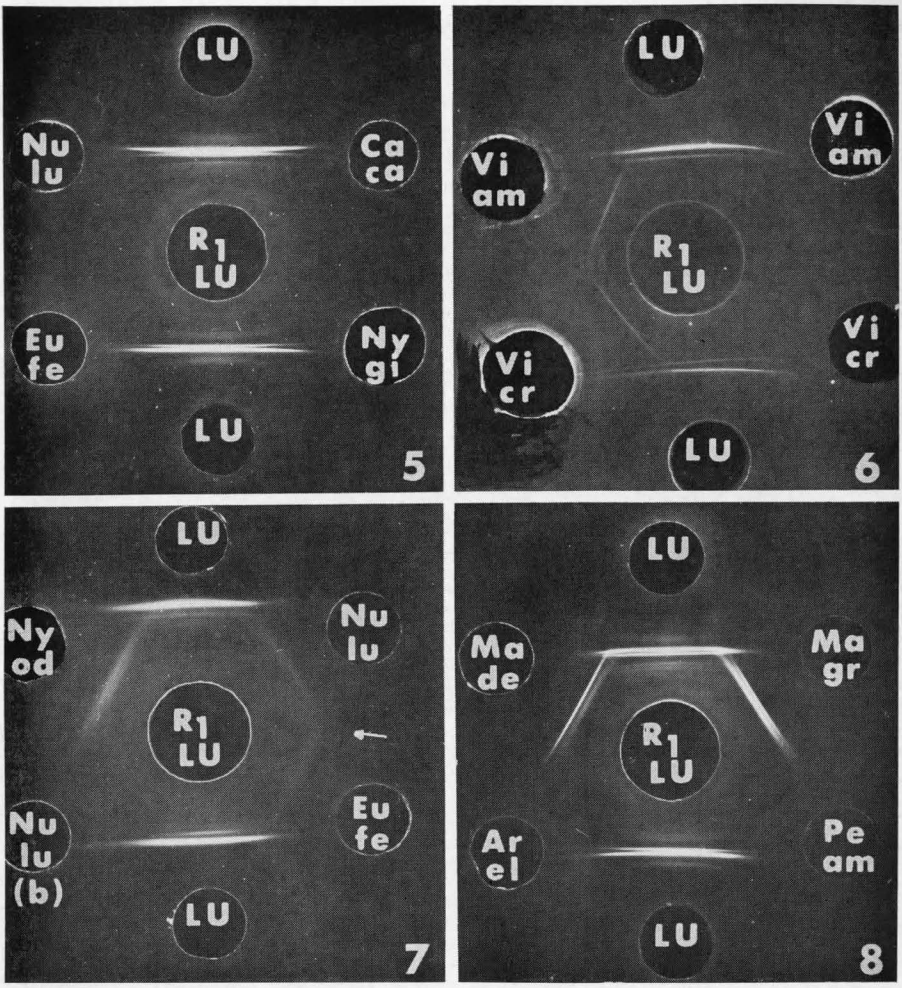

Fig. 5–8. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Nelumbo lutea* (R_1) serum and antigen extracts of Nymphaeales and other Angiosperm taxa.—KEY TO SYMBOLS: Fig. 5. Ca ca, *Cabomba caroliniana*; Ny gi, *Nymphaea gigantea*; Eu fe, *Euryale ferox*; Nu lu, *Nuphar luteum* subsp. *polysepalum*. (All from nondelipified extracts.)—Fig. 6. Vi am, *Victoria amazonica*; Vi cr, *Victoria cruziana*. (Nondelipified extracts at right, delipified extracts at left.)—Fig. 7. Nu lu, *Nuphar luteum* subs *variegatum*; Eu fe, *Euryale ferox*; Nu lu (b), *Nuphar luteum* subsp. *variegatum* (Nondelipified extract.); Ny od, *Nymphaea odorata*.—Fig. 8. Ma gr, *Magnolia grandiflora*; Pe am, *Persea americana*; Ar el, *Aristolochia elegans*; Ma de, *Magnolia delavayi*. (All from nondelipified extracts.)

the explanation may be, this phenomenon suggests that although *Nelumbo* and the other Nymphaeales taxa share proteins bearing more or less related determinant sites, these proteins may be quite different in their physico-chemical configurations.

The serological data emphasize the isolated nature of *Nelumbo vis-à-vis* other Nymphaeales taxa as based on morphological and anatomical evidence. This is particularly so when we observe that the serological reactions produced against the Nymphaeales taxa were of the same nature as those produced by members of the more distant families of the Annonales and Ranunculales. It is tempting, with the serological information at hand, to support the propositions made by Li (1955) and Takhtajan (1959, 1969) and to segregate *Nelumbo* to a separate order not directly related to Nymphaeales. However, additional tests using antisera prepared against other taxa of the Nymphaeales will have to be carried out in order to confirm the serological distinctness of *Nelumbo*. These tests for which antisera are now being produced, will also clarify the serological relationships of other Nymphaeales taxa and determine if the serological distinctness of *Nelumbo* is unique and not repeated by other members of that order.

CROSS REACTIONS WITH OTHER FAMILIES OF ANGIOSPERMS

The results of the preliminary survey indicate that the range of serological affinities of *Nelumbo* supports the proposed relationship as determined on the basis of morphological and anatomical evidence. The data presented here emphasize the primitive status of *Nelumbo* within the Angiosperms, but the wide range of reactivity appears to limit the usefulness of the serological data to ascertain more precisely the taxonomic position of this taxon. Although the similar type of reaction produced by all the taxa does not allow for a ranking of relative affinities, reactions involving primitive monocots appear to be of a different nature and possibly indicate less serological affinities.

Within the Dicotyledoneae, the survey indicates that *Nelumbo* shows serological affinities with the most primitive members of the Annoniflorae (sensu Thorne, 1968) or with the Magnoliales, Ranunculales and Papaverales of Cronquist (1968) and Takhtajan (1969). Here, the relationships as suggested by the serological data parallel well those proposed by Cronquist (1968) and are broader than those suggested by Takhtajan (1969) who derives *Nelumbo* from the Illiciales-Ranunculales stock. The negative tests obtained so far with the two species of Schisandraceae (Illiciales or Illicineae) need not cause concern at this point. Further tests of additional critical taxa of this order, such as *Illicium*, need to be done to clarify the serological position of this group. A small amount of crude extract prepared from a few seeds of *Drimys winteri* Forst. (Winteraceae) was tested and gave no reaction with anti-*Nelumbo* sera. Although this result needs confirmation, it might be significant because it has been suggested that the Illiciales have been derived directly from ancestors of the Winteraceae, which is possibly the most primitive family of the Magnoliales (Takhtajan, 1969).

The negative tests obtained with taxa of the Berberidaceae are more sur-

prising and a more involved survey of this family is in order. Berberidaceae have been included in the Papaverales by Cronquist (1968) and there are

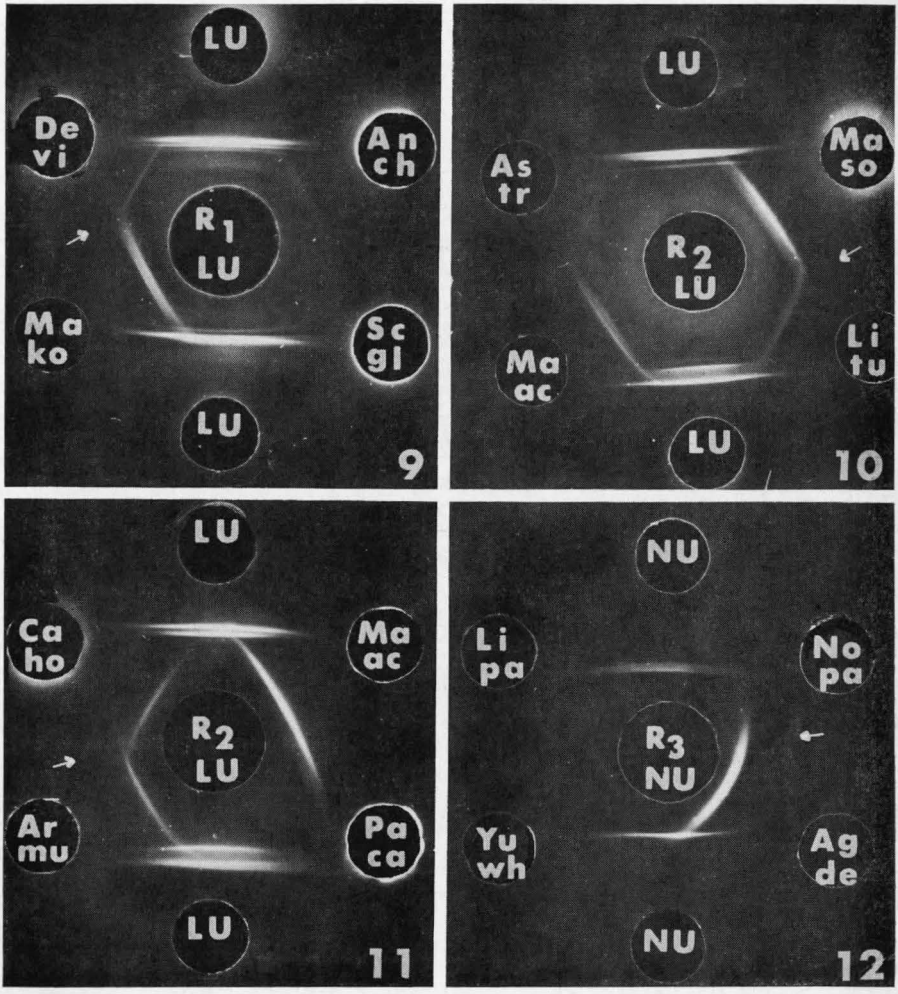

Fig. 9–12. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Nelumbo lutea* sera (R_1, R_2) or anti-*N. nucifera* serum (R_3) and nondelipified antigen extracts of Angiosperm taxa.—KEY TO SYMBOLS: Fig. 9. An ch, *Annona cherimola*; Sc gl, *Schisandra glabra*; Ma ko, *Magnolia kobus*; De vi, *Degeneria vitiensis*.—Fig. 10. Ma so, *Magnolia soulangeana*; Li tu, *Liriodendron tulipifera*; Ma ac, *Magnolia acuminata*; As tr, *Asimina triloba*.—Fig. 11. Ma ac, *Magnolia acuminata*; Pa ca, *Paeonia californica*; Ar mu, *Argemone munita*; Ca ho, *Caltha howellii*.—Fig. 12. No pa, *Nolina parryi*; Ag de, *Agave deserti*; Yu wh, *Yucca whipplei*; Li pa, *Lilium parryi*. (Arrows indicate spur formation.)

also close morphological, anatomical and biochemical relationships between this family and Ranunculaceae (Cronquist, 1968; Takhtajan, 1969; Thorne, 1968).

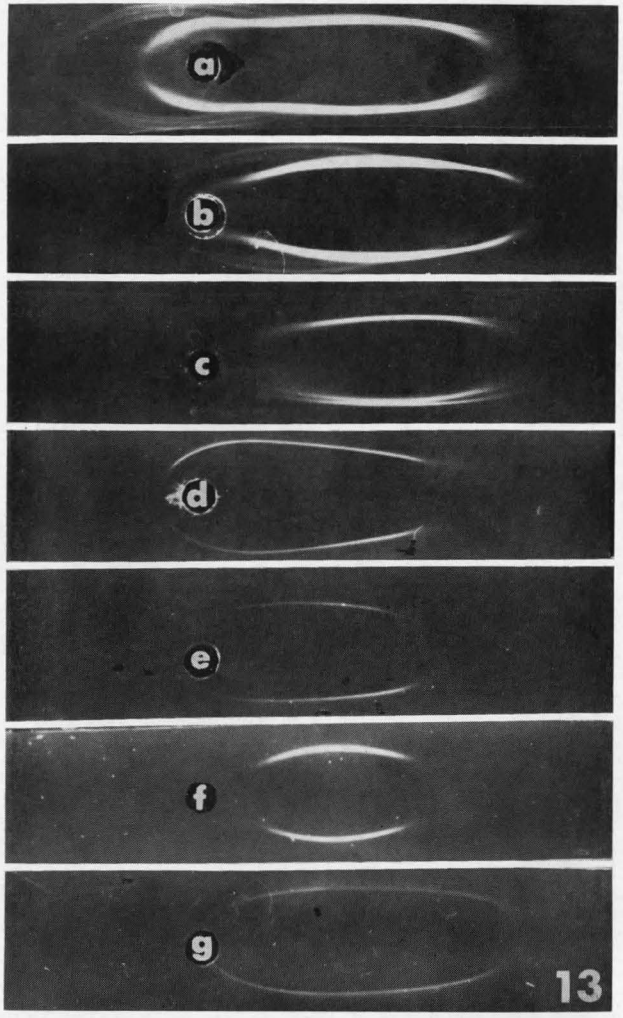

Fig. 13. Immunoelectrophoresis spectra resulting from reactions between anti-*Nelumbo lutea* (R_1) serum and antigen extracts of *Nelumbo* and other Nymphaeales taxa: a. *Nelumbo lutea*-1; b. *N. nucifera*-1; c. *N. nucifera*-1; d. *Victoria amazonica*-1; e. *Nuphar luteum* subsp. *polysepalum*; f. *Nymphaea gigantea*-1; g. *Brasenia schreberi*. (a–b, nondelipified extracts; c–g, delipified extracts.)

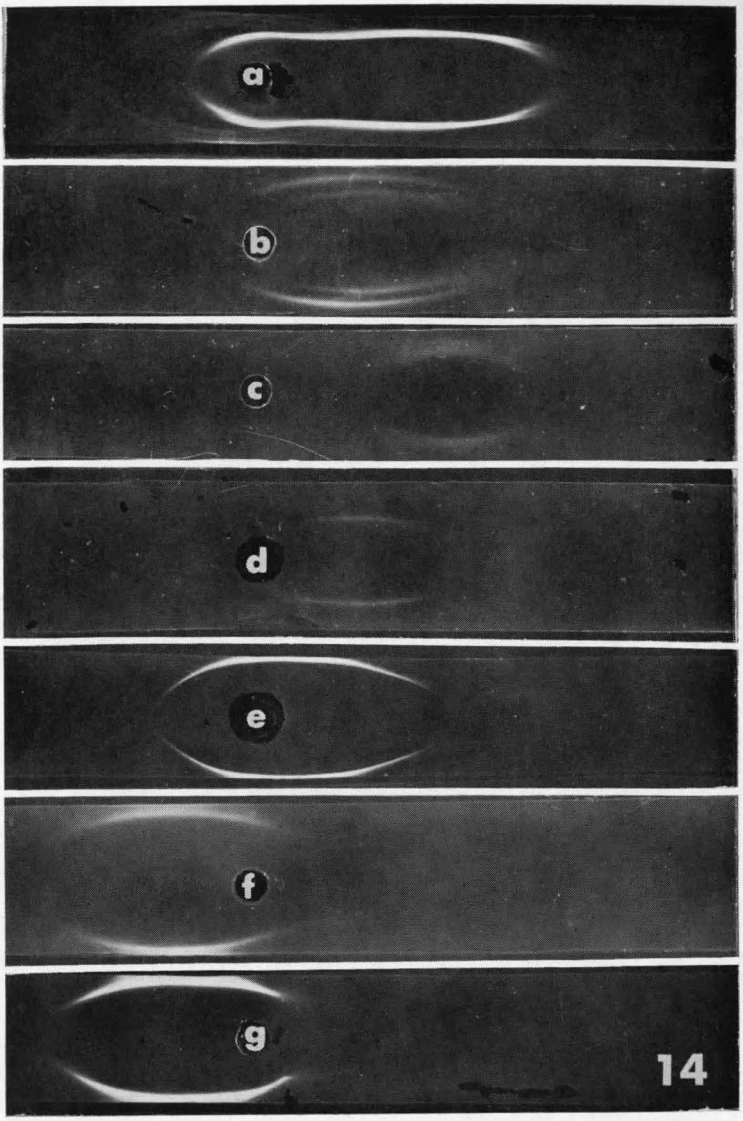

Fig. 14. Immunoelectrophoresis spectra resulting from reactions between anti-*Nelumbo lutea* (R_1) serum and nondelipified antigen extracts of selected taxa of Angiosperms: a. *Nelumbo lutea*-1; b. *Magnolia kobus*; c. *Degeneria vitiensis*; d. *Caltha howellii*; e. *Papaver californicum*; f. *Yucca whipplei*; g. *Agave shawii*.

Our efforts are now directed at establishing the range of reactivity of anti-*Nelumbo* sera against various more advanced angiospermous taxa. The usefulness of the serological approach will be inversely correlated to the range of reactivity obtained from such a survey. The data at hand already seem to indicate that, among dicots, "peripheral" taxa such as the derived and more specialized Aristolochiineae or Aristolochiales, Paeoniaceae (possibly of common origin with Dilleniaceae) and Crossosomataceae are excluded, as are also members of the superorder Hamamelidiflorae (Thorne, 1968). However, critical taxa need to be examined. For instance, we have not tested as yet the more primitive and vesselless members of the Hamamelidiflorae, *Trochodendron* and *Tetracentron*.

Both positive and negative tests were obtained with taxa of the Alismataceae and Liliaceae (Tables 2 and 3). All the reactions were weak and

TABLE 3. *Species giving no reaction against anti-*Nelumbo *sera.**

DICOTYLEDONEAE
- Crossosomataceae
  - *Crossosoma californicum* Nutt.
- Paeoniaceae
  - *Paeonia californica* Nutt.
- Aristolochiaceae
  - *Aristolochia elegans* Mast.
  - *Aristolochia grandiflora* Sw.
  - *Asarum caudatum* Lindl.
- Schisandraceae
  - *Schisandra glabra* (Brickell) Rehder
  - *Kadsura japonica* (L.) Dunal.
- Lauraceae
  - *Persea americana* Mill.
  - *Persea indica* Spreng.
- Berberidaceae
  - *Podophyllum emodi* Wall.
  - *Podophyllum peltatum* L.
  - *Berberis amplectens* (Eastw.) Wheeler
  - *Berberis (Mahonia) bealei* Carr.
  - *Berberis piperiana* (Abrams) McMinn.
- Hamamelidaceae
  - *Corylopsis glabescens* Franch & Zucc.
  - *Corylopsis spicata* Sieb. & Zucc.
  - *Hamamelis virginiana* L.
  - *Liquidambar styraciflua* L.
- Eucommiaceae
  - *Eucommia ulmoides* Oliv.

MONOCOTYLEDONEAE
- Alismataceae
  - *Alisma subcordatum* Raf.
  - *Alisma triviale* Pursh. (2)
  - *Baldellia ranunculoides* (L.) Parl.
  - *Echinodorus macrophyllus* (Kunth) Micheli
- Amaryllidaceae
  - *Allium campanulatum* Wats.
  - *Allium unifolium* Kell.
  - *Brodiaea elegans* Hoover
- Iridaceae
  - *Iris douglasiana* Herb.
- Liliaceae
  - *Lilium parryi* Wats.
  - *Lilium humboldtii* Roetz & Leichtl.
  - *Zigadenus fremontii* Torr.
- Cyperaceae
  - *Carex pansa* Bailey

*If more than one accession was studied, number is indicated in parentheses.

difficult to visualize and indicate that the seed possess very low amounts of the antigens responsible for these reactions. It may also suggest that the lack of reaction with some of the taxa, such as *Alisma*, may be due to low antigen concentration, undetected under the experimental procedure followed in this study, rather than to total absence. In view of the weak and diffuse reaction patterns produced by these primitive monocots, the strong and anomalous partial identity reaction shown by the taxa of the Agavaceae

was unexpected. Agavaceae are considered rather specialized taxa among the Liliales and possibly derived from the Liliaceae (Cronquist, 1968).

It is now well established that, to be comparable, the serological data have to be obtained from the same plant organ or tissue (Kloz *et al.*, 1960; Alston and Turner, 1963). Although the present study uses seeds as the source of antigenic material, the constitution and composition of these among the wide range of orders and families tested is not the same. One of the main differences lies in the presence or absence of endosperm and perisperm. Clearly, this might have some bearing in the serological reactions and, as the data accumulate, it may give us some indication of the influence of these seed differences upon the interpretation of the serological data.

SUMMARY

Seed antigens of a number of taxa of the Angiosperms were compared serologically using antisera against *Nelumbo nucifera* and *N. lutea.* Double immunodiffusion tests did not disclose differences among twelve accessions of *Nelumbo lutea* and *N. nucifera* but immunoelectrophoresis analyses showed that there were small changes in the net electric charge among antigens of the two species. Protein extracts of other Nymphaeales taxa which had not been subjected to delipification did not react with anti-*Nelumbo* sera but delipified antigen extracts produced one precipitin band showing partial identity with *Nelumbo.* Similar partial identity reactions were also obtained with taxa of the Dicotyledoneae families Magnoliaceae, Degeneriaceae, Annonaceae, Ranunculaceae, Calycanthaceae, Papaveraceae and one species of Lauraceae (*Umbellularia*). No reactions were detected with taxa of the following families: Berberidaceae, Hamamelidaceae, Aristolochiaceae, Schisandraceae, Paeoniaceae, Crossosomataceae and Eucommiaceae. Among the Monocotyledoneae, slight and diffuse reaction patterns were observed with some of the taxa of the Alismataceae, Hydrocharitaceae, Butomataceae and with *Veratrum* (Liliaceae), while species of the Agavaceae gave an unusually strong reaction of partial identity. Immunoelectrophoresis tests demonstrated the heterogeneity of the antigens involved in these reactions.

ACKNOWLEDGMENTS

I am most grateful to the following institutions and persons for supplying seed samples for this survey:

Beal-Garfield Botanic Garden, East Lansing, Michigan; Canada Department of Agriculture, Central Experimental Farm, Ottawa; Longwood Botanical Gardens, Kennett Square, Pennsylvania; Missouri Botanical Gardens, St. Louis; Botanic Garden, Munchen, Nymphenburg, Germany; New York Botanic Garden, The Bronx; Strybing Arboretum, San Francisco; University of Washington Arboretum, Seattle; O. E. White Research Arboretum, University of Virginia, Boyce; E. O. Beal, Western Kentucky University, Bowling Green; C. R. Bell, North Carolina Botanical Gardens, Chapel Hill; R. B. Clarkson, West Virginia University, Morgantown; C. D. K. Cook, Botanic Garden, University of Zurich, Switzerland; R. K. Godfrey, Florida

State University, Tallahassee; A. Hotchkiss, University of Louisville, Kentucky; E. V. Komarek, Tall Timbers Research Station, Tallahassee, Fla.; F. Landa, Santiago, Chile; P. A. Nutt, Longwood Botanical Gardens, Kennett Square, Penn.; J. Parham, Department of Agriculture, Fidji; D. E. Stone, Duke University, Durham, N.C.; J. W. Thieret, University of Southwest Louisiana, Lafayette; R. D. Thomas, Northeast Louisiana State College, Monroe; R. E. Wager, Panama City, Fla.

I am very grateful to Drs. J. E. Canright, University of Arizona; E. O. Beal, Western Kentucky University; R. B. Clarkson, West Virginia University; D. E. Fairbrothers, Rutgers University; and M. F. Moseley, Jr., University of California, Santa Barbara, for much advice and support during the initial phase of this research. I am particularly indebted to Professor Armen Takhtajan for discussing the project with me during his visit to the Rancho Santa Ana Botanic Garden in October, 1968, and to my esteemed colleague Robert F. Thorne for many hours of discussion concerning the taxonomic implications of the serological data.

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