

BIOCHEMICAL HETEROPHYLLY AND FLAVONOID EVOLUTION IN NORTH AMERICAN POTAMOGETON (POTAMOGETONACEAE)¹

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ABSTRACT

Morphologically heterophyllous species of *Potamogeton* also commonly display biochemical heterophylly with respect to flavonoid compounds. Generally, floating leaves contain an assortment of flavonoids, whereas submersed leaves often exhibit reduced flavonoid profiles. In strictly submersed (homophyllous) species, two patterns occur. Linear-leaved species have few flavonoids and their biochemical profiles resemble those of submersed leaves of heterophyllous species. Broad-leaved homophyllous species possess flavonoid profiles more similar to those of the floating leaves of heterophyllous species. Numerical analysis of these chemical data is consistent with phylogenetic relationships within the genus derived independently on the basis of morphological and chromosomal data. Glycoflavones, which are probably maintained in floating leaves because of their UV filtering ability, exhibit the most pronounced biochemical heterophylly in *Potamogeton*. The lack of glycoflavones in submersed leaves of heterophyllous species and in linear-leaved homophyllous species is attributable to the ability of naturally colored water to significantly absorb harmful UV radiation. These observations provide strong support for earlier hypotheses suggesting the importance of flavonoid evolution in the conquest of exposed terrestrial habitats by plants.

THE PONDWEED GENUS *Potamogeton* (Potamogetonaceae) includes approximately 100 species worldwide (Cook et al., 1974) representing a wide range of interspecific morphological and ecological diversity. Pondweeds can be divided into two morphological groups (see Les and Sheridan, 1990), the heterophyllous species (with both floating and submersed foliage) and homophyllous species (entirely with submersed foliage). Morphological variation within these groups provides for a variety of growth habits in the genus (Fig. 1). The conspicuous phenotypic distinction between floating and submersed leaves in *Potamogeton* is regarded as a classical example of heterophylly.

The phenolic biochemistry of aquatic plants has been a fairly neglected area of research. Reznik and Neuhausel (1959) reported differences in anthocyanin production and colorless anthocyanin pseudobase synthesis between terrestrial and aquatic plants. The inability of several aquatic plants to carry out certain phenol glucosylation reactions was reported over 25 years ago (Pridham, 1964).

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In discussing the secondary chemistry of aquatic plants, McClure (1970) remarked that emergence from the water significantly alters plant metabolism, and noted that several fundamental differences occur in phenolic biochemistry between aquatic and terrestrial plants. He also hypothesized that some secondary compounds in hydrophytes may be adaptations linked to their aquatic existence. Generally, however, the essential function of widely distributed flavones and flavonols in either aquatic or terrestrial plants has remained "a mystery" (Harborne, Mabry, and Mabry, 1975). Although flavonoids have been implicated as antioxidants, enzyme inhibitors, toxic substance precursors, pigments, light screens, and regulators of plant growth and development, it has been difficult to pinpoint specific features of flavonoid evolution within the angiosperms (McClure, 1975). Recent ecological interpretations have revived interesting hypotheses regarding the potentially important evolutionary role of flavonoids as ultraviolet light screens (e.g., Miller, 1988).

Potamogeton is an ideal model for pursuing questions relating to phytochemical and evolutionary aspects of submergence in hydrophytes. Heterophylly allows for biochemical comparisons between submersed and emergent foliage within a single individual and genotype. Furthermore, comparisons can be made

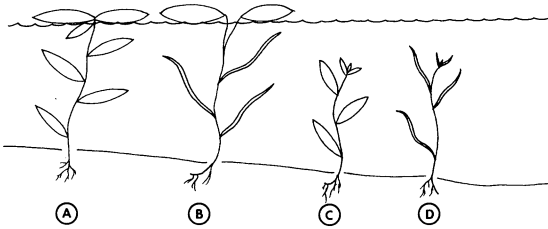


Fig. 1. Basic morphological types in *Potamogeton*. A, indistinct heterophylly with morphologically similar floating and submersed leaves. B, distinct heterophylly with morphologically dissimilar floating and submersed leaves. C, broad-leaved homophylly. D, linear-leaved homophylly.

between heterophyllous and homophyllous species as a means of identifying possible phytochemical correlations to these specific growth habits. Although the morphological and anatomical aspects of heterophylly have been given considerable attention, putative "physiological heterophylly" involving phenolic pigments in aquatic plants (Reznik and Neuhäusel, 1959) has not been studied in any detail.

We conducted a flavonoid survey of 17 North American species of *Potamogeton* to investigate the possibility of biochemical heterophylly in the genus, and to determine whether specific patterns of flavonoid distribution characterize different morphological groups. We also explore the systematic significance of flavonoid distribution in *Potamogeton* and discuss the pertinence of our results to the hypothesis that flavonoid evolution in plants may have been a significant evolutionary factor facilitating their terrestrialization.

MATERIALS AND METHODS—Flavonoid analysis—Fresh leaf material of 17 pondweed species (Table 1) representing 14 of 26 subsections, three of four sections, and both subgenera of the genus was collected in the field, rinsed thoroughly, and dried at 80 C for 24–48 hr. Floating and submersed foliage of heterophyllous species was separated prior to drying. In *Potamogeton natans*, the blades and petioles of the floating leaves were analyzed separately (see Discussion). Dried leaf samples were ground into a fine powder using a Waring commercial blender. Equal amounts (20 g) of powdered leaf material were used for samples whenever possible. In a few cases (e.g., submersed leaves of *P. natans*), smaller samples (4–18 g) were available. Each sample was extracted twice; the first 24-hr extraction using 85% methanol (MEOH), and the second 24-hr extraction using 50% MEOH. Products of each extraction were filtered, evaporated to

TABLE 1. Vouchers for species of *Potamogeton* surveyed for foliar flavonoids in floating (f) and/or submersed (s) leaves

<i>Potamogeton alpinus</i> Balbis: Catfish Lake, Wisconsin (f,s), Les 457 (UWM). <i>P. amplifolius</i> Tuckerman: Catfish Lake, Wisconsin (f,s), Les 459 (UWM); Pine Lake, Wisconsin (f,s), Les 483 (UWM); Cedar Lake, Wisconsin (s), Les 451 (UWM); Devil's Lake, Wisconsin (s), Les 455 (UWM). <i>P. crispus</i> L.: Earhart Road Pond, Michigan (s), Les 336 (UWM). <i>P. epihydrus</i> Raf.: Catfish Lake, Wisconsin (f,s), Les 461 (UWM). <i>P. foliosus</i> Raf.: Eau Galle Reservoir, Wisconsin (s), <i>Godshalk 13-18f</i> (UWM); Pond near Jackson, Wisconsin (s), <i>Burkett s.n.</i> , August 11, 1988 (UWM). <i>P. gramineus</i> L.: Little Muskie Lake, Wisconsin (f,s), Les 480 (UWM); Wildcat Lake, Wisconsin (s), Les 481 (UWM). <i>P. illinoensis</i> Morong: Lucas Lake, Wisconsin (f,s), Les 478 (UWM); Wallace Lake, Wisconsin (f,s), <i>Sheridan 88-005</i> (UWM); Pond near Eagle, Wisconsin (f,s), <i>Sheridan 87-001</i> (UWM). Cedar Lake, Wisconsin (s), Les 450 (UWM); Devil's Lake, Wisconsin (s), Les 456 (UWM); <i>P. natans</i> L.: Lucas Lake, Wisconsin (f,s), Les 479 (UWM); Pond near Eagle, Wisconsin (f,s), <i>Sheridan 87-002</i> (UWM); Wallace Lake, Wisconsin (f,s), <i>Sheridan 88-017</i> (UWM); Pond near Anvil Lake, Wisconsin (f), Les 470 (UWM). <i>P. nodosus</i> Poir.: Cedar Creek, Wisconsin (f,s), Les 479 (UWM); Eau Galle Reservoir, Wisconsin (f), <i>Godshalk 40a</i> (UWM); Pond near Novi, Michigan (f,s), Les 476 (UWM). <i>P. pectinatus</i> L.: Cedar Lake, Wisconsin (s), Les 448 (UWM); Eau Galle Reservoir, Wisconsin (s), <i>Godshalk 45p</i> (UWM). <i>P. perfoliatus</i> L.: Wildcat Lake, Wisconsin (s), Les 482 (UWM). <i>P. pusillus</i> L.: Silver Lake, Wisconsin (s), Les 474 (UWM). <i>P. richardsonii</i> (Benn.) Rydb.: Big Cedar Lake, Wisconsin (s), Les 452 (UWM). <i>P. robbinsii</i> Oakes: Devil's Lake, Wisconsin (s), Les 454 (UWM). <i>P. spirillus</i> Tuckerman: Catfish Lake, Wisconsin (f,s), Les 460 (UWM). <i>P. vaseyi</i> Robbins: Silver Lake, Wisconsin (s), Les 475 (UWM). <i>P. zosteriformis</i> Fern.: Catfish Lake, Wisconsin (s), Les 477 (UWM).

dryness, and combined for each sample. Extracts were spotted onto Whatman 3MM chromatography paper. Two-dimensional chromatograms were obtained by development in chromatocabs using standard TBA and HOAC systems (Mabry, Markham, and Thomas, 1970; Markham, 1982). The R_f values and color under ultraviolet (UV) light (with and without ammonia fumes) were recorded for spots resolved on the paper chromatograms. Spots were clipped from the paper and 10–20 replicates of each were combined to yield suitable concentrations for UV spectral analysis and subsequent acid hydrolysis. Flavonoids were eluted from the paper in 100% HPLC grade MEOH for 24–48 hr on a shaker table. Extracts were filtered and evaporated to dryness, respotted on Brinkman Polyamide 6 thin-layer chromatography (TLC) plates, and checked for purity after development in a 1,2-dichloroethane:methyl ethyl ketone:MEOH:water:formic acid (100:50:41:8:1 v/v) system. The UV spectra of compounds were recorded using a Beckman 35

TABLE 3. Basic data matrix and coding of biochemical characters used in numerical study of *Potamogeton* species. Data for *P. praelongus* taken from Roberts and Haynes, 1986

Species (tissue)		Character								
		1	2	3	4	5	6	7	8	9
<i>P. alpinus</i>	(floating)	1	1	0	1	1	0	1	1	0
	(submersed)	1	1	0	1	1	0	1	1	0
<i>P. amplifolius</i>	(floating)	0	1	0	1	1	0	1	1	0
	(submersed)	0	0	0	0	0	0	0	0	0
<i>P. epihydrus</i>	(floating)	1	1	0	1	1	0	0	1	0
	(submersed)	0	0	0	0	0	0	0	0	0
<i>P. gramineus</i>	(floating)	1	1	1	1	1	1	0	1	0
	(submersed)	0	1	1	0	1	1	0	1	0
<i>P. illinoensis</i>	(floating)	1	1	0	1	1	0	0	1	0
	(submersed)	0	1	0	0	1	0	0	1	0
<i>P. natans</i>	(floating)	1	1	1	1	1	1	1	1	1
	(submersed)	0	1	0	0	0	0	0	1	0
<i>P. nodosus</i>	(floating)	1	1	0	1	1	0	1	1	1
	(submersed)	0	0	0	0	0	0	0	0	0
<i>P. spirillus</i>	(floating)	1	1	1	1	1	1	0	1	0
	(submersed)	1	1	1	1	1	1	0	1	0
<i>P. vaseyi</i>	(submersed)	0	1	0	0	0	0	0	0	0
<i>P. crispus</i> ^a	(submersed)	1	1	1	0	0	0	1	1	0
<i>P. foliosus</i> ^a	(submersed)	0	0	1	0	0	0	0	1	0
<i>P. pectinatus</i> ^a	(submersed)	1	1	0	0	0	0	0	1	0
<i>P. perfoliatus</i> ^a	(submersed)	0	1	1	0	1	0	1	1	0
<i>P. praelongus</i> ^a	(submersed)	1	1	1	0	1	0	1	1	0
<i>P. pusillus</i> ^a	(submersed)	0	1	0	0	0	0	1	1	0
<i>P. richardsonii</i> ^a	(submersed)	0	1	1	0	1	0	1	1	0
<i>P. robbinsii</i> ^a	(submersed)	1	1	0	0	0	0	0	1	0
<i>P. zosteriformis</i> ^a	(submersed)	1	1	1	0	0	0	1	1	0

^a Homophyllous species.

1. apigenin aglycones/glycosides: absent (0); present (1). 2. luteolin aglycones/glycosides: absent (0); present (1). 3. chrysoeriol aglycones/glycosides: absent (0); present (1). 4. apigenin 6-C-glycosyl flavones: absent (0); present (1). 5. luteolin 6-C-glycosyl flavones: absent (0); present (1). 6. chrysoeriol 6-C-glycosyl flavones: absent (0); present (1). 7. O-glycosylation: absent (0); present (1). 8. O-glucuronosylation: absent (0); present (1). 9. 7-methylation: absent (0); present (1).

UV/VIS scanning spectrophotometer following standard procedures (Markham, 1982). Aqueous extracts of all compounds were completely hydrolyzed in 0.1 N trifluoroacetic acid (TFA) and subsequently evaporated to dryness. The hydrolyzate was partitioned in water/ethyl acetate to separate sugar and aglycone fractions. Aglycones were cochromatographed with standards on the polyamide TLC system described above, and sprayed with NA reagent (Markham, 1982) for visualization and verification of B-ring oxidation patterns. Sugars were identified by cochromatography with standards using a Fotodyne high performance radial chromatography (HPRC) system incorporating Brinkman Avicel Microcrystalline Cellulose MN 400 precoated plates developed in an ethyl acetate:pyridine:water (3:1.5:1 v/v) system (Les et al., 1989). Sugars were visualized by spraying dried plates with a solution of ethanolic aniline/phthalic acid, and warming until bands appeared. Following hydrolysis, suspected C-glycosyl flavones were chromatographed with 15% HOAC on cellulose plates (described above), and sprayed with NA

to check for Wessely-Moser rearrangement products (Mabry et al., 1970; Markham, 1982). Following acid hydrolysis, suspected glucuronides (nonhydrolyzed products) were subjected to enzyme hydrolysis using Sigma G-1758 beta-glucuronidase, chromatographed with 15% HOAC on cellulose plates, and sprayed with NA to check for hydrolysis.

Numerical analyses—A cluster analysis of flavonoids was performed for the 17 pondweed species studied. Nine flavonoid characters were scored for occurrence in floating and submersed foliage of both the heterophyllous and homophyllous species. These were used to construct a euclidean distance matrix using MVSP (Kovach, 1986). An average linkage cluster analysis of the euclidean distance matrix was carried out using the UPGMA algorithm of MVSP (Kovach, 1986).

RESULTS—Twelve flavone aglycones and glycosides were isolated from the pondweed species studied (Table 2). Six of eight heterophyllous species exhibited some degree of bio-

chemical heterophyly associated with flavonoid compounds. (Our examination of only submersed leaves for a ninth heterophyllous species, *P. vaseyi*, prevented an assessment of biochemical heterophyly in that species.) Although common in floating leaves, glycoflavones are typically absent in submersed foliage of heterophyllous species and also in many submersed species (Table 2).

The distribution of nine flavonoid characters (Table 3), delimits the heterophyllous species as a cohesive group by their floating leaf chemistry assessed by numerical analysis (Fig. 2). Of the homophyllous species studied, the chemistry of the broad-leaved group is most similar to that of the floating leaves of heterophyllous species (Fig. 2). The chemistry of the submersed leaves of six heterophyllous species, however, is most similar to that of the linear-leaved group of homophyllous species (Fig. 2).

The flavonoid constituents of floating leaf blades, floating leaf petioles, and submersed (phyllodal) leaves of *P. natans* differed in the population studied with eight compounds occurring in floating blade tissue, four compounds in floating petiole tissue, and one compound in submersed leaf tissue (Table 4).

DISCUSSION—Our documentation of the lack of flavonols, prevalence of luteolin and chrysoeriol derivatives, and presence of glycoflavones in *Potamogeton* agrees precisely with earlier biochemical studies of the genus (Boutard et al., 1972, 1973; Roberts and Haynes, 1986) and of the subclass Alismatidae in general (Crawford, 1978; Williams and Harborne, 1988). We further report the occurrence of isoorientin in *P. richardsonii* not detected by Roberts and Haynes (1986).

Intraspecific flavonoid variation is extensive in plants (Bohm, 1987) but was previously unreported for the genus *Potamogeton*. In *Potamogeton*, flavonoid compounds display some degree of quantitative intraspecific variation, possibly attributable to differential environmental factors (Roberts and Haynes, 1986). From wide interpopulational sampling, however, Roberts and Haynes (1986) found there to be no qualitative geographical flavonoid variation among even ampho-Atlantic populations sampled of several pondweed species. Similarly, we observed essentially no qualitative flavonoid glycoside variation among populations sampled of any particular species. From this study and that of Roberts and Haynes (1986), intraspecific flavonoid variation in *Potamogeton* appears to be almost exclusively associated with unsubstituted aglycones.

Biochemical heterophyly in *Potamogeton*,

TABLE 4. Biochemical differences between floating leaf blade, floating leaf petiole, and submersed leaf tissues of *Potamogeton natans* (Les 479). + = present, - = undetected (absent)

Flavonoid	Floating leaf blade	Floating leaf petiole	Submersed leaf
Luteolin	+	+	-
7-OCH ₃ luteolin	+	-	-
Apigenin 6-C-glucoside	+	-	-
Apigenin 7-O-glucuronide	+	-	-
Luteolin 7-O-glucoside	+	-	-
Luteolin 7-O-glucuronide	+	+	+
Luteolin 6-C-glucoside	+	+	-
Chrysoeriol 6-C-glucoside	+	+	-

manifested by different flavonoid glycoside distributions between the floating and submersed foliage of morphologically heterophyllous species, is reported here for the first time. This observation may have important evolutionary implications if the observed biochemical patterns are genetically based. Reznik and Neuhausel (1959) reported quantitative flavonoid variation in species of *Hydrocotyle*, *Houttuynia*, and *Saururus* with normally emerged foliage possessing higher quercetin concentrations than foliage subjected to 20 days of submergence in 50 cm of water. McClure (1970) found that photoconditions have a greater effect upon 3'4'-dihydroxylated compounds (such as quercetin) than on 4' hydroxylated compounds in aquatic plants. Because of the wide occurrence of luteolin (3'4' dihydroxylated flavonoid) compounds in *Potamogeton*, these factors must be addressed. Although we observed some degree of quantitative flavonoid variation in our study, we believe that qualitatively, the differential expression of flavonoids between floating and submersed foliage of morphologically heterophyllous species is genetically based and does not simply reflect environmentally induced phenotypic plasticity. Convincing evidence for the genetic maintenance of biochemical heterophyly is provided by our analysis of *P. nodosus* collected from a shallow stream. In this population, the floating leaves and the submersed leaves occurred alongside one another at the same level in the flowing water, only a few millimeters under the water surface. Therefore, the leaves in this habitat were subjected to the same environmental factors such as light, depth, current, and nutrient levels. Chemical analysis of the plants from this population, however, revealed marked biochemical heterophyly. High concentrations of six compounds occurred in the floating leaves (20 g sample), whereas flavonoids were completely undetectable in the

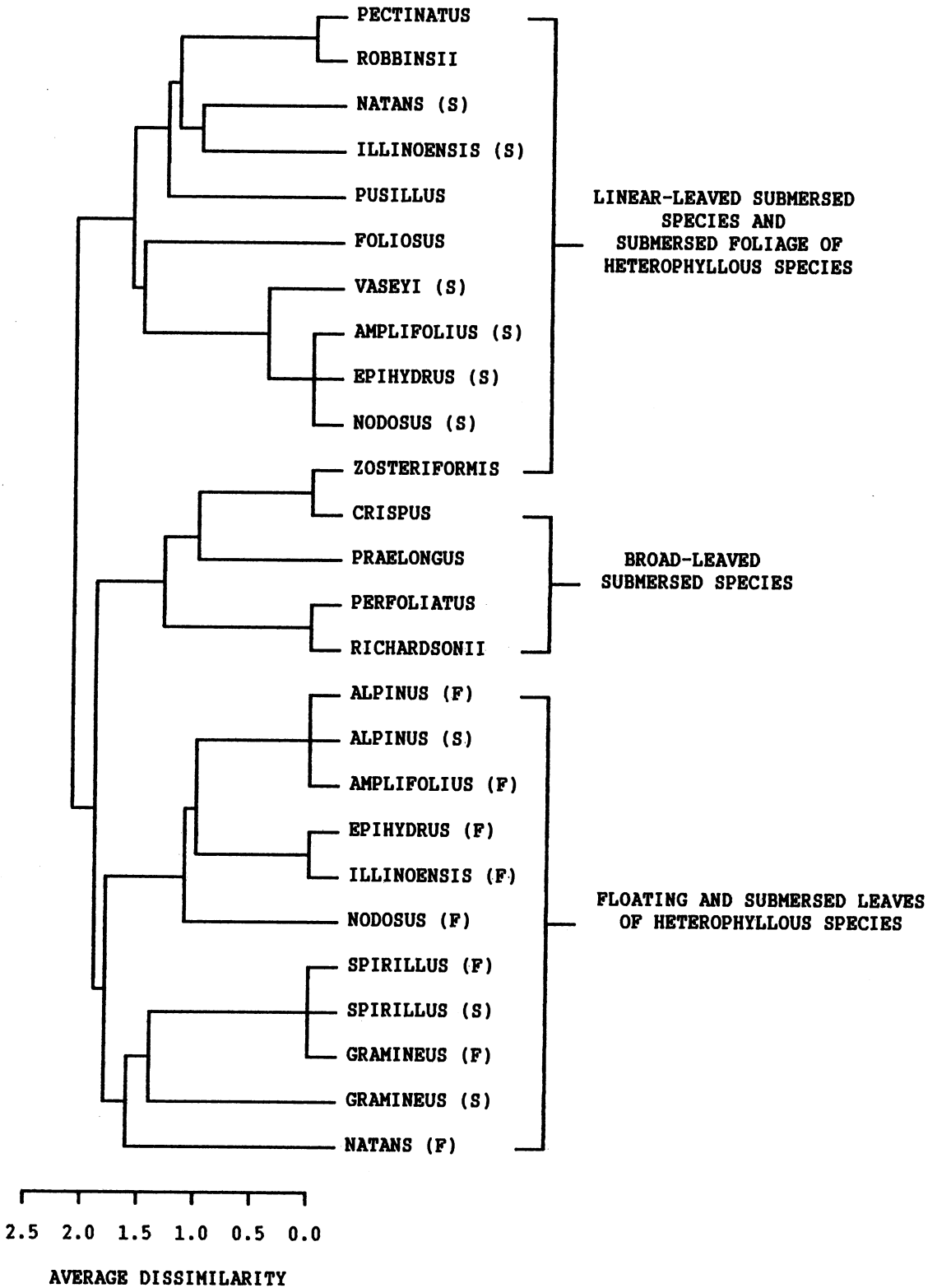


Fig. 2. UPGMA dendrogram of euclidean distances derived from flavonoid characters (Table 3) scored for 18 *Potamogeton* species. F = floating leaf chemistry; S = submersed leaf chemistry.

submersed leaves (20 g sample). This same pattern was observed consistently in other populations of the species surveyed. Similarly, plants of *P. illinoensis* exhibited biochemical heterophyly in a population where submersed leaves were produced in water only 5–10 cm deep. Although environmental influences were not as easily ruled out for the remaining collections of heterophyllous species, other evidence similarly points to a genetic basis for the commonly observed biochemical heterophyly. Geographically different collections of other species also consistently displayed the same pattern of biochemical heterophyly, a good argument for genetic control in those species. Also, several of the heterophyllous species (e.g., *P. amplifolius*, *P. epihydrus*, *P. spirillus*, and *P. alpinus*) were collected from the same lake, within relatively close proximity to one another. Although the overall lake conditions such as water depth and clarity were similar for these species, two exhibited biochemical heterophyly, whereas two did not. In *P. spirillus*, a species that was collected at relatively deep parts of the lake, the submersed leaves contained the same amount and variety of compounds as the floating leaves. In this case, the water depth had no apparent effect on either quantitative or qualitative flavonoid profiles of submersed leaves. Stranded mudflat forms of *P. nodosus*, *P. natans*, and *P. illinoensis* (with aerial floating leaves alone) possessed flavonoid profiles identical to those of collections with normal floating leaves (in contact with the water surface). Accordingly, we believe that the major aspects of biochemical heterophyly as well as morphological heterophyly in *Potamogeton* are controlled genetically.

What then is the evolutionary significance of biochemical heterophyly in *Potamogeton*? To answer this question we consider the possible adaptive significance of flavonoids as ultraviolet radiation screens in plants.

Foliar glycoflavones as ultraviolet screens in plants—Flavonoids have long been implicated as ultraviolet (UV) radiation screens in plants (Markham, 1988). Their stability under intense UV irradiation (Kaneta and Sugiyama, 1971) and their high UV absorbance in epidermal tissue (Caldwell, 1971) provide circumstantial evidence for this conclusion. The role of glycoflavones as UV radiation screens was proposed originally by Swain (1970) with respect to properties of their absorption spectra. The two peak absorption maxima of glycoflavones (257–70 nm; 335–349 nm) coincide with empirically determined peaks associated with mutagenesis and cellular death such as maxi-

mum thymine dimerization of DNA at 260 nm and maximum photodestruction of NAD and NADP at 340 nm (Swain, 1975). Although found in many plant tissues, glycoflavones are most common in aerial tissues (Chopin and Bouillant, 1975) where exposure to UV radiation is most severe.

Presently, precise details of glycoflavone distribution in the algae are uncertain. Markham (1988) questioned the authenticity of flavonoid reports from algae other than Charophytes which are known to possess only glycoflavones. Chopin and Dellamonica (1988) also reported glycoflavones in Chaetophoracean and Chaetosiphonacean algae. Because most algae lack flavonoids, Swain (1975, p. 1119) concluded that the unique occurrence of glycoflavones in Charophyte algae "... would obviously benefit those algae which were emerging from an aquatic environment onto the land." This opinion was more recently revived by Valant-Vetschera (1985) who attributed a similar adaptive function to glycoflavones. She proposed that because of their occurrence in some algae, glycoflavones may have functioned as a prerequisite for the colonization of land in the course of plant evolution. A similar suggestion has also been made with respect to all secondary metabolites in aquatic plants (McClure, 1970). Attempts to support this hypothesis beyond such circumstantial evidence, however, met with varied levels of success. A comparison of glycoflavone concentrations in top and bottom halves of a 0.4 m high *Chara* plant predicted greater concentrations in the upper part of the plant, but the experiment was inconclusive (Swain, 1975; Markham, 1988). It was noted that the results were understandable due to the lack of biochemical differences among *Chara* cells. Experiments using a 0.5 cm layer of a $10 \mu\text{g}^{-1}$ aqueous solution of vitexin (a glycoflavone), however, were successful in screening *Alternaria* cultures against harmful exposures of UV light at 264 nm (Swain, 1975).

Central to this discussion, is a consideration of the UV absorbing properties of water with the above hypotheses assuming the ability of water to substantially absorb UV radiation. Pure (distilled) water is actually ineffective at absorbing ultraviolet radiation with only 2.1% absorption of 380 nm wavelength light occurring in a one-meter column of distilled water (Hutchinson, 1957). Although this observation may raise serious questions concerning the validity of Swain's (1975) hypothesis, the apparent contradiction can be readily resolved. Unlike distilled water, natural waters are highly effective as UV screens with one meter of lake

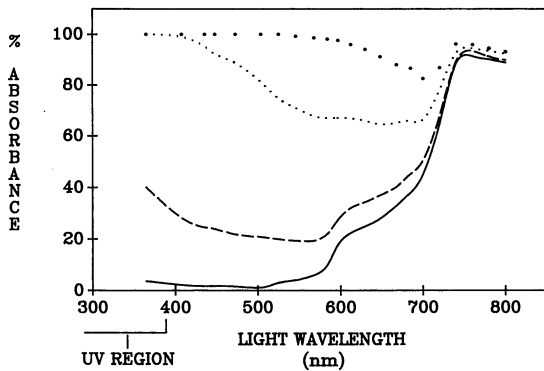


Fig. 3. Percent light absorption at different wavelengths in a one-meter column of water settled of particulates. Solid line = spectrum for distilled water (color index = 0); dashed line = spectrum for Crystal Lake, Wisconsin (color index = 0); small dotted line = spectrum for Adelaide Lake, Wisconsin (color index = 28); large dotted line = spectrum for Helmut Lake, Wisconsin (color index = 264). Plotted from data in James and Birge, 1938.

water (which has been settled of particulate matter) absorbing up to 100% of 365 nm wavelength light (Fig. 3). The greater UV absorption by natural water is a function of dissolved organic matter which can be in high enough concentrations to effectively filter UV even in water that appears clear to the naked eye.

The ability of natural water to screen against harmful UV radiation is a reasonable explanation for the lack of flavonoids in most algae and their occurrence in Charophyte algae, a group implicated evolutionarily with the ancestral complex leading to land plants. Our work with *Potamogeton* provides supporting evidence for this conclusion. Unlike the biochemically similar cells of *Chara*, heterophyllously in *Potamogeton* provides a much better model for studying biochemical differentiation between foliage exposed to intense UV radiation (i.e., the floating leaves) and foliage protected by UV filtering water (i.e., the submersed leaves). With our discovery of biochemical heterophylly in *Potamogeton* we suggest the possibility that flavonoid compounds perform adaptive functions associated with certain aspects of the environment. We observed a wider variety of flavonoid glycosides in floating leaves (3–6 compounds) compared to submersed leaves (0–4 compounds) in heterophyllous species. The foliage of submersed homophyllous species contained from 1–5 glycosides. A more definite pattern, however, is apparent when only glycoflavones are considered. Two glycoflavones (isovitexin and isoorientin) occurred in the floating leaves of all eight heterophyllous species examined, whereas isovitexin occurred in the submersed foliage of only

two of the species and isoorientin in the submersed foliage of only four of the species. In nine submersed homophyllous species, isovitexin is absent entirely and isoorientin occurs in only three (Table 3; Roberts and Haynes, 1986). The three homophyllous species containing isoorientin (*P. richardsonii*, *P. perfoliatus*, *P. praelongus*) occur within two allied broad-leaved subsections which are probably related closely to the heterophyllous species (Les and Sheridan, 1990). Flavonoid compounds in these species are detectable but occur in extremely low concentrations compared to floating leaf profiles. Accordingly, the presence of isoorientin in these homophyllous species is surely relictual. Isoscaparin occurred in floating leaves of three heterophyllous species, was absent in the submersed leaves of one of the species, and was lacking entirely in submersed homophyllous species. From these results we conclude that the general loss of glycoflavones in submersed foliage of *Potamogeton* species and their wide retention in floating foliage is indicative of their function as UV filters in floating leaves. The common loss of glycoflavones in submersed foliage and typical absence in submersed species may reflect an adaptive shift towards more efficient cycling of metabolic energy. The retention of glycoflavones in a few homophyllous species, however, demonstrates that adaptation to a submersed existence does not preclude the synthesis of these compounds (although concentrations were much lower in these species). Indeed, glycoflavones are found in several groups of submersed aquatic plants including *Ceratophyllum* (Les, 1983a) and *Cabomba* (Giannasi, 1988; D. H. Les, unpublished data). The pattern of flavonoid distribution in *Potamogeton* can be used in conjunction with other data to propose a generalized overview of flavonoid evolution in plants relating to their terrestrialization (Fig. 4). This scheme follows Swain's (1970; 1975) assessments that the evolution of UV screening glycoflavones represented an important step leading to plant terrestrialization. The emergence of land plants coincided with the evolution of many other types of flavonoid compounds. In addition to glycoflavones, flavonols and 8-hydroxy flavonoids (absorbing at longer wavelengths of 360–400 nm) may have provided additional protection against destruction of light-sensitive compounds such as FAD (Swain, 1975). By the time the angiosperms had evolved, the biosynthetic pathways for production of all major types of flavonoids existed. Our results substantiate the predicted loss of flavonoids in the submersed foliage of several heterophyl-

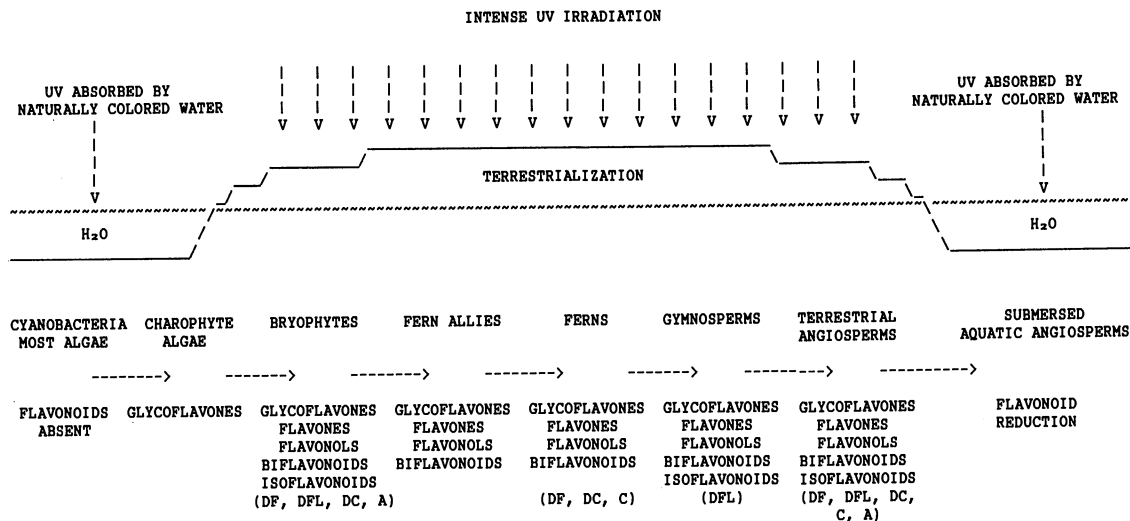


Fig. 4. Generalized scheme of flavonoid evolution accompanying major group radiations in the colonization of land by plants (from data summarized in Harborne, 1988, and this paper). Hypothetically, the UV screening properties of glycoflavones and other flavonoids contributed to plant survival in open landscapes where UV radiation was intense. Along with the return of terrestrial plants to the water is a characteristic reduction of their flavonoid profiles. Minor flavonoids are abbreviated: A = aurones, C = chalcones, DF = dihydroflavones, DFL = dihydroflavonols, DC = dihydrochalcones.

lous aquatic species. The retention of flavonoids in submersed foliage of some *Potamogeton* and other species may indicate that specific biological factors associated with a particular aquatic species may result in a different response to selection for retention of flavonoids. These factors may involve selection for UV screening compounds in shallow water habitats, or may involve other flavonoid functions entirely unrelated to their UV absorbing capacities.

Certainly, we do not imply that all flavonoids have been retained in angiosperms specifically because of their UV filtering ability. The various properties of these compounds undoubtedly perform many other functions that have been discussed elsewhere. We do, however, support the concept that a primary function of glycoflavones is to filter UV radiation. Given this assumption, the seemingly random distribution of glycoflavones throughout the plant kingdom may actually represent an ecological pattern. Harborne (1967, p. 246) noted that aside from grasses, glycoflavones are "otherwise uncommon in the monocots." Similarly, Bate-Smith (1968) noted the prevalence of glycoflavones in the monocots, especially in the Poaceae, Iridaceae, and Liliaceae. The occurrence of glycoflavones in grasses may reflect their ecological role as plants of open habitats where exposure to harmful UV radiation would be greater than for understory plants. Similarly, the occurrence of glycoflavones in the Cyper-

aceae and Lemnaceae (Chopin and Bouillant, 1975), also correlates with the exposed nature of their usual habitat. Glycoflavones occur in more than 50 angiosperm families including both monocots and dicots (Valant-Vetschera, 1985; Chopin and Dellamonica, 1988). The ecological aspects of this hypothesis could be tested further by comparing glycoflavone distributions within groups of related species that occupy habitats with different relative degrees of UV exposure.

Systematic significance of flavonoid evolution in Potamogeton—Because of the apparent variety of flavonoids in *Potamogeton*, their similarity among putatively related species, and their difference among putatively unrelated species, Roberts and Haynes (1986) remarked that such data should be important for improving the classification of the genus. We have found, however, that several factors limit the systematic use of flavonoid data in the group. A major difficulty involves the lack of precise evidence pinpointing the origin of submersed homophyllous species. In a recent study reexamining the classification of *Potamogeton*, we emphasize that there is insufficient evidence to determine whether the ancestors of submersed homophyllous species were also homophyllous or if they evolved from heterophyllous species by the loss of floating leaves (Les and Sheridan, 1990). Cluster analysis demonstrates a high degree of biochemical similarity among linear-

leaved homophyllous species and the submersed leaves of many heterophyllous species (Fig. 2). This observation offers some support for the hypothesis that at least some linear-leaved species possibly evolved from heterophyllous species by the loss of floating leaves. The clustering relationship of these groups, however, is primarily based upon their common lack of flavonoids rather than their shared occurrence of particular suites of compounds. If flavonoids are indeed primarily adaptive in floating foliage, then this pattern in submersed foliage could as easily reflect a convergent loss of compounds as it could a phylogenetic relationship. It is also possible that some patterns of flavonoid distribution are related to polyploidy (see Giannasi and Crawford, 1986) which is prevalent in *Potamogeton* (Les, 1983b).

Interestingly, the biochemical intermediacy of the broad-leaved homophyllous species between the linear-leaved homophyllous species and heterophyllous species by cluster analysis (Fig. 2) is consistent with patterns of relationship depicted both by dendrograms and cladograms derived from morphological data (Les and Sheridan, 1990). The clustering of *P. zosteriformis*, a linear-leaved homophyllous species, with broad-leaved submersed species cautions that intermediary stages undoubtedly exist in this proposed sequence.

With these noted precautions in mind, we have interpreted the flavonoid data to support the following assessment of evolutionary relationships in *Potamogeton*. Biochemical heterophylly in morphologically heterophyllous species, and similar losses of flavonoids in their submersed foliage and in submersed homophyllous species, supports the hypothesis that some homophyllous pondweed species evolved from heterophyllous ones by loss of floating leaves. The biochemical similarity of the foliage of broad-leaved homophyllous species and the floating leaves of heterophyllous species indicates their potentially close evolutionary relationship. Because an adaptive function as UV screens is attributed to glycoflavones, their presence in broad-leaved submersed species is assumed to be relictual and indicative of a phylogenetic tie to heterophyllous species. Heterophyllous species that retain a diversity of flavonoids in both floating and submersed foliage are regarded biochemically to be the more primitive species in the genus. The ubiquitous occurrence of glycoflavones in the floating leaves of all heterophyllous species examined may indicate an evolutionary relationship, or may reflect convergence due to the adaptive significance of these compounds. Flavonoid data support the hypothesis

that linear-leaved homophyllous species do not represent the primitive group of pondweeds (Les and Sheridan, 1990). They do not, however, clarify the question of whether homophyllous broad-leaved species or heterophyllous species represent the most primitive group. For this question, a flavonoid survey of the related genus *Groenlandia* (see Les and Sheridan, 1990) may provide more conclusive data. These conclusions are summarized in a scheme that reflects our concept of probable evolutionary relationships between morphological groups in *Potamogeton* (Fig. 5).

The nature of phyllodal leaves in Potamogeton natans—The homology of phyllodal monocot leaves and dicotyledon petioles was proposed over a century ago and is still avidly supported by several authors (e.g., Cronquist, 1988). Morphological transition series and unusual vascular bundle configurations of leaves in genera such as *Sagittaria* and *Potamogeton* have provided compelling evidence for acceptance of the "phyllode theory" (Arber, 1920). Perhaps the most widely recognized occurrence of phyllodal leaves in *Potamogeton* is in the submersed foliage of *P. natans*. Phyllodal submersed leaves occur sporadically in *P. lucens* (Arber, 1920) and we have commonly observed them in the related species *P. illinoensis* (Sheridan 88-005-UWM).

Potamogeton natans possesses phyllodal submersed leaves almost exclusively, but a morphological transition series (including some submersed leaves with rudimentary blades) is often evident if enough plants are examined (Fig. 6). From this series, it is reasonable to conclude that the submersed phyllodes of *P. natans* are derived from floating leaves by loss of the distal lamina, i.e., that morphologically, they represent petioles. We examined the petiole and blade of floating leaves and the submersed phyllodal leaves of *P. natans* independently to observe whether the submersed leaves were similar biochemically to the floating leaf petioles. Our analysis identified a total of eight compounds in the floating leaf blade, four compounds in the floating leaf petiole, and only one compound in the submersed leaves (Table 4). The flavonoid differences among these tissues indicate that although submersed leaves may be similar morphologically to floating leaf petioles, they differ from them biochemically. This is probably a reflection of some aspect of physiological specialization in submersed foliage.

The biochemical series also provides evidence pertinent to the origin of linear-leaved homophyllous species. The linear-leaved

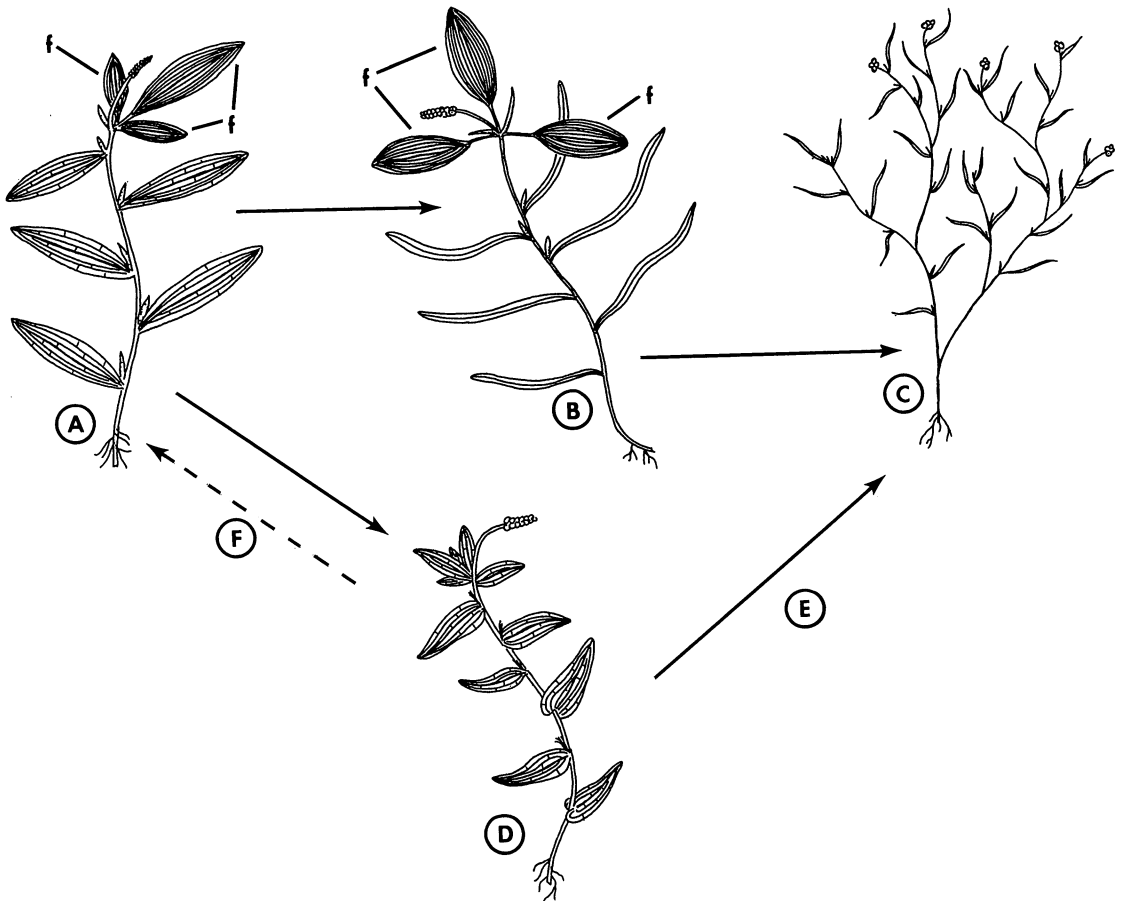


Fig. 5. Hypothetical trends of morphological and biochemical evolution in *Potamogeton*. A, indistinct heterophyllous and diverse flavonoid profiles characterized ancestral pondweed species (e.g., *P. alpinus*). B, morphological and biochemical specialization led to distinct heterophyllous with flavonoid reduction in submersed foliage (e.g., *P. epihydrus*). C, homophyllous linear-leaved species with reduced flavonoid profiles (e.g., *P. foliosus*) may have arisen from distinctly heterophyllous ancestors by loss of floating leaves. D, broad-leaved homophyllous species with diverse flavonoid profiles (e.g., *P. richardsonii*) may have arisen from indistinctly heterophyllous ancestors by loss of floating leaves. E, linear-leaved homophyllous species possibly arose from broad-leaved homophyllous species by morphological specialization and flavonoid reduction. F, Alternately, broad-leaved homophyllous species were ancestral and gave rise to heterophyllous species by morphological specialization of floating and submersed foliage. f = floating leaves.

homophyllous species of subgenus *Coleogeton* possess strictly phyllodal leaves. Examined anatomically, the structure of the submersed leaf of *P. natans* and that of *P. pectinatus* (subgenus *Coleogeton*) is nearly identical (see Ogden, 1974, pp. 74–75). If submersed leaves in *P. natans* are derived from floating leaves, then their loss of flavonoids indicates the possibility of a biochemical reductionary sequence in *Potamogeton*. Specifically, species with a wide variety of flavonoids (such as heterophyllous and broad-leaved homophyllous species) are probably ancestral to those with diminutive flavonoid profiles (such as homophyllous linear-leaved species). Furthermore, these observations strengthen the likelihood that some linear-leaved homophyllous species (such as

members of *Coleogeton*) were ultimately derived from heterophyllous ones. Therefore, the biochemical data support the conclusions of major evolutionary trends in the genus made on the basis of chromosomal data (Les, 1983b) and morphology (Les and Sheridan, 1990).

CONCLUSIONS—Biochemical heterophyllous in *Potamogeton* provides evidence in support of Swain's (1970; 1975) hypothesis that glycoflavone evolution was an important step in the terrestrialization of plants. Selection to retain UV screening glycoflavones in foliage of aquatic plants directly exposed to sunlight (e.g., floating leaves) is probably much greater than for submersed foliage because of the capability of naturally colored water to absorb harmful UV

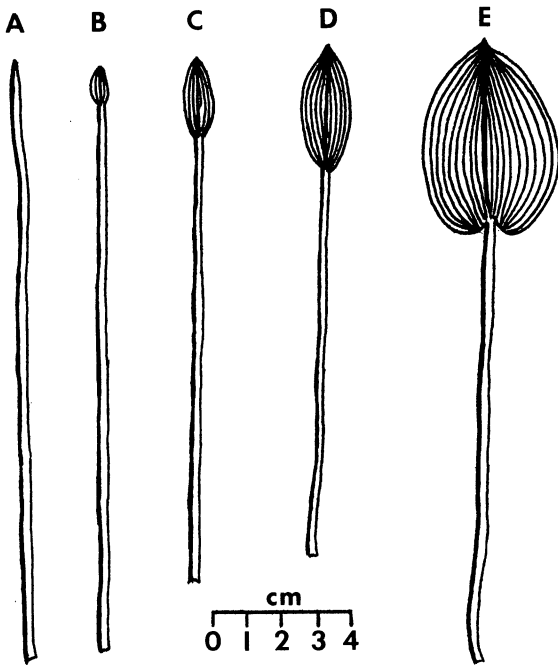


Fig. 6. Leaf variation in *Potamogeton natans* (drawn from Les 479-UWM). A, phyllodal submersed leaf. B, C, small-bladed submersed leaves. D, small-bladed floating leaf. E, "normal" floating leaf.

radiation. In *Potamogeton*, a wide variety of flavonoids, including glycoflavones, occurs in floating leaves of all eight heterophyllous species examined. The submersed foliage of heterophyllous species and of homophyllous species is characterized by a highly reduced flavonoid profile. We interpret this pattern as a result of compound losses from ancestral species with diverse flavonoid profiles. The origin of homophyllous pondweed species has probably involved different evolutionary pathways. Biochemical data indicate that some linear-leaved homophyllous species may have evolved gradually from heterophyllous species or from broad-leaved homophyllous species, whereas others have probably arisen directly from biochemically heterophyllous species by loss of floating leaves. These conclusions are consistent with hypotheses derived independently by analyses of morphological data. It would be informative evolutionarily to survey heterophyllous water plants of other genera to determine whether similar patterns of biochemical flavonoid heterophyly exist elsewhere in the angiosperms. Glycoflavone occurrences in aquatic and terrestrial plants should be reevaluated ecologically to ascertain whether these compounds occur more frequently in species of open habitats than in those of shaded or submersed habitats.

When the earth was first colonized by land plants, low atmospheric ozone and oxygen concentrations resulted in potentially destructive UV levels at the surface, and probably promoted flavonoid evolution in the emerging terrestrial flora (Markham, 1988). Ironically, the culturally enhanced depletion of earth's ozone layer is predicted to result in future increases of UV- β penetration (Brasseur and Hitchman, 1988). If such trends continue, they will undoubtedly influence the course of biochemical evolution in plants. Aquatics and other species that have lost the ability to synthesize UV filters such as flavonoids may be destined to face accelerated extinction rates.

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