The flavonoids of *Arnica frigida* and *A. louiseana* (Asteraceae)

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Received January 22, 1986

DOWNIE, S. R., and K. E. DENFORD. 1986. The flavonoids of *Arnica frigida* and *A. louiseana* (Asteraceae). Can. J. Bot. **64**: 2748–2752.

Forty-six populations within the *Arnica frigida* Meyer ex Iljin – *louiseana* Farr complex were examined for flavonoid content. Seven glycosides were isolated and six identified. Members of this complex were found to produce primarily quercetin and kaempferol 3-*O*-glycosides. Flavonoid profiles obtained from *A. frigida* ssp. *frigida* were the most diverse and in some cases the most depauperate. Within this taxon, flavonoid diversity appears to have accompanied high morphological variability. The occurrence of flavonoid-depauperate plants in unglaciated Alaska suggests that flavonoids are of value in identifying refugia. Flavonoid profile similarities between *A. frigida* ssp. *frigida* and *A. frigida* ssp. *griscomii* corroborate morphological evidence as to the close affinity between these two taxa.

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La teneur en flavonoïdes a fait l'objet d'études dans 46 populations à l'intérieur du complexe *Arnica frigida* Meyer ex Iljin – *louiseana* Farr. Sept glucosides furent isolés dont six identifiés. Les membres de ce complexe produisent principalement les 3-*O*-glycosides: quercétine et kaempférol. Les profils des flavonoïdes obtenus d'*A. frigida* ssp. *frigida* sont parmi les plus variés et en certains cas, les plus pauvres. À l'intérieur de ce taxon, la diversité en flavonoïdes parait avoir accompagné une variabilité morphologique élevée. La présence de plantes appauvries en flavonoïdes dans l'Alaska non glacié suggère que les flavonoïdes ont une certaine valeur dans l'identification des refuges. Les similarités des profils de flavonoïdes entre *A. frigida* spp. *frigida* et *A. frigida* spp. *griscomii* appuient l'évidence morphologique de la grande affinité entre ces deux taxons.

[Traduit par la revue]

Introduction

The *Arnica frigida–louiseana* complex (Asteraceae) has been previously recognized as one species, *A. louiseana,* with three infraspecific taxa: subspecies *frigida, griscomii,* and *louiseana* (Maguire 1943). An investigation into the morphology, cytology, and phytogeography of this complex supports the recognition of these taxa now as *A. frigida* ssp. *frigida* Meyer ex Iljin, *A. frigida* ssp. *griscomii* (Fernald) S. R. Downie, and *A. louiseana* Farr (Downie and Denford 1986). These three taxa have distinct geographic distributions: *A. frigida* ssp. *frigida* is found from eastern Siberia, Alaska, and Yukon, east to the Mackenzie River, N.W.T., with isolated populations east of the Mackenzie River and in northern British Columbia; *A. frigida* ssp. *griscomii* is extremely localized in Gaspé, Quebec, and in northwest Newfoundland; and *A. louiseana* is restricted to high elevations in the Rocky Mountains of Alberta and British Columbia (Downie and Denford 1986).

The allopatric distribution of the taxa as affected by historical factors, the occurrence of four chromosome races, the close relationship between the disjunct subspecies of *A. frigida* and the presence of both apomictic and amphimictic forms suggest that this complex might be profitably examined through a study of its flavonoid chemistry.

Flavonoids have been used extensively to support taxonomic revisions (Gornall and Bohm 1980) and to document changes in chemical complexity (both in flavonoid number and structural diversity) as a result of geographical isolation (Mears 1980; Wolf and Denford 1984*a*), hybridization (Wolf and Denford 1984*b*), polyploidy (Levy and Levin 1971, 1975), or plant migrations (Mastenbroek *et al.* 1983). In addition, flavonoids can provide useful data for inferring phylogenetic relationships (Stuessy and Crawford 1983). Chemosystematic investigations in *Arnica* have dealt primarily with floral chemistry, e.g., *A. montana* L. and *A. chamissonis* Less. (Borkowski *et al.* 1966; Willuhn *et al.* 1983; Merfort 1984, 1985; Kostennikova *et al.* 1985), while others have dealt with leaf flavonoids, e.g., *A. montana* (Saner and Leupin 1966), in which kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-glucogalacturonide were found. Wolf (1981) was able to identify 12 glycosides and 14 free aglycones in *Arnica* subgenus *Austromontana* with the glycosides of quercetin 3-*O*-gentiobioside and quercetin 3-*O*-diglucoside (viscosin[1]) being nearly ubiquitous in occurrence. Other common glycosides were found to be quercetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside with the most frequently occurring free aglycone in the subgenus being apigenin-6-*O*-methyl ether.

Chromatographic and ultraviolet spectral analyses were carried out on 46 populations representing the three taxa. Populations examined are listed in Table 1. No attempt was made to examine infrapopulational variability.

Materials and methods

Determination of flavonoid constituents within the *Arnica frigida–louiseana* complex was accomplished using the modified procedures of Mabry *et al.* (1970), Ribéreau-Gayon (1972), Neuman *et al.*[2], and Markham (1982). Plants collected in the field were air-dried or oven dried in paper bags with only leaf material being used in this study. Fresh leaf material from greenhouse-propagated plants was also analyzed and compared with dried material. There were no observable flavonoid differences between fresh and dried material.

For each population, 15–20 g (dry weight) of ground leaf material was extracted with approximately 500 mL of 85% aqueous methanol (MeOH). The slurry was placed on a shaker for 24 h, filtered through a Buchner funnel, and reextracted with another 500 mL of 85% MeOH. The same procedure was repeated twice more with 50%

[1]A diglucoside of quercetin with unusual chromatographic characteristics and an unknown sugar linkage. Isolated first from *A. viscosa* (Wolf 1981).

[2]Neuman, P., B. Timmerman, and T. J. Mabry. 1979. Laboratory manual for the systematic identification of flavonoids. University of Texas, Austin.

Printed in Canada / Imprimé au Canada

TABLE 1. Collections used in flavonoid analysis

Taxon	Locality and voucher
A. frigida ssp. *frigida*	CANADA: B.C.: Summit Lake, Stone Mtn. Prov. Park, *SRD 452*; Summit Lake, Stone Mtn. Prov. Park, *SRD 525*; YUKON: Km 32.5, Hwy. 9, *SRD 469*; Km 34.5, Hwy. 9, *SRD 470*; Km 38.5, Hwy. 9, *SRD 471*; Km 73.5, Dempster Hwy., *SRD 474*; Km 75, Dempster Hwy., *SRD 476*; Km 76, Dempster Hwy., *SRD 478*; Km 80, Dempster Hwy., *SRD 477*; Km 1717.5, Alaska Hwy., Kluane Park, *SRD 628*; Km 1912, Alaska Hwy., SSE Beaver Creek, *SRD 674*. U.S.A.: ALASKA: Mile 258, Richardson Hwy., 12 km S. Delta Junction, *SRD 503*; Mile 250, Richardson Hwy., *SRD 504*; Mile 254, Richardson Hwy., *SRD 662*; Donnelly Dome, *SRD 519*; Mile 259, Richardson Hwy., *SRD 660*; Mile 231, Richardson Hwy., Darling Creek Crossing, *SRD 663*; Mile 193, Richardson Hwy., *SRD 666*; Mile 84.8, Steese Hwy., *SRD 505*; Mile 89, Steese Hwy., *SRD 650*; Mile 99.5, Steese Hwy., Fish Creek Crossing, *SRD 657*; Mile 106, Steese Hwy., Eagle Summit, *SRD 506*; Mile 115, Steese Hwy., *SRD 656*; Mile 39, Elliott Hwy., 30 miles SE Livengood, *SRD 508*; Mile 39.3, Elliott Hwy., *SRD 508A*; Healy, *SRD 509*; Hwy. 3, 1 km N Denali Park entrance, *SRD 642*; Mile 246, Hwy. 3, S Healy, *SRD 644*; Mile 256, Hwy. 3, *SRD 645*; Mile 13, Hwy. 8 (Denali Hwy.), *SRD 515*; Mile 22, Hwy. 8, Tangle Lakes Campground, *SRD 516*; Mile 11, Hwy. 8, *SRD 517*; Mile 106.5, Glenn Hwy., Caribou Creek, *SRD 514*; Mile 1412, Alaska Hwy., SE Delta Junction, *SRD 668*; Hwy. 1, 12 miles S Tok, *SRD 638*; Mile 102.5, Hwy. 1, *SRD 639*; Mile 67.5, Hwy. 1, Carison Creek, *SRD 640*; Mile 40, Taylor Hwy., *SRD 475*
A. frigida ssp. *griscomii*	CANADA: QUEBEC: Mt. Saint-Alban, Forillion Natl. Park, *SRD 531*; NEWFOUNDLAND: SW Port Au Choix, *SRD 533*; Pointe Riche, *SRD 534*
A. louiseana	CANADA: ALBERTA: Moraine Lake, Banff Natl. Park, *SRD 449*; Peyto Lake, Banff Natl. Park, *SRD 450*; Columbia Icefields. Jasper Natl. Park, *SRD 544*; Bald Hills, Jasper Natl. Park, *SRD 546*; Bald Hills, Jasper Natl. Park, *SRD 547*

MeOH and the four filtrates were combined and evaporated *in vacuo* until about 100 mL of filtrate remained. The aqueous fraction was partitioned against a three-volume excess of chloroform to remove low-polarity contaminants such as chlorophylls, xanthophylls, fats, terpenes, and some flavonoid aglycones. This process was repeated until no colour remained in the solvent. Subsequent chromatographic analysis revealed that the chloroform fraction contained no flavonoids and it was discarded. The solvent-extracted aqueous layer, containing the flavonoid glycosides, was again reduced *in vacuo* to remove all traces of chloroform. This aqueous phase was partitioned further with ethyl acetate (EtOAc). Equal volumes of EtOAc (to water) were added to a separatory funnel and partitioned; this was repeated until the extracting solvent was clear. These two fractions were separated, reduced *in vacuo* to approximately 25 mL, and subsequently used in paper chromatography. Chromatography revealed that the flavonoid content of the EtOAc fraction was identical with that of the water fraction so this last partitioning step was abandoned.

The aqueous fraction were separated by standard paper chromatography techniques using the solvents BAW (butanol – acetic acid – water; 4:1:5; upper phase) and 15% acetic acid (HOAc) (Mabry *et al.* 1970). For a preliminary assessment of flavonoid diversity within the complex, one sheet of Whatman No. 3MM (46 × 57 cm) paper was used per population. In this method, the flavonoids present in a population show up as spots when viewed under ultraviolet light (366 nm). To heighten the sensitivity of detection one chromatogram per population was treated with NH_3 vapour and NA (Naturstoffreagenz A; diphenyl – boric acid – ethanolamine complex) spray reagent. These methods produce colour changes of structural significance in addition to aiding in locating minor constituents. All colour changes and spots were recorded on these chromatograms.

After this preliminary survey, 20 sheets of 3MM paper were run per population. Equivalent spots from each paper chromatogram were cut out, combined, and eluted in 80% MeOH for 24 h on a shaker. In addition, because the absence of a compound may be due to low flavonoid concentration, equivalent areas on the chromatogram where a flavonoid was presumed to occur were also cut out and eluted. This was done by comparing the chromatogram with the absent compound(s) with ones in which most compounds were present. Afterwards, the solution was filtered and the filtrate evaporated *in vacuo* to approximately 10 mL. These now-concentrated extracts obtained from flavonoids which were weakly concentrated on a single sheet of paper, or obtained from areas on a chromatogram from which a flavonoid was presumed to exist but could not be seen, were spotted on another chromatogram and rerun. These results and those obtained from the preliminary flavonoid analysis were used to compare taxa and prepare the presence–absence table.

Flavonoid extracts were subsequently purified by chromatography through a 5.5 × 60 cm column of Polyclar AT polyamide packed in the elution solvent. The column was first eluted in 100% MeOH and monitored using a UV lamp. The polarity of the solvent was gradually increased by adding water. Final purification was achieved on smaller columns (2.5 × 30 cm) packed with Sephadex LH-20 and eluted with 100% MeOH.

Fraction purity was assessed using thin-layer chromatography on polyamide, cellulose, and silica gel coated plates. Solvent systems for polyamide consisted of water – methyl ethyl ketone (MEK) – MeOH – acetyl acetone (13:3:3:1) or chloroform–MeOH–MEK (9:4:1) for glycosides and benzene–MEK–MeOH (60:26:14) for nonpolar flavonoids (see footnote 2). Solvent systems for cellulose consisted of 40% HOAc for aglycones to 15% HOAc for glycosides. Solvent systems for silica gel consisted of EtOAc – MEK – formic acid – water (5:3:3:1) for glycosides and toluene – ethyl formate – formic acid (5:4:1) for the aglycones (Randerath 1963).

Once isolated, flavonoids were identified using one-dimensional descending chromatography, cochromatography with standards, and standard spectral and hydrolytic procedures (Mabry *et al.* 1970; Markham 1982). The solvents Forestal (HOAc–water–HCl; 30:10:3) on Whatman No. 1 paper (Ribéreau-Gayon 1972) and toluene – ethyl formate – formic acid (5:4:1) on silica gel thin-layer plates (Randerath 1963) were used for aglycone identification after acid hydrolysis. The cleaved sugars were isolated by chromatography in isopropanol–*n*-butanol–water (140:20:40) (Smith 1969) and identified by cochromatography with standards and their colour reaction with aniline hydrogen phthalate solution after spraying and developing (Ribéreau-Gayon 1972).

Voucher specimens of material used in the present investigation are on deposit at the University of Alberta Herbarium (ALTA).

Results

Seven flavonoid glycosides (five flavonols, one flavone, and one unknown) were isolated from *A. frigida* and *A. louiseana*: quercetin 3-*O*-galactoside; kaempferol 3-*O*-glucoside; quercetin 3-*O*-diglucoside (viscosin); kaempferol 3-*O*-galactoside; quercetin gentiobioside, and apigenin 7-*O*-glucoside. Only two sugars, glucose and galactose, are associated with the

TABLE 2. Collection number and locality, ploidy level, and distribution of flavonoids in the *Arnica frigida–louiseana* complex

	Locality and collection No.	2*n*	Flavonoid*						
			1	2	3	4	5	6	7
Arnica frigida ssp. *frigida*	Alaska: 504, 506, 514, 519, 645, 656, 662, 668	38	+		+				
	Alaska: 508, 508A, 663, 666	38	+	+	+				
	Yukon: 470, 471, 474, 674	57	+	+	+				
	Alaska: 475, 638, 639, 640	57	+	+	+				
	Alaska: 505, 509, 515, 516, 517, 642, 644, 650	38	+	+	+		+		
	Yukon: 469, 477, 478	57	+	+	+		+		
	Yukon: 476	57	+	+	+	+			
	British Columbia: 452, 525	95	+	+	+	+			
	Alaska: 503, 660	38	+	+	+		+	+	
	Alaska: 657	38	+	+	+			+	
	Yukon: 628	57	+		+	+			
Arnica frigida ssp. *griscomii*	Quebec: 531	76	+	+	+				
	Newfoundland: 533, 534	76	+	+	+		+		
Arnica louiseana	Alberta: 449, 450, 544	76	+	+	+	+		+	
	Alberta: 546, 547	76	+	+	+	+		+	+

*1, quercetin 3-*O*-galactoside; 2, kaempferol 3-*O*-glucoside; 3, quercetin 3-*O*-diglucoside; 4, kaempferol 3-*O*-galactoside; 5, quercetin gentiobioside; 6, apigenin 7-*O*-glucoside; 7, unknown.

flavonoids. Sugar attachments are all at the 3 position in the flavonols and the 7 position in the flavone.

Compound 7 (the unknown) was found in such minute quantities that isolation and identification could not be done. However, the following preliminary information is available: R_f values, 0.42 BAW and 0.50 15% HOAc; colour reactions, purple UV, green UV and NH_3, and orange NA. Quercetin 3-*O*-diglucoside (viscosin), first reported in *Arnica* subgenus *Austromontana* (Wolf 1981), is characterized by the following spectral, R_f, and colour data: spectral data, MeOH, 258, 269*sh*, 348; NaOMe, 274, 340*sh*, 405; $AlCl_3$, 263, 295*sh*, 405; $AlCl_3$ and HCl, 260, 295*sh*, 356; NaOAc, 273, 390; NaOAc and H_3BO_3, 264, 374; R_f values, 0.26 phenol; 0.42 BAW, 0.41 15% HOAc, and 0.22 H_2O; colour reactions, purple UV, yellow UV and NH_3, and orange NA.

The distribution of flavonoids in the three taxa, their associated ploidy level, and collection locality are presented in Table 2. Quercetin 3-*O*-galactoside and quercetin 3-*O*-diglucoside were ubiquitous and kaempferol 3-*O*-glucoside was present in all but 8 collections of a total of 46 populations. The most complex flavonoid profile was exhibited by two collections in *A. louiseana* with compound 7 being unique. *Arnica frigida* ssp. *frigida* profiles were the most diverse and in some cases the most depauperate. Two profile types were found in *A. frigida* ssp. *griscomii*, one in Quebec and the other in northwestern Newfoundland, with both of these types common in subspecies *frigida*. Within the *A. frigida–louiseana* complex six major flavonoid profile types can be distinguished. This information, along with an indication as to whether the collection is a diploid or polyploid, is illustrated in Fig. 1.

Discussion

Flavonoid profiles of the *Arnica frigida–louiseana* complex are relatively simple, with two to six compounds found per population. The prevalence of quercetin and kaempferol glycosides is not unusual for they are widely distributed throughout the angiosperms (Harborne 1975); however, they do suggest a somewhat primitive biochemical profile (Harborne 1972). The lack of glycoside variability in the Asteraceae (Harborne 1977) is reflected in this complex for we see only two sugars occurring, glucose and galactose. The presence of quercetin 3-*O*-diglucoside (viscosin) in both *A. frigida* and *A. louiseana* and quercetin gentiobioside in only the former negates Wolf and Denford's (1984*a*) claim that these compounds are only found in *Arnica* subgenus *Austromontana*.

Narrow endemics are generally characterized by a reduced flavonoid profile composed largely of methylated aglycones, while in contrast, wide-ranging species within the same genus, occurring in a number of different habitats, are characterized by a high flavonoid diversity and few methylated aglycones (Mears 1980; Wolf and Denford 1984*a*). Mears (1980) has further reported in *Parthenium* a significant positive correlation of total number of flavonoids per taxon with area of distribution. An increase in flavonoid number with a corresponding increase in area can be contrasted to isolated island populations derived from mainland taxa in which we see both fewer and structurally simpler compounds produced (Mabry 1974). The depletion of the ancestral profile in narrow endemics may be due to such factors as the founder effect, autogamy (leading to a reduction in gene flow), and low environmental heterogeneity. The possibility that taxa exhibiting a reduced flavonoid profile represent the remnant of a refugial entity has been suggested by Denford (1973). The survival of *A. frigida* and *A. louiseana* during the Pleistocene in refugia has been previously discussed (Downie and Denford 1986).

Considering the distribution of chromosome races in *Arnica*, Barker (1966) showed that no well-developed sexual species ($2n = 38$) occurs in a glaciated area and no well-developed polyploid species occurs in an unglaciated area. *Arnica frigida* was determined to have a large sexual element in unglaciated Alaska (Downie and Denford 1986). In this respect, populations exhibiting only compounds 1 and 3 were found scattered throughout this area. The occurrences of these flavonoid-depauperate plants in unglaciated Alaska further corroborate the concept that flavonoids are of value in establishing

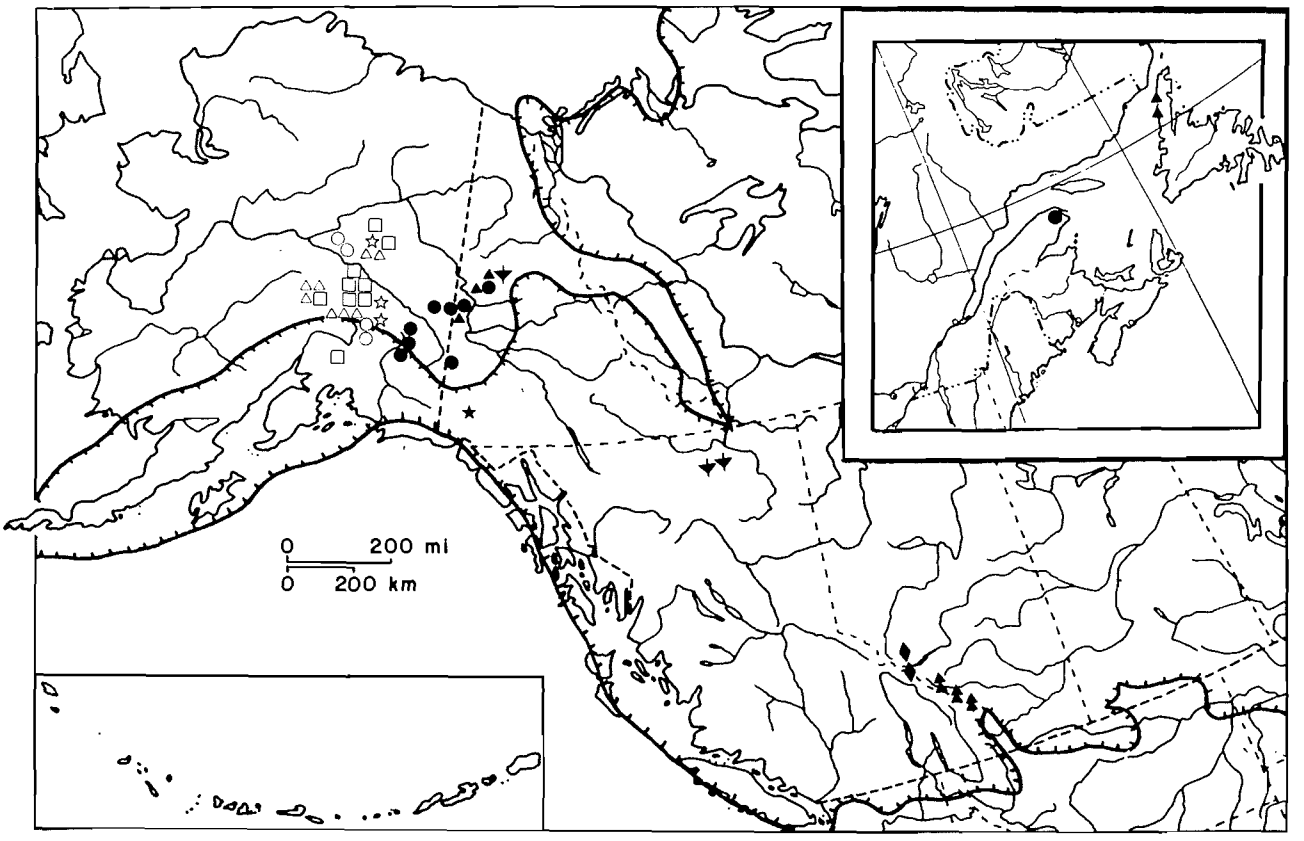

FIG. 1. The distribution of flavonoid profile types and associated ploidy level in the *Arnica frigida–louiseana* complex with respect to Pleistocene glacial limits (solid line). Open symbols, 2*n* (diploid); closed symbols, 3*n*, 4*n*, or 5*n* (polyploid). Flavonoid profile characterization: □, compounds 1 and 3; ○●, compounds 1, 2, and 3; △▲, compounds 1, 2, 3, and 5; ▼, compounds 1, 2, 3, and 4; ▲, compounds 1, 2, 3, 4, and 6; ♦, compounds 1, 2, 3, 4, 6, and 7; ☆★, miscellaneous.

plant refugial boundaries, as suggested by Denford (1973).

The polyploid species of *A. frigida* ssp. *frigida* appear to have radiated geographically and ecologically from their presumed refugium in unglaciated Alaska. Through dispersal and adaptation to different habitats, ecotypic variation, including chemical variation, would occur. With the prevalence of apomixis in this species (Barker 1966) and the large amount of morphological variability exhibited, much of the flavonoid variation probably reflects genetic heterogeneity from population to population. Considerable flavonoid populational variation has already been found in *A. cordifolia* (Wolf and Denford 1983). In contrast, *A. frigida* ssp. *griscomii* and *A. louiseana*, consisting of only one or two chromosome races, respectively, exhibit little morphological and flavonoid variability. Curiously, plants collected from similar geographic areas in unglaciated Alaska showed dissimilar flavonoid profiles, indicating the genetic discontinuity of amphimictic elements.

The lack of any correlation between flavonoid profile and chromosome number is not unusual and has been observed in other taxa (Glennie *et al.* 1971; Wolf *et al.* 1979; Wolf and Denford 1983).

The close morphological similarity between *A. frigida* ssp. *frigida* and *A. frigida* ssp. *griscomii* strongly infers that *A. frigida* was at one time confluent across North America. With the eradication of most of the central part of its range during Pleistocene glaciations, subspecies *griscomii* survived as a restricted biotype and can best be regarded as a paleoendemic. The presence of only one chromosome race ($2n = 76$), low morphologic variability, a rather stable invariant flavonoid profile, and a highly restrictive nature of these plants to specific calcareous habitats suggest that few biotypes survived glaciation. The presence of two flavonoid chemotypes in eastern Canada, both representative of patterns found within *A. frigida* ssp. *frigida*, is interesting for it confirms the relationship between the two disjuncts and the presence of two biotypes, one on the mainland in Gaspé, Quebec, and the other in northwestern Newfoundland. However, the rarity of subspecies *griscomii* precludes a wide survey of populations to ascertain the actual extent of flavonoid diversity.

Allopolyploids sometimes produce nonparental phenolics with structures produced by combining parental biosynthetic capacities (Mears 1979); thus newly created endemics may have a more diverse chemistry than their progenitors (Levy and Levin 1975). *Arnica louiseana* represents a taxon with the most complex chemical profile with five or six flavonoids present. Whether *A. louiseana* represents a novel entity created through the events of hybridization or polyploidy which, as previously suggested, may account for its complex chemical profile, or a paleoendemic which survived the Pleistocene either as present-day *A. louiseana* or its ancestor is difficult to ascertain. A further study into the genetic diversity of these present-day entities, utilizing allozymes, would shed more light on the origin and evolution of these taxa.

Acknowledgments

Financial support from the Boreal Institute of Alberta for Northern Studies and a Natural Sciences and Engineering

Research Council of Canada grant to K.E.D. are gratefully acknowledged.

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