

To the Graduate Council:

I am submitting herewith a thesis written by Alan B. Adams entitled "A Study of Trillium cuneatum and T. luteum." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

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Thesis
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A STUDY OF
TRILLIUM CUNEATUM AND T. LUTEUM

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Alan B. Adams

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ABSTRACT

A systematic study was made of two taxa of the genus Trillium subgenus Phyllantherum. Morphological, biochemical, ecological, and distributional data were analyzed in order to reevaluate past taxonomic treatments of Trillium cuneatum and T. luteum.

Morphological studies revealed the similarities of the two taxa. It was noted that T. cuneatum exhibited great plasticity with respect to morphology, whereas T. luteum showed more homogeneous features. This same relationship was noted with the flavonoids of the two taxa with T. cuneatum showing more variability than T. luteum. The chemistry of the two taxa was unique in that quercetin and kaempferol compounds identified portrayed significant glycoside diversity. A survey of past literature dealing with similar phenolic compounds resulted in an elucidation of the color variation seen within allopatric populations of T. cuneatum and led to a belief that T. luteum originally evolved from T. cuneatum as a homozygous recessive form which was favored due to more efficient sexual reproduction accompanying apomictic reproduction.

Scanning electron micrographs revealed different types of pollen grains which appeared to have taxonomic significance. The abaxial surfaces of the bracts apparently

were identical. Photomicrographs were useful also in revealing spiders as possible pollinating agents within the genus. Predator--prey relationships and cryptic coloring are discussed as possible factors affecting reproductive success of the plants.

Recent taxonomic treatments were reviewed and possible taxonomic considerations were considered. Based on the results of this study and past work of other botanists, it was concluded that the two taxa should be recognized as distinct species in keeping with traditional treatments. It is believed that the two taxa represent a good case of directional selection resulting in divergent evolution. Whether this divergence will result in more speciation in the future or whether the two taxa will merge into a single unit via introgressive hybridization, is a stimulating academic consideration.

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INTRODUCTION

Trillium is a genus of rhizomatous perennials that develops one to several shoots each spring. Upon the shoot are located three leafy organs and a single flower that usually has three sepals, three petals, two whorls of three stamens, and a tri-locular gynoecium with axile anatropous ovules. These plants are located in the remnants of the Arcto-Tertiary forests, which consist of moist deciduous or coniferous woods restricted to three areas of the northern hemisphere: eastern Asia, the Pacific Northwest, and eastern North America. The flower may be pedunculate or sessile, and this character constitutes a major dichotomy within the genus with the sessile-flowered plants theoretically being derived from the pedicilate group. This evolutionary reduction is believed to have been monophyletic.

Two recent studies were carried out at Vanderbilt University on the sessile-flowered Trillium subgenus Phyllantherum by John Freeman (1969) and James Murrell (1969) in which many of the taxonomic problems inherent to this subgenus were solved. Both these studies were intensive and commendably executed, however, the very thoroughness of their research resulted in the creation of many new areas of study within this group of plants. Indeed, the studies were such as to provide an impetus for future

research and were used as a focal point for initiating this study of two of the sessile-flowered taxa of Trillium.

The two taxa included in this study are common to east Tennessee, west North Carolina, southeast Kentucky, and northwest Georgia and have long been a source of taxonomic confusion. Both of the taxa are seemingly morphologically similar except for one noticeable difference, the petals of one (Trillium cuneatum) are red, whereas those of T. luteum are yellow. Also, the two are allopatric (i.e., geographically distinct) over much of their ranges. Within populations of T. cuneatum located outside the distribution of T. luteum, an occasional yellow-flowered form does occur. This plant has been accorded the rank of form (T. cuneatum forma luteum) and does not correspond to T. luteum (Muhl.) Harbison.

At the beginning of this study it was believed that a detailed examination of the flavonoids of these two taxa might possibly yield data useful to future classification schemes. This was indeed found to be the case. Reexamination of preestablished distribution patterns, scanning electron microscopic work with pollen grains and leaf surfaces, and some elucidation of plausible reproductive mechanisms and vectors of dispersal have provided some insight into possible modes of origin not only of T. luteum, but also of the other non-red forms of this subgenus (i.e., T. albidum and T.

discolor). In addition, an explanation for the color variation seen within populations of T. cuneatum is discussed.

CHAPTER I

GENERAL CONSIDERATIONS

Generic And Subgeneric Affinities

Trillium has generally been considered to be a member of the tribe Parideae (Liliaceae), which consists of four genera, all of which are restricted to the temperate forests of the northern hemisphere. Medeola (Gronov) L. is a monotypic genus restricted to the deciduous forests of the eastern United States. It possesses an aerial stem and has an umbellate inflorescence above two whorls of leaves. Scoliopus Torr. is a genus of two species both of which are native to the west coast extending from the northern redwood forest to the western slopes of the Cascade Mountains. It is characterized by a nearly sessile umbellate inflorescence with one to several flowers on long slender pedicels. Paris (Tourn. ex Rupp.) L. is a polymorphic Eurasian genus of approximately twenty species characterized by four or more parted flowers. Medeola is theoretically ancestral to the other three genera with Trillium supposedly representing the most reduced member of the alliance having a rhizome, usually having no foliage leaves, and possessing a single flower. "Verticelled foliation supposedly links the tribe Parideae through Medeola to subfamily Asparagoideae and to the Dioscoreaceae" (Freeman, 1969).

Taxonomic problems begin at the family level with some botanists removing the tribe from the Liliaceae and placing the four genera in the Smilacaceae or Convallariaceae, whereas Hutchinson (1959) and Porter (1959) have treated this alliance of plants as a distinct family (Trilliaceae). Recently, the intratribal alliance of the Parideae has been criticized. The hypothetical reduction series is based on morphological similarities, and the belief that teratological forms express ancestral "throwbacks." I have witnessed four bracts on a Trillium which resembles Paris, and on occasion two whorls of bracts have been observed that are very reminescent of the whorled foliage of Medeola (Weston, 1916). Scoliopus and Medeola have a haploid number of seven while Trillium and Paris both possess a haploid number of five. In Japan Trillium exhibits a polyploid series and Paris is found as a tetraploid in the Himalayas (Hara, 1971). Berg (1962) has presented embryological data strongly contradicting the Parideae alliance. He believes that Medeola and Scoliopus are unrelated to Paris and Trillium as well as to each other, but holds that the latter two genera are closely aligned.

The genus Trillium is believed to have existed intact within the remnants of the Arcto-Tertiary forest for the last 40 to 50 million years (Stebbins, 1971). This remarkable stability could be related to the five pairs of chromosomes found in every Trillium population tested within

North America. Stebbins (1950) states that these chromosomes are among the largest found in the plant kingdom.

Freeman (1969) has set up three informal divisions within the subgenus Phyllantherum which perhaps are presented best in his tabular form:

Group I--The Trillium recurvatum group. . . . Delimited by the basally recurved sepals, strongly incurved anthers and slender elongated rhizomes--occurring only in the eastern United States.

Group II--The Trillium sessile group. . . . Delimited from Groups I and III by the predominance of species with prominently prolonged anther connectives, very sharply angled ovaries and usually linear stigmas--sharing with Group III characteristically thick compact rhizomes. A relatively heterogeneous group--each species standing well apart from others. . . . Limited to the eastern United States, except for T. petiolatum.

Group III--The Trillium maculatum group. . . . characterized by the predominance of species with lateral anther dehiscence, anther connectives little prolonged, if at all, and stigmas usually subulate or thickly subulate. Essentially a residual group including species not referable to either I or II, but nevertheless exhibiting close affinities for one another; constituents rarely occur sympatrically, but often grow with members of I and/or II. Includes species of both eastern and western United States.

Trillium cuneatum and T. luteum are placed within Group III.

Morphology

The sepals of T. cuneatum and T. luteum are oblong and alternate with the slightly clawed petals. The stamens of the outer whorl are slightly larger than the inner, and both display two types of anther dehiscence, lateral or

introse. Although most structures are usually glabrous, the veins on the abaxial surface of the bracts are occasionally pubescent near the scape. The obovate, foliaceous bracts are always sessile and usually mottled. In four cases T. luteum plants were observed with non-mottled bracts, and although this condition was not noted with any T. cuneatum plants, it has been reported. Variations in the amount and/or intensity of leaf mottling showed no correlations when contrasted between the two taxa, among individual populations, or within populations.

One interesting feature was noted with respect to the mottling of one T. luteum plant. On the regularly mottled leaf pattern were yellow and dark green spots on a light green background. This phenomenon seems to be identical to that described by Vig (1973) on the leaves of Glycine max. These mosaic patterns have led some geneticists to conclude that crossing over is occurring during mitosis as well as in the first division of meiosis. One result of mitotic crossing over is that one-half of the daughter cells may be rendered homozygous recessive for any locus distal to the centromere and point of exchange. Recessives that were previously masked by dominant alleles in the parent cells become homozygous and are found as recessive phenotypes in daughter cells. Single and twin spots found in many organisms might be a type of genetic exchange in somatic tissues that resulted in cells that

were homozygous recessive for chlorophyll production. Non-meiotic crossing over may possibly provide information regarding the genetic structure of organisms in which meiotic cycles do not occur or in which the meiotic cycle is difficult to observe.

Freeman (1969) states that T. cuneatum represents the most variable taxon within the subgenus Phyllanthum. I also have noted this polymorphism in gynoecea, petal shapes and colors, leaf shapes, and especially in stigmas and type of anther dehiscence. Apparently, this variation is not attributable to physiographic provinces or other environmental factors. In many cases all the variability referred to above may be found within one population. Within populations sympatric with T. cuneatum, T. luteum also is found to display this plasticity at several locations, but T. luteum exhibits marked morphological homogeneity relative to T. cuneatum within allopatric populations. Often within one population T. cuneatum plants were found that had introse dehiscence while other plants displayed lateral dehiscence. Within the same populations were found plants with thickly subulate, coalescent stigmas as well as other specimens with divergent or spreading stigmas that were not as subulate. Within allopatric populations of T. luteum were found stamens possessing only introse dehiscence and thickly subulate, coalescent stigmas with very papillose surfaces. Pictures of T. luteum and T. cuneatum are presented in Figures 1 and 2.



Figure 1. Cluster of Trillium luteum plants.



Figure 2. Trillium
cuneatum.

Pollination And Seed Dispersal

Due regard should be given to both pollination mechanisms and vectors of seed dispersal when considering natural plant distributions, especially when it is probable that the taxa being considered are competing for the same resources. One of the chief factors affecting the reproductive success of competing species could well be the mechanisms of cross-pollination and incomplete self-fertilization with hybridizing (Lewis, 1967), as well as non-hybridizing (Levin, 1975), populations. Seed dispersal might possibly explain movements of gene frequencies in sympatric populations.

The pollination ecology of the genus Trillium is little understood and has not been thoroughly studied to date. According to Berg (1958) two types of insect-pollinated flowers are found in the genus: bee-pollinated flowers with a pleasant fragrance, and fly pollinated flowers with an ill scent. Patrick (1975) has recently conducted work on the pollination activity of five species of Trillium. Beetles (Coleoptera) were found to be the most common effective pollinators followed by flies (Diptera), thrips (Thsanoptera), wasps (Hymenoptera), and true bugs (Hemiptera). Representatives from all five of the orders listed above were found to visit T. cuneatum (T. luteum was not included in this study). Patrick noted ". . . that the coloration criterion for the beetle polli-

nation syndrome, namely that flower colors are dull, is reasonably met by . . ." the sessile-flowered Trillium that he studied in that they were all deep purple to greenish purple. However, fragrances could also be a factor affecting visitation of pollinators, and Patrick notes that several fragrances are detectable within the genus. Fukuda (1967) and Ihara (from Patrick, 1975) also have concluded that beetles are the most probable cross-pollinating agents of Trillium. Spiders of two families were noted as being phytophagous by Patrick and were found on Trillium plants in many instances. Although not enough data was available to conclude that spiders are pollinators, Patrick concluded that they certainly possess the capability.

Self-pollination has been reported in T. recurvatum (Robertson, 1896) and T. chloropetalum (Brandt, 1916). Ihara (from Patrick, 1975) has confirmed self-pollination with T. sessile.

Berg (1958) believes that seed dispersal was originally by endozoochory (i.e., dispersed by birds) and has presently become myrmecochorous (i.e., dispersed by ants). Some adaptive features associated with myrmecochory displayed by reduced species of sessile-flowered Trillium include an aril or elaisome, on the seed, a reduced peduncle, and a reduction of the scape. The first two features are found with T. luteum and T. cuneatum.

Past Taxonomic Treatments

Systematic treatments of these two taxa have been inconsistent and often have inadvertently resulted in creating even more confusion rather than clarifying any of the taxonomic problems. For example, Fernald (1950) used ambiguous characters for separating T. luteum from T. cuneatum. He noted that the leaves of T. luteum were more mottled than those of T. cuneatum. Personal observation has resulted in the unavoidable conclusion that this character is not correct (both exhibit extreme variability with respect to mottling) and is also impossible to quantify. Also, Fernald recorded petals as being 4 to 6 cm long in T. luteum versus 4 to 11.5 cm long in T. cuneatum. I have consistently found petals longer than eight cm in T. luteum populations, a few approaching ten cm in length, and on occasion whole clusters of T. luteum all with petals longer than six cm. Likewise, I have noticed that the petals will grow in length considerably after anthesis if the plant remains healthy. These characters appear to be variable and inconsistent, and would therefore be difficult characters for taxonomic purposes.

Some workers have treated T. cuneatum and T. luteum as distinct species (Freeman, 1969), others have treated them as varieties of the same species (Radford, et al., 1968), and Sarota (1969) has treated the two taxa as indistinguishable from one another. Sarota examined six populations

differing in flower scent, flower color (two red, two yellow, and two variable), and degree of geographical isolation. Using coefficients of variability based on morphological and cytological data, she concluded that the populations were very similar in morphology, breeding mechanisms, ecology, and karyotypes and were, therefore, not sufficiently differentiated to warrant taxonomic recognition.

On the other hand, Freeman (1969) recognized them as distinct species for the following reasons: 1) the essentially allopatric distributions of the two taxa with T. luteum being common to ". . . the Ridge and Valley and Appalachian provinces of eastern Tennessee and western North Carolina," and T. cuneatum being distributed in ". . . the Blue Ridge and Piedmont provinces from North Carolina into Alabama, northward . . . into southern Kentucky and southwestward through the Upper Coastal Plain of Mississippi to the Mississippi River." He noted, however, that the distributions do overlap in some areas and populations with plants exhibiting intermediate characters are found within these restricted regions of sympatry; 2) color differences of petals, stamens, and gynoecia; 3) difference in odor with T. cuneatum being spice-scented and T. luteum possessing a lemon scent; 4) to change T. luteum from a distinct species to a taxon of lower rank would result in even greater confusion within the literature where the name is well known, accepted, and used in botanical works; 5) any

nomenclatural change would be ". . . premature in the light of present knowledge of interspecific relationships."

Recent chemical work on T. cuneatum and T. luteum has likewise been inconclusive for solving the taxonomic problems. Paper chromatographic work was done by Murrell (1969) in which he found 51 fluorescent compounds in T. luteum and T. cuneatum of which seven were unique to the former, eight were unique to the latter, and 36 were found in both. Although these data are subject to wide interpretation, Murrell felt that they stress the similarities of the two taxa and, therefore, tend to support the point of view that T. luteum and T. cuneatum are not significantly different. It should be noted, however, that Murrell decided to divide the sessile-flowered Trillium into two groups--one possessing much interspecific variation (*i.e.*, much biochemical variation), the other showing little variation of phenolic compounds. Trillium cuneatum and T. luteum were classified with the latter. Thus, the case could be made that any biochemical differences between the two taxa are significant.

On the other hand, Murrell and Channell (1973) have published gas chromatographic data on fragrance variation between T. cuneatum and T. luteum that tend to stress the differences of the two taxa. Twelve compounds were isolated of which only two were common to both taxa, yet even these two showed a three-fold quantitative difference.

Quantitative variations have been shown to be reliable taxonomic criteria within genetically distinct individuals (von Rudloff, 1969; Hunt and von Rudloff, 1973; etc.). Although only one compound was positively identified (the alcohol linalool), it is believed that this is the substance responsible for the lemony odor of T. luteum. The authors concluded that these data alone were not sufficient to render T. luteum a taxonomic species; however, it does provide some support to Freeman's ranking of T. luteum at the specific level. Channell and Murrell point out that the evidence from chemical studies ". . . emphasizes the importance of a synthesizing approach to the final solution of this taxonomic problem, using information from all possible sources."

The distributions of T. cuneatum and T. luteum are shown in Figure 3. Although two populations of T. cuneatum were found within Knox County, Tennessee, these are not shown on the map because in both cases it is known that T. cuneatum was transplanted from an area outside the allopatric range of T. luteum. Locations of intermediate type plants are shown also in Figure 3 and listed on page 24. Freeman (1969) refers to intermediate type plants that are located where the Cumberland River cuts through the Cumberland Plateau but no sites are specified and the nature of the morphological variation is not discussed. Murrell and Channell (1973) and Sarota (1973) discussed variation in which fragrance is not typically

Figure 3. Distribution map of Trillium cuneatum and Trillium luteum.

The distribution of T. cuneatum is indicated by lines running in a southwest or northeast direction, whereas the distribution of T. luteum is indicated by lines running in a southeast or northwest direction. Intermediate populations are noted by circles with crosses. Specific locations of plants utilized in this study are listed on page 24.

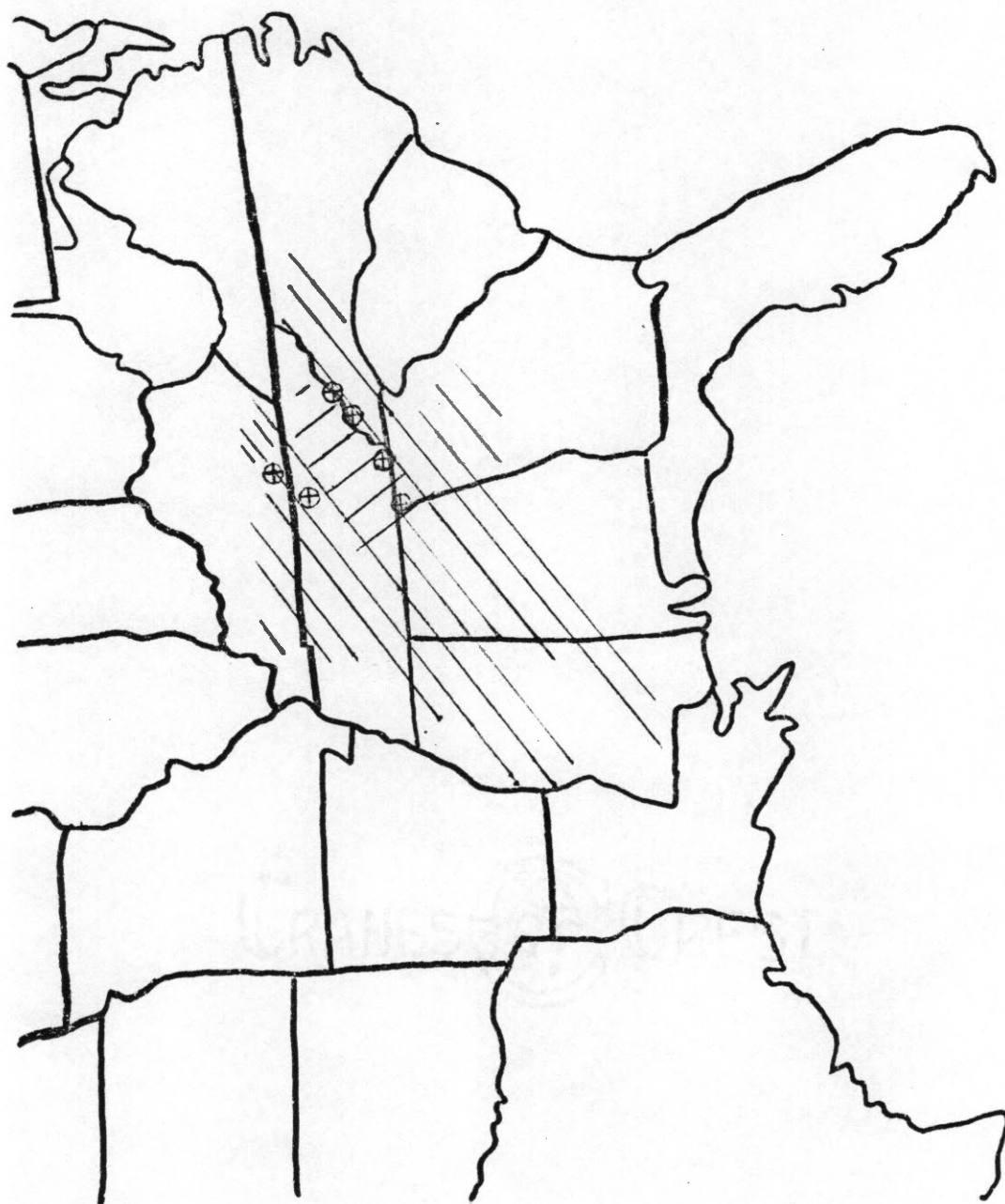


Figure 3

lemony with yellow plants nor spicy with T. cuneatum. Many of the plants seemed to possess no odor at all. I have likewise noted this odor variability but also have observed that the color variation within the petals is considerably higher within sympatric populations than in pure populations of T. cuneatum.

Non-flavonoid Chemical Constituents

A recent, thorough review of the chemical constituents of Trillium is presented by Murrell (1969), and his work will be discussed briefly. Saponins appear to be found throughout the genus (Marker, 1947). The only protein work known is that of Hall (1961) who utilized gel electrophoresis to correlate high protein content to teratology in T. grandiflorum with both teratological bracts and petals. Raphides (minute crystals of calcium oxalate) and chelidonic acid appear to occur throughout the genus.

CHAPTER II

THE USE OF FLAVONOIDS IN TAXONOMIC WORK

General Review

The application of chromatographic techniques, especially paper chromatography, to botanical problems has resulted in rapid and convenient methods for studying biochemical relationships between taxa, evolutionary pathways, ecological differentiation, and other features of difference. Phenolic substances have proven very useful as criteria in taxonomic work.

Flavones, flavanones, and flavonols are so common throughout the plant kingdom that one might question the validity of the preceding paragraph. For example, Lemnaceae and Asteraceae both contain flavone and flavonol glycosides, yet one could hardly say that they are closely related (McClure, 1966; Bierner, 1974). On the other hand, clear relationships can often be ascertained from the distribution of aglycones as is seen with Dugaldia and Helenium. Bierner (1974) has discovered that Dugaldia (a group of plants formerly grouped with Helenium) contains flavonols, whereas Helenium contains only flavones.

The distribution of some of the more unusual flavonoids can also be taxonomically significant. For instance,

it is characteristic for many of the genera of the family Rutaceae to have polymethoxyflavones due to a system in which methionine acts as a methyl donor (Geissman and Grout, 1969). Likewise, 6-methoxyflavonoids seem to be common only to certain genera of the Asteraceae (Mears, 1973).

Elucidation of phenolic constituents has often proven quite useful in proving the existence of and analyzing hybrid complexes. Additive effects have been noted in which compounds present in one taxon but not the other are found in intermediate quantities within F₁ hybrids (i.e., Mendelian codominance). Hybrid complementation was first noted by the work of Alston and Turner (1962, 1963, 1965), and is very well documented in the classic paper of Smith and Levin (1963) who worked with the Asplenium complex. Although introgressive work with flavonoids is possible, its significance is less accepted and more difficult to prove.

"Also interspecific hybridization is one means of subjecting coadapted gene complexes to a manifestly different genetic milieu" (Levy and Levin, 1971). One can not rule out the possibility of hybridization and introgression resulting in a loss of parental compounds or a gain of novel substances. With speciation resulting from hybridization, additivity of parental compounds might not always be the case, as is seen with the amphidiploid Lasthenia microglossa (Bohm, et al., 1974).

The determination of inter- and intrapopulational chemical variation is requisite to relevant chemosystematic work because chromatographic patterns can provide stable and reliable taxonomic criteria only if there is a relatively low level of intraspecific variability. Low levels of variability should be found when individuals are sampled from the same or different geographic area, when populations are sampled at the same time of the year, or at different stages of their life cycle. When repeatable qualitative variation occurs, it is indicative of genetic differences, not environmentally induced differences between taxa showing pattern variation (Brehm, 1966). If a compound's presence or quantity is influenced by factors other than genetics, (e.g., seasonal, climatic, or environmental factors), then the compounds usefulness to systematics is considerably lessened, if not completely abolished. There is a need to establish parameters of variability in pattern components (i.e., phenolic content). The fact that flavonoids can yield consistent chromatographic patterns is illustrated well by the work of McClure and Alston (1966) with clone cultures of Lemnaceae. These repeatable phenolic patterns were systematically significant because each taxon portrayed unique compounds.

Identifying phenolic compounds not only avoids the necessity of making tenuous assumptions, but also allows for a more meaningful assessment of the systematic implications

of the chemical data. This is exemplified with Crawford's work with Chenopodium (Crawford and Dorn, 1974) in which two species exhibited spots with identical R_f values and the same color reactions, yet spectral analysis revealed that the spot of one taxon was a glycoside of quercetin and that of the other a glycoside of isorhamnetin.

Past Work With Trillium

The only previous identification of flavonoids within the genus has dealt with two anthocyanins. Asbury (1974) has worked with the two anthocyanins of T. cuneatum plus other sessile-flowered species of Trillium. One of these pigments has been characterized as cyanidin 3- di-arabinoside, and the other pigment also has been identified as a cyanidin type. Kozuka (1963) found five pedicellate species and one sessile-flowered species to possess two anthocyanins with identical R_f values in one organic solvent system. Murrell (1969) found that all the members of the sessile-flowered subgenus have two anthocyanins with identical R_f values in two solvent systems (one organic, the other aqueous). Based on these results with paper chromatography, it seems quite likely that only two anthocyanins are found within the genus and that they differ only by the nature of the sugar constituents (Murrell, personal communication). As far as is known, no flavonoids besides the cyanidins have been identified within the genus previous to this study.

CHAPTER III

FLAVONOID STUDIES

Materials And Methods

General. Flavonoid studies were conducted on 18 populations of T. luteum, 11 populations of T. cuneatum, and 7 populations of intermediate type plants. Voucher specimens of the populations studied are deposited in the University of Tennessee Herbarium and are listed in Table I. Most plants were pressed in the field, dried, and stored until needed. No significant qualitative variation in flavonoid content was detected in plants collected before flowering, at anthesis, or shortly after flowering; therefore most plants were collected at anthesis for taxonomic purposes.

For paper chromatography and spectral analysis, the techniques of Mabry, Markham, and Thomas (1970) and Crawford (1973) were utilized. Phenolics were extracted from dried leaf material two to four times in 85 per cent methanol over a 48 hour period, and each extraction was filtered through Whatman 1 MM paper. The extract was then concentrated with a rotary evaporator in vacuo. Fresh material was extracted with two to three changes of boiling 95 per cent ethanol, filtered, and concentrated. Extracts were

TABLE I

POPULATIONS OF TRILLIUM EXAMINED FOR FLAVONOIDS
AND SCANNING ELECTRON MICROSCOPY

T. luteum

1. Tennessee: Blount Co. At Bote Mt. trailhead between Townsend and Cades Cove on Foothills Parkway. ABA 1
 2. Tennessee: Blount Co. On the north facing slope of Rich Mt.^{a,b} ABA 5
 3. Tennessee: Bledsoe Co. Two-tenths of a mile west of Summer City on Hwy. 30 on west side of Walden's Ridge just below crest. ABA 22
 4. Tennessee: Bledsoe Co. Nine miles north of Pikeville on Hwy. 127.^b ABA 27
 5. Tennessee: Bledsoe Co. Thirteen miles south of Pikeville on east side of Hwy. 127.^b ABA 30
 6. Tennessee: Anderson Co. Located along creek in deciduous forest three-tenths of a mile off Hwy 441 3.6 miles north of junction with Hwy. 61.^b ABA 34
 7. Tennessee: Campbell Co. Five miles north of Caryville on Hwy. 25. ABA 9
 8. Tennessee: Campbell Co. Eight miles north of Caryville on Hwy. 25. ABA 8
 9. Tennessee: Cumberland Co. Fifteen miles south of Crossville on Hwy. 127. At the headwaters of the Sequatchie River.^{a,b} ABA 28
 10. Tennessee: Knox Co. On the University of Tennessee woodlot near Hwy. 129 approximately 1 mile south of the Tennessee River.^{a,b} ABA 7
 11. Tennessee: Knox Co. Plant collected in 1931 on Cherokee Bluffs. Whitson NC
-

Table I (continued)

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- | | | |
|-----|---|---------------|
| 12. | Tennessee: Knox Co. Ijams Park. ^b | <u>ABA 26</u> |
| 13. | Tennessee: Morgan Co. Five miles north of Harri-
man on Hwy. 27. | <u>ABA 23</u> |
| 14. | Tennessee: Sequatchie Co. On Hwy. 8 approximately
one mile west of Hwy. 127. ^{a,b} | <u>ABA 29</u> |
| 15. | Tennessee: Sevier Co. Between Gatlinburg and Cher-
okee Orchards on bank of Le Conte Creek. ^b | <u>ABA 17</u> |
| 16. | Tennessee: Sevier Co. On U.S. 441 approximately
eight miles south of Gatlinburg. ^b | <u>ABA 18</u> |
| 17. | Tennessee: Sevier Co. Plant collected in 1934 at
a site just above Gatlinburg (2500'). <u>Jennison 311</u> | |
| 18. | Tennessee: Sevier Co. Five-tenths of a mile north-
east of Sugarlands Ranger Station. ^b | <u>ABA 32</u> |

T. cuneatum

- | | | |
|-----|--|---------------------|
| 19. | Georgia: Dade Co. Just west of Interstate 59,
approximately nine miles south of Tennessee state
line. ^b | <u>ABA 455</u> |
| 20. | Georgia: Dade Co. On Hwy. 143 between Trenton and
Cloudland Canyon State Park. ^{a,b} | <u>ABA 456</u> |
| 21. | Kentucky: Christian Co. Approximately eleven miles
north of Tennessee state line along Hwy. 41. ^{a,b} | <u>Phillipe 453</u> |
| 22. | Kentucky: Whitley Co. On Hwy. 904, 3.9 miles east
of junction with Hwy. 92. | <u>ABA 404</u> |
| 23. | Tennessee: Davidson Co. In dry woods one mile
from Wilson Co. line on south side of Interstate
40. ^{a,b} | <u>ABA 457</u> |
| 24. | Tennessee: Davidson Co. On Hwy. 70 located 6.5
miles east of Pegram Station. ^b | <u>ABA 462</u> |
| 25. | Tennessee: Davidson Co. Along Hillwood Drive
approximately one-fourth mile south of Hwy. 70. At
junction of Forestwood. ^b | <u>ABA 461</u> |
-

Table I (continued)

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- | | | |
|-----|---|---------------------|
| 26. | Tennessee: Giles Co. Plant collected in 1949 northwest of Campbellville along Factory Creek. | <u>Sharp 10913</u> |
| 27. | Tennessee: Williamson Co. On East Fork Road nine-tenths of a mile from Hwy. 106. ^{a,b} | <u>ABA 458</u> |
| 28. | Tennessee: Wilson Co. Cedar glade located two miles south of junction of Hwy. 66 with Interstate 40. ^b | <u>Phillipe 453</u> |
| 29. | Tennessee: Van Buren Co. On Hwy. 30, seven miles west of Piney Creek. | <u>ABA 401</u> |

Sympatric Populations

- | | | |
|-----|--|----------------|
| 30. | North Carolina: Graham Co. On Hwy. 129 approximately one mile northeast of junction with Hwy. 28 in moist cove by waterfall. | <u>ABA 801</u> |
| 31. | North Carolina: Graham Co. Slickrock Creek | <u>ABA 807</u> |
| 32. | Tennessee: Hamilton Co. On Hwy. 148 going over Lookout Mt. | <u>ABA 816</u> |
| 33. | Tennessee: Morgan Co. Frozen Head State Park located seven miles north of Hwy. 62. | <u>ABA 802</u> |
| 34. | Tennessee: Marion Co. Near Jasper | <u>ABA 817</u> |
| 35. | Tennessee: Scott Co. On south side of Oneida at city limits along stream. | <u>ABA 808</u> |
| 36. | Tennessee: Scott Co. Three miles west of Interstate 75 on south side of Hwy. 63. | <u>ABA 815</u> |
-
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^aPopulations examined by scanning electron microscopy.

^bPopulations that had petals extracted and examined for flavonoid content.

spotted directly on Whatman 3 MM 46 X 56 cm chromatographic sheets, and the chromatograms were developed by the descending method with the first dimension being an organic phase of tertiary butanol, acetic acid, and water (solvent system 1) and the second dimension consisting of an aqueous phase of 15 per cent acetic acid (solvent system 2). Since flavonol glycosides were not completely separated with this procedure, solvent systems 3 and 4 were used when necessary (see page 37).

Following development in both dimensions, chromatograms were viewed alone and in the presence of ammonia fumes under long wave length UV light (360 nm), color characteristics of the spots were noted, and R_f values were computed. Compounds were eluted from the paper with 100 per cent MeOH and purified by streaking the compound on a sheet of Whatman 3 MM paper and developing the chromatogram in an appropriate solvent system. The streaks were eluted with spectroscopic grade methanol for not more than 15 minutes, UV spectra were run on the isolated compounds, and spectral maxima and inflections were recorded.

For analysis of flavonoid glycosides, pure compounds were either completely hydrolyzed by refluxing in 6 per cent HCl at 100 degrees C for at least one hour, or partially hydrolyzed by refluxing for varying time intervals with either 6 per cent HCl or 20 per cent HOAc. The resultant hydrolysate was then taken to dryness to free the sugar

moiety and aglycone of acid and then redissolved in 5 per cent methanol. This solution was then partitioned with ethyl acetate and water to separate the aglycone from the sugar. The ethyl acetate fraction was spotted and chromatographed in two dimensions, the R_f values of the aglycone were determined, and, if possible, the spot was eluted and analyzed spectrally.

The lower fraction containing the sugar moiety was evaporated to complete dryness and a volatile trimethylsilyl ether derivative was made by successively adding five-tenths ml pyridine, two-tenths ml hexamethyl disilazane, and one-tenth ml of trimethyl chlorosilane. After this mixture was given sufficient time to react, it was evaporated to dryness, redissolved in heptane, and filtered through paper. This filtrate was then injected into a gas-liquid chromatograph. In the midst of this study, however, it was decided to use Tri-Sil manufactured by Pierre Chemical Company for making derivatives of the sugars rather than the above procedure. One-half ml of this formulation was simply mixed with the dry sugars and then centrifuged at 275 X g for 20 minutes. The supernatant was removed for direct GLC analysis. This latter method was considerably easier and resulted in a significant reduction of contaminant peaks that had been observed with the previous method.

A Bendix 2600 GLC equipped with a six ft X 0.25 in column packed with acid-washed silanized chromosorb W coated with 3 per cent SE-52 was used for qualitative and quantitative work with sugar moieties. Unknowns were identified by comparing their retention times to those of known standards. If there was any uncertainty of a sugar's identity, known sugars were mixed with the unknown in question and run simultaneously. All derivatives were run at 170 degrees C unless sugars with overlapping retention times were observed, in which case the temperature was lowered to 155 degrees C to improve resolution. R_t values were computed using β -glucose as the standard since its retention time is longer than that of other sugars. For quantitative analysis areas under the curves were estimated by multiplying one-half the base times the height of the peaks.

Column chromatography was also utilized in this study for the isolation of larger quantities of flavonoids. Columns were constructed by mixing polyamide powder (pencro Nylon 66-D) with celite analytical filter aide (1:2 v/v) and swirling in distilled water for 12 hours. The resulting slurry was poured into the column, which was plugged with glass wool and powdered cellulose or a fritted disc. After the adsorbent had settled, it was drained of excess water and washed in 50 per cent methanol overnight in order to remove low molecular weight polyamide

material, which would have otherwise been eluted during the chromatographic run. Extracts obtained from fresh material were dissolved in a minimum of aqueous methanol and carefully applied to the column. Elution was initiated with 100 per cent water and as soon as visible bands were eluted from the column, successive solutions of 25, 50, 75, and 100 per cent MeOH were applied. Seventy-five to 100 ml fractions were collected, analyzed in solvent systems 1 and 2, and flavonoid content of each fraction was recorded. In one case a linear gradient was run going from zero to 50 per cent MeOH over a 12 hour period. Several compounds, which were unobtainable in sufficient quantities due to the immense amounts of chlorophyll and carotenoids extracted with them by previous techniques, were isolated by this method in sufficient quantities for complete analysis. In the case of compounds number 18 and 53, 350 ml fractions were necessary in order to obtain sufficient quantities of the compounds for complete analysis.

Chromatographic Data. Paper partition chromatography was introduced by Bate-Smith (1948), and it was soon determined that besides separation, chromatograms may provide data relevant to structural determination. For example most flavonoids possess characteristic colors (Harborne, 1960), and thus, structural information may be obtained by viewing color characteristics under UV light with and without the presence of ammonia vapors. Glyco-

sylation at the 3-position results in both types of flavonol glycosides appearing purple with UV light; however, kaempferol 3-glycosides appear green with ammonia and quercetin 3-glycosides appear yellow-green under the same conditions due to ionization of an additional hydroxyl group shifting the absorption more with quercetin than kaempferol. Quercetin 3,7-glycosides may be distinguished from quercetin 3-glycosides in that the former turn bright yellow upon exposure to ammonia vapors, the latter yellow-green. The aglycones of quercetin and kaempferol appear yellow in UV light as do compounds with a free hydroxyl at the 3-position and a glycoside at the 7-position (Harborne and Williams, 1975; Mabry, et al., 1970; Crawford, 1973).

The type of constituent plus its point of substitution results in predictable effects upon its movement in solvent systems 1 and 2 during paper chromatography (Bierner, 1971; Averett, 1970; and Mabry, et al., 1970). Likewise, if conditions are held constant, various components will emerge at reproducible and characteristic times from a column depending upon concentration of the solvent. With gas-liquid chromatography the time from the detection of the solvent to the center of the peak is known as retention time or R_t . This value is analogous to an R_f value in paper chromatography or the elution volume in column chromatography. In all three of the above mentioned cases,

the figures may be useful for tentatively qualifying an unknown compound.

With paper chromatography the number of hydroxyl groups will affect the movement of flavonoids. This is easily noted with the B-ring where the fewer the number of hydroxyls, the higher the R_f value in both the organic and aqueous phases. For example kaempferols will move faster than quercetins in both solvent systems.

With the addition of sugars, the compounds migrate slower in the organic phase and faster in the polar, aqueous phase. This mobility varies with the number of sugar molecules attached and with respect to the position of attachment. With flavonols, increasing glycosylation at the 3-position lowers the R_f in a regular fashion with organic solvents and increases it in aqueous systems. Flavonol 3,7-diglycosides respond in the same manner as their corresponding 3-diglycosides, but move even slower in the organic phase and faster in the aqueous phase. Variation in the structure of the sugar may also affect R_f 's with pentosides possessing higher R_f values than the corresponding hexosides.

Column chromatography offers a method for large scale isolation and purification of flavonoid compounds. Polyamide functions by hydrogen bonding between the phenolic groups and the amide groups in the interior of the particles as well as on the surface, and so, the column has a capacity

much higher than one based simply on adsorption (Seikel, 1962). By starting with 100 per cent water as a solvent and by replacing the aqueous content of this eluting solvent gradually with alcohol, flavonoid compounds may be separated from lipids, chlorophyll, carotenoids, and other compounds, while the flavonoid compounds are eluted in high quantities. Also, different classes of flavonoids may be resolved, and indeed, the rate of elution of a compound can give information regarding the structure. For example, the more sugars attached to a flavonoid, the higher its solubility in water; therefore, compounds with four sugars will theoretically be eluted from the column before compounds with three sugars, which in turn will come off previous to molecules with disaccharides. Likewise, a greater number of hydroxyl groups on the flavonoid nucleus will result in a greater number of hydrogen bonds with the polyamide, giving slower elution rates. So, not only is polyamide adsorbent useful for initial purification of crude extracts, but also may yield qualitative data. In other words definite correlations may be found between elution volume and the structure of a compound with polyamide column chromatography.

Results

A composite chromatogram of the two taxa of Trillium examined is shown in Figure 4. Numbers were assigned to spots in order to facilitate discussion, and the compounds

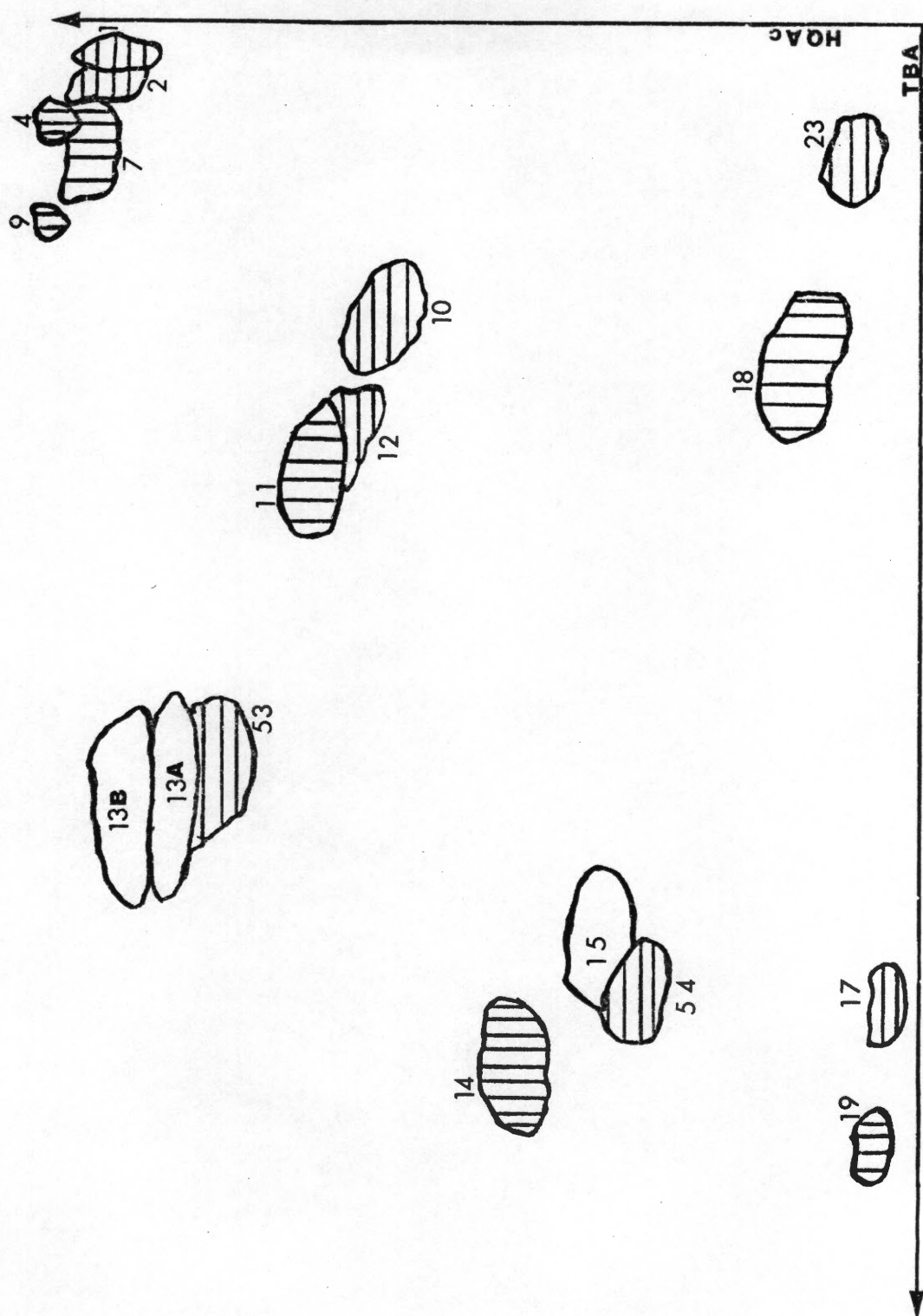


Figure 4. Composite chromatogram of the compounds analyzed.
Kaempferol compounds are shaded vertically and quercetin horizontally.

found to be synonymous in both taxa based on chromatographic and spectral data were assigned identical numbers. Quercetin, kaempferol, and possibly isorhamnetin skeletons were identified; quercetin compounds are shaded horizontally and kaempferol compounds vertically in Figure 4. The sugars glucose, galactose, arabinose, and rhamnose were hydrolyzed from the flavonoid skeletons. Mono-, di-, tri-, and possibly one tetrasaccharide were identified.

UV Analysis. The basic flavonoid skeleton plus the position of attachment of sugars were confirmed with UV spectral analysis. Initial spectral work with flavonoids was begun in the 1920's with anthocyanidins (Schou, 1927) and expanded considerably in the 1950's (Jurd, 1962). The techniques were standardized and clarified significantly in 1970 (Mabry, et al., 1970), and these procedures were utilized in spectral analysis consistently throughout this study. Chromatographic data are presented in Table II, and the absorption maxima (λ max) and inflections for each compound are listed in Table III. Compound 54 will be discussed in detail, and the spectral data for remaining compounds will be referred to only with respect to their unique features.

Compound 54 appeared purple with UV light and turned yellow-green with ammonia. Upon analysis with a UV spectrophotometer (450-230 nm), two major absorption peaks were observed; one at 359 nm and the other at 259 nm.

TABLE II

IDENTITY AND CHROMATOGRAPHIC PROPERTIES
OF THE FLAVONOIDS OF TWO TAXA OF TRILLIUM

IDENTITY	COLOR ^a		R _f VALUES			
	UV	+NH ₃	1 ^b	2 ^c	3 ^d	4 ^e
1. Quercetin 3,7-O-arabinogalactorhamnogluco-side ^f	P	BrY	.03	.65	.59	.0
2. Quercetin 3-O-glucoside-7-arabinogalactoside	P	BrY	.05	.67	.59	0
4. Kaempferol 3,7-O-arabinogalactoglucoside ^f	P	Gr	.06	.78	.73	0
7. Kaempferol 3,7-O-glucogalactoarabinoside ^f	P	Gr	.15	.77	.73	0
9. Kaempferol 3,7-O-glycoside	P	Gr	.19	.79	.73	0
10. Quercetin 3-O-arabinogalactoglucoside	P	YGr	.27	.57	.54	.08
11. Kaempferol 3-O-arabinogalactoglucoside	P	Gr	.42	.67	.58	.12
12. Quercetin 3-O-rhamnoarabinoside ^f	P	YGr	.40	.6	.4	.16
14. Kaempferol 3-O-galactoglucoside	P	Gr	.66	.48		

Table II (continued)

IDENTITY	COLOR		R _f VALUES			
	UV	+NH ₃	1	2	3	4
13A. Fisetin 3-O-glycoside ?	FlB	FlYGr	.57	.72	.58	
13B. 4' trihydroxyflavone 3, 7-O-glycoside ?	FlB	FlYGr	.56	.82	.58	
15. Isorhamnetin 3-O-glycoside	P	YGr	.57	.41		
17. Quercetin	Y	Y	.55	0		
18. Kaempferol 7-O-glucoside	Y	Y	.2	.13		
19. Kaempferol	Y	Y	.81	.05		
23. Quercetin 7-O-glycoside	Y	Y	.1	.08		
53. Quercetin 3-O-glycoside	P	YGr	.58	.6		
54. Quercetin 3-O-galactoglucoside	P	YGr	.5	.41		

^aP = purple; Y = yellow; Fl = fluorescent; Gr = green; B = blue; Br = bright.

^bSolvent 1 = tertiary butanol:glacial acetic acid: water (3:1:1 v/v).

^cSolvent 2 = water:glacial acetic acid (17:3 v/v).

^dSolvent 3 = phenol:water (approximately 4:1 v/v).

^eSolvent 4 = chloroform:methanol:butanone:water (55:22:20:3 v/v).

^fExact sequence of sugars unknown at this time.

TABLE III
ABSORPTION MAXIMA (nm) OF FLAVONOIDS

	MeOH	NaOMe	AlCl ₃	AlCl ₃ /HCl	NaOAc	H ₃ BO ₃
1.	265,231 ^a , 300 ^a ,359	271,427	271,299 ^a , 364,436	262,358, 400	260,415	256,382
2.	260,273 ^a , 290,362	273,420	273,297 ^a , 334,438	264,303 ^a , 367,404	260,290 ^a , 379,416	260,285 ^a , 374
4.	288,319 ^a , 351	270,358 ^a , 401	275,301 ^a , 353,399	274,299, 350,397	266,294 ^a , 352 ^a ,400	268,324 ^a , 354
7.	267,288 ^a , 329 ^a ,351	269,296 ^a , 361 ^a ,401	254 ^a ,275, 301 ^a ,352, 400	254 ^a ,275, 300 ^a ,352, 398	260 ^a ,269, 404	268,280 ^a , 355
9.	268,346	272,299 ^a , 364 ^a ,407	264 ^a ,272, 300 ^a ,350, 397	264 ^a ,271, 348,396	267,292 ^a , 356 ^a ,401	267,289 ^a , 318 ^a ,352
10.	258,272 ^a , 361	272,413	272,435	268,361, 400	271,322 ^a , 390	264,381
11.	267,349	278,326 405	274,301 ^a , 350,396	273,302 ^a , 347,396	389,301 ^a , 276	268,299 ^a , 351
12.	255,268 ^a , 290 ^a ,358	278,315 ^a , 420	263,305 ^a , 427	267,301 ^a , 362,399	271,307 ^a , 412	251,281 ^a , 379

TABLE III (continued)

	MeOH	NaOMe	AlCl ₃	AlCl ₃ /HCl	NaOAc	H ₃ BO ₃
13A.	284, 298 ^a , 326	272, 386	255 ^a , 292 ^a , 355	285, 298 ^a , 326	272 ^a , 281 ^a , 300 ^a , 347	283, 345
13B.	272, 278 ^a , 296, 323	285, 320 ^a , 377	272, 278 ^a , 295 ^a , 323	273, 277 ^a , 294 ^a , 323	273, 278 ^a , 320 ^a , 325 ^a	272, 279 ^a , 289 ^a , 343
14.	267, 283 ^a , 298 ^a , 349	278, 325, 368 ^a , 404	272, 300, 345, 390	271, 300 ^a , 345, 392	270, 300 ^a , 362	266, 290 ^a , 350
15.	259, 265 ^a , 292 ^a , 357	279, 332 ^a , 415	268, 297, 362, 398	269, 295 ^a , 361, 400	270, 297 ^a , 415	268, 295 ^a , 395
18.	268, 320, 365	269, 354, 451, dec.	269, 304 ^a , 353, 420	267, 302 ^a , 349, 418	268, 323, 367	266, 320
53.	260, 267 ^a , 298 ^a , 355	283, 315 ^a , 404	273, 299 ^a , 335, 430	272, 297 ^a , 350, 400	273, 312, 399	262, 294 ^a , 372
54.	258, 268 ^a , 297 ^a , 359	280, 420	274, 304 ^a , 435	267, 298 ^a , 361, 400	272, 322, 399	261, 297 ^a , 379

^aInfection.

These two peaks fell within the ranges common for flavonoids with Band I (300-380 nm) being due to absorption of the B-ring cinnamoyl system and Band II (240-280 nm) associated with absorption involving the A-ring benzoyl system. The absorption peak of a quercetin aglycone is 370 nm whereas a glycosidic substitution at the 3-position causes a hypsochromic shift (i.e., to shorter wavelengths) of 12-17 nm in Band I. These data, along with the peak and shoulder between 250-275 nm suggest that compound 54 is a quercetin with substitution occurring at the 3-position.

The reagents sodium methoxide (NaOMe), aluminum chloride (AlCl_3), hydrochloric acid (HCl), sodium acetate (NaOAc), and boric acid (H_3BO_3) have increased the value of spectral data considerably since they produce characteristic shifts in the maxima indicative of the location of functional groups within the flavonoid molecule. Five additional spectra were conducted on each compound using the above reagents with the initial MeOH spectrum serving as a reference.

A strong base ionizes to some extent all hydroxyl groups and is used for the detection of free 3 and/or 4'-hydroxyl groups. Sodium methoxide will cause a 40-65 nm bathochromic shift (i.e., to longer wavelengths) in Band I with no decrease in intensity of the peak if a 4'-hydroxyl is present. However, a 3-hydroxyl causes the compound to be base sensitive and resultant decomposition

causes a decrease in the intensity of Band I. Compound 54 exhibited a 61 nm bathochromic shift with an increase in intensity. The spectrum was run again after 15 minutes with no signs of peak degeneration. It was concluded that the flavonoid had a free 4'-hydroxyl and that it was substituted at the 3 position.

Aluminum chloride forms acid stable complexes with hydroxyl groups at C-3 or C-5 (Jurd and Geissman, 1956) and acid labile complexes with flavonoids that contain ortho-dihydroxyl systems (Markham and Mabry, 1968). These complexes result in a 45 nm bathochromic shift due to a complex with the C-4 keto group and the C-5 hydroxyl plus a bathochromic shift of 35 nm associated with the 3', 4' complex. These shifts are additive and, therefore, should result in an 80 nm bathochromic shift. With the addition of AlCl_3 to a methanol solution, compound 54 showed a 77 nm bathochromic shift indicating that hydroxyl groups were located at the 5, 3', and 4' positions. With the addition of HCl , the 3', 4' complex is broken resulting in a hypsochromic shift of 35 nm. This compound showed a 35 nm hypsochromic shift with the addition of HCl confirming the presence of a 3', 4'-dihydroxyl system.

Since NaOAc has one more carbon than NaOMe , it is a weaker base and ionizes only the more acidic hydroxyl groups. It is used for diagnosis of the 7-hydroxyl group since Band II is affected. A 5-20 nm bathochromic shift

of Band II will be observed with the addition of NaOAc if the 7-hydroxyl is free. Compound 54 showed a 13 nm bathochromic shift in Band II with the addition of fused NaOAc indicating the presence of a free hydroxyl.

In the presence of NaOAc, H_3BO_3 will chelate with all ortho-dihydroxyl groups except at C-5, 6 and Band I will exhibit a 12-30 nm bathochromic shift due to this chelation. A 20 nm shift upfield was observed with the addition of H_3BO_3 thus confirming a 3', 4'-dihydroxyl system.

From the above results it is concluded that compound 54 has hydroxyl groups at the 5, 7, 3', and 4' positions, no hydroxyl constituents at the 6, 8, or 5' positions, and that the 3 position is substituted. This particular type of phenolic molecule is known as a quercetin. Spectral analysis further indicated that compounds 12, 10, and 53 had similar flavonoid skeletons, differing only by the type and nature of the constituents at the 3 position. Compounds one and two likewise had identical spectra except that the addition of NaOAc resulted in no bathochromic shift of Band II; thus, it was concluded that both the 3 and 7 positions were substituted.

Compounds 14 and 11 agreed with the spectra of compound 54 in all aspects except that the addition of $AlCl_3$ resulted in approximately a 45 nm bathochromic shift of Band I (41 and 47 nm, respectively), whereas the addition of HCl yielded a spectrum almost identical to the $AlCl_3$ spectrum. It is believed that these two compounds have

no hydroxyl at the 3' position and are thus kaempferol type compounds. Compounds 4, 7, and 9 were likewise found to be kaempferol type compounds differing only from the above in that the NaOAc spectrum showed no shift in Band II indicating that the 7 position is substituted.

Compound 18 was also found to be a kaempferol type compound but was unique in that the 3 position was free whereas the 7 position was blocked. Sodium methoxide brought about slow decomposition (i.e., alkali-sensitive) of the peaks pointing to free 3, 4'-hydroxyl groups. The AlCl_3 spectrum exhibited a bathochromic shift of 55 nm due to competition for the formation of the complex between the 5 and 3-hydroxyls with the C-4 keto group. The addition of HCl caused only a two nm shift indicating that the previous shift with AlCl_3 was not due to an ortho-dihydroxyl system at the 3', 4' positions.

Compounds 13 A and 13 B are believed to be flavonols lacking 5-hydroxyls. This belief is based on their λ_{max} for Band I (326 and 328 nm respectively) and color characteristics in UV light (light blue) and with ammonia (fluorescent yellow-green). With the addition of AlCl_3 compound 13 A exhibited a 29 nm shift upfield, and this shift was nullified by the addition of HCl. It was thus concluded that this compound had a 3', 4'-dihydroxyl system and lacked a 5-hydroxyl. Compound 13 B showed no shift with AlCl_3 , and thus, only a 4'-hydroxyl is present or else the 3' or

4' position is blocked. A 19 nm bathochromic shift with H_3BO_3 confirmed the ortho-dihydroxyl of compound 13 A. The NaOAc spectrum showed the 7 position to be blocked in 13 B but was inconclusive in the case of 13 A.

Compound 15 exhibited a quercetin type spectrum with MeOH but only a 45 nm shift was observed with the addition of $AlCl_3$, and no change was detected with the addition of HCl. It is believed that this compound has both the 3 and 4' or 3' positions blocked and could be an isorhamnetin (i.e., a methyl ether at the 3' position) type compound; however, the H_3BO_3 spectrum was not consistent with this belief. Unfortunately, there was not enough compound for hydrolysis or to recheck the results.

Hydrolysis. Acid hydrolysis will cleave all oxygen-linked sugars on a flavonoid compound; however, the rate of hydrolysis can vary considerably depending on the size of the sugar and its position of attachment. For example, pentosides such as arabinose are hydrolyzed more rapidly than hexoses such as glucose and galactose. Sugars attached at the 3 position are removed more rapidly than those attached to the 7 position and by varying the time allowed for hydrolysis or by using a weaker acid (e.g., HOAc), it is possible to isolate intermediate 7-glycosides. Alkaline hydrolysis will distinguish flavonol 3-glycosides from 7-glycosides with only 7-glycosides being cleaved by the base (Harborne and Williams, 1975).

Complete acid hydrolysis was conducted with compound 54 yielding an aglycone and sugar residue. The aglycone was developed chromatographically and found to be yellow under UV light indicating the presence of a free 3-hydroxyl. The compound also had R_f values identical to quercetin, and thus, complete hydrolysis was confirmed. Similar results were obtained with compounds 10, 1, 2, and 12 with complete (more than one hour) acid hydrolysis; however, only compound 10 yielded enough material for spectral analysis.

Complete hydrolysis of compound 11 also yielded a yellow spot with R_f values in solvents 1 and 2 identical to those of kaempferol. Spectral analysis confirmed that the compound was kaempferol, and spots 11, 9, 7, and 4 yielded similar results.

Partial acid hydrolysis of compound 2 was conducted yielding a yellow spot with R_f values identical to spot 23, therefore confirming hydrolysis of the 3 position and not the 7 position. Negative results were obtained with alkaline hydrolysis. Partial hydrolysis was also conducted on compounds 54, 10, and 11 by refluxing for a shorter time period with HCl (approximately 20 minutes) than is used for complete hydrolysis. Insufficient compound was isolated for partial hydrolysis of spots 1, 4, 12, or 15. No hydrolysis was conducted on spots 53, 13 A, 13 B, or 9 due to quantitative factors.

Although not enough compound was isolated to obtain spectra for compounds 17, 19, and 23, an educated speculation concerning their structures can be made. All three spots were yellow under UV light and so probably have free hydroxyls at the 3 positions. Spot 17 had R_f values similar to quercetin, and spot 19 had R_f values identical to kaempferol. Also, quercetin glycosides were hydrolyzed to spots similar to 17, as were kaempferol glycosides hydrolyzed to spots identical in appearance to spot 19. Partial hydrolysis of spot two leads one to believe that spot 23 is a quercetin 7-glycoside with at least one sugar identical to those at the 7 position in compound two.

Analysis of Sugar Moieties. Results of sugar analysis are given in Table IV with R_t values for each sugar listed. The sugar residue of spot 54 was made into a derivative and injected into a gas-liquid chromatograph. Partial hydrolysis of the compound with HCl gave much more galactose than glucose; however, when complete hydrolysis was affected, galactose and glucose were found to be present in a one to one ratio. It was concluded that the compound was quercetin 3-O-galactoglucoside. Partial hydrolysis of spot two with HOAc gave glucose, whereas full hydrolysis resulted in glucose, galactose, and arabinose. Spot two was identified as quercetin 3-O-gluco-7-O-arabinogalactoside.

TABLE IV
R_t VALUES OF SUGAR MOIETIES

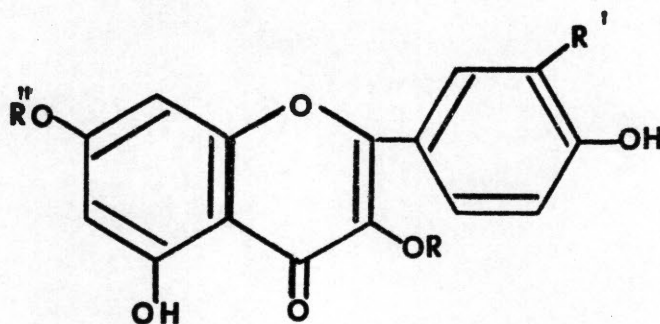
SUGAR	R _t OF KNOWN SUGARS	COMPOUNDS											
		1	2	4	7	10	11	12	14	15	18		
beta glucose	1	1	1	1	1	1	1	1	1	1	1		
alpha glucose	.687	.679	.676	.680	.669	.680	.680		.679	.666	.689		
galactose	.497		.559		.569	.500	.505		.500	.500			
	.583	.595	.608	.606	.613	.595	.601		.593	.587			
	.704	.709	.724	.721	.728	.707	.716		.709	.700			
arabinose	.202		.217	.210	.223	.204	.211	.207					
	.235	.241	.248	.244	.256	.238	.241	.237					
	.259	.261	.266	.261	.272	.265	.274	.264					
rhamnose	.215	.217						.214					
	.294	.261						.280					

Compounds 10, 1, and 12 were found to be quercetin 3-0-arabinogalactoglucoside, quercetin 3-0-gluco-7-9-arabinogalactorhamnoside¹, and quercetin 3-0-rhamnoarabinoside¹, respectively. Likewise, spots 18, 11, 7, and 4 were found to be kaempferol 7-0-glucoside, kaempferol 3-0-arabinogalactoglucoside, kaempferol 3,7-0-arabinogalactoglucoside¹, and kaempferol 3,7-0-arabinogalactoglucoside¹. Compounds 7 and 4 exhibit identical sugars and spectra but were separable chromatographically; therefore, the sequence of sugars must be the only difference between the two compounds. Molecular structures are presented in Figures 5 and 6.

Analysis of Elution Volumes. The elution volumes for known compounds are given Table V where it is seen that elution rates are consistent with previous structural determinations (see methods, page 32). Compounds with four and three sugars at both the 3 and 7 positions (i.e., flavonol 3,7-polyglycosides) were eluted initially with 100 per cent water, flavonol 3-triglycosides at 75 per cent water, and flavonol 3-diglycosides were eluted at 50 per cent water. Kaempferol glycosides were eluted before quercetin glycosides with identical sugars.

Perhaps it is appropriate to mention that upon spotting the second and third fractions (100 per cent water), it was discovered that only flavonol 3,7-polyglycosides were present. Before drying the fractions the color of

¹Exact sequence of sugars and attachment unknown.



R = arabinogalactoglucoside; R' = R'' = H; kaempferol 3-O-arabinogalactoglucoside.

R = galactoglucoside; R' = R'' = H; kaempferol 3-O-galactoglucoside.

R = OH; R' = H; R'' = glucoside; kaempferol 7-O-glucoside.

R = arabinogalactoglucoside; R' = OH; R'' = H; quercetin 3-O-arabinogalactoglucoside.

R = galactoglucoside; R' = OH; R'' = H; quercetin 3-O-galactoglucoside.

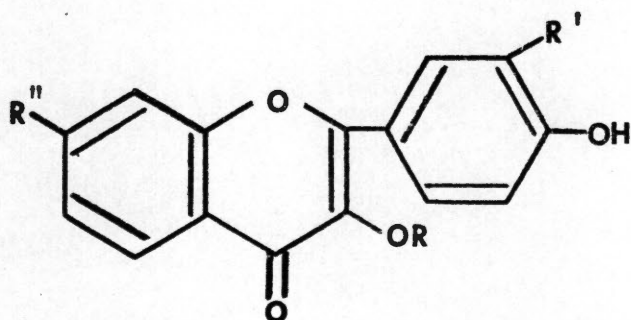
R = arabinorhamnoside; R' = OH; R'' = H; quercetin 3-O-arabinorhamnoside.

R = OH; R' = OH; R'' = glycoside; quercetin 7-O-glycoside.

R = unknown; R' = OCH₃?; R'' = H; isorhamnetin 3-O-glycoside.

R = glucoside; R' = OH; R'' = arabinogalactoside; quercetin 3-O-glucoside-7-O-arabinogalactoside.

Figure 5. Kaempferol, quercetin, and isorhamnetin structures with various constituents as shown above.



R = unknown; R' = OH; R'' = H; Fisetin 3-O-glycoside?

R = unknown; R' = H; R'' = unknown; 4'-trihydroxyflavone
3,7-O-glycoside?

Figure 6. Fisetin and 4'-trihydroxyflavone with various constituents as shown above.

TABLE V

FLAVONOID COMPOSITION OF 75-100 ML FRACTIONS OBTAINED BY POLYAMIDE COLUMN CHROMATOGRAPHY

SOLVENT	FRACTION NUMBER ^a	FLAVONOIDS												
		1	2	4	7	9	13A	13B	11	10	12	23	18	53
H ₂ O	1													
H ₂ O	2	+	+	+	+									
H ₂ O	3	+	+	+	+	+	+	+	+					
H ₂ O	4	+	+	+	+	+	+	+	+					
25% MeOH	5	+	+	+	+	+	+	+	+	+	+	+		
25% MeOH	6		+		+				+	+	+	+	+	
25% MeOH	7		+		+				+	+	+	+	+	
50% MeOH	8		+		+						+	+	+	+
50% MeOH	9										+	+		+
50% MeOH	10													+
50% MeOH	11													+
75% MeOH	12													+

^aFraction volume was varied occasionally to allow for separation of detectable bands.

the solution was noted as being bright yellow. This color was intensified upon drying. These same compounds were found to be the major constituents of alcoholic extracts from the petals of T. luteum and it is quite possible that these flavonol 3,7-glycosides contribute significantly to the yellow color of T. luteum petals.

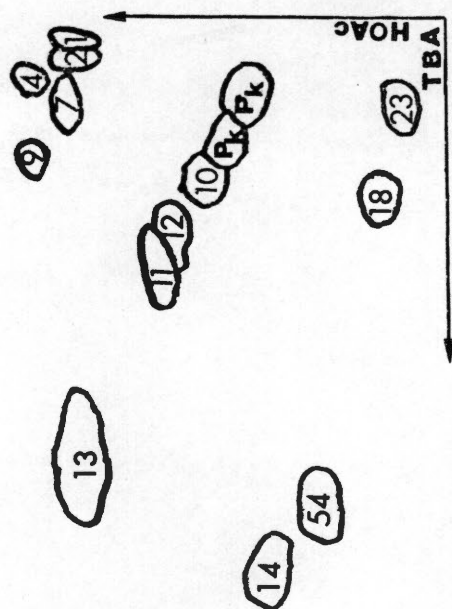
Discussion

Little intraspecific qualitative variation of flavonoids from the extracts of foliaceous bracts was noted within populations of T. luteum except that compound one was often not detectable in significant quantities², and the presence of compound 15 was noted in only one population (see Figure 7). No definite conclusions can be drawn about quantitative variation of flavonoids since this study did not deal directly with this aspect. However, comments can be directed toward relative amounts of identical compounds in different taxa based on visual observations of chromatograms with the aid of a UV light. Although significant quantitative variation was observed in T. luteum plants, 3,7-O-glycosides were always obviously present in significant amounts. On the other hand, flavonol 3-O-trisaccharides were often absent or observed only as trace components. The latter compounds were not fully characterized until 40 grams of fresh material were

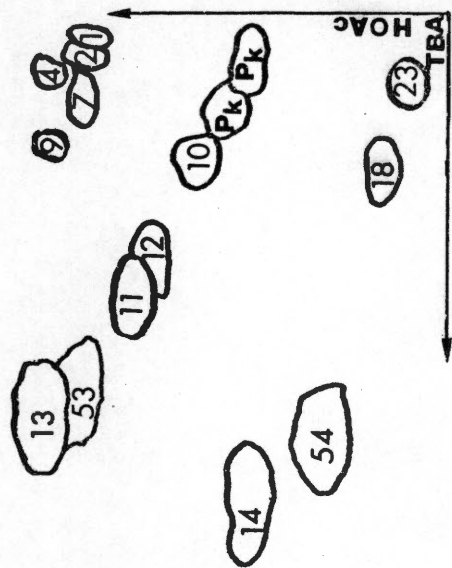
²Difficult to resolve due to overlap with spot two.

Figure 7. Chromatographic profiles of the bracts and petals of Trillium luteum and Trillium cuneatum. The numbers correspond to those listed on pages 36 and 37, and P_k represents the anthocyanins.

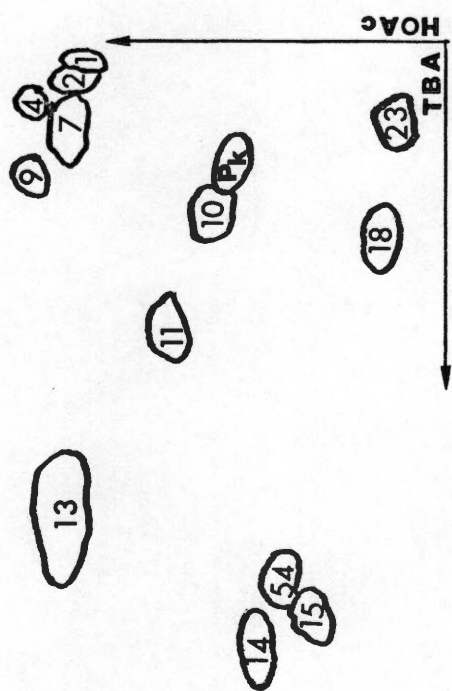
- A. Bracts of Trillium luteum.
- B. Bracts of Trillium cuneatum.
- C. Petals of Trillium luteum.
- D. Petals of Trillium cuneatum.



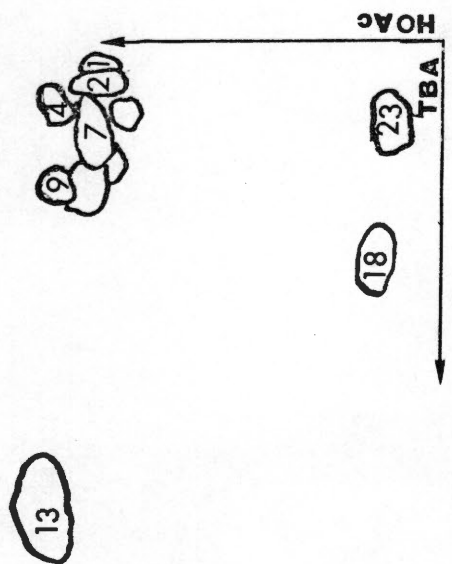
B



D



A



C

Figure 7

extracted and chromatographed through a column of polyamide (see methods, page 29).

Although the flavonoid profiles of the bracts of T. luteum and T. cuneatum were quite similar, some qualitative differences were observed. Compound twelve was never detected in T. luteum, but was detected in several instances in T. cuneatum. Trillium cuneatum often showed considerable quantitative variation, but flavonol 3-trisaccharides were always easily detected. The flavonol 3,7-polysaccharides were usually present in T. cuneatum, yet often were detectable only as trace components. Murrell (1969) noted that two anthocyanins were present in the bracts of T. cuneatum and only one was found in the bracts of T. luteum. This was found to be true except when large amounts of fresh T. luteum bracts were extracted in which case two pink spots were detected.

The petals of the two taxa exhibited significant qualitative differences in flavonoid content. Trillium luteum possessed only 3,7-glycosides (compounds one through nine) and 7-glycosides (compounds 18 and 23) with the 3,7-polyglycosides being the major quercetin and kaempferol constituents; however, T. cuneatum petals contained 3-triglycosides (compounds ten and eleven) and 3-diglycosides (compounds 14, 54, and 53) as the major quercetin and kaempferol components. All five of these

compounds were not noted, or noted only as traces, in T. luteum petals. In addition the T. cuneatum petals contained two anthocyanins not present in T. luteum petals.

Kaempferol and quercetin show the most typical $C_6(A)-C_3-C_6(B)$ hydroxylation pattern in which $C_6(A)$ is phloroglucinol-derived, and ring B is 4'- or 3', 4'-dihydroxylated. These trihydroxy flavones usually occur in plants as glycosides with 3-glycosides being the most common and 7-glycosides also being abundant. Since quercetins and kaempferols are the most common flavonols and since they are found throughout the plant kingdom, then perhaps the absence of these flavonols would be more significant than their presence with respect to classification schemes (see Chapter II). On the other hand, specific characteristics of flavonol molecules such as glycosylation or methoxylation patterns may provide useful taxonomic characters.

Durkee and Harborne (1973) have found that with species or genera within the Brassicaceae glycosidic patterns rather than distribution of flavonol aglycones are likely to be of the most taxonomic value for distinguishing groups. Perhaps of more interest is the recent work of Bohm, Saleh, and Ornduff (1974) in which it was noted that once the flavonol level of oxidation is achieved within the plant group in question (i.e., Lasthenia), a great potential for glycoside diversity is created. One

of the conclusions drawn by the authors was: "The variety of glycosylations seen in Lasthenia might prove useful as a generic characteristic. It is obvious that further taxonomic discussion requires an understanding of the basis of glycosylation differences."

It is quite possible that a unique aspect of the genus Trillium may be the glycoside diversity of quercetin and kaempferol compounds. According to Harborne and Williams (1975) only seven flavonol trisaccharides have been fully characterized with respect to type of bonding (α or β) and whether the sugars are branched or linear. Most trisaccharides are obviously related to disaccharides and di- and triglycosides have been found to co-occur. For instance in Primula sinensis, kaempferol 3-gentiotri-
oside is found with the bioside. With Solanum tuberosum 3-glucosylrutinosides occur with the 3-rutinosides and 3-di-glucosides (Harborne, 1967). In the genera Chamaesaracha and Leucophysalis, Averett (1970) found kaempferol and quercetin occurring in pairs as 3-monoglucosides, 7-monoglucosides, 3,7-diglucosides, 3-rutinosides, and 3-rutinoside-7-glucosides. My results concur with these previous studies with kaempferol and quercetin pairs being noted with compounds 19 and 17, 18 and 23, 14 and 54, 11 and 10, and this would probably also be true with the 3,7-polyglycosides if they had been characterized more thoroughly. One point that should be made is that the

trisaccharides identified in the two taxa of Trillium were composed of three different sugars (glucose, galactose, and arabinose).

The compound isolated that had four sugars attached glycosidically is especially worthy of discussion. The only known flavonoid with four sugars is kaempferol 3-sophorotrioside-7-rhamnoside from Solanum tuberosum seed (Schmid and Harborne, 1973), which has a linear trisaccharide at the 3 position and rhamnose at the 7 position. Compound one showed not only the presence of four sugars, but four different sugars (glucose, galactose, arabinose, and rhamnose). More work must be done with this compound before definite statements can be made concerning its significance, but it certainly points to a unique aspect of the biochemistry of these two taxa.

The most common carbohydrate component of flavonoids is glucose with galactose and rhamnose being less frequent. The pentose arabinose is present only in biosides or triosides, or in the case of T. luteum and T. cuneatum in a tetrasaccharide, and arabinose is never attached directly to the aglycone (Bandyukova and Avanesov, 1972). All the above were found to be true with the two taxa studied except that arabinose was more common than rhamnose. Apparently, glucose is the sugar attached directly to the flavonoid nucleus at the 3 position in all cases examined and arabinose is found as the terminal sugar in the trisaccharides.

Bohm, et al. (1974) suggested that within Lasthenia an increase in glycoside diversity seems to be correlated with evolutionary reduction. On the basis of comparisons of paper chromatograms of several pedecilate and non-pedecilate species, it appears that this could well be the case within the genus Trillium. The two sessile-flowered taxa worked with in this study showed much more flavonoid variety and exhibited greater quantities of 3,7-polyglycosides than any of the pedecilate species of Trillium. This could support the theory that sessile-flowered species were derived from pedecilate species; however, more work is necessary before definite conclusions can be made with respect to this theory or the glycoside diversity within the genus Trillium.

Glycosylation has indeed found taxonomic applicability in this study. The differences in glycosylation noted in the petals of T. luteum and T. cuneatum might be a result of different enzymatic rates rather than enzyme specificity. Enzyme kinetics might be such within T. luteum petals that once a flavonol molecule appears within the cell, it is immediately glycosylated at the 7 position. This statement is made with the assumption that glycosylation is a terminal process (Barber, 1962). Barber also found that with leaf extracts of Phaseolus aureus the rate of enzymatic incorporation of L-rhamnose-C¹⁴ was 75 per cent complete approximately two hours after the reaction was initiated.

This work has been confirmed by Larson (1971); Barber and Chang (1968); Sutter, et al. (1972); and von Brederode and von Nigtevecht (1973). It may be assumed then that the reactions occur relatively fast, and so once a flavonol aglycone is synthesized, it is rapidly glycosylated. Another factor which might be considered with respect to glycosylation differences could be an operator gene which acts as an off-on switch for a series of genes controlling glycosylation. The genes might be controlled by the presence or absence of a regulator gene, or maybe the action or non-action of a regulator gene.

GLC and Sugar Sequencing. The use of a gas-liquid chromatograph in the analysis of sugars was a unique aspect of this study in that this instrument has not been used to any great extent in past flavonoid research. The potential for this type of chromatography in future studies appears to be astounding. The amount of compound needed for analysis is approximately one-tenth the amount necessary for sugar analysis with paper or thin-layer chromatography. The time involved once the process has been standardized is approximately one-half that of other methods. The problem of glucose and galactose showing identical R_f values with paper or thin-layer chromatography is nonexistent with GLC where the two sugars are readily resolved. Perhaps of even more importance, however, is the usefulness of GLC in determining the position (i.e., sequencing) of sugars on a flavonoid nucleus. By using

simple quantitative methods and determining the ratio of the areas under the curves and comparing the ratio to those of known proportions of sugars, one can ascertain with fair certainty what the proportions of the unknown sugar moieties are. If one is working with a compound that has R_f values that suggest a disaccharide, then complete hydrolysis should yield the two sugars in a predetermined one to one ratio (i.e., if they are different sugars). Partial hydrolysis should result in a lesser proportion of the sugar attached directly to the flavonoid nucleus than the sugar attached to the molecule only by the other sugar. This same method may be used to determine the position of attachment with selective hydrolysis (e.g., HOAc hydrolyzing the 3 position before the 7 position).

Utility of Flavonol Glycosides. Plants tested late in the season (late July to early August) showed few if any flavonol glycosides. This raises the question of whether or not the plants have found some function for the flavonol glycosides. The hydrolysis of glycosides in association with infection has been documented by Levin (1971). Brederude and Nigtevecht (1973) point out that in Melandrium album, the lack of glycosylation is accompanied by small, atypical flowers whereas glycosylation of the the 7 position is associated with normal petal morphology. Glycosylation is a mechanism for

rendering a phenolic aglycone chemically inactive while also allowing for increased solubility in cell sap. Perhaps glycosylation of flavonols in Trillium is a mechanism for storing flavonoids until needed to fight an infection or to maintain petal integrity. More plants should be tested for flavonoids late in the season in order to confirm their presence or absence.

Genetic Considerations. Scott-Moncrieff and her co-workers discovered that simple biochemical effects in pigment synthesis are a result of single gene differences (Scott-Moncrieff, 1936; Lawrence and Scott-Moncrieff, 1935). Harborne (1965 a) notes that these studies with anthocyanin inheritance in higher plants were used by Beadle in formulating his one gene--one enzyme hypothesis. A single gene difference may change the flavonoid phenotype, and indeed, the flavonoid class as well as patterns of hydroxylation, methylation, glycosylation, and acylation (Levin, 1971). Harborne has even commented that more is known about the inheritance of flavonoids than of any other plant constituent. Generalizations with respect to flavonoid inheritance may be made due to the fact that the genetic control of the synthesis and structural modifications have been documented in numerous species (Levin, 1971; Levy and Levin, 1971).

Based on the many shades of petal color seen in T. cuneatum (purplish red, pink, dirty brown, reddish green,

greenish yellow, and rarely a bright yellow), one might be led to believe that the control of pigment concentration is polygenic. Lawrence and Scott-Moncrieff (1935) found that the C₁₅ flavonoid precursor may go different routes with the increase of one pigment resulting in a concomittant decrease in another (see Figure 8). Likewise, when flavonols occur together with anthocyanins, ". . . they have a bluing effect on flower color if present in sufficient amount, since the two classes of pigments form weak pigment-copigment complexes in the cell sap" (Harborne, 1965). Kaempferol glycosides may alter flower color significantly from maroon to mauve with increasing concentration (Harborne and Sherratt, 1961). It seems reasonable to assume that quercetin glycosides also will alter the color of anthocyanins. The fact that these flavonol glycosides were found in the petals of T. cuneatum provides a very reasonable explanation for the color variation seen within pure T. cuneatum populations. Although the color is certainly a factor of anthocyanin concentration, it is also directly affected by the concentration of flavonol glycosides. Indeed, the reddish-purple T. cuneatum petals showed an abundance of kaempferol 3-glycosides (compounds 14 and 11) and quercetin 3-glycosides (compounds 10, 53, and 54).

The proposed pathway of flavonoid biosynthesis is shown in Figure 8. These sequences are based mostly on

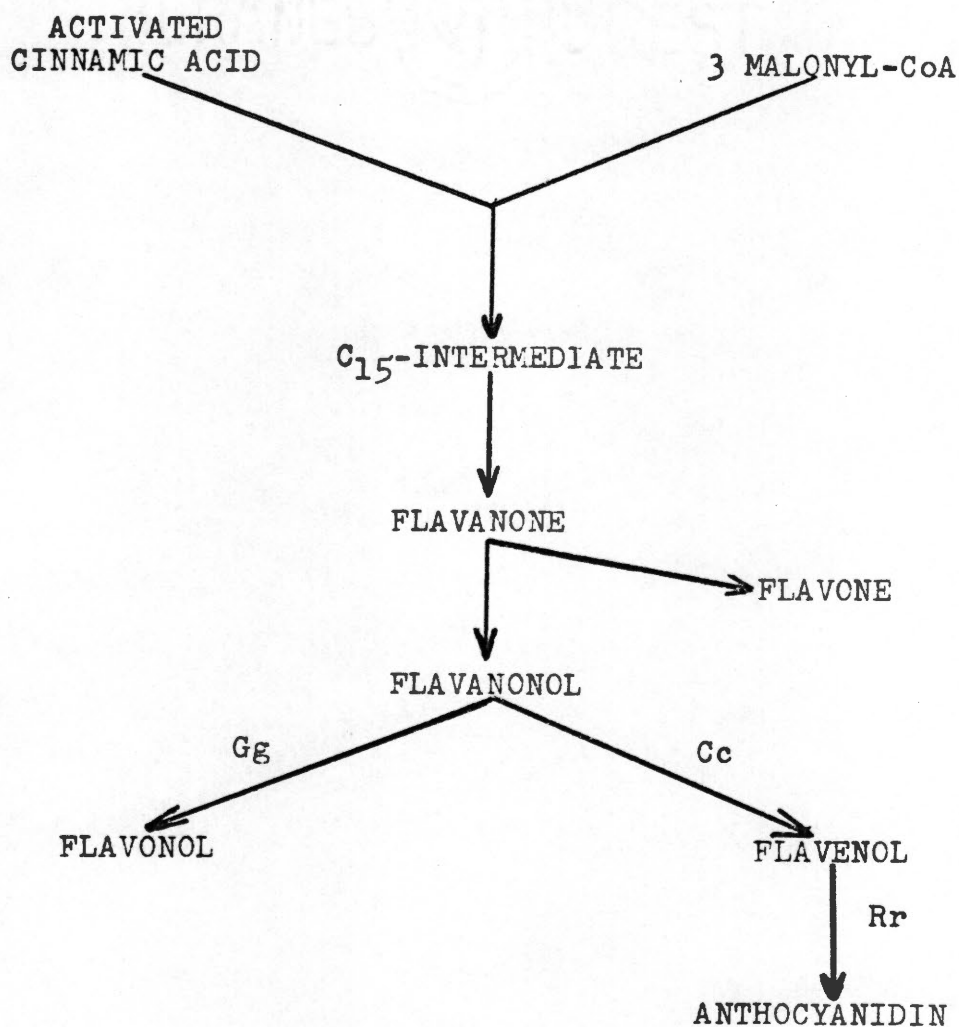


Figure 8. The biosynthetic pathway of flavonoids based on studies with radioactive tracers. The genes discussed in the text are represented as single letters and presented as being heterozygous dominant.

radioactive labeling studies with C^{14} which are discussed by Hess (1975). Strong support for the validity of this pathway may be found in work with maize (Kirby and Styles, 1970) and is reviewed by Ribereau-Gayon (1972). It is obvious that a number of genes are involved in flavonol and anthocyanin production, and the fact that these genes must be present in the dominant form has been shown by Briggs (1966). Plants homozygous for the recessive allele of any one of these genes lack anthocyanin pigments in the organ in question. This basic pattern of flavonoid biosynthesis appears to be common to all higher plants as shown by numerous genetic, physiological, and chemical studies (Harborne, 1967). If the homozygous recessive condition does occur (i.e., rr or cc), then the production of flavonols would continue and probably would occur in a higher concentration than would be the case with the presence of a dominant allele for anthocyanin production. The phenotypic result of this homozygous recessive condition would very likely be yellow flowers, as in T. luteum. The fractions from column chromatography definitely showed that flavonol 3,7-diglycosides appear yellow in visible light. The presence of flavonol glycosides being associated with yellow color is not necessarily a major find restricted to this study. Mayer and Cook (1943) state that hydroxyls at the 3' and 4' positions produce a deep yellow shade with flavonols. In the white petals of Anaphalis margaritacea

only flavones were isolated by Favreau and Raymond (1970), yet the yellow silky fiber was found to contain the flavonols kaempferol and quercetin. Of more recent interest is a note by Durkee and Harborne (1973) that three yellow-seeded varieties of rape were found to contain 7-glucosides while brown seeded varieties lacked them. There has been some doubt expressed, however, concerning the contribution of quercetin and kaempferol to the yellow color of flower petals as noted by Harborne (1965 b). He points out that these compounds have been isolated from white, ivory, and cream flower petals. My results tend to support the view that flavonol glycosides contribute at least in part to the yellow color of T. luteum petals. It is not believed that they are the only pigments responsible for this color.

CHAPTER IV

SCANNING ELECTRON MICROSCOPY

Studies were conducted on the pollen grains and abaxial leaf surfaces of Trillium luteum and T. cuneatum. Pollen grains were used because they have been found to be taxonomically, ecologically, and phylogenetically important (Echlin, 1968). The effect of various preparative techniques differ from species to species, ". . . therefore, it is important in any systematic pollen research to ascertain the amount of change in the material so that the data can be placed in proper perspective for comparative interpretation" (Hanks and Fairbrothers, 1970). Preparation for SEM involves placing specimens in a vacuum and also coating them with a conductive surface since they are usually not conductive with respect to secondary electrons. Both processes may result in distortion and misrepresentation of real pollen grains.

Voucher specimens utilized in SEM studies are listed in Table II (page 36). Four populations of T. luteum and five populations of T. cuneatum were examined with populations selected from diverse geographical areas. Specimens were mounted on standard three-fourth inch aluminum studs. Pollen grains were attached to double-faced Scotch

tape; the spider (see page 76) was attached by liquid graphite as were the leaf sections. The mounted specimens were then coated with vaporized carbon and gold in vacuo using a Denton vacuum coater equipped with a random rotating head. The specimens were viewed with an ETEC Auto-scan model Scanning Electron Microscope and the results are presented in Figure 9.

Differences were detected between T. cuneatum and T. luteum with respect to ultrastructure of the pollen grains, yet not enough populations were observed in order to reach concrete conclusions. It appears, though, that the exine surface of T. luteum is much more consistent than the surface of T. cuneatum pollen grains. Patrick (1975) described the pollen grains as being globose with the exine possessing evenly distributed wart-like projections. This description seems to conform with the photomicrographs except for the indentations often observed with the SEM. These indentations are believed to be artifacts of preparation techniques. The wart-like projections appear to be coarse verrucae. Although mono-aperturate (Kapp, 1969), the furrow has escaped detection at this time.

No differences were noted on the abaxial surfaces of the bracts. Small puberulent hairs are found on the veins of the bracts of both taxa, and these hairs portray striations at high magnifications.

Figure 9. Scanning electron microscope photomicrographs of pollen grains and leaf surfaces of Trillium cuneatum and Trillium luteum.

- A. Trillium cuneatum pollen grain (2,500X).
- B. Trillium cuneatum pollen grain (8,000X).
- C. Trillium luteum pollen grain (2,500X).
- D. Trillium luteum pollen grain (8,000X).
- E. Representative abaxial leaf surface of both Trillium cuneatum and Trillium luteum showing vein (80X).
- F. Striated protrusion on vein (900X).

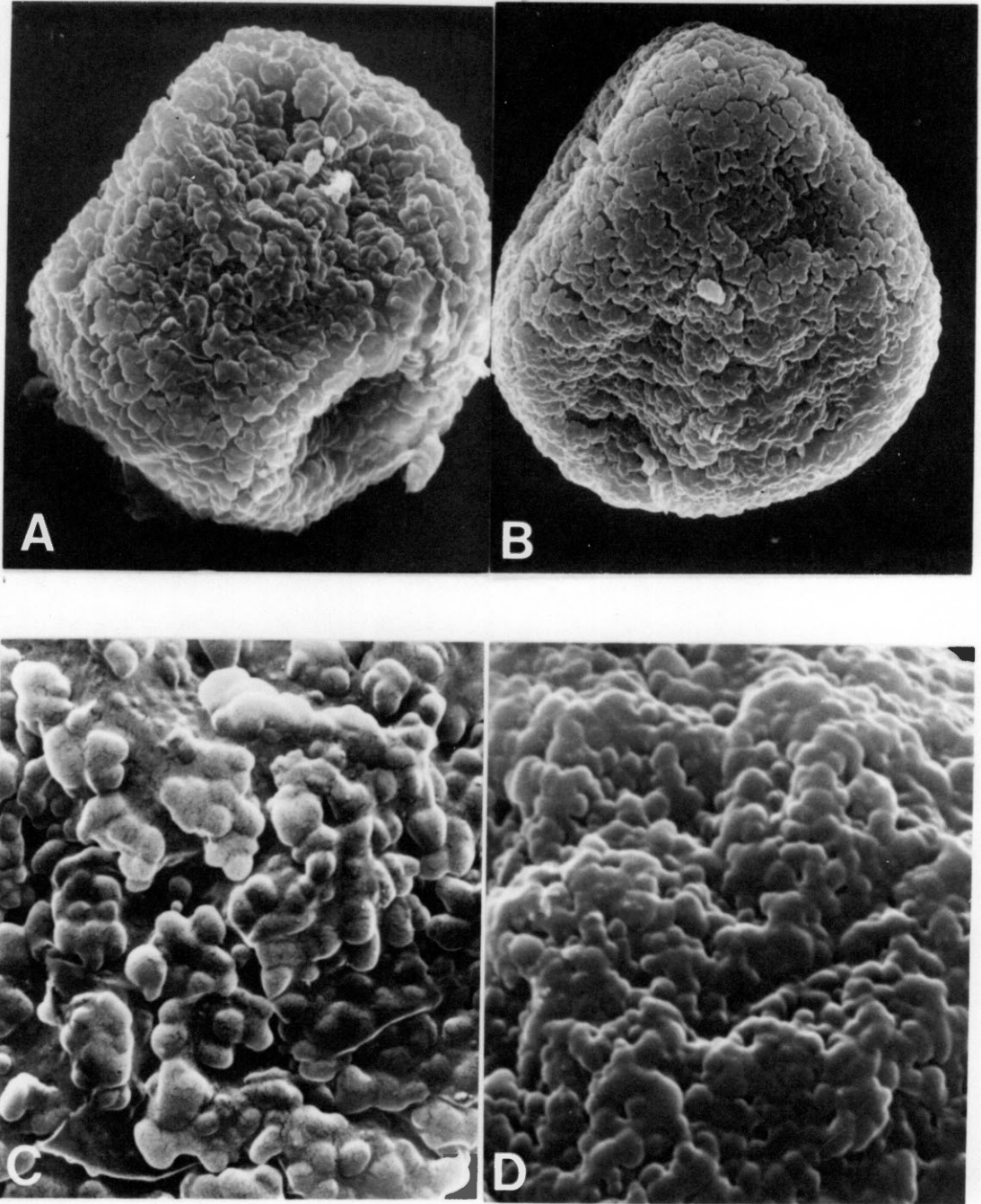


Figure 9

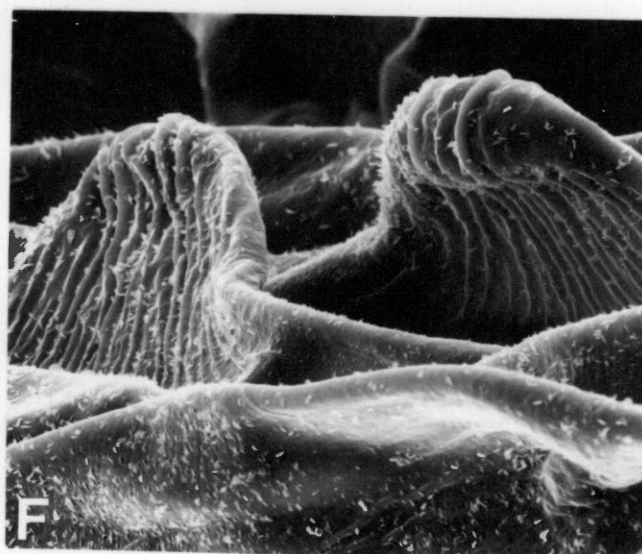
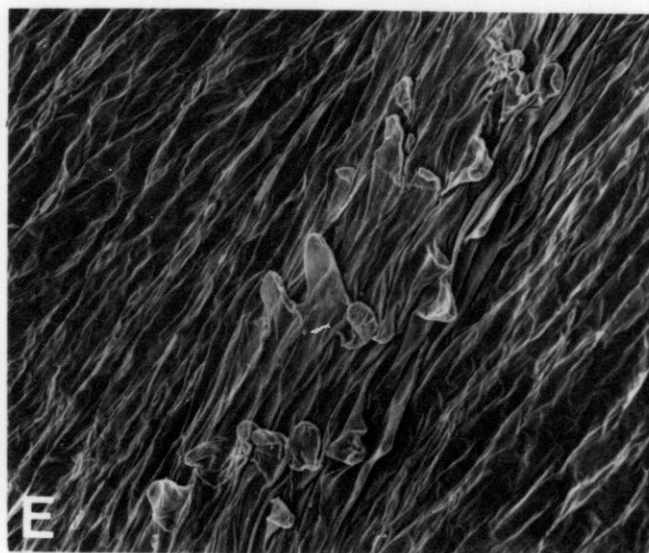


Figure 9 (continued)

Six other taxa of Trillium were observed with SEM, and the pollen grain ultrastructure was different within each taxon. As stated on the preceding page, more populations should be observed before decisive statements are made concerning variations of pollen grains, but it appears that these structures might prove to have potential systematic significance.

CHAPTER V

POSSIBLE CAUSES OF CURRENT DISTRIBUTIONS OF TRILLIUM CUNEATUM AND TRILLIUM LUTEUM

Petal Color. The variation of flower color noted in Trillium cuneatum is not found only with this species of Trillium but in fact has been noted by Freeman and Murrell (1969) in every species of sessile-flowered Trillium in the eastern United States. Within taxa that show this variation, no taxonomic significance based on color differences can be derived; yet, Freeman states that color of floral parts in Trillium is most important in a population sense, especially when plants possessing a distinctive color are found to have a well-defined geographic range. Trillium discolor, T. luteum, and T. albidum occur in ranges where red forms are rare if not completely absent (Freeman, 1969). In T. luteum this was found to be true within the region of allopatry except for the Sequatchie Valley where T. luteum is found with T. recurvatum.

One might conclude that color differences could well be a chief factor in affecting distribution. Perhaps some vector of pollination is attracted only to yellow plants, or maybe some predator exists within the areas

that contain the white or yellow species that preys on insects that normally pollinate Trillium. In such a case survival could be dependent upon some mechanism to protect pollinators. For instance, a yellow insect would be readily conspicuous on a red petal, but inconspicuous on a yellow petal. If the continuation of the yellow form depended upon the fertilization of only homozygous recessive gametes (i.e., yellow with yellow), then this predator--prey relationship would provide a means of explaining the spread of a yellow variety into a region, or the lack of success of the red form within that region. This is especially significant when one considers the hypothesis that evolutionary success is dependent upon reproductive efficiency when two organisms are competing for the same resources.

The possibility of yellow petals favoring cross-pollination within a physiographic province is demonstrated by a spider in the Misuminops genus of the Thomisidae family. This spider is white or sometimes yellow when observed on T. luteum plants, and in several instances it was observed traveling from one plant to another. At first observation its presence was not noticed due to the fact that its color is very close to that of T. luteum petals and the white veins on the lower surfaces of the bracts. A specimen of the spider was collected and mounted on a pedestal for SEM observations. Pollen grains were

detected in several areas on the spider (see Figure 10), and the pollen is unmistakably that of T. luteum. These pictures prove that the spider, which is known to travel from one plant to another, also has the potential for carrying pollen grains with it. It does not prove that the spider is a vector of cross-pollination, yet it certainly presents a strong possibility. Even if this spider were a vector of cross-pollination, it would be hard to prove that it was the agent responsible for the success of T. luteum within the Tennessee Valley. This spider does, though, show quite effectively how cryptic coloring could affect the reproductive success of one taxon over another (e.g., T. luteum over T. cuneatum) resulting in directional selection.

Apomictic Reproduction. Another possibility that might explain the present distribution of the two taxa is that T. luteum could reproduce by self-pollination. If indeed T. luteum is apomictic, then a natural mechanism exists for allowing only homozygous recessive zygotes to be formed. Once a homozygous recessive form rises, self-pollination would allow for its perpetuation and would eventually result in a higher degree of homogeneity within a population. The occurrence of the red-flowered T. recurvatum within the Sequatchie Valley might be explained by the fact that it is known to be apomictic. There would be no need for cross-pollination, and therefore, the color

Figure 10. Sequence of photomicrographs showing that Trillium luteum pollen grains are carried by this spider. The arrows in A and B point to pollen grains.

- A. Full view of spider (20X).
- B. Two pollen grains shown on spider's leg (40X).
- C. Higher magnification of the two pollen grains shown in B (940X).

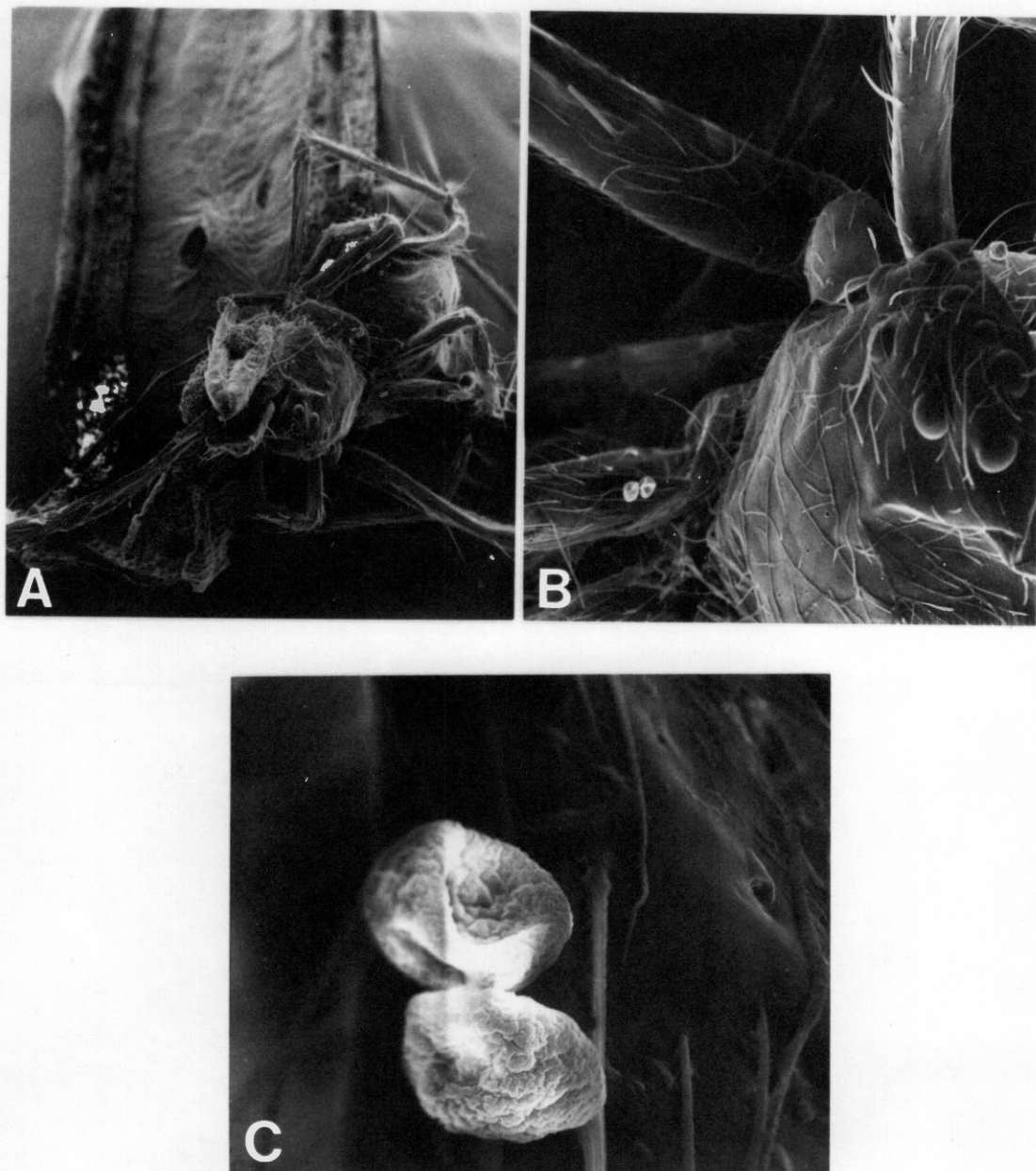


Figure 10

of a pollinator would be of no consequence with respect to distribution. The possibility of T. luteum self-pollinating might perhaps best be shown with Figure 11 where one can readily see anther sacs in close proximity to stigmatic surfaces.

Self-pollination as a mechanism of spreading a yellow-flowered form of Trillium has actually been observed in a population of T. recurvatum located near Nashville, Tennessee. Channell (personal communication) has pointed out a large yellow-flowered T. recurvatum with three much smaller yellow-flowered individuals growing within two feet of the larger plant. No other yellow forms were observed anywhere in the vicinity of these four plants. Upon digging up the large plant, it was discovered that the three smaller plants were in no way attached to it. The possibility of three pollen grains with recessive genes for anthocyanin production pollinating three eggs of the larger plant seems highly unlikely, especially since no other yellow forms were observed in the area. It appears that the yellow-flowered small plants arose from the larger one (see Figure 12) as a result of apomictic reproduction, which allowed for the perpetuation of the homozygous recessive genes. More work is certainly needed within this area, yet the likelihood of these two hypotheses (i.e., pollination and apomictic reproduction) explaining the divergence of T. luteum from T. cuneatum is certainly promising.



Figure 11. Trillium luteum with two stamens removed.



Figure 12. Trillium recurvatum forma shayi.

CHAPTER VI

TAXONOMIC CONSIDERATIONS

There are several ways that one might treat Trillium luteum with respect to T. cuneatum: 1) as being indistinguishable; 2) as a form; 3) as a variety; and, 4) as a distinct species. The differences and similarities of T. luteum and T. cuneatum are listed in Table VI. One glance at the table makes it immediately obvious that the two taxa are distinguishable from one another. Form is a rank reserved for variation seen within a population due to simple genetic variability. Some genes have a low frequency and seldom are expressed phenotypically. Allopatric distribution rules out this ranking, and therefore, the status of variety must be considered. Morphologically and geographically the two taxa seem to fit well into the rank of variety, especially since they appear to be hybridizing in sympatric populations. When chemical differences are considered, though, one might be led to believe that T. luteum and T. cuneatum are between varietal and specific levels of classification.

This intermediate position is not unique in the plant kingdom as exhibited by the following quote by Grant (1971):

TABLE VI

COMPARISON OF DISTINCTNESS AND SIMILARITIES
OF TRILLIUM LUTEUM AND TRILLIUM CUNEATUM

DIFFERENCES	
<u>Luteum</u>	<u>Cuneatum</u>
Allopatric in East Tennessee	Allopatric in many physiographic provinces
Different terpene chemistry; lemon odor	Different terpene chemistry; spicy or variable odor
Lack of flavonol 3-glycosides within petals	Presence of flavonol 3-glycosides within petals
Lack of cyanidins within petals	Presence of cyanidins within petals
Pollen grains with overlapping verrucae	Pollen grains with coarse, non-overlapping verrucae
Homogeneity with consistent introse dehiscence; stigmas never divergent, always together	Polymorphic with mostly lateral and some introse dehiscence; stigma often divergent and not as papillose
SIMILARITIES	
Identical karyotypes	
Lack of 100 per cent barriers to hybridization	
Morphological similarities	
Existence of yellow form within allopatric populations of <u>T. cuneatum</u>	

The fact that not all systems of breeding populations fall into two clear-cut alternative categories, races and species, but that some have intermediate and transitional properties, was emphasized by Darwin (1859), who concluded from this that species are units which can be defined only in an arbitrary way.

This situation is particularly evident in plants, which may reproduce asexually as well as sexually and, therefore, create semi- or microspecies. In the early stages of divergence, populations may be found as geographical races occupying adjacent territories and interbreeding freely in their zones of contact, whereas in later stages the populations located within sympatric areas have created barriers to cross-fertilization and are, therefore, distinct species. Trillium luteum and T. cuneatum seem to represent two taxa currently at an intermediate stage between varietal and specific status connected by a reduced amount of interbreeding and gene flow. Within the areas of allopatry, each taxon exhibits distinct characters and a trend toward uniqueness (e.g., difference in terpene chemistry, synthesis versus non-synthesis of anthocyanins, and production versus lack of production of flavonol 3-glycosides within the petals). Grant (1971) has used the term semispecies to refer to populations that are neither good races nor good species but are connected by a reduced amount of interbreeding and gene flow; this situation seems to apply well to T. luteum.

"Semispecies can develop not only as entities on the way to becoming species, but also as products of hybridization between previously well-isolated species" (Grant, 1971). This could well be the case with T. luteum and T. cuneatum in which populations in areas of sympatry give the appearance of hybrid swarms or introgressing populations. A barrier to interbreeding between T. luteum and T. cuneatum apparently has not become 100 per cent effective at this point in time, and therefore, a major question is whether T. cuneatum is moving into the Tennessee Valley or whether the allopatric range of T. luteum is expanding. It is possible that the cause of the establishment of T. luteum is no longer present, in which case the former condition would probably be true. A third alternative is that the two taxa have stabilized their distributions with respect to each other allowing primary speciation within the regions of allopatry to continue. It is my belief that the last situation (i.e., status-quo) is the most probable because the range of T. luteum is more-or-less restricted to distinct physiographic provinces (the Tennessee and Sequatchie Valleys) with regions of sympatry and intermediate populations occurring at the borders of these areas. These regions of overlap occur where the Cumberland River dissects the Cumberland Plateau, where the Sequatchie River emerges from the Cumberland Plateau into northern Alabama, and also where the

Little Tennessee River cuts through the Unaka Mountains. The fact that T. luteum is found on the southeast side of the Smokey Mountains well into North Carolina could possibly be taken as evidence that this taxon is increasing its range.

Semispecies is not a recognizable working unit within botanical systems of nomenclature. A classification of subspecies, as is commonly found and accepted within the Asteraceae, might be the most appropriate classification for T. luteum; however, this would be inconsistent with the ranking of other taxa within the genus Trillium, or even within the Liliaceae. It is probably better to retain T. luteum as a distinct species until more is known regarding its past and possible future distribution. In other words, the determination of whether the range is shrinking or growing might be the best criterion for delimiting the two taxa, yet this specification is presently unknown. If the uniqueness of T. luteum is being lost via introgressive hybridization with T. cuneatum, which is slowly spreading into the Tennessee Valley via natural means or transplants by people, then it would be best to consider T. luteum as a variety of T. cuneatum. On the other hand if T. luteum is holding or increasing its range, then the rank of species would be the most appropriate. Because most workers recognize T. luteum as a distinct species, and because a change in rank to a lower

position would cause confusion, and because the ranking of species appears to be the best level from which future work might be directed, I feel that T. luteum should be maintained at the specific level at this time.

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