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The Biogeographical Distribution of Duncecap Larkspur (*Delphinium occidentale*) Chemotypes and Their Potential Toxicity

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Abstract Larkspurs (*Delphinium* spp.) are poisonous plants found on rangelands in western North America. Larkspur's toxicity has been attributed to the norditerpenoid alkaloids, which are divided into two main structural groups: the highly toxic (*N*-methylsuccinimido) anthranoyllycoctonine type (MSAL type) and the less toxic 7,8-methylenedioxylycoctonine type (MDL type). Plants high in the MSAL-type alkaloids are thought to be the most toxic to cattle, and the concentrations of these alkaloids have been used as a predictor of plant toxicity. Duncecap larkspur, *Delphinium occidentale*, occurs throughout much of the Intermountain West and Northwestern United States. Specimens from field collections and herbaria deposits were evaluated taxonomically and chemically. Two distinct alkaloid profiles were identified: one that contains the MSAL-type alkaloids and one that contains little, if any, MSAL-type alkaloids. Thus, plants with these two alkaloid profiles should differ in their toxic potential. Each profile was unique in its geographical distribution. These findings have important implications in grazing management decisions on *D. occidentale*-infested rangelands, and they demonstrate that botanical classification alone is not a good indicator to determine the toxic risk of *D. occidentale*.

Keywords Larkspur · MSAL-type alkaloids · Norditerpene · *Delphinium occidentale* · Methyllycaconitine

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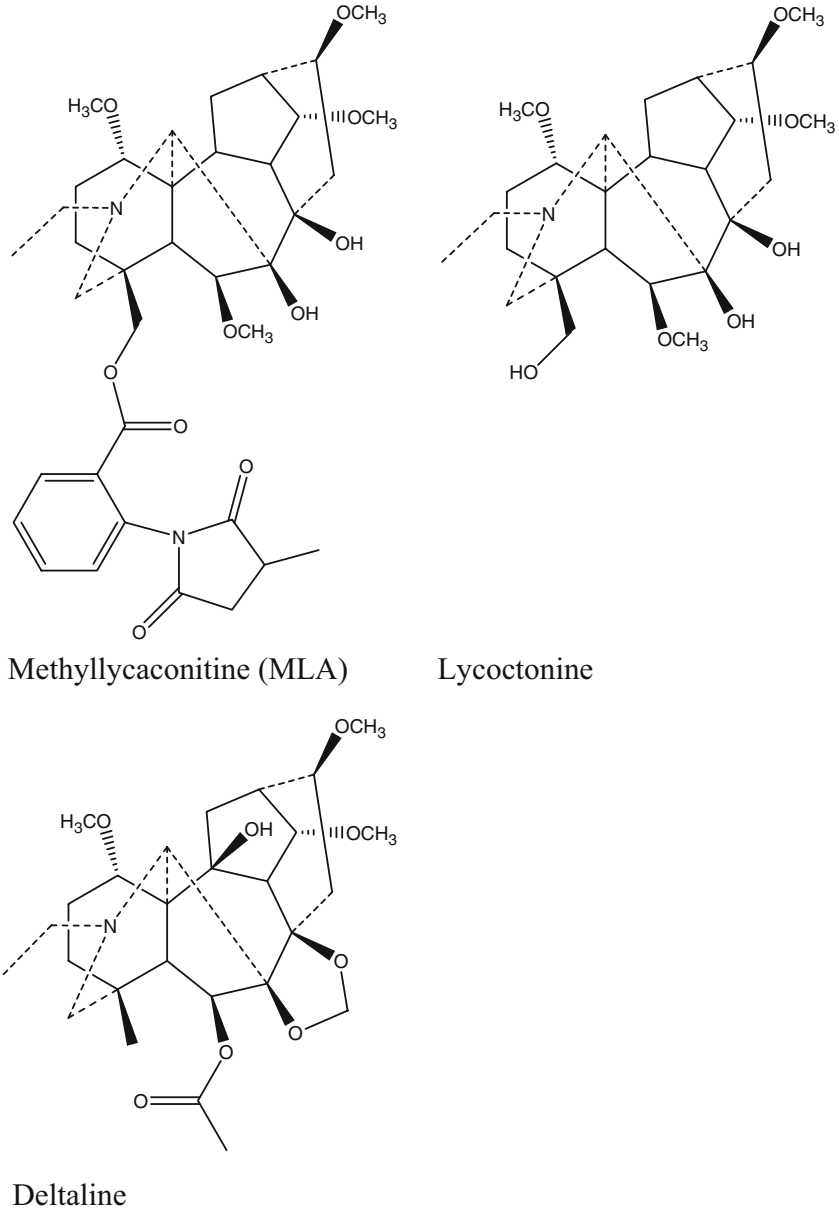
Introduction

Larkspurs (*Delphinium* spp.) are poisonous plants on rangelands in the western USA. They are responsible for significant losses to the cattle industry and are the subject of extensive research (Marsh et al. 1916, 1934; Pfister et al. 1999, 2002). Total cost to the livestock industry from cattle deaths attributed to larkspur poisoning is estimated to be millions of dollars annually (Nielsen et al. 1994). Larkspurs are divided into three groups based principally upon their height: low larkspurs, plains larkspurs, and tall larkspurs. The tall larkspurs are responsible for a greater number of cattle losses than either the plains or low larkspur.

Larkspur-induced poisoning in cattle is attributed to the diterpenoid alkaloids that can represent up to 3% of the plant dry weight. There are two main structural groups of norditerpene alkaloids, the *N*-(methylsuccinimido) anthranoyllycoctonine type (MSAL-type) and the 7,8-methylenedioxylycoconine type (MDL-type) norditerpenoid alkaloids (Fig. 1; Olsen et al. 1990). The MSAL-type are approximately 20 times more toxic than the MDL-type based upon the LD_{50} of the individual compounds in a mouse model (Manners et al. 1993, 1995; Panter et al. 2002). Acute larkspur poisoning has been attributed to the MSAL-type alkaloids (Aiyar et al. 1979; Pfister et al. 1999), and plants high in the MSAL type are thought to be the most toxic to cattle. The concentrations of these alkaloids have been used as a predictor of plant toxicity (Pfister et al. 2002; Ralphs et al. 2002). The most abundant member of the MSAL-type alkaloids in the tall larkspurs is methyllycaconitine (MLA; Gardner et al. 2002).

Two synopses of the genus *Delphinium* found in North America have been published, the first by Ewan (1945) and the second by Warnock (1995, 1997). Three tall larkspur species (*Delphinium barbeyi*, *Delphinium occidentale*, and

Fig. 1 Structure of select norditerpene alkaloids in *Delphinium occidentale*



Delphinium glaucum) of particular interest received different taxonomic treatment by both authors. Recent research from standard systematics (Welsh and Ralphs 2002), chemotaxonomy (Gardner et al. 2002), and molecular markers (Li et al. 2002) support the treatment given to these three species by Ewan (1945). All of these species can contain the MSAL-type alkaloids with MLA being the major member.

An observation of particular interest made by Gardner et al. 2002 was the identification of two alkaloid profiles in *D. occidentale*. One alkaloid profile lacked or displayed small amounts of the MSAL-type alkaloids, whereas the other profile displayed large amounts of the MSAL-type alkaloids. The objective of this study was to determine the extent of these two profiles throughout the geographical distribution of *D. occidentale*. We report here that *D. occidentale* has two definable chemotypes, with distinct geographical boundaries, that should differ in potential toxicity. These results have implications for grazing management decisions in *D. occidentale*-infested rangelands, and they demonstrate that taxonomic classification alone is not a good indicator to determine the toxic risk of *D. occidentale*.

Methods and Materials

Plant Materials Analytical samples were prepared from plant material collected from herbarium specimens and resident populations of *D. occidentale*. Herbarium specimens were provided by the Intermountain Herbarium at

Utah State University, the Stanley L. Welsh Vascular Plant Herbarium at Brigham Young University, the University of Colorado Museum Herbarium, the Rocky Mountain Herbarium at the University of Wyoming, the University of Washington Herbarium, and the Herbarium at Oregon State University. Specimens of questionable identification were verified to be authentic *D. occidentale* specimens by staff at the Intermountain Herbarium at Utah State University or the Stanley L. Welsh Vascular Plant Herbarium at Brigham Young University. Leaf and flower material were sampled from herbarium specimens and subsequently ground by using a Retsch mixer mill MM301 (Haan, Germany).

Field samples of *D. occidentale* populations (1,464 plants representing 118 accessions) were collected in the summer of 2007 and 2008. Accessions were collected throughout the geographical distribution of *D. occidentale* including the states of Utah, Idaho, Montana, Wyoming, Colorado, Nevada, and Oregon. Samples immediately were placed on dry ice after collection and stored at −80°C upon return to the laboratory. Samples were frozen for possible use in subsequent research. Samples were freeze-dried and ground to pass through a 2-mm screen using a Wiley mill.

Sample Extraction and Alkaloid Analysis Individual plant samples were extracted and analyzed by electrospray mass spectrometry by using procedures previously described (Gardner et al. 1999). In summary, 25-mg plant material from herbarium samples was extracted in 6 ml of methanol for 16 h. Reserpine (125 µg) was added as an internal reference. The sample was mixed then centrifuged. A 200-µl sample was diluted into 800 µl of 1:1 methanol/1% acetic acid. For plant samples collected in the field in the summers of 2007 and 2008, 100-mg plant material was extracted in 6 ml of methanol for 16 h. Reserpine (500 µg) was added as an internal reference. The sample was mixed then centrifuged. A 50-µl sample was diluted into 950 µl of 1:1 methanol/1% acetic acid.

Mass spectra were recorded for each sample over a range of 150–800 *m*/*z* and averaged across all scans taken at 40% of peak height (total ion current). Data were calculated by recording the abundance of all ions above a relative area of 0.1%. The amount of a compound (as represented by a single mass unit) detected was calculated based on the relative abundance of the internal standard reserpine (MH^+=609). The resulting mass spectral data were reduced and tabulated to a final set of quantitative values for 57 different protonated molecules by using a method similar to that reported by Gardner et al. (2002) (Table 1).

Data Analysis Each sample was assigned into group A (samples with MLA concentrations greater than 100 µg/mg) or group B (samples with MLA concentrations less than 100 µg/mg). This cutoff was chosen because it clearly separated the two alkaloid profiles observed previously by Gardner et al. (2002). MANOVA and discriminant analysis of the two assigned groups were performed as a pairwise comparison by using BioNumerics 4.6 (Applied Maths, Inc.). Two parameters were reported: (1) *L* (Wilk's Lambda likelihood ratio test) is the likelihood of the obtained discrimination with the assumption that the groups are drawn from the same population. A low *L* value infers that the groups are likely to be drawn from different populations. (2) *P* is the probability that a random grouping of the groups would yield the same degree of discrimination. *t* tests were performed by using Microsoft Excel. All multivariate and univariate statistical comparisons were made from plants of the same developmental stage.

Results

Field collections (1,464 specimens representing 118 accessions) of *D. occidentale* were made in the summer of 2007 and 2008. Samples were representative of the reported geographical distribution of *D. occidentale.* In addition, 599 herbarium specimens of *D. occidentale* from the cooperating herbaria were sampled. These specimens were collected from the late 1800s to the current year, and they also were representative of the reported geographical distribution of *D. occidentale*. All samples were analyzed by electrospray mass spectrometry. The resulting mass spectral data were reduced and tabulated to a final set of quantitative values for 57 different masses (Table 1).

Initially, each sample was scored for the presence of MLA (>100 µg/mg) or reduced amounts of MLA (<100 µg/mg). A total of 698 samples were identified that contained greater than 100 µg/mg MLA. A total of 1,365 samples were identified that contained less than 100 µg/mg MLA. To confirm that these two groups were unique, multivariate statistical methods (MANOVA and discriminant analysis) were used to test for grouping. The pairwise MANOVA revealed that the two groups were different (*P*= 0.001%, *L*=0.15). Discriminant analysis also was performed by comparing the two groups as a pairwise comparison. Discriminant analysis showed clear separation of the two groups based upon multiple alkaloids. The five most important discriminants were the following masses: *m*/*z* 683 (MLA), 715, 739, 753, and 699. These two multivariate methods demonstrated that the two groups were different. As a result, samples with greater than 100 µg/mg MLA will hereafter be termed chemotype A and those samples that have less than 100 µg/mg MLA will hereafter be termed chemotype B. Representative mass

Table 1 Comparison of total alkaloid extract from *Delphinium occidentale* chemotypes A and B

m/z[a]	Possible alkaloid(s)[b]	Chemotype A[c]	Chemotype B[c]
365	Unknown	17	16
386*	Delnuttaline, geyeridine	9	14
397	Unknown	16	16
404*	Unknown	65	46
414	11,13-Diacetyhetisine	15	20
428*	Geyerine	1	54
432*	Unknown	15	26
434*	8-Acetylkarasamine	9	15
436*	14-Acetylleroyine, delelatine	22	28
446	Unknown	54	88
450*	Delpheline, occidentaline	15	28
452*	Dictyocapinine, chasmanine	13	38
454*	Delcosine, delectinine	23	77
464*	6-Dehydrodeltamine	11	21
466*	Deltamine, 14-dehydrobrowniine	56	89
468	Delsoline, lycoctonine	190	218
476*	Unknown	41	58
480*	Barbeline, 14-acetyldelcosine	11	23
482*	Delphatine	50	165
492	Barbinidine	19	23
494	Dictyocarpine	180	175
496*	14-Acetyldelectinine, 6-epi-pubescenine	17	32
498*	Unknown	26	59
500	Unknown	7	4
504*	Unknown	5	11
508*	Deltaline	739	1120
510*	14-Acetylbrowniine	56	114
524*	Unknown	14	30
526	Unknown	1	4
527	Unknown	9	9
536	14-Acetlydictyocarpine	33	23
538	Unknown	36	25
546	Unknown	9	13
550	Unknown	30	31
552	Glaucedine	245	183
564	Glaucerine	52	72
568	Unknown	4	5
578	Barbisine, glaucenine	163	307
587*	Anthranolyllycoctonine	10	0
598*	Glaucephine	16	26
633*	Unknown	15	5
641	Unknown	24	23
649	Unknown	11	10
665*	Unknown	14	3
667*	Barbinine	11	1
669*	14-Deacetylnudicauline, 16-deacetylgeyerline	34	3
675*	Unknown	9	2
683*	Methyllycaconitine	656	4
689	Unknown	6	4

Table 1 (continued)

m/z[a]	Possible alkaloid(s)[b]	Chemotype A[c]	Chemotype B[c]
691*	Unknown	21	6
697*	Unknown	3	0
699*	Unknown	14	1
711*	Geyerline, nudicauline, acetylgrandiflourine	38	1
715*	Unknown	13	1
739*	Unknown	21	1
753*	Unknown	45	1
773*	Unknown	9	1

[a] Masses followed by an asterisk are different between the two chemotypes ($P<0.05/57=0.0009$). Analyses were performed using a *t* test with pairwise comparison of means

[b] Alkaloid identification taken from Pelletier et al. (1981, 1989), Joshi et al. (1988, 1989), Kulanthaivel et al. (1988, 1990), and Manners et al. (1996, 1998)

[c] Relative alkaloid concentrations per 100 mg of plant material. Concentrations were normalized to an internal reserpine standard

spectra of the two chemotypes are shown in Fig. 2. Chemotype A contains the MDL- and MSAL-type alkaloids including MLA, while chemotype B contains principally the MDL-type alkaloids and very little, if any, MLA.

A *t* test was used to identify masses whose concentrations are different between the two chemotypes. The mean concentration of each mass for each chemotype and the possible alkaloid each mass may represent are listed in Table 1. A conservative approach was taken for identifying masses whose concentrations are different by using the Bonferroni *P* value at $\alpha=0.05/57=0.0009$. By using these criteria, 36 masses were identified whose concentrations were different between the two chemotypes. The concentrations of 16 masses were greater in chemotype A and the concentrations of 20 were greater in chemotype B. The alkaloids that accumulated to greater amounts in chemotype A were other MSAL-type alkaloids in addition to MLA, while those alkaloids that accumulated to greater amounts in chemotype B were the MDL-type alkaloids.

The geographical distribution of each chemotype was investigated by state and counties within each state. Table 2 shows the number of samples analyzed from each state, county, and the number of samples assigned to each chemotype. A distribution map of the two chemotypes is shown in Fig. 3. Most counties had only samples of one chemotype. However, a number of counties such as Caribou (ID), Lincoln (WY), Sublette (WY), and Fremont (WY) have samples of each chemotype, indicating that they serve as a transition zone from south to north. Although these counties have plants with both chemotypes, individual populations from field collections in each county were either chemotype A or B in most cases. Likewise, counties that are in the transition zone from east to west, such as Juab and Box Elder counties of Utah, also have samples of both chemotypes but are spatially separated: chemotype A to the west and chemotype B to the east.

Discussion

Previous research has demonstrated that the MSAL-type alkaloids are more toxic than the MDL-type in a mouse model (Manners et al. 1993, 1995; Panter et al. 2002; Welch et al. 2008). The MSAL-type alkaloids are thought to be the most toxic to cattle, and their concentrations have been used as a predictor of plant toxicity (Ralphs et al. 2002; Pfister et al. 2002). Consequently, current management recommendations for grazing cattle on larkspur containing ranges are based primarily on the concentration of MSAL-type alkaloids (Pfister et al. 2002; Ralphs et al. 2002).

In considering these facts, a finding of particular interest made by Gardner et al. (2002) was the identification of two alkaloid profiles in *D. occidentale*. One profile lacked or displayed small amounts of the MSAL-type alkaloids, and another that displayed larger amounts of the same alkaloids. However, no information regarding the relative distribution of these two profiles was provided. Therefore, the objective of this study was to define the distribution of these two profiles throughout the geographical distribution of *D. occidentale* by using field collections and herbarium specimens.

Two chemotypes of *D. occidentale* were identified, thus supporting the preliminary finding of Gardner et al. (2002). Chemotypes A and B contain similar MDL-type alkaloids but at different concentrations (Table 1). However, no MDL-type alkaloid was chemotype specific, and differences in the MDL-type alkaloids between chemotypes were due to the large sample size. Localized populations of each chemotype may not contain statistically different amounts of the MDL-type alkaloids. The major MDL-type alkaloids in both chemotypes based upon concentration are those with masses (*m/z*) 454, 468, 482, 494, 508, 552, and 578 (Table 1). Deltaline ($MH^+=508$) is the major MDL-type alkaloid in most samples of both chemotypes; however, the

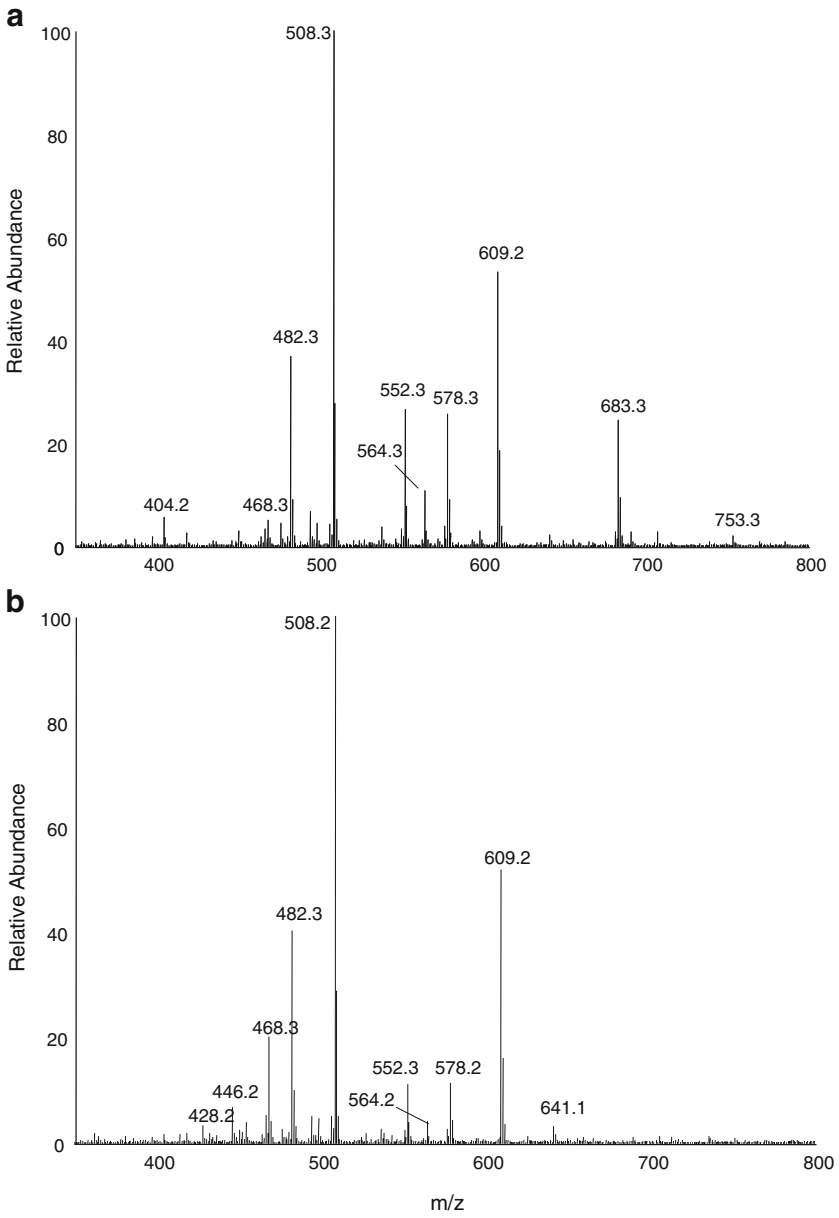


Fig. 2 Electrospray mass spectra from representative samples from each *Delphinium occidentale* chemotype

ratio of deltaline to other MDL alkaloids varies among locations. For example, in some locations masses (*m*/*z*) 454, 552, and 578 may be the major MDL-type alkaloid. Chemotypes A and B contain different concentrations of the MSAL-type alkaloids. Concentrations of the other MSAL-type alkaloids such as nudicauline, geyerline, 14-deacetynudicauline, and others are different between the two chemotypes but like the MDL-type alkaloids are not chemotype specific. This is due to the fact that some of these minor MSAL-type alkaloids are not found in all populations of chemotype A. Methyllycaconitine (MH^+= 683) concentration, which was used as the basis for assigning the two groups, was chemotype specific by definition. Three conclusions can be drawn from these data.

First, plants that represent chemotype B containing very low amounts or no detectable MSAL-type alkaloids would likely pose little risk to grazing cattle based upon current models and recommendations. Current management recommendations state that plants with greater than 3-mg MSAL-type alkaloids per gram plant material pose the greatest risk to cattle (Pfister et al. 2002). Alternatively, plants that represent chemotype A containing the MSAL-type alkaloids may pose considerable risk to cattle based upon current models and recommendations. However, poisoning is always dependent upon the dose and duration. Chemotype B plants contain the less toxic MDL-type alkaloids. Therefore, at the proper dose and duration, there is a possible risk of poisoning livestock.

Table 2 The biogeographical distribution of each *Delphinium occidentale* chemotype by state and county

State	County	Total	Chemotype A	Chemotype B
Colorado	Garfield	3		3
	Grand	8		8
	Jackson	19		19
	Pitkin	1		1
	Rioblanco	16		16
	Routt	26		26
Idaho	Bannock	15	12	3
	Bear Lake	89		89
	Blaine	7	7	
	Boise	5	5	
	Bonneville	31	31	
	Canyon	1	1	
	Caribou	67	7	60
	Cassia	19	19	
	Clark	9	8	1
	Clearwater	7	7	
	Custer	13	13	
	Elmore	1	1	
	Franklin	00	12	88
	Fremont	32	32	
	Idaho	4	4	
	Lemhi	15	15	
	Madison	2	2	
	Payette	2	2	
	Teton	9	9	
	Twin Falls	20	20	
	Valley	1	1	
Montana	Beaverhead	45	44	1
	Gallatin	55	54	1
	Madison	26	26	
	Park	35	34	1
	Stillwater	2	2	
	Sweet Grass	10	10	
Nevada	Elko	58	58	
	Eureka	6	6	
	White Pine	20	20	
Oregon	Baker	2	2	
	Union	9	9	
	Wallowa	15	15	
Utah	Box Elder	34	26	8
	Cache	303		303
	Carbon	2		2
	Daggett	14		14
	Davis	1		1
	Duchesne		10	10
	Emery	14		14
	Juab	44	8	36
	Millard	38		38
	Rich	15		15
	Salt Lake	31	1	30
	Sanpete	89		89
	Sevier	38		38
	Summit	58		58
	Tooele	16		16
	Uintah	22		22
	Utah	52	2	50
	Wasatch	67		67
	Weber	23		23
	Albany	30		30
Wyoming	Carbon	45		45
	Converse	1		1
	Fremont	10	6	4
	Hot Springs	1		1
	Lincoln	87	28	59
	Natrona	2		2
	Park	26	25	1
	Sublette	90	27	63
	Sweetwater	2		2
	Teton	92	87	5
	Uinta	1		1

Second, in general, each chemotype had a distinct distribution with defined boundaries (Table 2 and Fig. 3). In some cases, the chemotypes are separated by notable geographic features. For example, the east to west transition is separated by the desert that runs north to south on the west side of the state of Utah (Fig. 3). On the other hand, the north to south transition zone of the two chemotypes is not separated by any notable geographic features. In fact, two populations in Lincoln County, Wyoming that represent the two different chemotypes occurred on the same watershed less than 5 miles apart.

Third, the data suggest that the qualitative nature of the alkaloid profiles in *D. occidentale* remains constant at a given location. This conclusion is supported by field collections that have the same qualitative alkaloid profiles as the herbarium specimens from identical locations that were collected up to 100 years earlier. In addition, the data suggest that the alkaloid composition of herbarium specimens is not modified as a result of long-term storage at room temperature. Thus, herbarium specimens may serve as useful resources in determining risk of other larkspur species. However, quantitative amounts of these alkaloids may vary among years, and quantitative assessment of the alkaloids over time due to environment and other factors merits further investigation. For example, in *D. barbeyi*, a closely related tall larkspur species, both the MDL- and MSAL-type alkaloids decline as the plant matures and

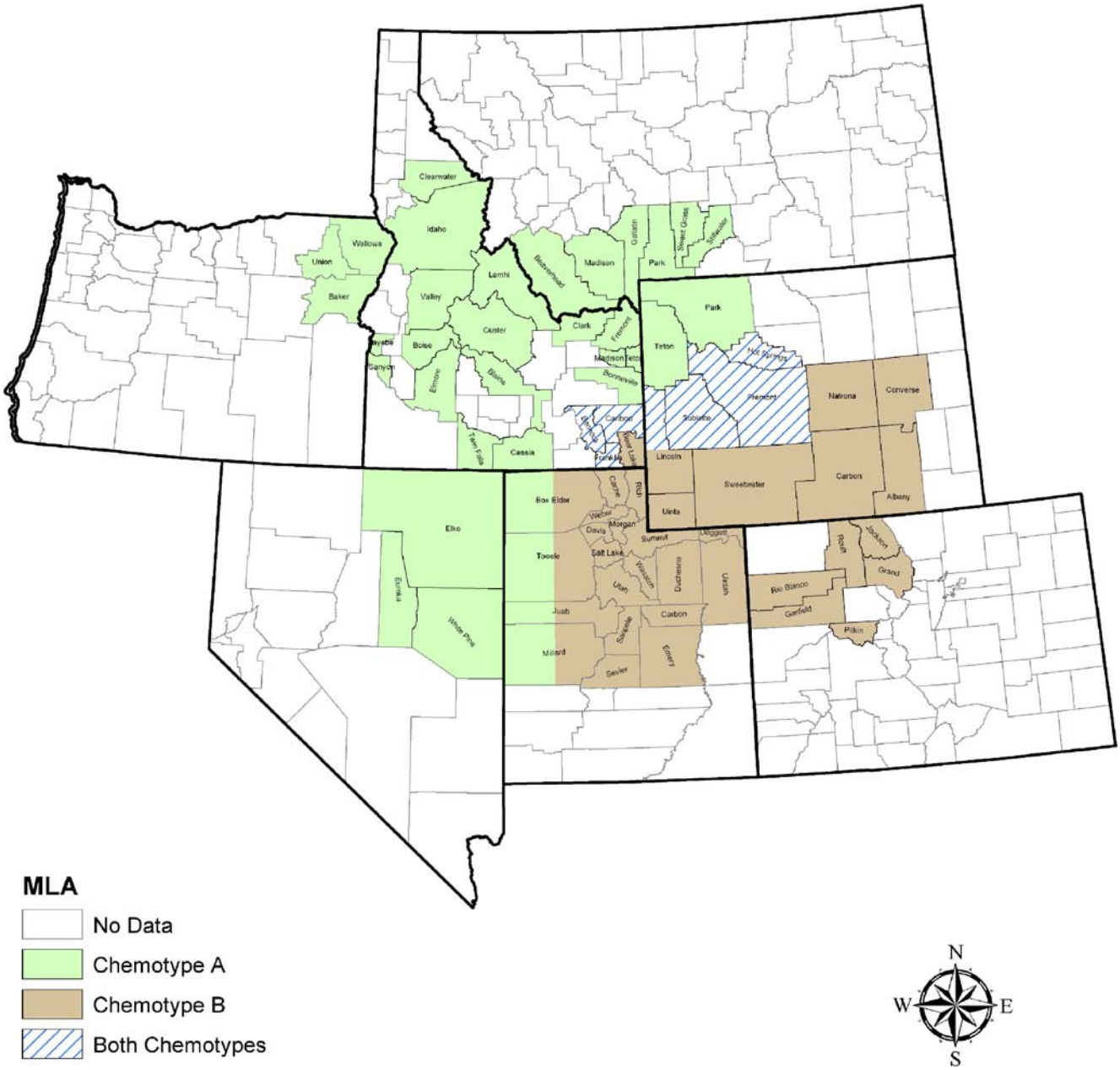


Fig. 3 Distribution map of chemotypes A and B throughout the geographical distribution of *Delphinium occidentale*

accumulate to different amounts in differing plant tissues (Ralphs and Gardner 2003). Additionally, environmental factors such as light, defoliation, and herbivory by insects have been shown to influence norditerpene alkaloid concentrations and pools (Ralphs et al. 1998a, b; Ralphs and Gardner 2001b). Such causes could be responsible for or play some part in the observed differences between the two chemotypes as well as their distribution.

We currently are not able to explain why there are two unique chemotypes of *D. occidentale* with such a defined geographical distribution, although some possibilities merit consideration. First, these chemotypes may represent unique *Delphinium* species or varieties, although no obvious morphological features used for taxonomic separation of the two groups have been noted. Second, the chemotypes may have originated due to environmental differences among locations. Previous research has shown that *D. occidentale* with chemotype A and *D. barbeyi* have distinct alkaloid profiles. However, when reciprocal gardens were established with these two species, the variation in profiles disappeared (Ralphs and Gardner 2001a). Third, these chemical profiles may be the result of natural phytochemical evolution of plants in the presence of herbivores. Frequency-dependent selection by herbivores may result in the evolution of differing chemical phenotypes (Schmeller et al. 1994). Furthermore, in certain

instances, plants with one chemotype have been shown to be more susceptible to herbivory than those of another chemotype (Berenbaum and Zangerl 1988; Bowers and Puttick 1988). It appears likely that plants with chemotype B may be more susceptible to herbivory due to their lack of methyllycaconitine, a potent insecticide (Jennings et al. 1986). Finally, chemotype B may be derived from hybridization between chemotype A of *D. occidentale* and *D. barbeyi* as suggested previously (Ralphs and Gardner 2001a; Gardner et al. 2002; Li et al. 2002). In fact, chemotype B may overlap with *D. barbeyi* in parts of Sevier, Sanpete, and Emery counties in Utah, all the counties in Colorado, and Albany and Carbon counties in Wyoming (Ralphs and Gardner 2001a; Gardner et al. 2002). Hybridization can result in either the gain of novel secondary compounds or the loss of secondary compounds (Orians 2000). If chemotype B were derived from hybridization, it is possible that a loss of function in the biosynthetic pathway leading to the MSAL-type alkaloids occurred, which could result in the accumulation of the MDL-type alkaloids. To address such possibilities, we are initiating further studies that include phylogenetic analysis and reciprocal gardens.

In summary, the MSAL-type alkaloids, such as MLA, are the primary factors responsible for the toxicity of larkspur plants. We report here that *D. occidentale* has two defined chemotypes: one (chemotype A) contains significantly more MSAL-type-alkaloids, and one lacks or contains very small amounts of the MSAL-type alkaloids (chemotype B). In general, the plants with these chemotypes grow in distinct geographical locations; however, there are counties that contain populations of both chemotypes. In addition, this study demonstrates that taxonomic identification of *D. occidentale* is not sufficient to determine risk. Lastly, based upon current toxicity models, the results from this study have implications for making management decisions for D. *occidentale*-infested rangelands. Additional research, however, is needed to determine the exact risk to livestock of each chemotype before these management recommendations can be further refined.

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