1971

Comparative Serology of the Order Nymphaeales. II. Relationships of Nymphaeaceae and Nelumbonaceae

Jean-Pierre Simon

Rancho Santa Ana Botanic Garden

COMPARATIVE SEROLOGY OF THE ORDER NYMPHAEALES II. RELATIONSHIPS OF NYMPHAEACEAE AND NELUMBONACEAE.

JEAN-PIERRE SIMON

Rancho Santa Ana Botanic Garden
Claremont, California 91711

INTRODUCTION

In a continuing effort to elucidate the serological relationships of taxa of the aquatic order Nymphaeales, I am reporting here additional data obtained from cross-reactions with antisera produced against species of *Nuphar*, *Nymphaea*, *Victoria* and *Euryale*. An earlier study established that *Nelumbo* was serologically isolated from the remaining species of the Order (Simon, 1970). In addition, the data showed that *Nelumbo* had serological affinities with members of the Magnoliales, Ranunculales and Papaverales which were of the same magnitude to those found between *Nelumbo* and the remaining taxa of the Nymphaeales.

MATERIALS AND METHODS

Extracts of representative species of Nymphaeales, Magnoliales, Ranunculales and other orders of Angiosperms, as listed in Tables 2 and 3 of Simon (1970), were investigated. Seeds of additional species of these and other orders of Angiosperms have since been available for this study (Table 3 and text). The preparation of antigen extracts, antisera and the serological techniques were as described by Simon (1970) with some modifications as described below.

In order to save reactants, preliminary comparative tests were carried out using the microimmunodiffusion technique (Crowle, 1961, p. 222). Those extracts giving positive reactions were reinvestigated using the standard ID method described earlier.

Three characteristics of antigen extracts of Nymphaeales taxa (with the exception of *Nelumbo*) need to be stressed:

1.—The protein content of the extracts was very low. Under the described conditions of extraction and before standardization (Simon, 1970), the protein content of delipified seed extracts of the taxa ranged from 0.20–0.35 mg/ml, as compared to 1.6–2.4 mg/ml obtained with *Nelumbo* under comparable extraction techniques.

2.—The antigen extracts were rather unstable when stored at –20 C. A heavy precipitate occurred upon defrosting of the extracts stored for as little

[325]
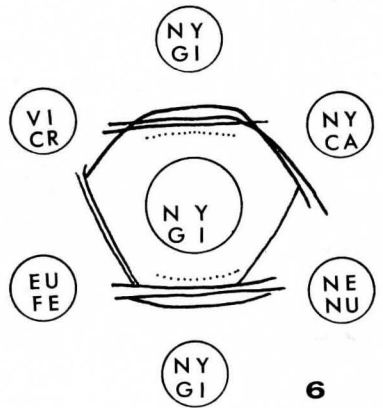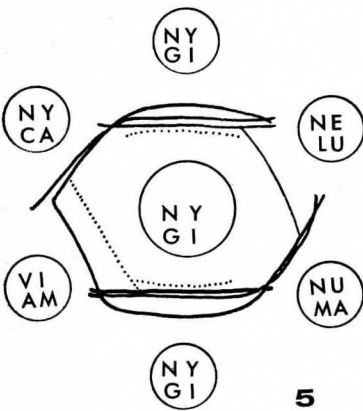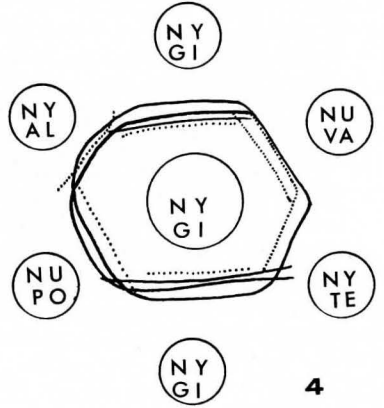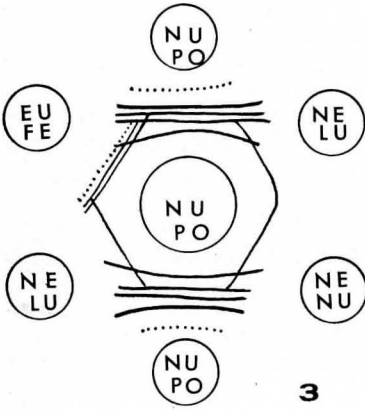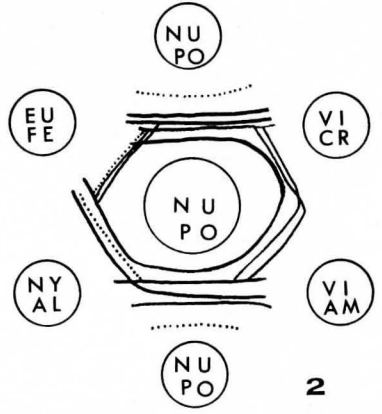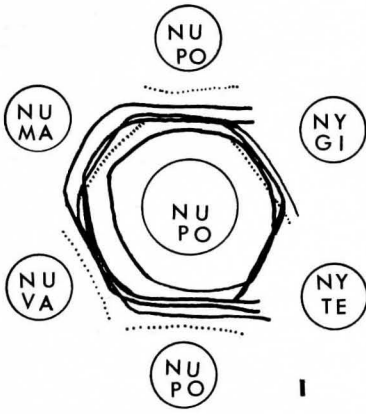
as two weeks. The addition of sucrose (Heitefuss et al., 1959), 10% w/v, to the dialyzed extracts increased the solubility and stability of the extracts up to a period of storage of two months without affecting ID or IE spectra. Additional length of storage was still detrimental under these conditions. Buffers of higher molarity and/or higher pH did not improve the stability of the extracts but affected the sharpness of the precipitin bands in ID tests. Extracts containing 10% sucrose were produced for this study and were used within two weeks following preparation.

3.—Antibodies were not readily elicited with the antigen extracts; while strong antisera were produced with *Nelumbo* extracts after only three series of injections (ca. 60 mg protein, Simon, 1970), a longer series of injections ranging from 7–10 (ca. 100–130 mg protein) was needed before a stabilization of the number of precipitin bands in ID tests was obtained with antisera produced against *Nuphar luteum* spp. *polysepalum*, *Nymphaea gigantea*, *Euryale ferox* and *Victoria amazonica*. Due to the extensive tests to be performed, pooled antisera from pairs of rabbits were used in this study.

RESULTS

CROSS REACTIONS BETWEEN NYMPHAEALES TAXA

The serological data involving taxa of *Nuphar*, *Nymphaea*, *Victoria*, *Euryale* and *Nelumbo* are presented in Tables 1 and 2 and Figs. 1–16.

Antisera produced against *Nuphar luteum* spp. *polysepalum* reacted similarly to extracts of the three subspecies of that taxon. Four precipitin bands were common to the subspecies and a fifth very faint band was also observed in some replicates of two of the three taxa (Table 1, Figs. 1–3). Species of *Nymphaea* shared two bands in common with *Nuphar* and one or two additional partial identity bands (type III of Ouchterlony, 1964) were also seen in the ID plates (Figs. 1, 2). Less serological affinity was shown by species of *Victoria* since these two species shared only one identity band with *Nuphar*. Two partial identity bands, and in some replicates one additional very faint band, were also observed in the ID spectra of *Victoria amazonica* and *V. cruziana*. Extracts of *Euryale* and *Nelumbo* reacted with anti-*Nuphar* sera giving only partial identity bands. The ID spectrum of *Euryale ferox* was characterized by two strong and one faint band while both *Nelumbo nucifera* and *N. lutea* produced only one such band (Table 1, Figs. 2, 3).

IE spectra confirmed the serological similarities of the three subspecies of *Nuphar luteum* (Fig. 13). At least 8 arcs (some of them rather faint) located in the anodal region of the IE spectrum were observed in these reactions. *Nymphaea gigantea* shared three of these arcs as seen in Fig. 13 c,

Fig. 1–6. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Nuphar luteum* ssp. *polysepalum* (NU PO Fig. 1–3) or anti-*Nymphaea gigantea* (NY GI, Fig. 4–6) sera (central wells) and antigen extracts of *Nymphaeales* taxa. Key to symbols: NU MA, *Nuphar luteum* ssp. *macrophyllum*; NY TE *Nymphaea tetragona*; NU VA, *Nuphar luteum* ssp. *variegatum*; EU FE, *Euryale ferox*; VI AM, *Victoria amazonica*; CI CR, *Victoria cruziana*; NY AL, *Nymphaea alba*; NE LU, *Nelumbo lutea*; NE NU, *Nelumbo nucifera*; NY CA, *Nymphaea capensis*.
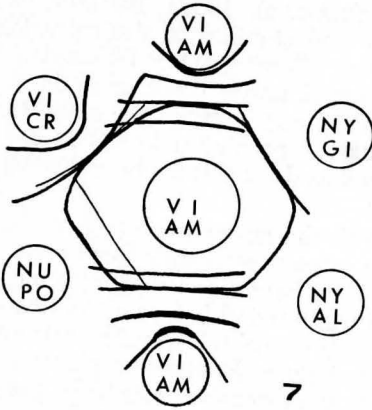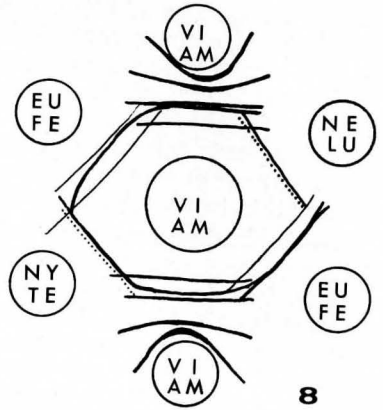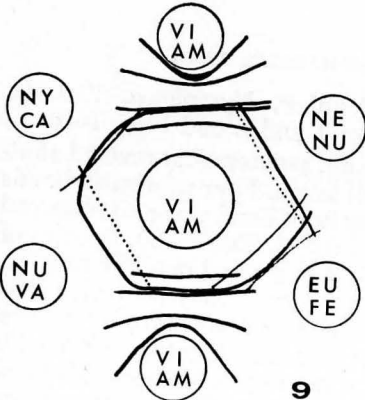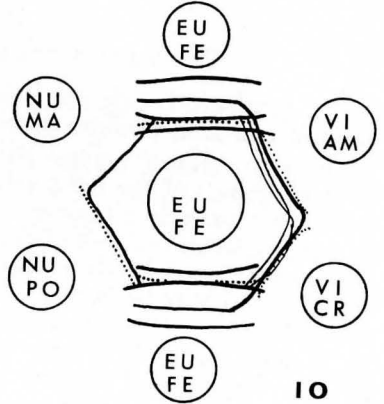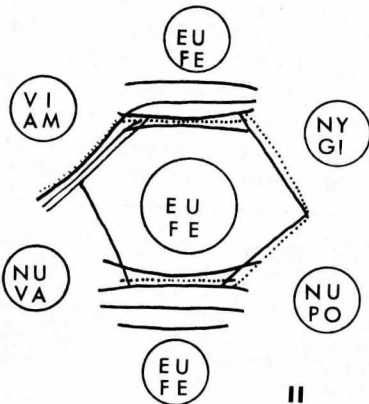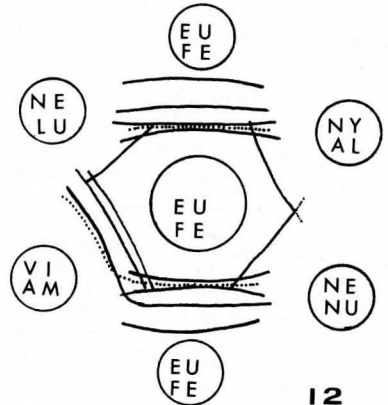
while the IE spectrum of *Nymphaea tetragona* showed only one arc positioned further towards the anode (Fig. 13 d). The positions of the arcs in both *Victoria amazonica* and *Euryale ferox* were rather similar and only one arc was observed in reactions involving *Nelumbo lutea* antigens (Fig. 13).

Antisera prepared against *Nymphaea gigantea* produced three distinct precipitin bands in the ID plates when reacted against *N. gigantea* antigens. One additional very faint band was observed in some replicates but not in others (Table 1, Figs. 4–6). However, other species of *Nymphaea* reacted giving two (*N. alba, N. capensis*) or only one identity band (*N. tetragona*) with this antiserum (Figs. 4, 5). Extracts of subspecies of *Nuphar luteum* produced two identity bands and the ID tests indicated that these were the product of the same antigens found in *Nymphaea* extracts. In the case of *Nuphar luteum* ssp. *variegatum* the second band was faint and was not observed in all the replicates. Additional faint partial identity bands were also observed in some cases (Figs. 4, 5). Both, *Victoria amazonica* and *V. cruziana* produced one identity and one partial identity rather faint band in the ID tests with anti-*Nymphaea* serum. *Euryale ferox* and both *Nelumbo* species produced only two and one partial identity band respectively (Figs. 5, 6).

IE analyses show serological differences among *Nymphaea* species as indicated by the number and variable position of the arcs produced in the IE spectra (Fig. 14). Four sharp and one diffuse arcs were produced in the test reaction. However only two of the former and a more intense anodal arc were observed in the IE spectrum of *Nymphaea alba* (Fig. 14 b). The Immunoelectropherogram of *Nymphaea tetragona* showed only a major leading anodal precipitin arc and a more diffuse one at the same position (Fig. 14 c). The IE spectra of both *Nuphar luteum* ssp. *polysepalum* and *Victoria amazonica* were markedly different from those produced by the *Nymphaea* taxa and from one another (Fig. 14 d–e). Finally, both *Euryale ferox* and *Nelumbo lutea* spectra were characterized by the presence of only one faint arc positioned towards the anode.

Antisera produced to *Victoria amazonica* gave five precipitin bands in the test reaction (Table 2, Figs. 7–9). *Victoria cruziana*, however, shared only three such bands with the former species but one additional type III band was also present in the ID plates. Extracts of species of *Nuphar*, *Nymphaea* and *Euryale* shared only one band in common with *Victoria* species (Figs. 7–9, Table 2). With the exception of *Nymphaea alba*, one additional faint partial identity band was observed in reactions involving *Nuphar* and *Nymphaea* antigen extracts and anti-*Victoria* serum. Two such bands were present in reactions with *Euryale ferox* and *Nelumbo* antigen extracts and in the latter case one of these type III bands was very faint (Figs. 8, 9).

Fig. 7–12. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Victoria amazonica* (VI AM, Fig. 7–9) or anti-*Euryale ferox* (EU FE, Fig. 10–12) sera (centra wells) and antigen extracts of Nymphaeales taxa. Symbols as for Fig. 1–6.
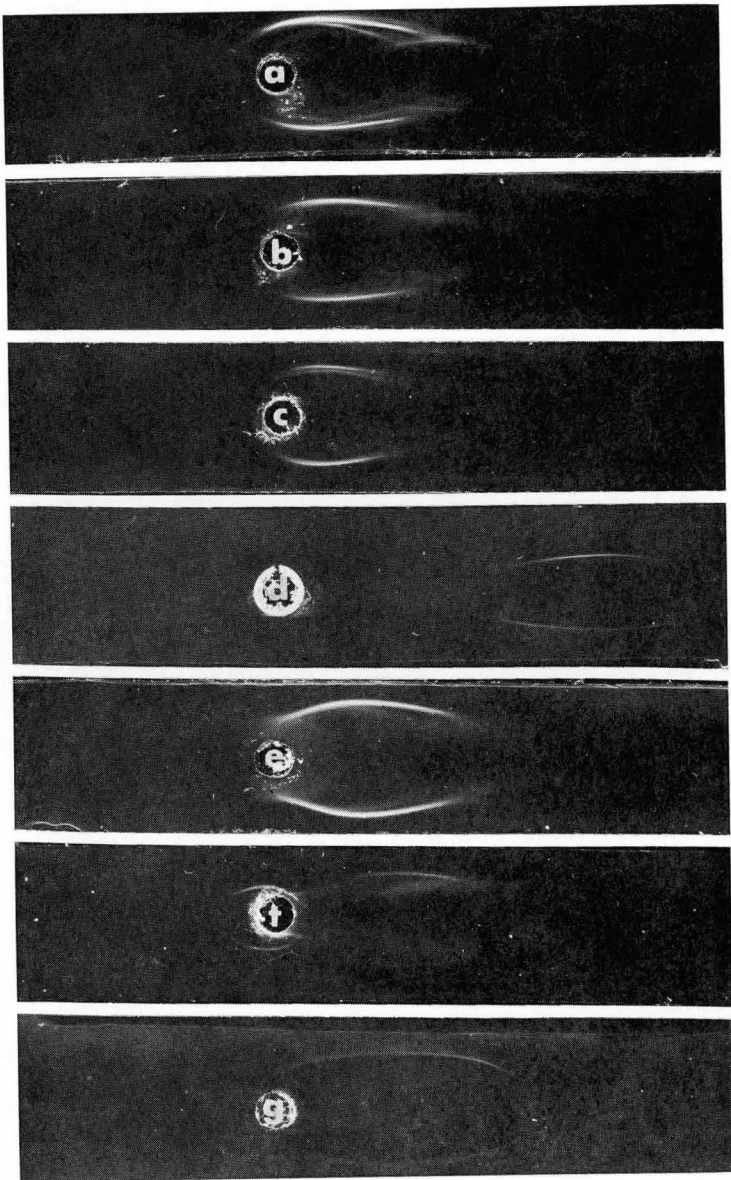
Fig. 13. Immunoelectrophoresis spectra resulting from reactions between anti-*Nuphar luteum* ssp. *polysepalum* serum and antigen extracts of *Nymphaeales*: a. *Nuphar luteum* ssp. *polysepalum*; b. *Nuphar luteum* ssp. *variegatum*; c. *Nymphaea gigantea*; d. *Nymphaea tetragona*; e. *Victoria amazonica*; f. *Euryale ferox*; g. *Nelumbo lutea-3*.

IE tests using anti-*Victoria amazonica* sera are shown in Fig. 15. Spectra of reactions involving *V. amazonica* and *V. cruziana* are markedly different in relation to the position of the arcs in the IE spectra (Fig. 15 a, b). In addition, the spectrum of *V. cruziana* appears to be lacking two of the anodal arcs positioned close to the origin in the *V. amazonica* test. The IE spectra of both *Nuphar luteum polysepalum* and *Nymphaea gigantea* were very similar in producing two anodal arcs. Two such arcs are also seen in the spectrum of *Euryale ferox* but in this case one of them is positioned further towards the anode (Fig. 15 e). Only one distinct arc is discernible in the IE spectrum of *Nelumbo lutea* but a weak and diffuse arc was also observed in some replicates to be located further towards the anode (Fig. 15 f).

TABLE 1. *Number and type of precipitin bands produced in reactions between anti-*Nuphar luteum *subsp.* polysepalum *or anti-*Nymphaea gigantea *sera and extracts of species of Nymphaeales. (Tests performed in Ouchterlony plates.)*

	ANTISERA			
ANTIGEN	*Nuphar luteum* ssp. *polysepalum*		*Nymphaea gigantea*	
	Type 1*	Type III	Type I	Type III
Nuphar luteum ssp. *macrophyllum*	4	1	2	—
Nuphar luteum ssp. *polysepalum*	4 (1)**	—	2	(1)**
Nuphar luteum ssp. *variegatum*	4 (1)	—	1 (1)	(1)
Nymphaea gigantea	2	1 (1)	3 (1)	—
Nymphaea alba	2	1	2	(1)
Nymphaea tetragona	1	1	1	1
Nymphaea capensis ssp. *zanzibariensis*	1	1 (1)	2	—
Victoria amazonica	1	2	1	1
Victoria cruziana	1	2 (1)	1	(1)
Euryale ferox	—	2 (1)	—	2
Nelumbo lutea	—	1	—	1
Nelumbo nucifera	—	1	—	1

*According to Ouchterlony (1964). Type I = identity band, Type III = partial identity, with spur formation.
**Number in parentheses refers to weak precipitin bands which were not observed in all replicates.

Reactions involving *Euryale ferox* antisera gave clear cut differences between that taxon and the remaining species of Nymphaeales (Table 2, Figs. 10–12). While four to five precipitin bands were observed in the test reaction in Ouchterlony plates only one distinct identity band was shared with species of *Victoria*. One additional such band, but very faint, was also observed in reactions involving *Victoria amazonica*. The rest of the *Victoria* spectra was made of two partial identity bands (Figs. 10–12). Species of *Nuphar, Nymphaea* and *Nelumbo* showed slight serological affinity with *Euryale* since only two to one type III bands were produced in the ID plates (Figs. 10–12).
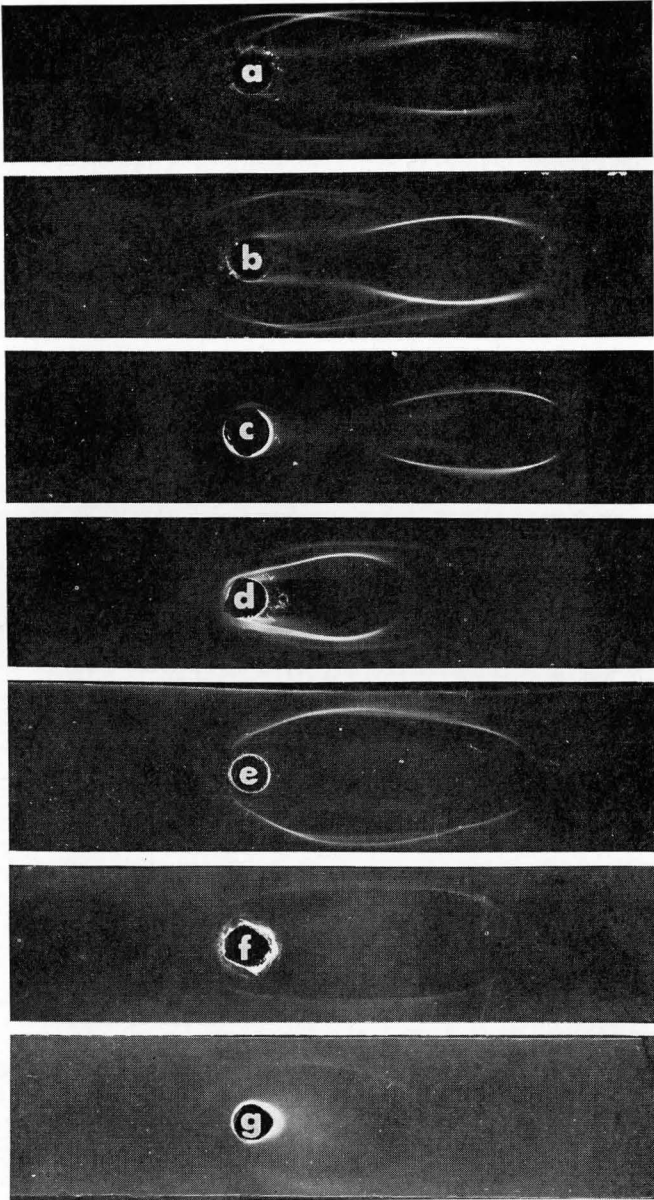
Fig. 14. Immunoelectrophoresis spectra resulting from reactions between anti-*Nymphaea gigantea* serum and antigen extracts of Nymphaeales: a. *Nymphaea gigantea*; b. *Nymphaea odorata*; c. *Nymphaea tetragona*; d. *Nuphar luteum* ssp. *polysepalum*; e. *Victoria amazonica*; f. *Euryale ferox*; g. *Nelumbo lutea*-1.

IE tests corroborate the results obtained with ID plates (Fig. 16). While the test reaction produced three distinct and two faint arcs in the anodal zone of the IE spectrum, tests with *Victoria amazonica* antigens showed three anodal arcs but the position and shape of two of them were different from those of the test reaction (Fig. 16 b). IE spectra involving *Nuphar luteum polysepalum, Nymphaea gigantea* and the two *Nelumbo* species showed only one anodal arc positioned close to the origin (Fig. 16 c–f).

In summary, stronger serological affinities are shown by species of *Nuphar* and *Nymphaea*. While the subspecies of *Nuphar luteum* are serologically very similar, species of *Nymphaea* show a wide range of differences. *Victoria amazonica* and *V. cruziana* are differentiated from one another and are more distantly related to the cluster formed by *Nuphar* and *Nymphaea*.

TABLE 2. *Number and type of precipitin bands produced in reactions between anti-*Euryale ferox *or anti-*Victoria amazonica *sera and extracts of species of Nymphaeales.* (*Tests performed in Ouchterlony plates.*)

	ANTISERA			
ANTIGEN	*Euryale ferox*		*Victoria amazonica*	
	Type I*	Type III	Type I	Type III
Euryale ferox	4 (1)**	—	1	2
Victoria amazonica	1 (1)	2	5	—
Victoria cruziana	1	2 (1)	3	1
Nuphar luteum ssp. *macrophyllum*	—	1	1	1
Nuphar luteum ssp. *polysepalum*	—	1 (1)	1	1
Nuphar luteum ssp. *variegatum*	—	1	1	(1)
Nymphaea gigantea	—	1 (1)	1	1
Nymphaea alba	—	1	1	—
Nymphaea tetragona	—	1	1	(1)
Nymphaea capensis ssp. *zanzibariensis*	—	1 (1)	1	1
Nelumbo lutea	—	1	—	1 (1)
Nelumbo nucifera	—	1	—	1 (1)

*According to Ouchterlony (1964). Type I = identity band, Type III = partial identity, with spur formation.
**Number in parentheses refers to precipitin bands not observed in all replicates.

Serologically, the closest taxon to *Euryale ferox* is *Victoria* but there are large differences between them. Only slight serological relationships are found between *Nuphar* or *Nymphaea* and *Euryale,* and *Victoria* appears to bridge the "gap" between these taxa. Finally, *Nelumbo* is serologically very isolated from any of the other genera of the Nymphaeales investigated to date.

CROSS REACTIONS WITH OTHER FAMILIES OF ANGIOSPERMS

In addition to the species tested against *Nelumbo* antisera by Simon (1970), several new taxa belonging to a number of families of Angiosperms were reacted against these antisera and with the four produced for this
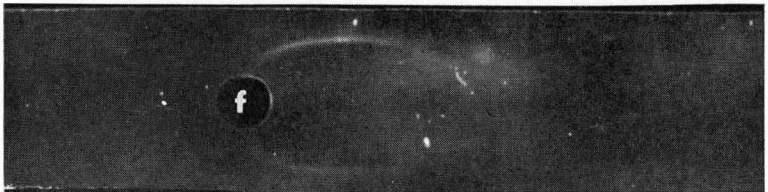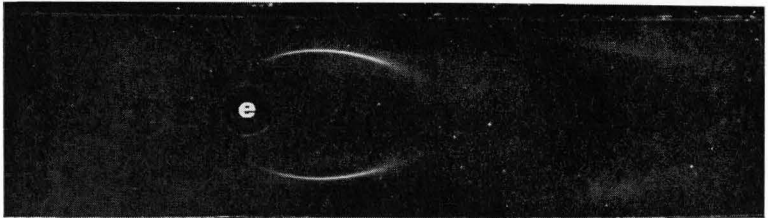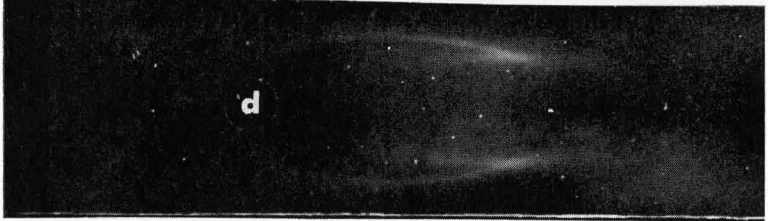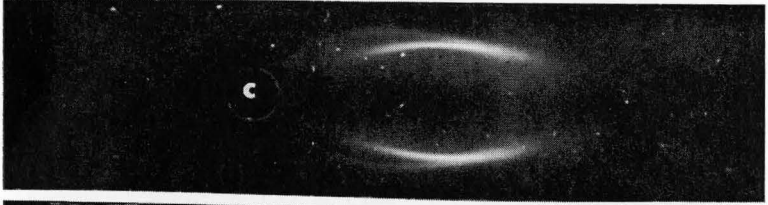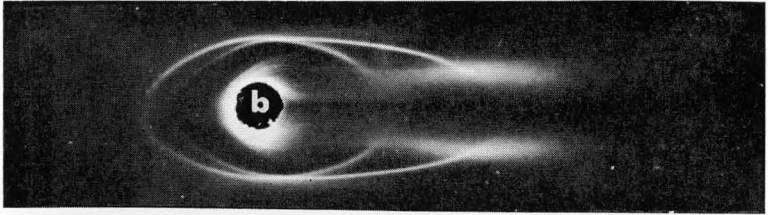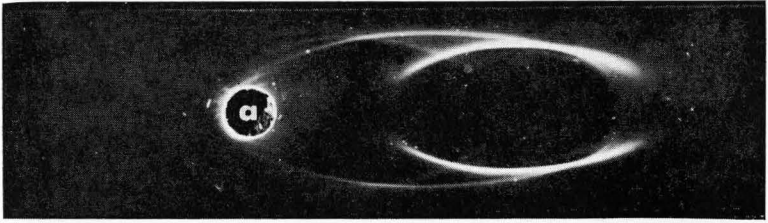
study. Table 3 lists those taxa which reacted with at least one of the antisera produced. All these reactions were characterized as reactions of partial identity in the ID plates (Type III of Ouchterlony, 1968; see Figs. 17–22). Most reactions produced only one precipitin band but there were several exceptions producing two (Figs. 18, 22). In this case, and with the exception of *Magnolia* species, the second band was weaker. The range of cross-reactions varied according to the antiserum tested. Antisera against *Nelumbo*, *Nuphar*, *Nymphaea* and *Euryale* were rather similar in producing reactions with most species of the Magnoliales, Ranunculales and Papaverales. Among the Ranunculales, it is noteworthy that although anti-*Nelumbo* sera failed to react with any of the species of Berberidaceae (see also Simon, 1970), the other three antisera reacted with either all of them (anti-*Nuphar*, *Nymphaea* sera) or partially (anti-*Euryale*). With the exception of *Victoria amazonica* antisera, all antisera reacted with some of the species of the Alismales and Liliales but not with others (Table 3, Figs. 17–22). The type of reaction produced by the primitive monocots, with the exception of Agavaceae, was similar to that reported by Simon (1970) using *Nelumbo* antisera. It was characterized by the formation of weak and diffuse banding pattern of low stainability with protein stains. Further tests indicated that the antigens responsible for these reactions probably were protein-polysaccharide complexes since the bands also stained with specific stains for polysaccharides such as the Schiff reagent and Alcian blue (Crowle, 1961, pp. 309–310). The diffuse pattern of banding was interpreted by Simon (1970) as indicative of a very low degree of serological affinity between *Nelumbo* and these primitive monocots. One additional fact appears to reinforce that suggestion. It was found that the precipitin band dissolved away when the ID plates were washed in saline, in concentrations ranging from 0.2 M to 1 M NaCl. The dissolution of the band was usually accomplished in less than six hours at 25 C and this phenomenon occurred only with these extracts. A partial loss of the band was also noticeable in reactions with species of the Agavaceae but here even overnight washings did not dissolve the aggregates completely. The solubility of antigen-antibody aggregates in saline of high molarity strongly suggests that the physicochemical configuration of the antigen determinant sites do not match in a mirror image those of the antibody. In this case the attraction forces keeping the lattice arrangement of antibody and antigen molecules from dissociating are most probably rather weak. It is the contention of the author and that of Leone* (personal communication) that this phenomenon probably indicates a low degree of serological correspondence between taxa of the Nymphaeales and the monocots.

---

*Dr. Charles A. Leone, Dean of the Graduate School, Bowling Green State University, Ohio.

Fig. 15. Immunoelectrophoresis spectra resulting from the reactions between anti-*Victoria amazonica* serum and antigen extracts of Nymphaeales: a. *Victoria amazonica*; b. *Victoria cruziana*; c. *Nuphar luteum* ssp. *polysepalum*; d. *Nymphaea gigantea*; e. *Euryale ferox*; f. *Nelumbo lutea*.
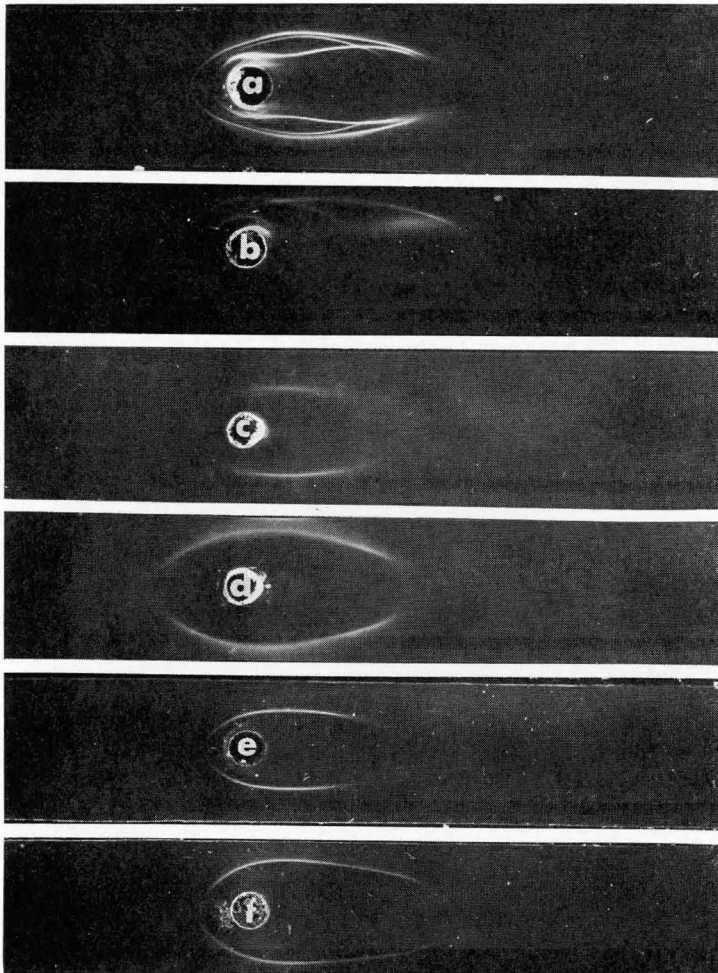
Fig. 16. Immunoelectrophoresis spectra resulting from reactions between anti-*Euryale ferox* serum and antigen extracts of Nymphaeales: a. *Euryale ferox*; b. *Victoria amazonica*; c. *Nuphar luteum* ssp. *polysepalum*; d. *Nymphaea gigantea*; e. *Nelumbo lutea*; f. *Nelumbo nucifera*.

The range of reactions obtained with *Victoria amazonica* antiserum was more restricted. With the doubtful exception of *Helleborus niger,* giving a very weak band in some of the replicates, none of the tested species of the Ranunculaceae, Berberidaceae, Papaveraceae, nor any of the monocot taxa (except Agavaceae) reacted with this antiserum.

Extracts from the following species, grouped by order, gave no reactions against any of the antisera produced for this study: Schisandrales-Illiciales:

Schisandra chinensis (Turcz.) Baill., *Schisandra glabra* (Brickell) Rehder, *Schisandra rubiflora* Rehder & Wilson, *Kadsura japonica* (L.) Dunal, *Illicium anisatum* L.; Laurales: *Persea americana* Mill., *Persea indica* Spreng.; Aristolochiales: *Aristolochia elegans* Mast., *Aristolochia grandiflora* Sw., *Asarum caudatum* Lindl.; Hamamelidales: *Corylopsis glabescens* F. & Z., *Corylopsis spicata* Sieb. & Zucc., *Hamamelis virginiana* L., *Liquidambar styraciflua* L.; Eucommiales: *Eucommia ulmoides* Oliv.; Trochodendrales: *Trochodendron araloides* Sieb. & Zucc.; Eupteleales: *Euptelea pleiosperma* Hook. f. & Thoms.; Dilleniales: *Hibbertia cuneiformis* (Labill.) Gilg., *Hibbertia scandens* (Willd.) Dryand.; Paeoniales: *Paeonia californica* Nutt., *Paeonia delavayi* Franch., *Paeonia lutea* Delav., *Paeonia officinalis* L.; Crossosomataceae: *Crossosoma californicum* Nutt.; Saxifragales: *Boykinia elata* (Nutt.) Greene, *Carpenteria californica* Torr., *Heuchera macrantha* Dougl. ex Lindl., *Lithophragma affinis* Gray, *Ribes roezlii* Regel, *Ribes californicum* H. & A., *Saxifraga californica* Greene; Rosales: *Acaena californica* Bitter., *Adenostoma fasciculatum* H. & A., *Cercocarpus betuloides* Nutt. ex T. & G., *Heteromeles arbutifolia* M. Roem., *Lyonothamnus floribundus* Gray, *Prunus virginiana* L., *Rosa gymnocarpa* Nutt. ex T. & G.; Leguminosae (Fabales): *Cercidium floridum* Benth., *Cercis occidentalis* Torr. ex Gray, *Acacia greggii* Gray, *Prosopis juliflora* (Sw.) D.C., *Lupinus densiflorus* Benth., *Medicago sativa* L., *Pisum arvense* L.; Caryophyllales: *Abronia villosa* Wats., *Amaranthus californicus* Moq. & Wats., *Calandrinia ciliata* (R. & P.) D.C., *Echinocactus viridescens* T. & G., *Eriogonum douglasii* Benth., *Mesembryanthemum nodiflorum* L., *Opuntia basilaris* Engelm. & Bigel., *Phytolacca americana* H. Walt., *Silene laciniata* Cav.; Alismales: *Alisma subcordatum* Raf., *Alisma triviale* Pursh., *Baldellia ranunculoides* (L.) Parl., *Echinodorus macrophyllus* (Kunth) Micheli; Liliales: *Iris douglasiana* Herb., *Allium campanulatum* Wats., *Allium unifolium* Kell., *Brodiaea elegans* Hoover; Cyperales: *Carex pansa* Bailey.

DISCUSSION

Serological relationships of the Nymphaeales

The serological data accumulated to date indicate that the genera of the Nymphaeales possess varying affinities to one another. On the one hand, a close relationship is found between *Nuphar* and *Nymphaea*. These taxa share two immunoprecipitin bands in common and the intergeneric differences are not larger than those found between some of the species of *Nymphaea*. One identity band was produced in cross-reactions between *Victoria* and the two genera, *Nuphar* and *Nymphaea*. Although the differences between *Victoria* and the two latter genera were larger than those found between *Nuphar* and *Nymphaea*, when considering the whole order, *Victoria* appears to be rather close serologically to these two genera. *Euryale ferox* stands further apart from the cluster formed by *Nuphar*, *Nymphaea* and *Victoria*, although *Euryale* appears to be closer to *Victoria* than to the other two genera. Finally, the isolated position of *Nelumbo* is indicated by the low degree of serological correspondence of this taxon with any of the

TABLE 3. *Cross-reactions of anti-Nymphaeales sera with taxa of other families of Angiosperms.*

SPECIES ANTIGENS	ANTISERA				
	Nelumbo	*Nuphar*	*Nymphaea*	*Euryale*	*Victoria*
DICOTYLEDONEAE					
Magnoliaceae					
Magnolia acuminata L.	+ 2*	+	+	+ 2	+
Magnolia campbellii Hook f. et Thoms.	+ 2	+	+	+	+
Magnolia delavayi Franch.	+ 2	+	+ w	+ 2	+
Magnolia grandiflora L. (2)**	+ 2	+	+	+ 2	+
Magnolia kobus D. C.	+ 2	+	+	+ 2	+
Magnolia obovata Thunb.	+ 2	+	+	+	+
Magnolia soulangeana Soul.	+ 2	+	+	+ 2	+
Magnolia sprengeri Pamp.	+ 2	+	+	+	+
Magnolia tripetala L.	+ 2	+	+	+ 2	+
Magnolia virginiana L.	+	+	+	+	+
Michelia champaca L.	+ w	+ w	+ w	+ w	+ w
Liriodendron tulipifera L.	+	+ w	+ w	+ w	+
Annonaceae					
Annona cherimola Mill.	+ w	+	+	+ w	+ w
Asimina triloba Dunal (2)	+ w	+ w	+ w	+ w	+ w
Polyalthia nitidissima Benth.	+ w	+ w	+ w	+ w	+ w
Rauwenhoffia leichhardtii Benth.	+ w	+ w	+ w	+ w	+ w
Degeneriaceae					
Degeneria vitiensis Bail. & Smith	+	+ w	+ w	+	+ w
Calycanthaceae					
Calycanthus floridus L.	+	+	+	+	+ w
Calycanthus occidentalis Hook. & Arn.	+ w	+ 2 w	+ 2 w	+	+ w
Winteraceae					
Drimys winteri Forst (2)	+ w	+	+	+	+ w
Tasmannia xerophila Parm. (2)	+ w	+ w	+ w	+ w	+ w
Tasmannia purpurascens (Vickery) A.C. Sm.	+ w	+ w	+ w	+ w	+ w
Eupomatiaceae					
Eupomatia laurina R. Br.	+ w	+ w	+ w	+ w	+ w

TABLE 3. *Cross-reactions of anti-Nymphaeales sera with taxa of other families of Angiosperms.*
(Continued)

SPECIES ANTIGENS	ANTISERA				
	Nelumbo	*Nuphar*	*Nymphaea*	*Euryale*	*Victoria*
DICOTYLEDONEAE (Cont'd)					
Himantandraceae					
Galbulimima baccata F. M. Bailey	+	+	+	+	+ w
Lauraceae					
Laurus nobilis L.	—	+ w	+ w	+ w	—
Umbellularia californica Nutt.	+	+ w	+ w	+	+ w
Ranunculaceae					
Aquilegia pubescens Cov.	+ w	+	+	+	—
Caltha howellii (Huth.) Greene	+	+	+	+	—
Clematis lasiantha Nutt.	+ w	+ w	+ w	+ w	—
Clematis ligusticifolia Nutt.	+ w	+ w	+ w	+ w	—
Delphinium variegatum T. & G.	+	+ w	+ w	+ w	—
Delphinium parryi Gray	+ w	+ w	+ w	+ w	—
Helleborus niger L. (2)	+	+ 2 w	+ 2 w	+	+ w ?
Ranunculus californica Benth.	+	+ 2	+ 2	+	—
Thalictrum polycarpum (Torr.) Wats.	+ w	+ w	+ w	+ w	—
Papaveraceae					
Argemone munita Dur. & Hilg.	+	+ w	+ w	+ w	—
Eschscholzia californica Cham.	+	+	+	+	—
Eschscholzia lobbii Greene	+	+ w	+ w	+	—
Papaver californicum Gray	+	+	+	+	—
Stylomecon heterophylla (Benth.) Tayl.	+	+ 2	+ 2	+ 2 w	—
Berberidaceae					
Podophyllum emodi Wall.	—	+ w	+ w	+ w	—
Podophyllum peltatum L.	—	+ w	+ w	+ w	—
Berberis amplectens (Eastw.) Wheeler	—	+ w	+ w	—	—
Berberis bealei Carr.	—	+ w	+ w	—	—
Berberis piperiana (Abrams.) McMinn.	—	+ w	+ w	—	—

TABLE 3. *Cross-reactions of anti-Nymphaeales sera with taxa of other families of Angiosperms.*
(Continued)

SPECIES ANTIGENS	ANTISERA				
	Nelumbo	Nuphar	Nymphaea	Euryale	Victoria
MONOCOTYLEDONEAE					
Alismataceae					
Sagittaria lancifolia L.	+ w d	+ w d	+ w d	+ w d	—
Sagittaria latifolia Willd.	+ w d	+ w d	+ w d	+ w d	—
Sagittaria platyphylla (Engelm.) Smith	+ w d	+ w d	+ w d	+ w d	—
Butomaceae					
Butomus umbellatus L.	+ w d	+ w d	+ w d	—	—
Hydrocharitaceae					
Ottelia alismoides (L.) Pers.	+ w d	+ w d	+ w d	+ w d	—
Liliaceae					
Lilium humboldtii Roetz & Leichtl.	—	+ w d	+ w d	—	—
Lilium parryi Wats.	—	+ w d	+ w d	—	—
Veratrum album L. (2)	+ w d	+ w d	+ w d	+ w d	—
Veratrum nigrum L.	+ w d	+ w d	+ w d	+ w d	—
Zygadenus fremontii Torr.	—	+ w d	+ w d	—	—
Agavaceae					
Agave deserti Engelm.	+ d	+ d	+ d	+ d	+ d
Agave shawii Engelm.	+ d	+ d	+ d	+ d	+ d
Agave utahensis Engelm.	+ d	+ d	+ d	+ d	+ d
Nolina parryi Wats.	+ d	+ w d	+ w d	+ w d	+ w d
Yucca brevifolia Engelm. in Wats.	+ d	+ d	+ d	+ d	+ d
Yucca whipplei Torr.	+ d	+ d	+ d	+ d	+ d

*2, indicates two precipitin bands in Ouchterlony plates; w, weak (faint) precipitin band(s); d, diffuse precipitin band.

**If more than one accession was studied, number is indicated in parentheses.
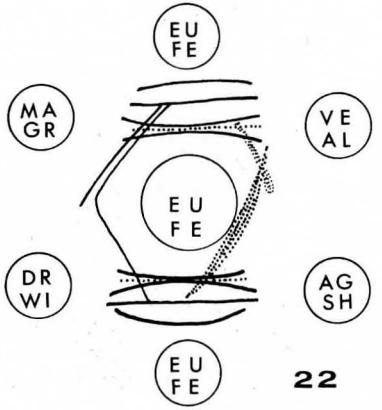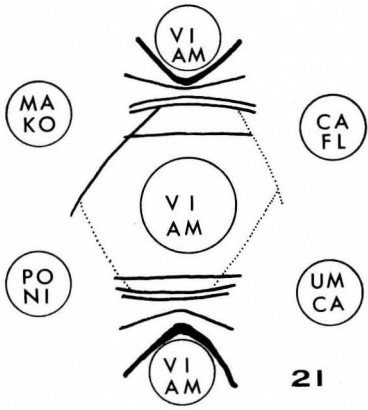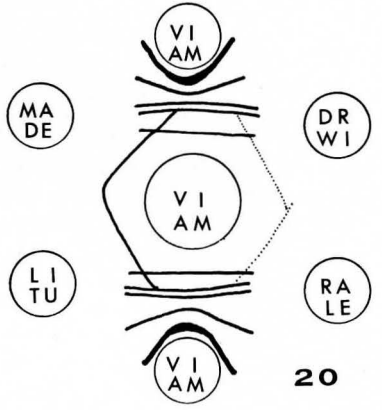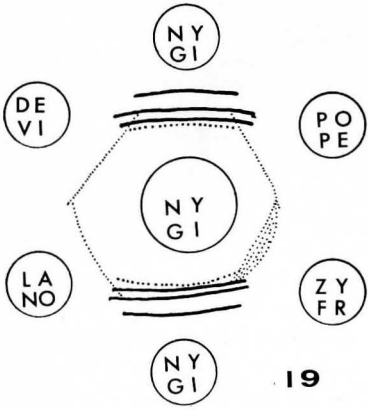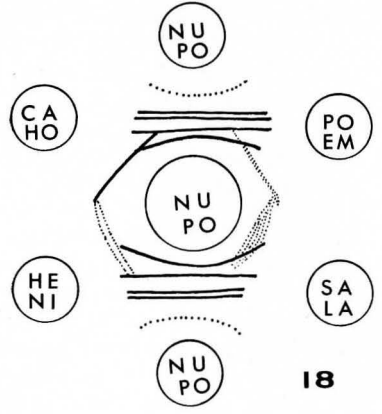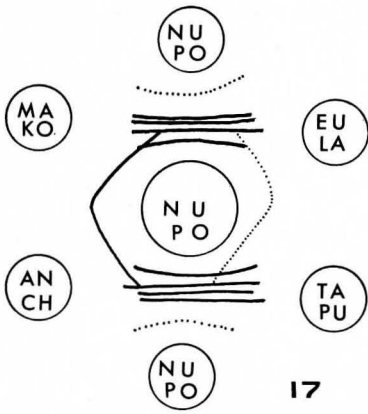
other species studied. A diagrammatic presentation of the relative serological relationships of the genera is presented in Fig. 23.

The close serological relationship of *Nuphar* and *Nymphaea* appears to correlate with the morphological, anatomical and biochemical similarities. In a recent communication, Goleniewska (1970) reported a comparative study of several morphological, anatomical and biochemical characteristics found in selected species of the Nymphaeales. On the basis of 20 morpho-anatomical leaf characteristics, she established an index of similarity from comparing each possible pair of species. The highest indices were obtained from comparisons of species of *Nuphar* (95%). Species of *Nymphaea*, involving both European (subgenus *Castalia*) and tropical species (subgenus *Lotus*), ranged from 100% to 75%. The latter figure was obtained from comparisons of species belonging to the two subgenera. Comparisons of species of *Nuphar* and *Nymphaea* gave indices ranging from 75% to 60%. Goleniewska (1970) also reported similarities in the alkaloids for the two genera. *Nymphaea* and *Nuphar* share a number of identified sesquiterpene alkaloids.

It is significant that representatives of three subspecies of *Nuphar* were serologically very similar. The serological survey lends support to the treatment of Beal (1956) who, on the basis of morphological and cytological evidence, reduced the North American and European species of *Nuphar* to one species, *N. luteum* (L.) Sibth. & Smith, with nine recognized subspecies. *Nuphar japonicum* DC., a stable endemic species from Japan, is reported to be morphologically and karyologically distinct and efforts are being made to obtain seeds of this species in order to establish its serological affinity.

By contrast, the species of *Nymphaea* tested showed a wide range of serological differences but these were not clearly correlated with the established taxonomy based on morphology. *Nymphaea* is rich in species and in urgent need of modern taxonomic revision. Conard (1905), the author of the only monograph of the genus, listed 34 species. Index Kewensis (1906–1966) listed 88 specific names having definite geographical ranges. Additional species have been described during the last decade. It is suspected, however, that many of these names are synonyms or represent intermediate forms resulting from hybridization between known species or cultivated varieties of existing species. The species analyzed in this study were few. Thus, in view of the state of taxonomic confusion in *Nymphaea*, a more involved serological analysis should be undertaken before one attempts to draw conclusions regarding intrageneric relationships.

The serological survey suggests a lower degree of relationship between the cluster formed by *Nuphar* and *Nymphaea* species and *Victoria* or *Euryale* taxa than that reported from the analysis of morphological and anatomical data. Goleniewska (1970) reported a range of similarity indices from 70 to 45% and 85 to 50% in comparisons between species of *Nuphar-Nymphaea* and *Victoria* and *Euryale* respectively. These index values are not markedly different from those found in comparisons between *Nuphar* and *Nymphaea* species. The alkaloid survey is less clear but does not indicate a close relationship between these pairs of genera. *Euryale* and *Victoria* shared only two unidentified compounds with some of the species of *Nuphar*
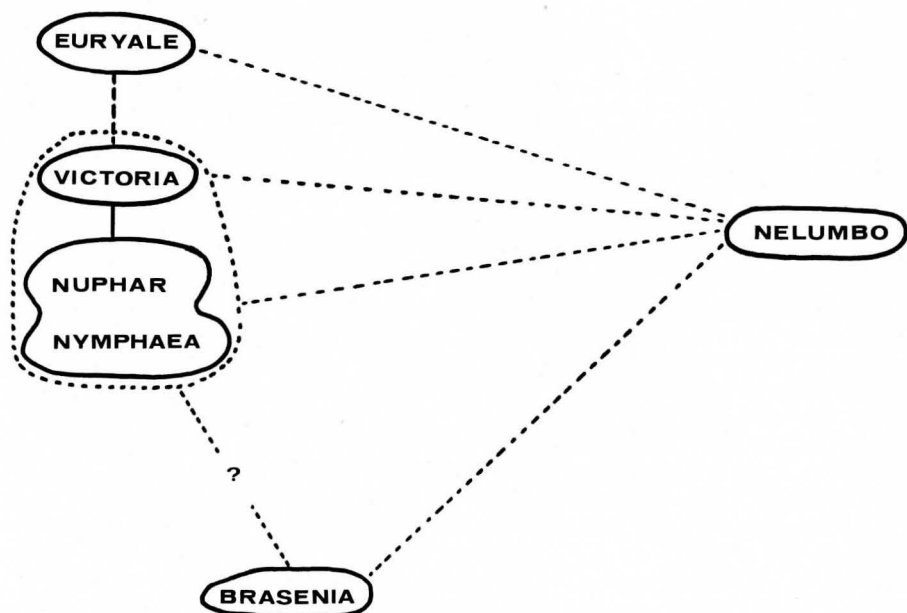
17

18

19

20

21

22

Fig. 23. Diagrammatic presentation of the relative serological affinities of genera of the Nymphaeales.

and *Nymphaea*, but not with other species. They lacked, however, any of the other identified sesquiterpene and thioalkaloids found in species of *Nymphaea* and *Nuphar*. On the basis of her study, Goleniewska (1970) supported the inclusion of *Nuphar*, *Nymphaea*, *Victoria* and *Euryale* in the family Nymphaeaceae. Contemporary taxonomists are in general agreement with this treatment (Cronquist, 1968; Takhtajan, 1969; Thorne, 1968) but Li (1955) has proposed that *Euryale* and *Victoria* should be segregated to a new order Euryalales on the basis of the distinct flower and vegetative morphology. The serological data indicate that these two tropical genera are relatively isolated but, in the context of the whole order Nymphaeales,

Fig. 17–22. Immunodiffusion patterns produced in Ouchterlony plates from reactions between anti-*Nuphar luteum* ssp. *polysepalum* (NU PO, Fig. 17–18), anti-*Nymphaea gigantea* (NY GI, Fig. 19), anti-*Victoria amazonica* (VI AM, Fig. 20–21) and anti-*Euryale ferox* sera (central wells) and antigen extracts of Angiosperm taxa. Key to symbols: MA KO, *Magnolia kobus*; AN CH, *Annona cherimola*; EU LA, *Eupomatia laurina*; TA PU, *Tasmannia purpurascens*; CA HO, *Caltha howellii*; HE NI, *Helleborus niger*; PO EM, *Podophyllum emodi*; SA LA, *Sagittaria latifolia*; DE VI, *Degeneria vitiensis*; LA NO, *Laurus nobilis*; PO PE, *Podophyllum peltatum*; ZY FR, *Zygodenus fremontii*; MA DE, *Magnolia delavayi*; LI TU, *Liriodendron tulipifera*; DR WI, *Drimys winteri*; RA LE, *Rauwenhoffia leichhardtii*; PO NI, *Polyalthia nitidissima*; CA FL, *Calycanthus floridus*; UM CA, *Umbellularia californica*; MA GR, *Magnolia grandiflora*; VE AL, *Veratrum alba*; AG SH, *Agave shawii*.

they may still be considered as clustering with the other genera of the Nymphaeaceae *sensu stricto*.

Although based on the comparison of different sets of characters, the data of both Goleniewska (1970) and Li (1955) suggest a very close relationship between *Victoria* and *Euryale*. The serological survey, however, indicates that these taxa are rather isolated from each other. The serological differences may be a reflection of the distinct geographical distribution of the genera. Whereas *Victoria* species are endemic to the Amazon Basin, *Euryale ferox* is distributed in eastern Asia, from far eastern Russia, south to China and west to Kashmir and Assam (Li, 1955; Goleniewska, 1970).

The serological data obtained to date emphasize the isolated position of *Nelumbo* vis-a-vis other Nymphaeales species and support the accumulated evidence based on morphological, anatomical and chemical characteristics which indicates that *Nelumbo* does not appear to be related directly to the other members of the Nymphaeales. For instance, Goleniewska (1970) found similarity indices on the order of 45% or less from the comparison of *Nelumbo* with the other species of the order. Khanna (1965, 1967) also reports fundamental anatomical differences between *Nelumbo nucifera* and other members of the order. Pollen morphology is also different, since *Nelumbo* is the only taxon possessing tricolpate pollen whereas all other species of the Nymphaeales have monocolpate pollen grains (Cronquist, 1968). Flower and vegetative morphology are also different (Li, 1955; Cronquist, 1968; Takhtajan, 1969) and the side-wall sculpture of the vessel elements reported in the roots of *Nelumbo* is the most advanced type found among the Nymphaeales species (Kosakai, 1968; Kosakai, Moseley and Cheadle, 1970). The chromosome number of *Nelumbo*, reported as $n=8$, $2n=16$, is not found in other species of Nymphaeales, with the exception of *Ceratophyllum* (Ceratophyllaceae) where a polyploid series with base $x=8$ has been reported (Fedorov, 1969; Darlington and Janaki, 1956; Wood, 1959). Takhtajan (1969) has further reported that the karyotype of *Nelumbo* is very different from that found in other species of Nymphaeales. In addition, *Nelumbo* is unique among the Nymphaeales in producing isoquinoline and aporphine types of alkaloids (see review by Goleniewska, 1970).

The evidence, including serology, certainly indicates that *Nelumbo* should *at least* be raised to family status within the Nymphaeales. Whether it should further be segregated to a separate order, i.e., Nelumbonales, as suggested by Li (1955) and Takhtajan (1969) is a matter of opinion but, the serological data would suggest that *Nelumbo* is *as isolated* from the remaining Nymphaeales taxa as it is from the Magnoliales and Ranunculales.

Information on the serological relationships of the Cabombaceae (i.e., *Cabomba* and *Brasenia*) is much needed. Our efforts to secure enough seeds for their study have been largely unsuccessful, as have been our attempts to raise antisera against *Brasenia schreberi*. Although some taxonomists include *Brasenia* and *Cabomba* in the Nymphaeaceae (Cronquist, 1968; Thorne, 1968) others segregate these genera to the Cabombaceae (Li, 1955; Takhtajan, 1969) on the basis of distinct flower and vegetative morphology. Goleniewska (1970) found very low indices of similarity, ranging from 50

to 25%, between these taxa and species of the Nymphaeaceae and Nelum-bonaceae. In addition, *Brasenia* and *Cabomba* are differentiated from the remaining species of the order in that they do not produce alkaloids (Goleniewska, 1970).

SEROLOGICAL RELATIONSHIPS OF NYMPHAEALES WITH OTHER FAMILIES OF ANGIOSPERMS

Serological affinities are shown between the Nymphaeales and Magnoliales, Laurales, Ranunculales and Papaverales (*sensu* Takhtajan, 1969; see Fig. 24). Within the Magnoliales, the analysis has expanded to cover species of Winteraceae, Annonaceae, Eupomatiaceae, Magnoliaceae, Himantandraceae and Degeneriaceae, and, therefore, give us a good coverage of the order. Because five of the six antisera produced for this study gave partial identity reactions with species of the Ranunculales and Papaverales, the negative tests obtained with *Victoria* antiserum were unexpected. This antiserum also failed to react with any of the antigen extracts of primitive monocots and, therefore, appeared to be rather specific. Additional rabbits are being immunized with *Victoria* antigens to ascertain possible variations in the specificity of the antisera.

The lack of reaction obtained with representative species of a number of orders supposedly derived from the primitive Magnoliales and Ranunculales circumscribes well the range of reactivity obtained with the Nymphaeales antisera (Fig. 24). However, the negative tests obtained with extracts of species of the Illiciaceae, Schisandraceae and Aristolochiaceae were unexpected and need to be discussed further.

Takhtajan (1969) has suggested that the Nelumbonales were probably derived from the Illiciales-Ranunculales stock (see also Fig. 24). Cronquist (1968) includes the Schisandraceae and Illiciaceae within the order Magnoliales. Furthermore, he indicates that the closest allies to the Ranunculales, within the Magnoliales, are found in the Illiciaceae and Schisandraceae. Similarly, Thorne (1968) classifies both families in a suborder Illicineae of his broader Annonales between the Winteriineae and the Magnoliineae. Unpublished serological data from Fairbrothers* (personal communication) suggest that there exists a definite, although distant, relationship between the Magnoliaceae and the Illiciaceae and Schisandraceae. From the present study, we have seen that the Magnoliaceae react rather strongly with all the antisera of the Nymphaeales. With this background of information, one would have expected, *a priori*, that antigen extracts of *Illicium, Schisandra* and *Kadsura* would also have reacted with the Nymphaeales antisera.

The Aristolochiaceae, as the Illiciales, possess spherical secretory cells (ethereal oil cells) and are, therefore, considered to be rather closely related to the Magnoliales. Thorne (1968) has included the Aristolochiaceae in a suborder of the Annonales, Aristolochineae, but other recent treatments consider Aristolochiales as a distinct, more advanced taxon derived from the Magnoliales (Cronquist, 1968; Takhtajan, 1969).

*Dr. David E. Fairbrothers, Rutgers University, New Brunswick, New Jersey.

The serological survey, therefore, confirms the widely accepted proposition that the Nymphaeales *sensu lato* are primitive taxa showing affinities with the Magnoliales (Annonales)-Ranunculales stock (Cronquist, 1968; Thorne, 1968; Takhtajan, 1969). The serological data do not support the suggestions made by Bate-Smith (1968) and Meeuse (1970) that the Nymphaeales (excepting *Nelumbo*) may be out of place in the "order Polyc.rpicae" (Magnoliales and Ranunculales). Their assumption of an independent origin of the Nymphaeales in respect of the Ranalian assembly is based on the presence of ellagitannins in *Nuphar luteum* (L.) Sm. (Bate-Smith, 1968). Ellagitannins are apparently of rare occurrence among primitive dicots. Among these, they have been found only in *Cercidiphyllum japonicum* Sieb. & Zucc. of the Cercidiphyllaceae, a family now considered included or closely related to the Hamamelidales (Cronquist, 1968; Thorne, 1968; Takhtajan, 1969).

As discussed earlier (Simon, 1970), the similar type of reactions produced by all the species of Magnoliales and Ranunculales does not allow for a definite ranking of relative affinities. Although differences in the intensity of partial identity reactions were observed (Table 3, Figs. 17–22), both strong and faint bands occurred with species of the same order or family. Clearly, differences in the intensity of the reactions may be due to variations in the relative amount of a given antigen and not to its specificity to a particular antibody. It is my contention that one should not place too much emphasis on quantitative differences, as observed in ID plates, for establishing relationships among taxa. Far more important is the obtaining of a critical comparative analysis of qualitative aspects of these antigens (i.e., position of the reaction in the agar arena, formation of spurs in comparison with other antigens, substrate affinity, etc.). This in turn may provide us with a better insight to the physicochemical configurations of the antigens (Crowle, 1961; Ouchterlony, 1964; Carpenter, 1965). As seen from the results of this study, the two-dimensional double immunodiffusion technique of Ouchterlony (1948) and Elek (1948) is particularly sensitive for demonstrating these differences.

Although slight serological reactions occurred between the Nymphaeales and some of the primitive monocots, the results are at best ambiguous. The anomalous type of reaction has been discussed above and by Simon (1970). To some extent the antigens responsible for these reactions have the properties of haptens, i.e., substances which by themselves do not induce antibody formation but that may react demonstrably with the appropriate antibody (Carpenter, 1965). For instance, carbohydrate components of the cell wall of certain bacteria possess hapten properties, and it may be very significant that the monocot antigens reported in the present study are most probably protein-polysaccharide complexes. However, these antigens, with the exception of the Agavaceae, did not react with antisera produced against *Prosopis* species (Fabaceae). Therefore, some degree of specificity must be assumed. It must be emphasized that none of these antigens reacted with normal sera, that is, sera taken from the rabbits before the initiation

of immunizations. This then would eliminate the possibility that lectins or lectin-like substances may be the cause of these anomalous reactions.

In the light of the serological tests reported here (as well as from unpublished data), the strong reactions of partial identity obtained with species of Agavaceae, particularly *Agave*, are also considered rather anomalous and should be looked upon with suspicion. These antigen extracts produced
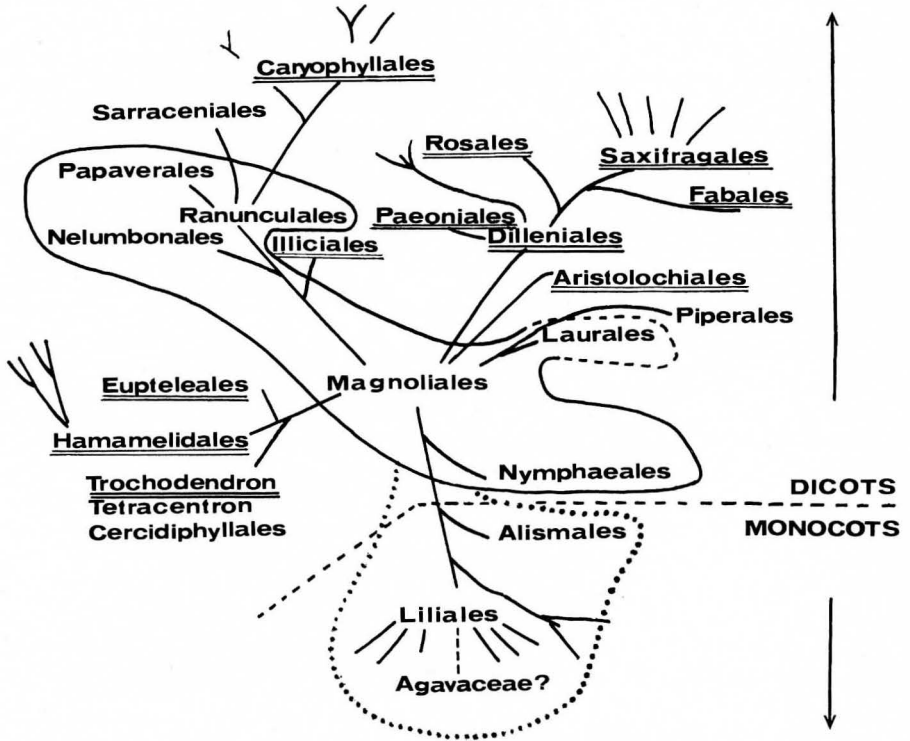

Fig. 24. Range of reactivity of antisera produced against Nymphaeales taxa. Orders enclosed within contour line gave positive reactions with most antisera. Underlined Orders are those for which representative taxa did not react with any of the antisera. Orders which are not underlined have not been investigated as yet. Point-contours of the monocot taxa indicate the anomalous type of reaction obtained with these taxa as discussed in the text. The "evolutionary tree" is adapted from Takhtajan (1969).

similar, although less intense, reactions with antisera produced against *Prosopis juliflora* DC. and *P. chilensis* (Mol.) Stuntz of the Fabaceae, a family relatively specialized among dicots and not considered related to the monocots. Furthermore, Lee and Fairbrothers (1970; and personal communication) have reported anomalous strong reactions between these antigens and antisera produced against various taxa of the Typhales.

Routine ID tests performed with extracts of species of Agavaceae and normal sera did not produce reactions in the Ouchterlony plates. However, Dr. D. W. Lee (personal communication) found that two of his normal sera reacted with these extracts giving a diffuse precipitin band in the plates. This reaction did not appear to be due to the presence of lectins since the hemagglutination tests performed by Lee gave negative results. Instead, these extracts lysed rabbit red blood cells. More extensive tests performed by us with all available normal sera disclosed that one of the 26 sera gave a weak diffuse precipitation pattern when reacted against extracts of *Agave* and *Yucca*. However, absorption tests using this normal serum indicated that the absorbed antigen extracts still reacted with the anti-Nymphaeales sera.

Even if we consider our results as an indication of some relationship between the Nymphaeales and primitive monocots, the data do not help us to clarify the issue whether or not monocots may have evolved from ancestors like Nymphaeales (Takhtajan, 1969). The recent discovery of primitive vessel elements in the root system of *Nelumbo* has led Kosakai, Moseley and Cheadle (1970) to conclude that this hypothesis is highly improbable. An assessment of the serological information at this point is rather premature and will have to wait for much supporting evidence. For instance, it would be extremely important to establish the range (and type) of reactivity of antisera produced against other primitive Ranalian species, i.e., *Magnolia, Schisandra, Drimys*, etc. Although some of these species have been studied serologically (see Fairbrothers, 1968, 1970), to my knowledge, no comparative tests using antisera produced against these taxa have been performed with species of monocots or with more advanced dicot families. A thorough and broader analysis of related orders may help us to elucidate some of the relationships of primitive dicots and monocots.

ACKNOWLEDGMENTS

I am particularly grateful to Dr. David E. Fairbrothers for sending material of *Magnolia, Michelia* and *Schisandra* and for allowing me to cite some of his unpublished data. I am also very much indebted to Dr. F. M. Moseley Jr., University of California-Santa Barbara, Dr. David W. Lee, The Ohio State University, Columbus, and to my esteemed colleague Robert F. Thorne for helpful discussions. I am appreciative of the technical help and assistance of Fred Oettinger and my wife Alicia.

SUMMARY

Antisera produced against *Nuphar luteum* ssp. *polysepalum, Nymphaea gigantea, Victoria amazonica* and *Euryale ferox* were reacted against seed antigens of a number of angiospermous taxa using immunodiffusion and immunoelectrophoretic tests. The serological data indicate that *Nuphar* and *Nymphaea* are serologically rather close to one another. *Victoria* shows some affinity with both the cluster formed by *Nuphar* and *Nymphaea*, and *Euryale*, but *Euryale* is isolated from the former genera. Only slight serological relationships are found between *Nelumbo* and the other genera of the Nymphaeales investigated to date. The four antisera produced partial identity reactions (Type III bands) with members of the orders Magnoliales (Annonales), Laurales, Ranunculales and Papaverales. These antisera also produced partial identity reactions with some of the monocot taxa investigated but not with others. These tests indicate, however, that monocots produce anomalous, possibly asystematic, reactions with antisera produced against the Nymphaeales. The taxonomic implications of the serological data are discussed in relation to other kinds of evidence.

LITERATURE CITED

Bate-Smith, E. C. 1968. Chemotaxonomy of *Nuphar luteum* (L.) Sm. Phytochemistry 7: 459.

Beal, E. O. 1956. Taxonomic revision of the genus *Nuphar* Sm. of North America and Europe. J. Elisha Mitchell Sci. Soc. 72: 317–346.

Carpenter, P. L. 1965. Immunology and Serology. W. B. Saunders Co. Philadelphia. 2nd Edition, 456 p.

Conard, H. S. 1905. The waterlilies: a monograph of the genus *Nymphaea*. Publ. No. 4. Carnegie Inst. Washington.

Cronquist, A. 1968. The evolution and classification of flowering plants. Houghton Mifflin Co. Boston. 396 p.

Crowle, A. J. 1961. Immunodiffusion. Academic Press. New York. 333 p.

Darlington, C. D., and E. K. Janaki. 1956. Chromosome atlas of cultivated plants. George Allen and Unwin Ltd. London. 397 p.

Elek, S. D. 1948. The recognition of toxicogenic bacterial strains in vitro. Brit. Med. J. 1: 493–496.

Fairbrothers, D. E. 1968. Chemosystematics with emphasis on systematic serology, pp. 141–174. *In* V. H. Heywood (ed.). Modern methods in plant taxonomy. Academic Press. New York.

———. 1970. Plant chemosystematic (macromolecules) research at Rutgers. Serol. Museum Bull. 43: 6–8.

Fedorov, A. A. 1969. Chromosome numbers of flowering plants. Acad. Sci. USSR. V. L. Komarov Botanical Institute. 926 p.

Goleniewska-Furmanowa, M. 1970. Comparative leaf anatomy and alkaloid content in the Nymphaeaceae Bentham and Hooker. Monogr. Bot. 31: 1–56.

Heitefuss, R., D. J. Buchanan-Davidson, and M. A. Stahmann. 1959. The stabilization of extracts of cabbage leaf-proteins by polyhydroxy compounds for electrophoretic and immunological studies. Arch. Biochem. 85: 200–208.

Index Kewensis Plantarum Phanerogamarum 1895, suppl. I-XIII, 1906–1966. Oxford.

Khanna, P. 1965. Morphological and embryological studies in Nymphaeaceae II. *Brasenia schreberi* Gmel. and *Nelumbo nucifera* Gaertn. Austral. J. Bot. 13: 379–387.

————. 1967. Morphological and embryological studies in Nymphaeaceae III. *Victoria cruziana* D'Orb. and *Nymphaea stellata* Willd. Bot. Mag. Tokyo 80: 305–312.

Kosakai, H. 1968. The comparative xylary anatomy of various Nymphaeaceae. M. A. Dissertation, Univ. Calif. Santa Barbara.

————, M. F. Moseley, Jr., and V. I. Cheadle. 1970. Morphological studies of the Nymphaeaceae: V. Does *Nelumbo* have vessels? Amer. J. Bot. 57: 487–494.

Lee, D. W., and D. E. Fairbrothers. 1970. Serological analysis of seed proteins from the Typhales and other Monocotyledons. Amer. J. Bot. 57: 753 (Abstract).

Li, Hui-Lin. 1955. Classification and phylogeny of Nymphaeaceae and allied families. Amer. Midl. Naturalist 54: 33–41.

Meeuse, A. D. J. 1970. The descent of the flowering plants in the light of new evidence from phytochemistry and from other sources. II. Suggestions for a holo-taxonomic major classification. Acta Bot. Neerl. 19: 133–140.

Ouchterlony, Ö. 1948. In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta Pathol. Microbiol. Scand. 25: 189–191.

————. 1964. Gel-diffusion techniques, pp. 55–78. *In* J. F. Ackroyd (ed.) Immunological methods. F. A. Davis, Philadelphia.

————. 1968. Handbook of immunodiffusion and immunoelectrophoresis. Ann Arbor Publ. Ann Arbor, Michigan. 324 p.

Simon, J. P. 1970. Comparative serology of the Order Nymphaeales I. Preliminary survey on the relationships of *Nelumbo*. Aliso 7: 243–261.

Takhtajan, A. 1969. Flowering plants. Origin and dispersal. Oliver & Boyd. Edinburgh. 310 p.

Thorne, R. F. 1968. Synopsis of a putatively phylogenetic classification of the flowering plants. Aliso 6 (4): 57–66.

Wood, C. E., Jr. 1959. The genera of the Nymphaeaceae and Ceratophyllaceae in the southeastern United States. J. Arnold Arbor. 40: 94–112.